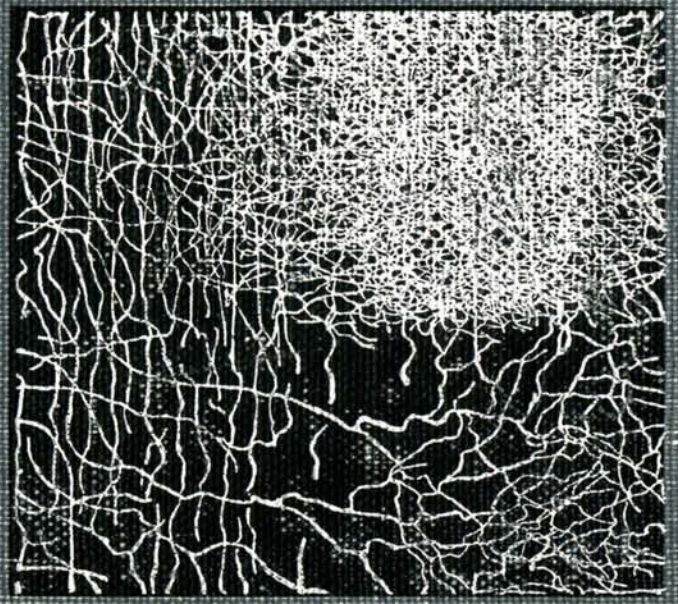
THE NEUROSCIENCES



THE NEUROSCIENCES

A STUDY PROGRAM

THE

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NEUROSCIENCES

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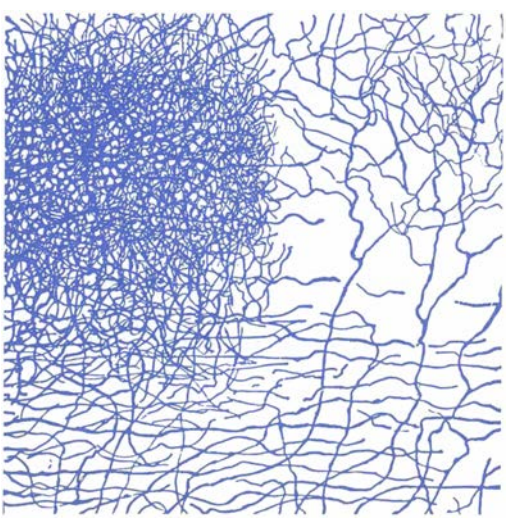

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FOREWORD

THE HUMAN BRAIN and the behavior correlated with its development, structure, and function present a problem that is both the most complex to which man can address himself through the use of scientific methods and the most urgent from the human viewpoint—urgent not only because of the academic challenge and the humanitarian commitment to relieve suffering caused by mental illness, but also because science, which sparks our ever-accelerating cultural evolution, results from thought processes and is uniquely human; if man could better understand these, he would accelerate the advance of science and make revolutionary discoveries about his own nature.

Formidable obstacles reduce the effectiveness of scientists' attack on these problems: the abysmal lack of knowledge about brain structure, chemical composition, and dynamics; the tendency of scientists to withhold their hard-earned opinions and speculative hunches; the tardiness of publication; the slowness and expense of conventional modes of storing and retrieving published information; the lack of critical evaluation of relevant information; and the specialistic approach that fails to see the forest for the trees and that thwarts meaningful communication among the different disciplines of neuroscientists.

To overcome these obstacles, sheer necessities of our culture demand more adequate means than have yet been devised. To help supply them, an international, interuniversity organization was formed in 1962, funded by Federal grants, sponsored by the Massachusetts Institute of Technology, and called the Neurosciences Research Program (NRP). Several dozen cooperating Associates assume responsibility for NRP and contribute the knowledge and judgment earned as eminent scientists in a wide array of disciplines, ranging from mathematics and physics through biochemistry and biology to neurology and psychology. They are aided by a small but strong Staff at the NRP Center in the Boston House of the American Academy of Arts and Sciences.

Not an institute, but a kind of investigative center, NRP has been acting through its Associates, consultants, and professional Staff to

garner, glean, and share the best obtainable facts and ideas about the nervous system, its cells, their organelles and molecules, as well as mathematical models and behavior and mental processes. NRP gathers this information less from the literature than from the minds that create seminal literature, primarily through bimonthly Work Sessions of guest experts who survey world research on topics of high conceptual content and relevance to the neurosciences. Resident scientists and interdisciplinary writer-editors then cooperate with the experts in writing evaluative summaries of these critical and synthetic symposia. Finally, NRP broadcasts the upshot of its findings through the *Neurosciences Research Program Bulletin,* distributed to libraries and qualified investigators, and through interdisciplinary books such as this.

This book is an integrated series of surveys of selected scientific fields of obvious or demonstrable relevance to an understanding of brain function. It is an outgrowth of the month-long Intensive Study Program (ISP) in the Neurosciences held in the summer of 1966 on the campus of the University of Colorado in Boulder. The substance of the ISP and its exact relationship to this book are detailed in the Preface, while the ISP purposes and procedures are discussed here.

The 1966 ISP and its intended successors are meant to further the unification of the disparate neurosciences and to draw attention to the wide range of emerging opportunities for pioneer research in advancing scientific understanding of human brain function, especially the biology of learning. Since the founding of the NRP, its Associates have unanimously favored the holding of such ISPs. Planning for the first ISP began in 1963, with the encouragement of Mr. I. Rogosin, and intensified after the National Institute of General Medical Sciences funded the conference. July 18, 1966, saw the Associates and Staff of the NRP joined by more than a hundred other selected scientists from around the world who represented many different professional backgrounds, including the fields of psychiatry, pharmacological psychology, physiological psychology, neurology, neuroanatomy, experimental embry-

ology, physiology, neurophysiology, cell biology, neurochemistry, immunochemistry, biochemistry, molecular biology, biophysics, physical chemistry, physics, and mathematics. The participants are listed at the back of the book.

A major part of the four-week program was the series of morning lectures, each a survey of an important field of research, particularly from the conceptual aspect, as seen by an expert in the subject. These morning lectures were supplemented by several special afternoon lectures, evening addresses, and film programs, but most afternoons were devoted to intradisciplinary and interdisciplinary discussions of the morning lectures, to a series of seminars, or to the four sessions of tutorial lectures on brain structure given by Walle J. H. Nauta, Sanford L. Palay, Jay B. Angevine, Jr., and David Bodian.

Almost all of the 153 scientific participants remained throughout the four weeks, thus assuring the desired interdisciplinary interaction and cross-tutoring. This solidarity demonstrated that the ISP enabled lecturers and listeners alike to participate in an intensive learning and reorientation experience by sharing views. For example, the program offered the participants who were neurophysiologists and psychologists an opportunity to review those topics in the basic sciences thought to be relevant by the chemists, to think through the relevance to their own fields, and to consult the lecturers for further discussion. Some carried out a re-evaluation of the work in their own fields of investigation with the possibility of upgrading their research programs by utilizing the new concepts and methods in closely related disciplines.

Meanwhile the biophysicists, physical chemists, and biochemists, who arrived with a tendency to regard the neurosciences as being in their infancy and lacking powerful simplifying explanations, left with an awareness of the great complexity of brain structure. Although little is yet known about the molecular mechanisms of major neuronal functions, such as the coupling between bioelectric, biosynthetic, and bioenergetic processes, molecular biology is being extended actively to the study of

nervous tissue. New ideas generated at the 1966 Boulder conference may point the way for some of these extensions.

Whatever their backgrounds, conferees made friendships and even partnerships that are already resulting in richer cooperative research projects controlling far more variables. Now the educational and integrative purposes of the ISP are being furthered by publication of the lectures in this book. On behalf of its readers and of the NRP, I have the pleasant duty as Chairman to express deepest thanks and appreciation to all who participated in and made this complex enterprise possible, including the chairmen and other NRP Associates; the other lecturers; the accomplished and promising scientists who participated as Fellows; the hard-working, dedicated members of the NRP Staff; President Detlev W. Bronk for his constant encouragement of the ISP since its inception, for his active participation during a portion of the conference in Boulder, and for making it possible for The Rockefeller University Press to publish the volume; the staff of The Rockefeller University Press; and the organizations listed on page xiv for the sponsorship and substantial financial support that made possible both NRP's first Intensive Study Program and this book.

FRANCIS O. SCHMITT

Brookline, Massachusetts
June 18, 1967

PREFACE

As PROFESSOR SCHMITT has said in the Foreword, this book grew out of the NRP's first Intensive Study Program in the Neurosciences. It is a reasonably faithful record of that month-long meeting. All but one of the 65 scheduled lectures given there are represented, together with two additional chapters, one based on an impromptu lecture given at Boulder and one written subsequently by an invitee whose previous commitments kept him from attending the meetings.

However, the lectures as given have in all cases been revised by their authors. Substantive changes were made in the light of the authors' experiences at the multidisciplinary discussions held throughout the month and as a result of critical thinking following the study program. Editorial changes have also been made, to increase the possibility of interdisciplinary communication. Thus, the reader is the beneficiary of the second as well as the first thoughts of the scientists who accepted invitations to survey the state of the art, the major issues, the major hypotheses, and the major findings in the fields selected.

A glance at the table of contents shows that the scope of the 1966 ISP was unusually wide, spanning more than half-a-dozen levels of organization, from that of molecules and macromolecular assemblies through those of organelles, cells, and cell assemblies, to the organ and organism. Yet its coverage of brain mechanisms of behavior was not encyclopedic. Many interesting approaches received little emphasis—for example, those which concentrate on phylogeny, ontogeny, maturation, and pathology, and those which exploit natural, mechanical, and mathematical models. Future NRP study programs may focus on some of these.

When this series of lectures was planned, the first step was to stand back from the ongoing process of science to try to anticipate where the greatest activity might occur during the next decade. In many areas of research, current results appear to be mainly descriptions of phenomena, often circumscribed by the limitations of particular technological devices. For such areas, an effort was made to ascertain the types of new

knowledge required to provide an adequate explanation of the observed phenomena. As a rule, this new information will come from a level of science more basic than that at which the observations were made and will require the development of more complete and effective theoretical understanding.

Those engaged in neuroscience research can be regarded as members of small task forces, each with specialized training and each attacking the problems of the nervous system at one level only. To those involved at one level the activities of other scientists are not wholly strange but usually are not understood in detail. Each major theme of the 1966 ISP was chosen with the expectation that, in the future, it will contribute to a greater knowledge of function at the next higher level. Some of these themes can be outlined to illustrate their anticipated relevance.

A major theme concerns the forces stabilizing macromolecules and factors influencing conformation change. This information has already provided a basis for the understanding of structure in biological systems and has helped explain the characteristics of chemical reactions involving macromolecules. These topics will certainly be relevant to developing insights into the structure and function of brain cells and their organelles.

There has been a tendency to oversimplify the behavior patterns of both neurons and glia. All cell types in the nervous system are complex and are concerned at various times with many different activities. Each of these activities is under some type of internal or external control, or both. Therefore, cellular control mechanisms were reviewed in general, even though little is known about how these are applied in the nerve cell itself. An investigation of factors controlling protein synthesis also appeared essential for future knowledge of enzymatic control of brain function.

The immediate future will certainly see intensive investigations of membranes, of transport mechanisms within nerve cells, and of the development and mode of action of other organelles. In pursuit of these

goals, biophysical and biochemical studies of processes that maintain structure and correlate with function will borrow from methods worked out for simpler systems.

Despite the recognition that the nerve impulse is important in conveying information over long distances within the nervous system, little is known about the mechanisms of encoding and decoding. Doubtless, the next few years will produce much work on the fundamental electrical and chemical processes by which information is mediated by brain cells. For this reason, the block of lectures on information processing was included.

Stimuli that act on the organism do not always lead to the same response. Sometimes a shift in the response pattern is the result of learning; sometimes it is also the result of major alterations in the general programing of the nervous system. For instance, during the identifiable and discriminable states of sleeping and waking there are gross differences in patterns of nervous activity; as a result, the behavior of the organism and the processing of information handling differ, although the environmental input may seem identical. We chose to consider some major activity patterns of whole nervous systems in anticipation of interpretations of localized processes within the framework of these background events.

Although many topics in physiological psychology could have been considered with profit, the topic of the biology of learning was chosen for special emphasis. The mechanisms of biological storage in the nervous system must be understood if an adequate explanation of information processing is to be developed.

All of these topics are important for the immediate future of the neurosciences, but there are obvious differences in the character of the research it is possible to do on them. For instance, those working on the biology of learning must admit that their efforts are the merest beginning and that they have not yet really found out how to apply the most powerful conceptual tools.

These notes suggest that a considerable effort was made to predict the future, to determine the most promising lines of research, and to focus on those fields of science most likely to augment the activities of the neighboring fields. The ultimate selection of topics may or may not have been wise, but certainly it was neither hasty nor capricious. This ISP was three years in the planning, and many sources of judgment were tapped, including more than a dozen consultants around the world, all NRP Associates, and more than a hundred NRP Work Session participants. Naturally, opinions expressed in the literature of each of the neurosciences were also used.

During the planning we adopted the technique of dividing the program into blocks, and the blocks into days. Each block and each day had a chairman drawn from the NRP Associates. At subsequent block and day planning meetings, topics were pinpointed and lecturers were nominated. These men were known to be expert in their fields, good at exposition, and open to multidisciplinary give and take. The day chairmen then recruited the speakers. Both before and during the ISP, individual conferences were held with the speakers to get their ideas about the subject they were to discuss and to develop a style of presentation consistent with our interdisciplinary goals.

This book is the result of the intellectual labor described—a more-or-less integrated series of surveys of selected fields in science that pertain to brain function. For reasons given in the introduction to the first two chapters, the ascent from molecule to behavior is preceded by a close look at brain components. Then come five sections that reflect the logic of selection already described. Finally, a section derived from special evening lectures presents views that are more evolutionary, historical, and philosophical.

Attempts to identify trends in science and to channel the activities of scientists who are noted for independence and creativity can be both presumptuous and futile. The editors obviously shifted this burden to a great many others, too numerous to thank individually. We especially

thank the NRP Associates who chaired the various blocks of lectures: Theodore H. Bullock, Robert Galambos, Albert L. Lehninger, Robert B. Livingston, and Francis O. Schmitt, as well as Manfred Eigen, who assisted us in planning certain parts of the program. They tolerated our impatience and instructed us in our ignorance. So did the chairmen of each day at Boulder, and indeed every lecturer and Fellow.

The editors have hoped for a wide distribution and use of the material in this book. The costs of publication, requiring a high price for each volume, would have interfered with this distribution had it not been for a welcome grant from The National Institutes of Health.

The credit for making this a handsome book, despite the economy and speed of its production, belongs to Messrs. William Bayless and Reynard Biemiller, Director and Assistant Director of The Rockefeller University Press. The precision and clarity of expression are significantly higher than they would have been but for the year-long labors, begun at Boulder itself, of the publisher's editor, Mrs. Helene Jordan, whom we join other authors in thanking for her tireless, thorough, and amiable professionalism.

GARDNER C. QUARTON *and* THEODORE MELNECHUK

Brookline, Massachusetts
June, 1967

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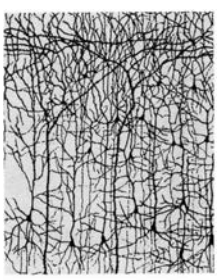

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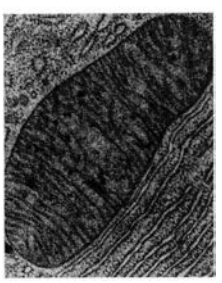

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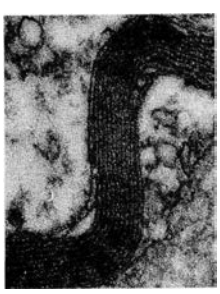

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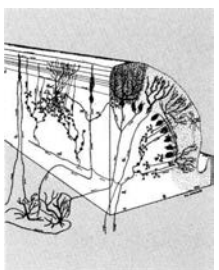

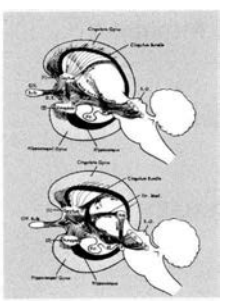

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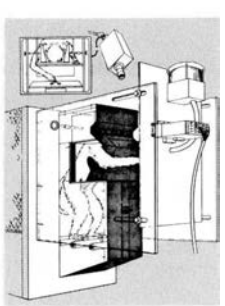
BRAIN CORRELATES OF LEARNING



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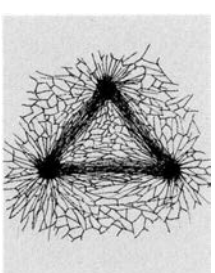

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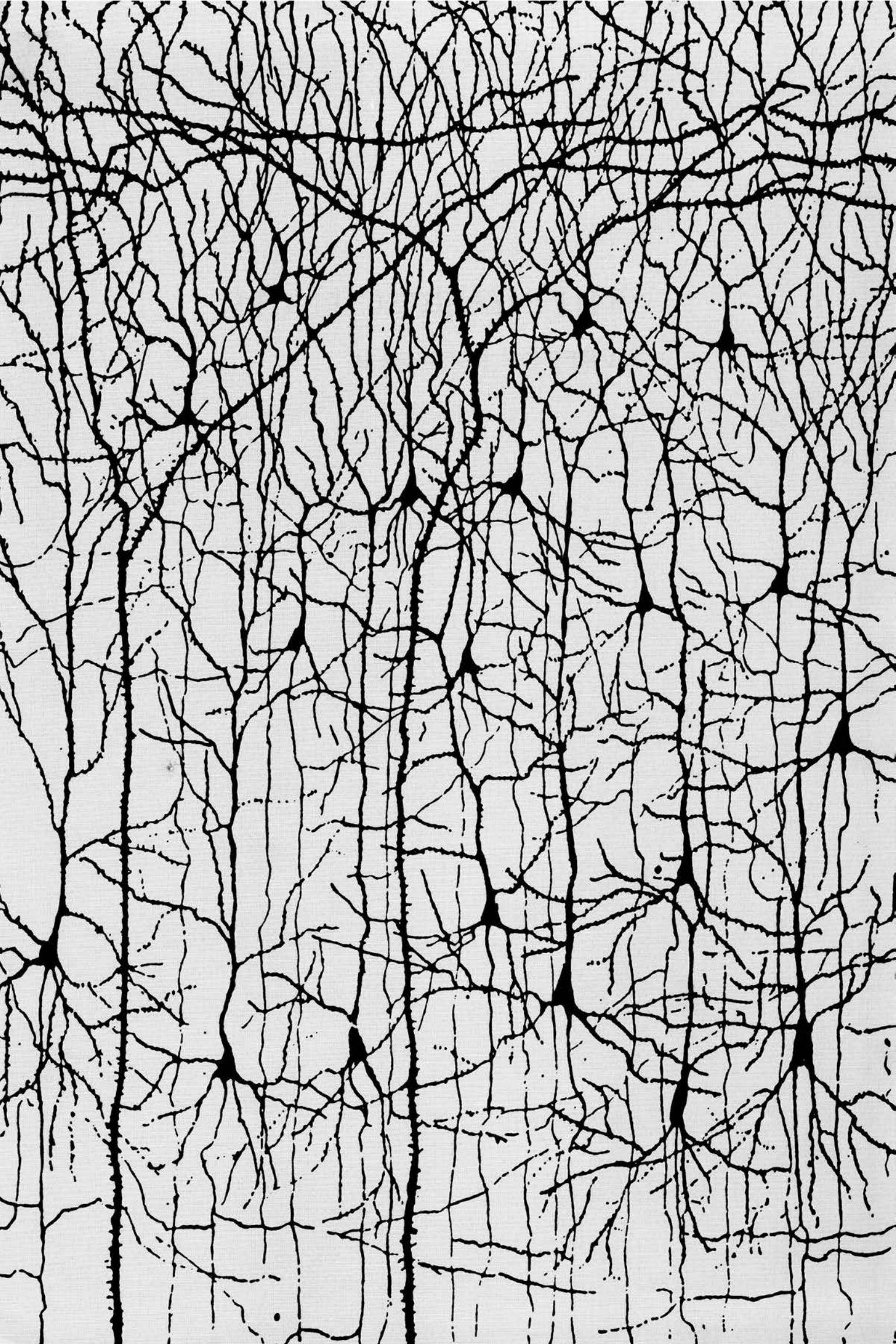
A STUDY PROGRAM



COMPONENTS OF THE NERVOUS SYSTEM

"The nervous system is not a random net. Its units are not redundant. Its organization is highly specific. . . ." PALAY, PAGE 31. *This drawing from Ramón y Cajal, based on a Golgi-stained preparation, illustrates the complexity and organization of dendritic branching in the superficial part of the visual cortex of a human infant.*

INTRODUCTION

THE FIRST SECTION of this study program gives the reader a close look at the cells and organelles of nervous tissue. Nervous system function is clearly rooted in nervous system structure, which therefore must be considered by any student of brain mechanisms of behavior.

Structure can be examined from several points of view. Gross anatomists, for example, study the parts of the nervous system—its regions and tracts. Any adequate presentation of so complicated a subject would take more space than can be given to a single contributory discipline in this selective survey. Luckily, the thorough presentation of gross anatomy in textbooks permits the omission of its systematic consideration here.

In this study program, then, the morphological emphasis is less on the connections between specialized brain regions than it is on the structure of specialized brain cells. This emphasis on components is intended to underline the structural complexity of cellular elements, a complexity that undoubtedly has implications for the understanding of function. In some interdisciplinary discussions the neuron is treated as a simple binary element in a switching network. This simplification is not acceptable. A discussion of the actual features of different cell types should be a necessary prerequisite to the development of hypotheses about the function of cellular aggregates or assemblies.

Recent years have witnessed a radical revision in scientific understanding of the structure and function of the identifiable subunits of the cell. Organelles can no longer be thought of as parts of a fixed cellular skeleton divorced from chemical changes within the cell; to the contrary, they play an active role in chemical mechanisms that control cell function. Another bridge between anatomy and molecular biology is provided by forces that stabilize molecular conformation and the conditions that permit assembly of molecules into biological structures.

Thus, the two papers in this section constitute an important introduction to the consideration of neural function at the molecular level, a major consideration in subsequent sections.

Neurons, Circuits, and Neuroglia

DAVID BODIAN

THE GREAT COMPLEXITY of nervous tissue, compared with other tissues of the body, has resulted from the evolution of the capacity of nerve cells to communicate with each other and with other cells. One of the great advances in the history of neurology is the discovery of the extraordinary variety of ways in which nerve cells intercommunicate. These studies are still under way. The forces of natural selection being what they are, it would seem that every conceivable capability of a cell to intercommunicate has had some adaptive advantage and has ultimately found expression.

Intercommunicating sets of nerve cells (neurons) appear as complex three-dimensional networks at any moment of time. Yet, the time dimension is crucial, both in relation to short-term events, or signaling, in the system, and in relation to long-term events. Some long-term events may be derived from the signaling, in that some aspect of the communication transaction may be stored (memory engram). Quite another type of long-term event must also be kept in mind, since the complex topography of neurons is the product of slow embryological transformations (see chapters by Edds and Ebert, this volume). Throughout its life, the neuron not only performs its role in a relatively stable systems-network; it also mirrors its life history of transformation by revealing a small degree of transforming and adaptive capability throughout life. Some of this adaptive capability is employed in experimental morphology, and I shall refer later to one or two examples.

Since whole cells are basic units of neural function, the light microscope is still an indispensable tool for neuroscientists, and we shall begin by dealing with information and ideas obtained largely by studying transparently thin sections of nervous tissue with the light microscope. The thin sections can be made to reveal specific cellular and intracellular elements by means of a host of staining methods, which selectively reveal some components while leaving others poorly stained or even invisible. The electron microscope now extends the range of observations to the near-molecular level. From two-dimensional light and electron microscopic pictures, one attempts to fill in both the third dimension and, of course, the time dimension. Wherever possible, I shall include pictures of actual material, in order to convey the nature of the study materials.

It is a truism that major themes of macrocosmic organization will be found in its microcosms. In this volume the themes of structure, specificity, and bonding recur at social, organismic, cellular, and molecular levels. Which level is most important seems to me to be an irrelevant question. Interplay of knowledge and concepts from level to level is the key to the profitable direction of investigative effort of those who would not be trapped within a fragment of a theory of higher nervous function. In biology the expert in any field must have a minimal understanding of the basic concepts and state of the art at each level of bio-organization. Unfortunately, the cellular level does not have the familiarity of the behavioral sciences, the quantitative precision of the molecular sciences, or even a terminology that combines both qualitative and steric, or spatial, concepts as elegantly as does chemical nomenclature. What microscopists must convey to each other is accepted or rejected on the basis of credibility of a complex image of a structure at a particular moment of time. New knowledge is acquired with difficulty, is slow of acceptance, and has often been widely ignored for years before its significance was finally realized.

For the most part, we shall not address ourselves to experts; instead, we shall try to present a picture of the cellular level of organization as it really looks in material that is legally dead, but alive with information. Above all, we hope to make clear that morphological diversity at the microscopic level is indicative of the evolution of a variety of structural-functional devices for transmitting a variety of signals from neuron to neuron, or from sets of neurons to other sets. Among these devices may be some that will offer a clue to higher nervous function, just as others at the close of the last century were shown to contain the clue to a rational framework for reflex neurophysiology.[1]

Surface geometry and function in neurons

Neurons are cells of complex geometry and of greatly variable size, in which the immediate component of communication transactions is the surface membrane. Within the complex geometry of the membrane, the interior, or cytoplasm, has a specialized metabolic center surrounding and including the nucleus, and is therefore called the perikaryon, or cell body. Extending away from the peri-

DAVID BODIAN Department of Anatomy, The Johns Hopkins University School of Medicine

karyon are a variable number of usually branched processes (Figure 1).

Most neurons are highly elongated and polarized in such a way that one pole normally receives information and the opposite pole transmits information to other nerve cells or to muscle or glandular cells. The receptive pole may be called the dendritic zone, because in many neurons it is branched in the form of a tree. The transmitting pole, which often possesses terminal branches called telodendria, is either the sum of a set of the terminal expansions of these branches, the synaptic bulbs, or is the terminal of an unbranched process. These endings are often called "boutons." The branched or unbranched process that connects the receptive pole with the transmitting pole is ordinarily an elongated process called the axon. The axon, which may vary in length from microns to meters, is capable of conducting a propagated response or nerve impulse at velocities up to 100 meters per second. The conducting axons, with their sheaths, are also called nerve fibers. In most neurons the axon may be said to originate at the point of origin of the propagated nerve impulse, which is near the junction of the receptive portion of the neuron and the axon. This region is also called the initial segment of the axon, or axon neck, since it is often constricted. The specification of an origin and termination of a nerve fiber is possible because conduction normally is unidirectional, although under experimental conditions the axon can be shown to conduct in both directions, away from the point of stimulation. By considering each functional component of the neuron in detail, we may hope to relate the variable shape, size, and microstructure of neurons to major membrane and cytoplasmic functions.[2–6]

PERIKARYON The embryonic cell about to become a neuron is spindle-shaped and is not greatly different from other cells around it. As soon as threadlike extensions begin to grow out from the nucleated cytoplasmic mass, the so-called perikaryon, two major developments take place. The cell no longer is capable of undergoing division, and the cytoplasmic structures associated with protein synthesis, the ribosomes (ribonucleoprotein particles), increase in proportion to the degree of outgrowth of processes, but for the most part remain in the vicinity of the nucleus. The perikaryon is thus the metabolic center of the nerve cell

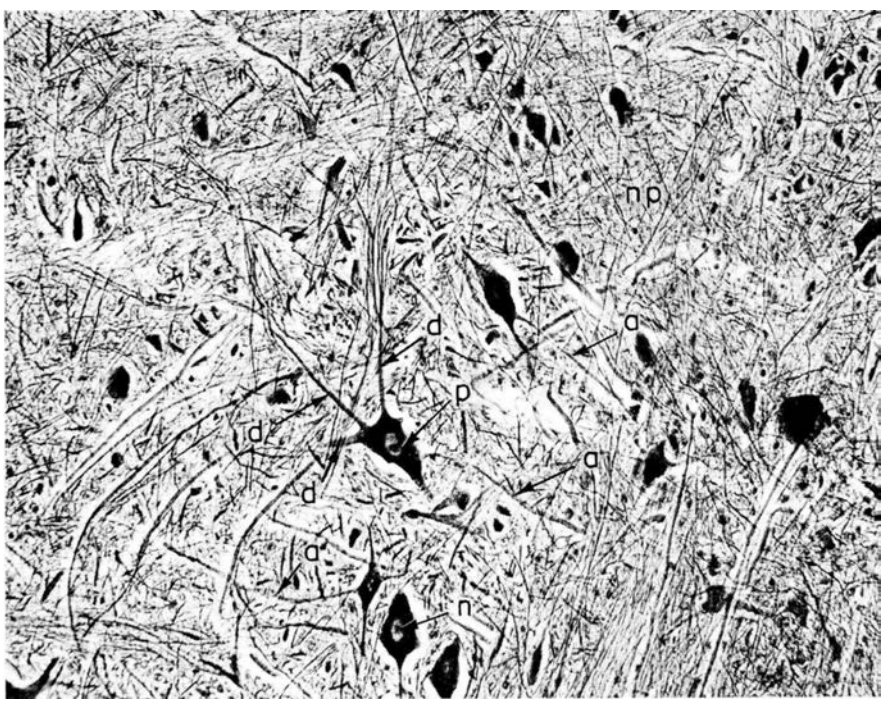


FIGURE 1 Cluster of neurons in the gray matter of the spinal cord of the monkey, illustrating a common arrangement of neuron cell bodies and their axon and dendrite processes. Much of the space between cell bodies is occupied by a feltwork consisting of these processes and a large number of incoming, branched axon terminals. The feltwork of axons and dendrites, within which neuron-to-neuron communication largely occurs, is known as the "neuropil." p—perikaryon. a—axon. d—dendrite. np—neuropil. n—nucleus of large neuron, probably a motoneuron. Paraffin section, 15μ. Bodian silver stain. X150. (The apparent space around the neuron cell bodies is a shrinkage artifact.)

and synthesizes proteins, such as enzymes, which must be transported to the distant reaches of the nerve cell processes. These may be several meters long, in some instances.[7]

The special importance of protein synthesis in nerve cells may be illustrated by two points. If, in certain neurons, the elongated axon process, which may contain the bulk of the neuron volume, is separated from the perikaryon, only the separated axon degenerates, while the ribosome aggregates (Nissl substance) of the perikaryon diminish in proportion, but may gradually be reconstituted as a new process grows out. In many ways both structure and metabolism revert to the embryonic stage and then repeat the process of maturation (Figure 2). It is also considered likely that, after an axon outgrowth, the very failure of nerve cells to divide is related to the high rate of specific protein synthesis, since in secretory cells in general the synthesis of the secretory product is usually incompatible with cell division. Axon amputation does not interrupt synthesis, and axon regeneration begins immediately, rather than de-differentiation and cell division. A large number of randomly growing sprouts arise from the proximal stump, and these are resorbed after one of them succeeds in finding an adequate pathway. The successful sprout then begins to mature, i.e., the caliber thickens and myelination begins.[8]

Figure 2 also illustrates three ways in which axon interruption has been used to analyze the connections of certain systems in the central nervous system (CNS).[9] The perikaryon of origin can be localized at a distance from the interruption of its axon because of the partial loss of its Nissl substance (retrograde chromatolysis). The fragmented degeneration products of the isolated and degenerating axon (Wallerian degeneration) can be followed along their entire course by special staining methods (Marchi and Nauta methods). Finally, degenerating synaptic bulbs are now beginning to be precisely localized on their neuron of termination in electron micrographs. Figure 3 shows a synaptic terminal six days after axon interruption. The degenerating synaptic bulb is still identifiable as to type (monosynaptic), although it stands in sharp contrast to its healthy synaptic bulb neighbors on a dendrite in the motoneuron column of the spinal cord. (Motoneurons send their axons into voluntary muscle fibers.)

Figure 4 shows actual photographs of the process of reaction and recovery of motoneuron perikarya to axon

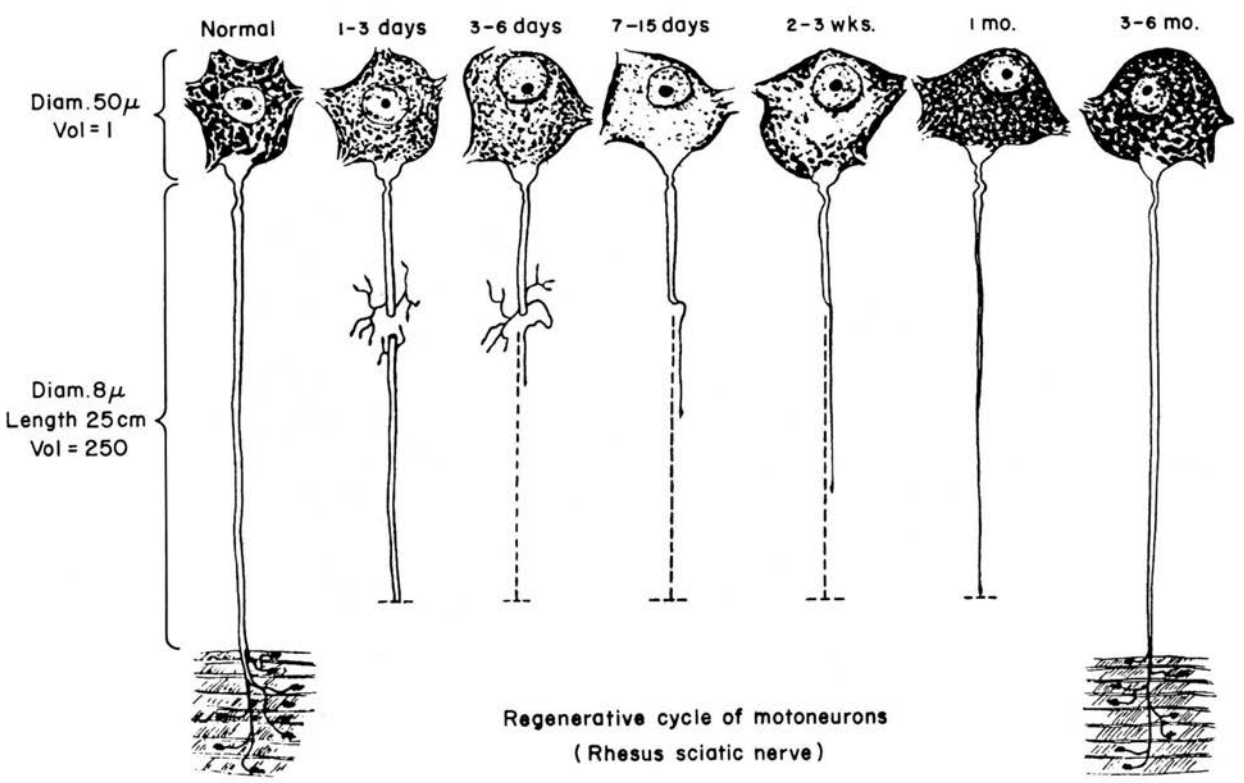


FIGURE 2 Diagram illustrating the cycle of changes in cytoplasmic Nissl substance (ribonucleoprotein), correlated with axon amputation and axon regeneration. At the height of reduction of Nissl substance (chromatolysis), the regeneration of the disproportionately large axoplasmic volume is barely under way. (From Bodian, Note 7)

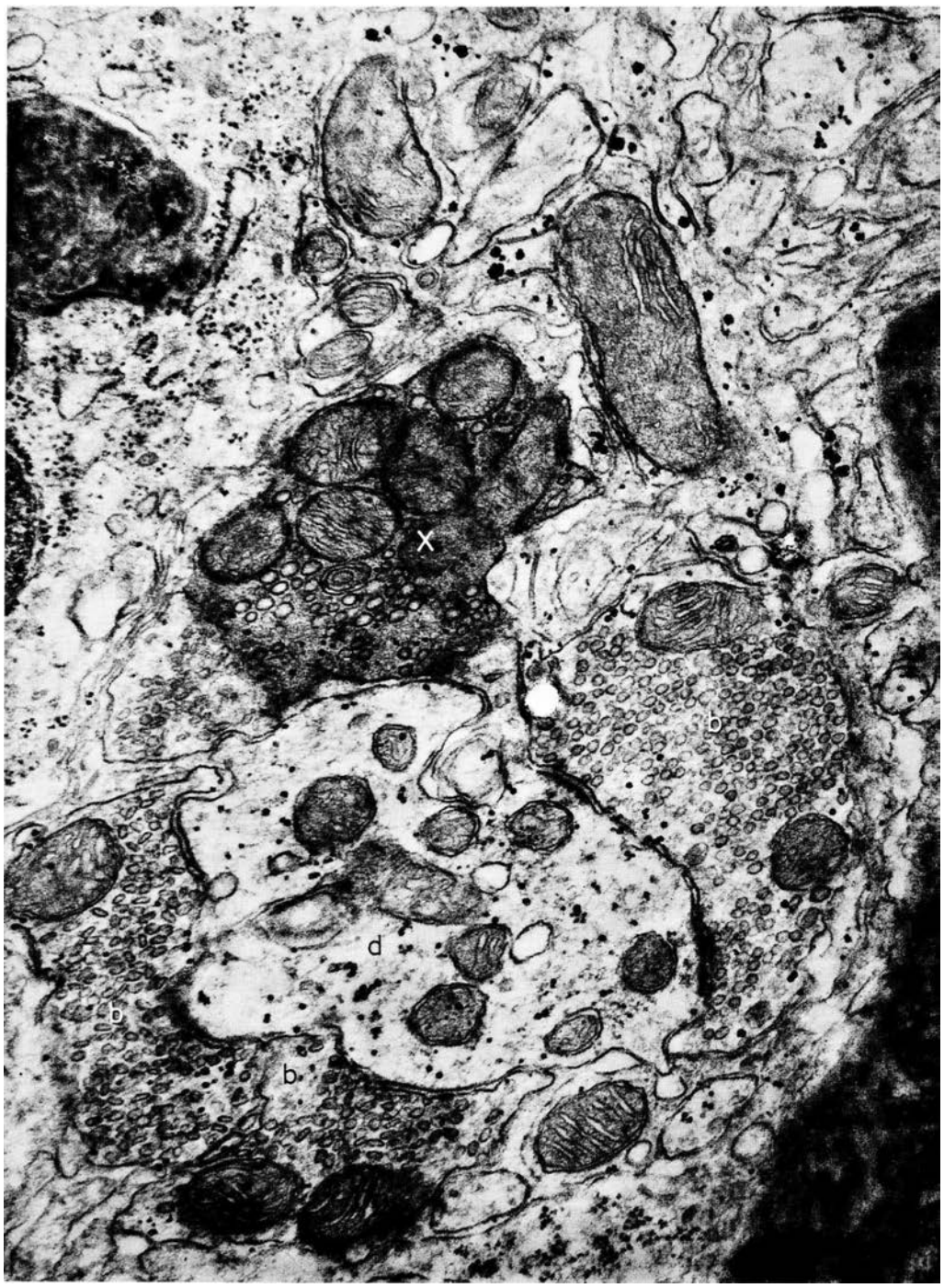


FIGURE 3 Electron micrograph of degenerating synaptic bulb in the lumbar spinal cord of the monkey. The parent axon (monosynaptic afferent fiber) was interrupted by section of the dorsal root six days previously. The degenerating synaptic bulb (x) stands in sharp contrast to its healthy synaptic bulb neighbors (b) on a dendrite (d) in the motoneuron neuropil of the spinal cord. X40,000.

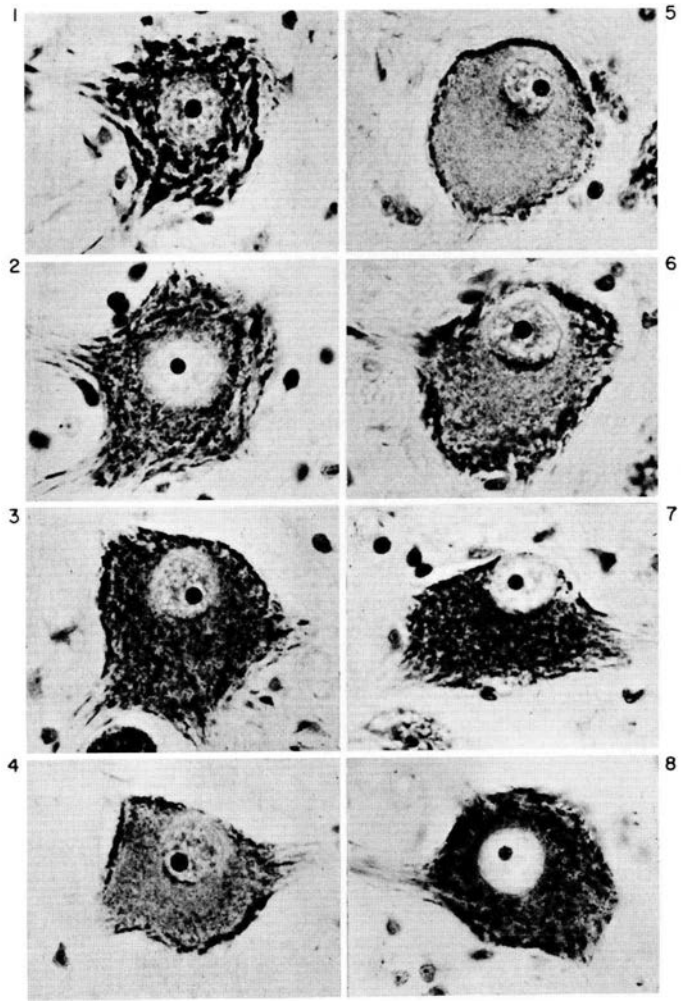


FIGURE 4 Photomicrographs of stages in the process of reaction and recovery of motoneuron perikarya after axon interruption. Earliest changes are shown in (2), 3 days after axon interruption, the height of chromatolysis in (4) and (5) about one week after interruption close to the perikaryon, and virtual recovery in (8) at 110 days after axon interruption. (From Bodian and Mellors, Note 10)

interruption.[10] Other neuron types behave differently. Some degenerate and disappear in a week or two. Others become smaller and have reduced amounts of stainable Nissl substance in the perikaryon for an indefinite period —six months or more. The utter dependency of some perikarya on their axons, while others show complete regenerative capacity, is not well understood.

The production of protein probably occurs in the perikaryon, and such products often must be transported for considerable distances within the nerve cell processes in order to be used or even exported at the tips of the processes. In connection with this function, the perikaryon is also the center of a cytoplasmic tubular system, the so-called neurotubules, which extend out into dendritic and axonic processes. These microtubules, which vary in diameter from 200 Å to 400 Å in caliber, are connected in the perikaryal cytoplasm with a system of channels, the endoplasmic reticulum, known to be involved in transport of secretory products of cells in general. Perhaps more characteristic of neuron cytoplasm than microtubules are the so-called neurofilaments, present in all parts of the cytoplasm, and having dimensions of 80 to 100 Å in caliber. Their role is not known, but the remarkable affinity of nerve cells for silver, which made possible much of our present knowledge of neurons, is attributed by some to the neurofilaments. In contrast to protein synthesis, the energy requirements for other functions, such as active ion transport, is high at both receptor and transmitter terminals of nerve cell processes, and the cytoplasmic structures associated with oxidative enzymes, the mitochondria, are often especially numerous in these sites, as well as in the perikaryon. All of these organelles are sensitive indicators of the health of the neuron, and their electron microscopic study is therefore affecting the field of neuropathology.[11]

Although the nucleated portion of the neuron is the center for outgrowth of processes and for protein synthesis, it may be located in the receptive pole of the neuron, as it is in most vertebrate neurons; within the course of the axon, as in vertebrate sensory ganglia; or within the transmitting pole of the cells, as in certain neurons that appear to possess no axons. *Thus, the location of the perikaryon has no constant relationship to the functional geometry of neurons in general, although in any specified type the location is fixed*[6] (Figure 5).

RECEPTIVE PORTION OF A NEURON Neurons may be divided into two general classes on the basis of type of input. Receptor neurons are those that receive and transduce environmental energy, such as light, heat, mechanical or electrical energy, and chemical stimuli. They have a geometrically simple dendritic zone, although in each case the membrane is specialized to respond to the specific stimulus. Synaptic neurons are those that receive already coded information from other neurons by means of synaptic contacts.

Much of the diversity found in the form of neurons is dependent on the complexity of branching and on variation in the length of processes extending from the receptive portion of synaptic neurons, the dendrites. In receptor neurons and in some small neurons the geometry of the receptive zone is relatively simple, and discharge of the nerve impulse may follow after input from one or a few sources. In large neurons of higher nervous centers, the branching of dendrites may greatly increase the receptive

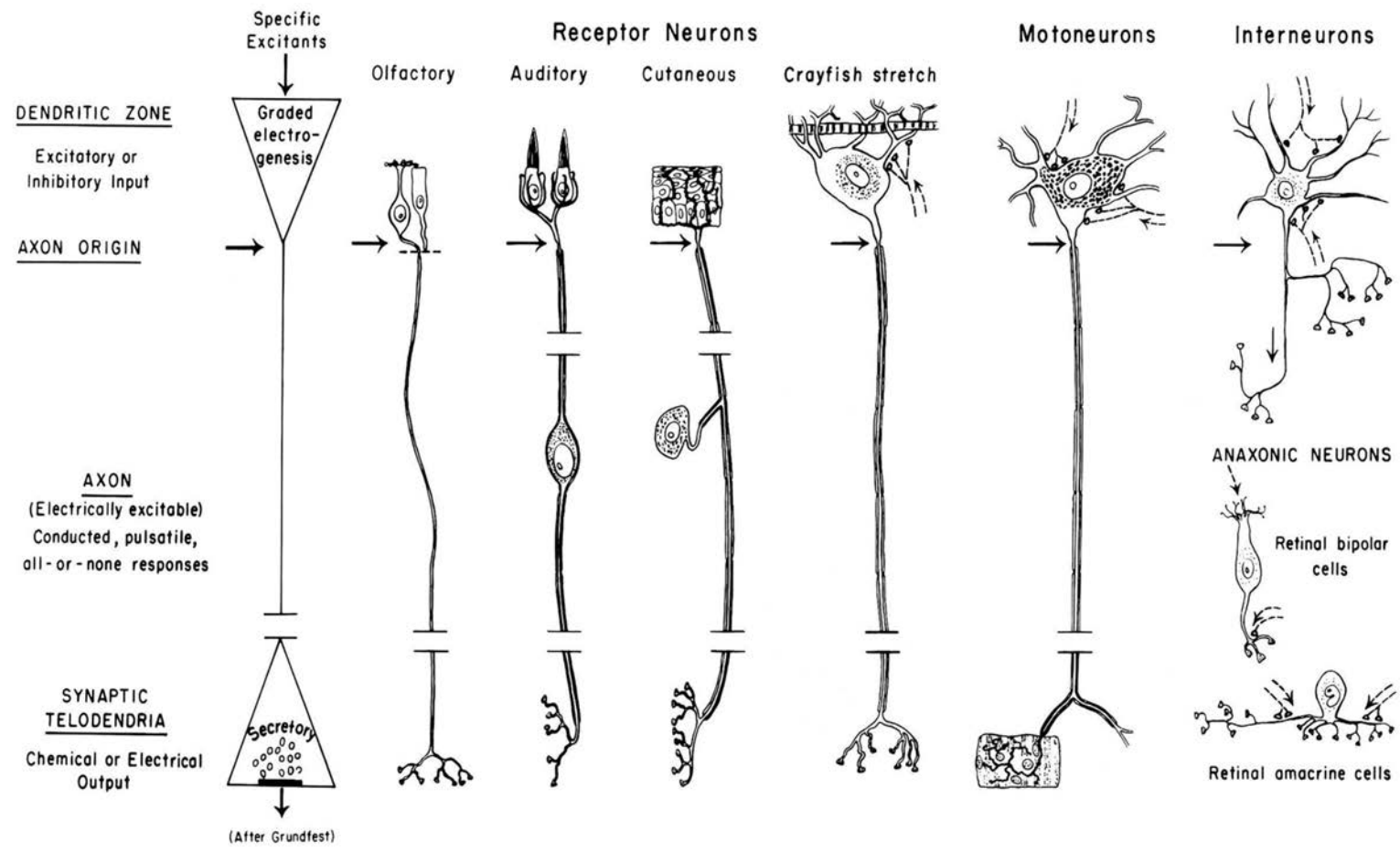


FIGURE 5 Diagram of a variety of receptor and effector neurons, arranged to illustrate the idea that impulse origin, rather than cell-body position is the most reasonable focal point for the analysis of neuron structure in functional terms in neurons with an axon process. In all axon-bearing neurons the four major points of interest (receptor zone, axon origin, conducting zone or axon, and transmitting or synaptic zone) conform to the functional diagram of the generalized neuron proposed by Grundfest.[2] The location of the nucleated portion of the cytoplasm, or perikaryon, however, does not have a constant relationship to the functional geometry of neurons in general, although in any specified type the location is fixed. Anaxonic neurons, in which the spike-conducting region is absent, may be considered as having processes of dendritic or telodendritic (pre-synaptic) character. (From Dowling and Boycott, Note 28)

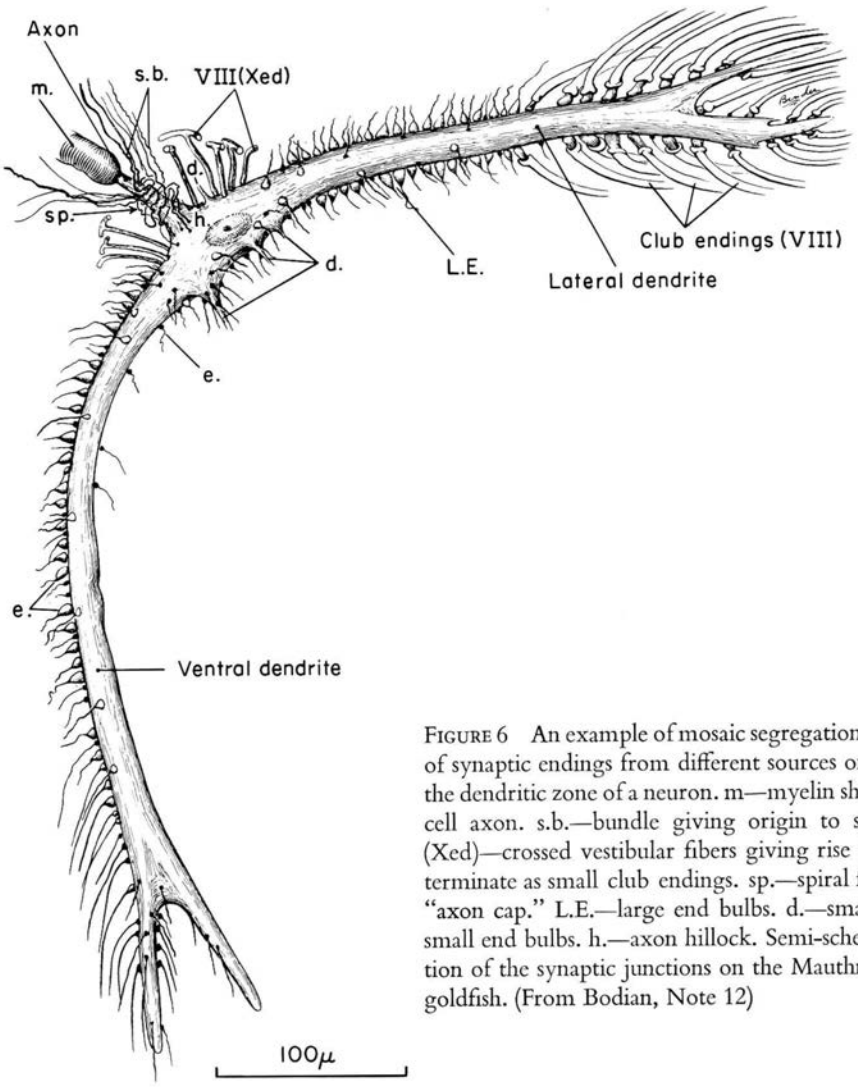


FIGURE 6 An example of mosaic segregation of different types of synaptic endings from different sources on specific parts of the dendritic zone of a neuron. m—myelin sheath of Mauthner cell axon. s.b.—bundle giving origin to spiral fibers. VIII (Xed)—crossed vestibular fibers giving rise to collaterals that terminate as small club endings. sp.—spiral fibers in region of "axon cap." L.E.—large end bulbs. d.—small dendrites. e.—small end bulbs. h.—axon hillock. Semi-schematic representation of the synaptic junctions on the Mauthner neuron of the goldfish. (From Bodian, Note 12)

surface and make possible inputs from a multiplicity of sources. Each neuron type can be recognized as a member of a class, but each is designed to play its own role. The "typical" neuron is a fiction, and the retina cannot be understood by assuming that its neurons look and act like motoneurons. The diversity, or so-called convergence, of sources of input finds a highly instructive expression in neurons such as the Mauthner cell of higher fishes (Figure 6). Here the geometry of the receptive surface is arranged in mosaic fashion, so that excitatory and inhibitory inputs are restricted to specific sites on the receptive surface in a constant arrangement.[12,13] The occurrence and frequency of firing of nerve impulses on the axon of such a cell is therefore achieved by the summing of a variety of local excitatory and inhibitory changes, as Furshpan and Furukawa have shown.[14] If a certain critical level of excitation is reached in the receptive zone, a nerve impulse will be generated near the initial axon segment. Thus, the receptive portion of a neuron is differentiated to respond to specific chemical or bioelectric stimuli, but it also serves as a mechanism that integrates converging inputs. Nevertheless, the existence of a receptive pole in a polarized neuron does not preclude the occurrence of occasional synaptic contacts on axons or axon terminals, so that the conventional scheme of a specialized receptor or dendritic zone is generally, but not universally, applicable.

INITIAL SEGMENT The axon or nerve fiber has its origin at some point on the receptive portion of neurons capable of discharging a propagated spike potential or nerve impulse. In this region, the spike potential also originates under natural conditions (Figure 5). This portion of the neuron, the initial axon segment, has two distinctive anatomical features that sharply differentiate it from the base

of dendritic processes. First, the ribosomes, the cytoplasmic structures most involved in protein synthesis, may be found in cytoplasm of perikaryon and dendrites, but do not extend into the initial segment, nor are they found in any portion of the axon. Second, the initial segment is often constricted to form the so-called axon neck of myelinated axons. This is also a characteristic of periodic loci along the axon called *nodes of Ranvier*. In some neurons the initial segment of an axon arises from a portion of the perikaryon conspicuously free from ribosomal material, the axon hillock. The axon may also arise from a dendritic process.

CONDUCTING PORTION The conducting portion, or axon, of a nerve cell is a delicate, cylindrical structure, varying greatly in length and caliber in different nerve cells. In some systems, especially at immature stages, axons may be packed together without intervention of other cellular elements (Figure 7). Such axons, often of extremely fine caliber (0.2μ), are called unsheathed axons, because most axons are enclosed in cellular sheaths that separate an axon from neighboring neuronal components. Axons that are enclosed by a single fold of a sheath cell are referred to as unmyelinated axons, and these also are usually extremely fine in caliber (Figure 8). Progressively larger axons in vertebrates tend to be ensheathed by increasingly numerous concentric wrappings of membranous processes of sheath cells, which become packed ultimately at intervals of 130 to 180 Å units.[15] The packed concentric folds of a sheath cell are referred to as the myelin sheath, and axons so ensheathed are called myelinated axons (Figure 9). Each sheath cell may have several membranous processes,[16] and their myelin tubes cover a portion of the axon that is relatively constant in length for axons of any specified caliber in any particular species. However, adjacent myelin segments on an axon are not quite in contact with each other, leaving a small unmyelinated gap, the node of Ranvier. At these interruptions of the myelin sheath, axon branches known as axon collaterals often arise at right angles. In the central nervous system, also, synaptic bulbs may occur as part of the structure of the unsheathed portion of the axon.[17] A variety of physiological properties are associated with axons of varying size and sheath characteristics. For example, the velocity of the nerve impulse is directly related to nerve fiber caliber, so that in general the highest conduction velocities are to be found in axons of greatest caliber and with the thickest myelin sheaths. A myelinated fiber of 20μ caliber may conduct at over 100 meters per second, whereas a 2μ fiber may conduct at only 10 meters per second.

The ultrastructure of axons is characterized by the absence of protein-synthesizing organelles, the ribosomes, and by the presence of a small number of mitochondria, reflecting relatively modest requirements of energy derived from oxidative metabolism. Extensions of the microtubular system of the cytoplasm are present and, as mentioned before, may be involved in the transport of materials synthesized only in the perikaryon, but needed throughout the axon and dendritic processes. Among those materials, essential enzymes, nitrogenous transmitter substances, and neurohormones first come to mind.

To summarize, the axon is electrically excitable and specialized for conduction of a self-propagating nervous impulse. It also serves the equally important function of transporting essential materials, synthesized in the perikaryon, to the transmitting terminals of sometimes distant axon telodendria.

TRANSMITTING PORTION The terminations of synaptic telodendria (Figure 5) are generally expanded or bulbous structures, but in special cases they may be club- or cup-shaped. Unmyelinated axons or their telodendria at times exhibit swellings along their course that make synaptic contacts with other neurons and that have structural and functional characteristics of synaptic bulbs. *The morphological characteristics of the transmitting portion are the most distinctive features of neurons.* At the fine structural level, transmitting synaptic bulbs reveal three characteristic morphological features, never seen in nonneuronal cells. This triad consists of an accumulation of microvesicles (synaptic vesicles) within the synaptic bulb, a specialization of the membranes at the synaptic interface to form sticky attachment plaques, an asymmetry of structure across the synaptic interface based upon the clustering of synaptic vesicles on the presynaptic side, and often a greater density of osmiophilic material on the postsynaptic side.[18]

One of the most significant findings at the electron-microscopic level, confirming earlier suggestions of qualitative differences among synaptic bulbs,[12] is the specific structural variation of synaptic vesicles, of attachment sites, and of mitochondrial concentration among synaptic bulbs of specific functional neuron groups. For example, differences in shape and size of synaptic vesicles can be used to identify the synaptic bulbs of inhibitory neurons as distinct from synaptic bulbs of excitatory function.[19–21] Synaptic bulbs of neurons associated with a specific transmitter substance, such as noradrenalin, are also characterized by special microvesicles of larger size and containing osmiophilic cores—those that can more easily be stained with osmium tetroxide.[22]

An important and common feature of the transmitting portion of a neuron is the way in which the discharge of the nerve impulse is distributed in a widespread manner according to the various branchings of the synaptic telo-

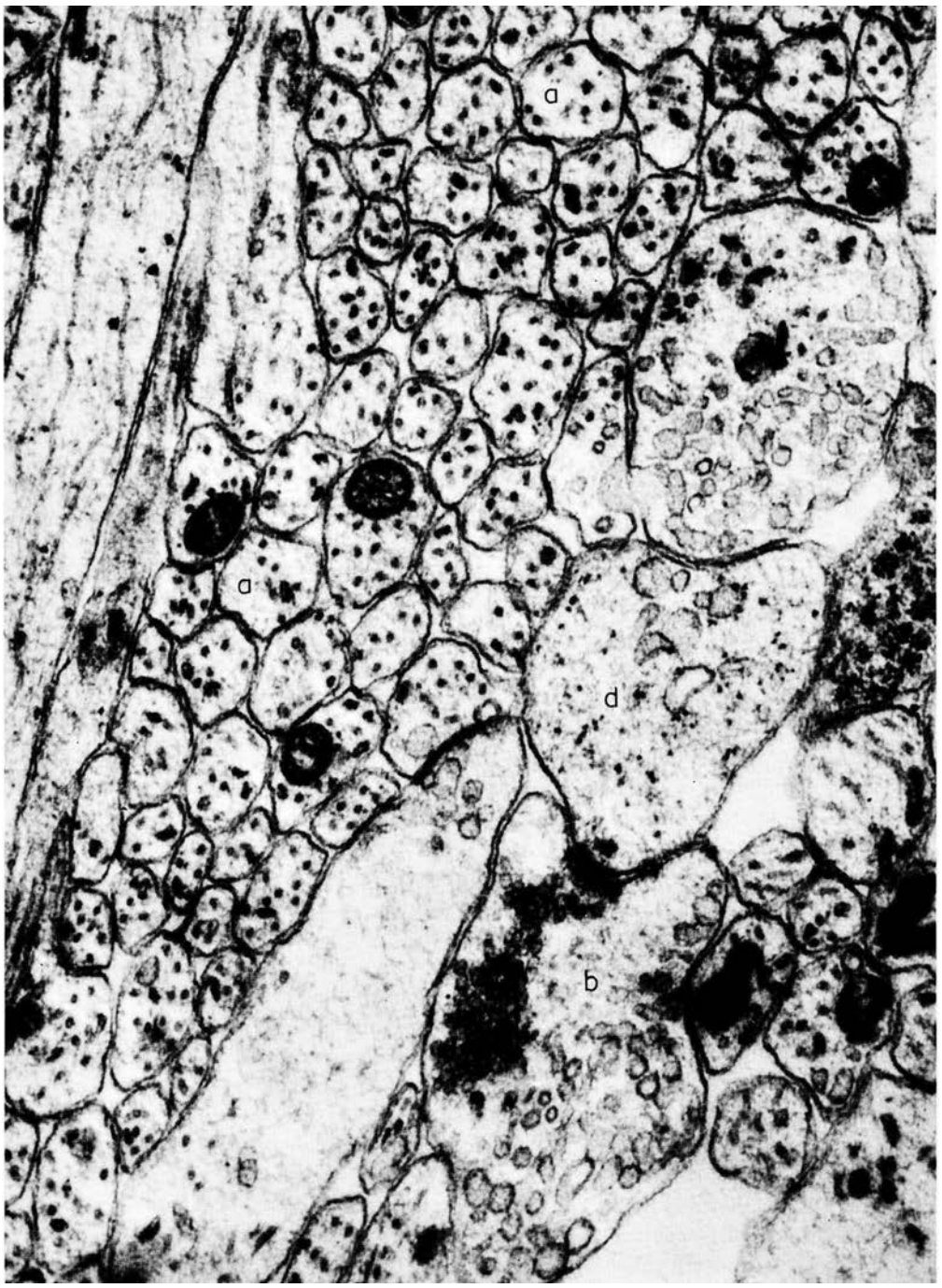


FIGURE 7 Electron micrograph of embryonic neuropil of the simian spinal cord, showing cross-sections of delicate, unsheathed axons (a), primitive dendrite (d), and primitive synaptic bulb (b). Most of these primitive axons are of the order of 0.2 micron in caliber, and will acquire sheaths as they enlarge. Reflex functions in this neuropil have begun (28 mm. stage). X54,000. (From Bodian, Note 27)

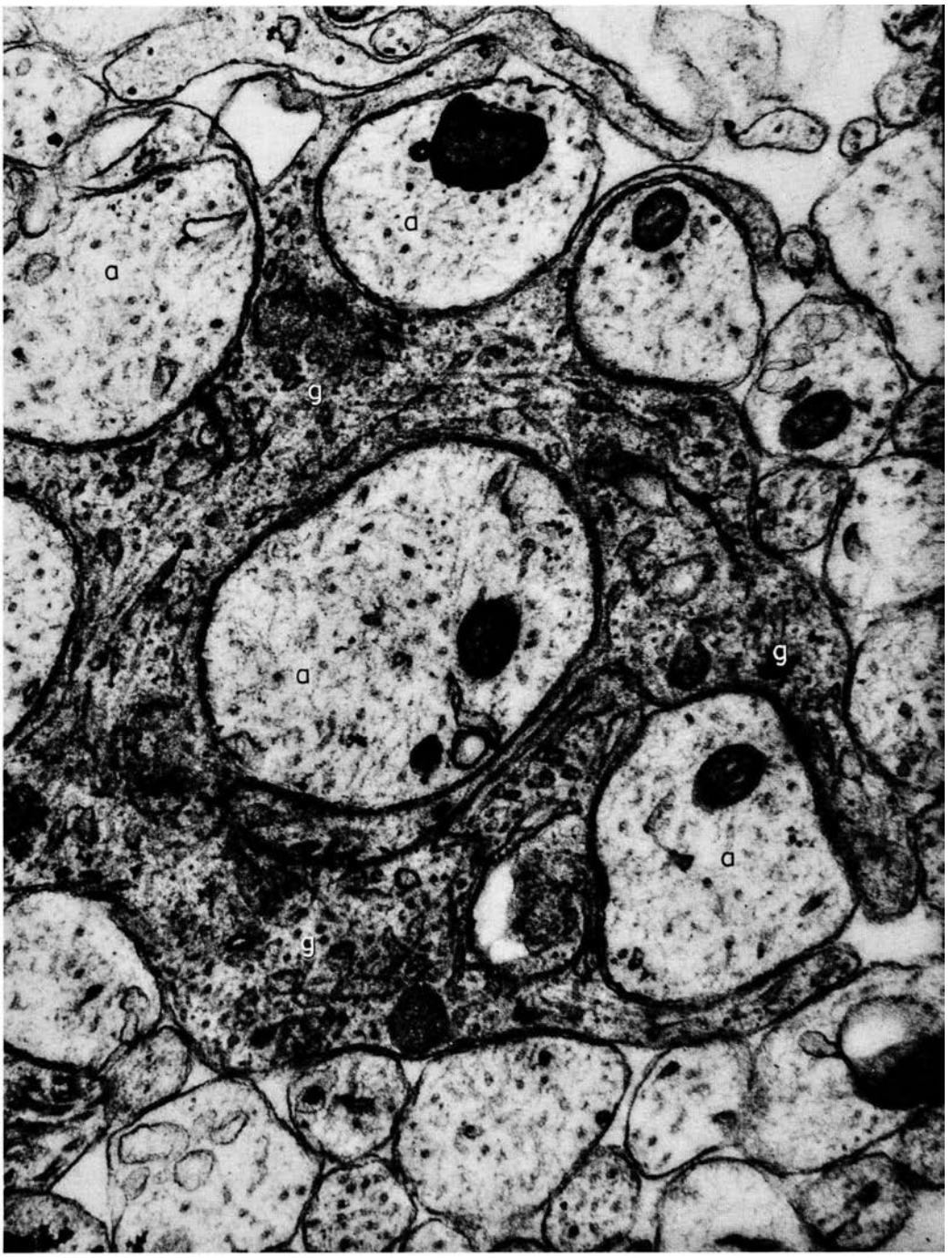


FIGURE 8 Early stage of sheathing of primitive axons in the neuropil of the simian spinal cord. Processes of a primitive neuroglial cell (g) envelope several primitive axons (a). 85 mm. embryo. X62,000.

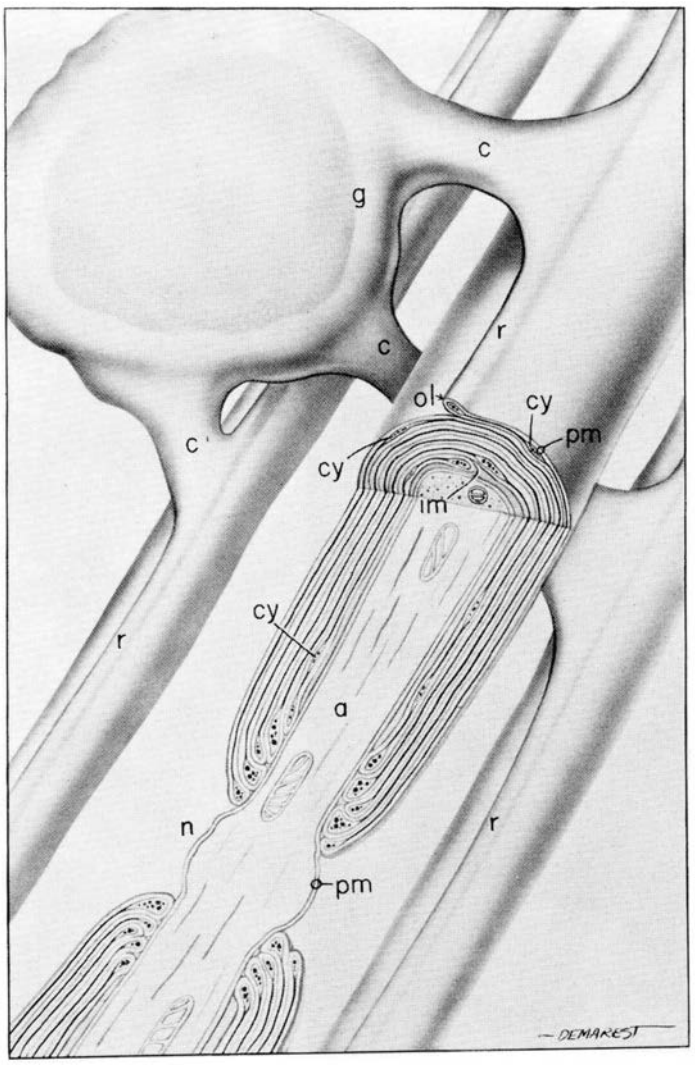


FIGURE 9 Diagram of myelinated axons, showing how processes (c) of a neuroglia cell (g) enwrap axons (a) by means of concentric membranous folds, which ultimately become packed at intervals of 130 to 180 Å units. (cy)—"trapped" cytoplasm of glial cell. (im)—inner mesaxon and inner loop of plasma membrane. (n)—bare portion of axon or node of Ranvier. (ol)—outer loop of plasma membrane. (pm)—plasma membrane. (r)—external ridge of myelin sheath. (From Bunge, et al., Note 16)

dendria, or so-called presynaptic elements. This divergence of output of a neuron and the breadth of receptive or postsynaptic surface, which makes possible the convergence of inputs from a variety of sources, is at the heart of the progressive increase in neural sophistication during evolution. The basic features of single neuron units are, however, the same throughout most of the animal kingdom.

For example, where refinement of control of muscle movement is required, as in finger movements, each motoneuron shows minimal branching of neuromuscular telodendria, so that only five or ten muscle fibers are controlled by one motoneuron. When massive contraction of a large muscle is involved, as in the gastrocnemius muscle, which produces extension of the foot and elevation of the whole body on the toes, a relatively small number of motoneurons, working in unison, control the function. Each motoneuron may here innervate more than 1000 muscle fibers. The same principle applies to the central nervous system, in which minimal arborization of axon telodendria may be associated with precisely defined channels of communication, whereas widespread arborization may be associated with the production of widespread effects by relatively few neuron units. Of course, the anatomical divergence expresses only ultimate capability, since conditions at the receptor, or postsynaptic, surface may sharply restrict the effective zone of a divergent telodendric arborization.

Functional groups of neurons and neuron circuits

We now understand in specific detail what the earliest students of nervous function apprehended only dimly—that segregation of functions is the essence of neural organization. The pattern of segregation, which is fixed for each species, takes the form of hierarchical levels of control, of functionally specific pools of neurons, or "centers," and of diffuse systems of neurons that interpenetrate with other systems to such an extent that their characterization lags far behind that of more circumscribed functional territories, such as the retina of vertebrates, for example. Nevertheless, it is within poorly localizable or "diffusely segregated" sets of neurons that some functions may reside, especially in the realm of higher-order functions. It is not too difficult to imagine that special engrammatic capability, beyond the capacity of neurons in general, may reside within a specific system of neurons with relatively diffuse localization.

Because of the basic integrative function of neurons, complex nervous systems, such as those of vertebrates, are arranged in a hierarchy of sets of neurons.[12] Segregated sets are often associated with a single functional role, such as control of eye movement. In Figure 10, each neuron represents a category of functional pools, including primary sensory pools, output or motoneuron pools, intermediate pools, and cortical, or highest level, pools.

In many functional pools there are large neurons mixed with small ones (Figure 1). The large ones send their axons outside the pool, are generally thought of as the major neuron component of the pool, and are referred to as Golgi Type 1 neurons. The small neurons have short axons, which ramify within the pool, and are known as internuncial neurons, interneurons, or Golgi Type II neurons. The motoneuron pool of the vertebrate spinal cord has been studied in great detail and exemplifies the way in

which the converging input to the motoneuron dendritic zone is derived both from extrinsic excitatory and inhibitory neurons and from interneurons that may intervene between the extrinsic input neuron and the output neuron of the motoneuron pool. Figure 11 shows a simplified diagram, and there is a variety of such interneuron relations in each macroneuron pool. In such settings, neuron interaction is subjected to modulating effects produced by the summation of a variety of excitatory and inhibitory inputs from extrinsic and intrinsic neurons.

In special cases, such as in the retina, cerebral cortex, and cerebellar cortex, a special feature of organization of neuron groups is encountered. Groups of neuron cell bodies and dendritic zones are arranged in layers, with input and output axons more or less perpendicular to the plane of lamination.[1,23] Without going into detail, one can note that this arrangement makes possible a high degree of organized lateral interaction of interneurons and of Type I neurons within single layers, as well as a mosaic arrangement of vertical units within the entire layered structure (Figures 12 and 13).

Within any system of neurons, the essential locus of neuron communication is the dendritic zone, where synaptic telodendria interweave with dendrites in a kind of feltwork known as the neuropil, often spaced at a certain distance from the cell bodies of the dendrites in question. The complexity of the neuropil can be fantastic, as shown in Figure 14, a photograph of a section through a motoneuron. Figure 14 shows the distribution of individual synaptic bulbs on the surface of a motoneuron in which shrinkage of the neuropil has made possible a clearer staining of the synaptic bulbs with silver salts. Even under the light microscope it is apparent that different functional centers show different characteristics of the neuropil or synaptic zone, but the electron microscope has revealed a whole new order of structural specialization, the key to all but the simplest of neural phenomena. In the motoneuron neuropil, for example, the key to the neuropil is not only the circuit made possible by the telodendria arriving at a motoneuron but, as established in electron micrographs, at least five distinct structural species of synaptic knobs, each with possibly distinct excitatory or inhibitory effects, finally terminate on the receptor surfaces of the motoneuron.[21]

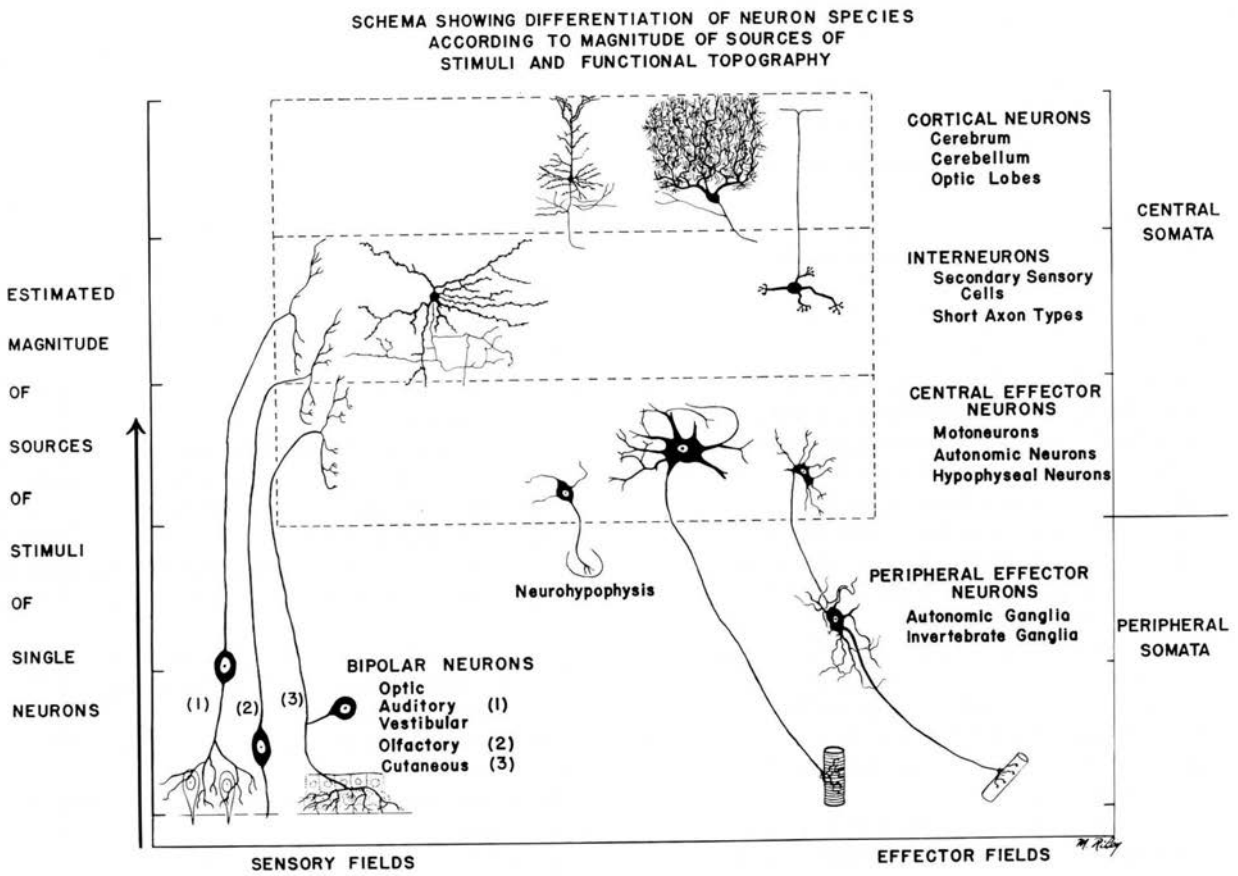


FIGURE 10 Major neuron types in the mammalian central nervous system, arranged according to general role, hierarchical level, and probable magnitude and diversity of sources of synaptic connections. (From Bodian, Note 12)

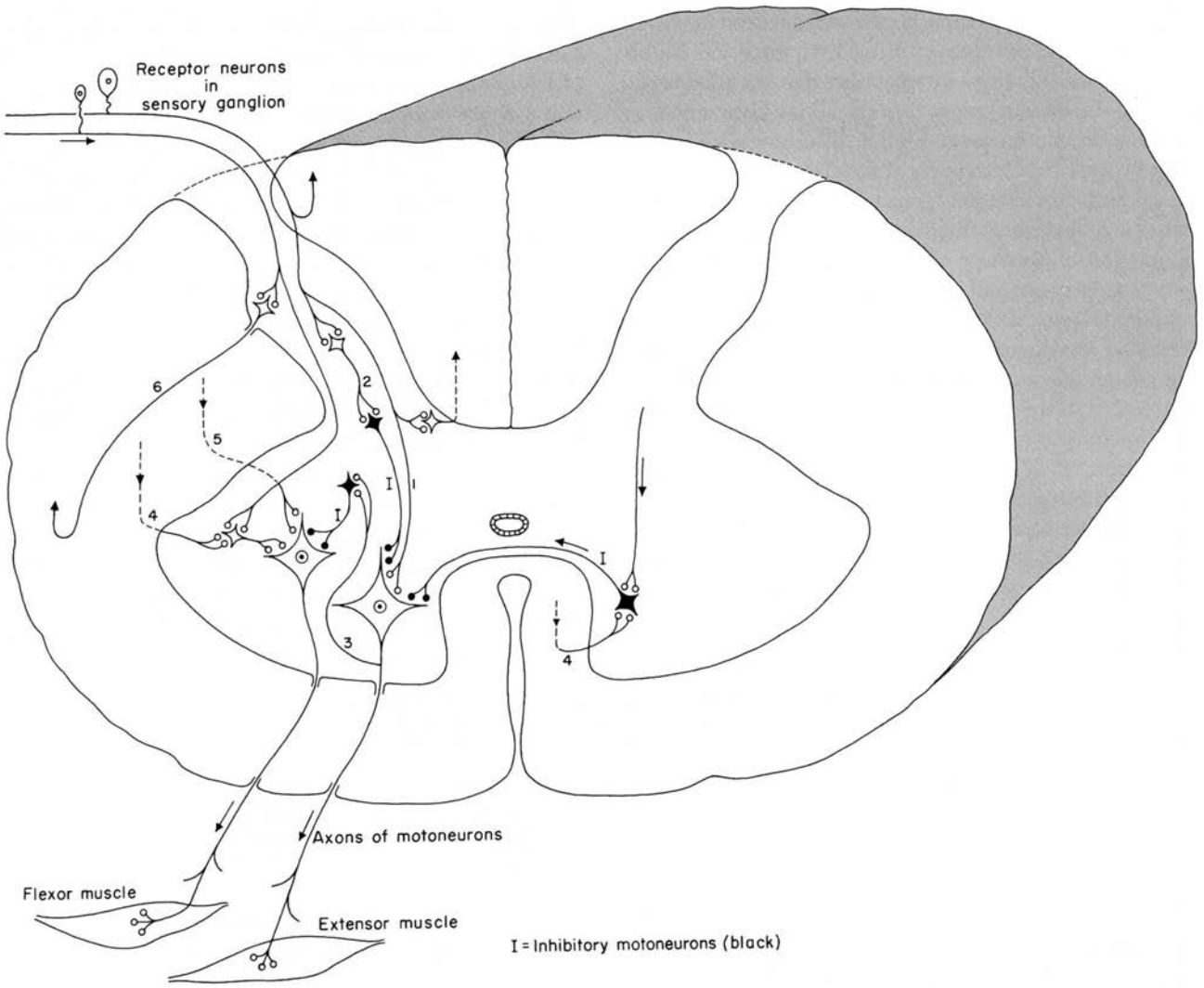


FIGURE 11 Diagram illustrating a few sample neuron circuits affecting the motoneuron pool, and exemplifying also the major aspects of neuron circuitry in general (divergence, convergence, delay paths, recurrent loops). A flexor motoneuron, representing a flexor motoneuron "pool," and an extensor motoneuron are shown, since these two pools have interrelated activity (contraction of extensor muscle is concurrent with inhibition of flexor muscle). Small neurons (interneurons) are part of the motoneuron pool or center, and intervene synaptically between receptor neurons and motoneurons, or between descending neurons from brain or upper spinal cord and motoneurons. Receptor neurons have *divergent* telodendria that make synapse with many neurons; motoneurons receive *convergent* synapses from many neurons.

1: Direct connection from muscle receptor neuron to extensor motoneuron, as in "stretch" reflex such as knee jerk. The "stretch" reflexes are the only known monosynaptic reflexes in vertebrates.

2: Delay pathway, involving two interneurons, and therefore a total of three synaptic delays. One of the interneurons (I) is inhibitory in its action on the motoneuron.

3: Recurrent loop from a motoneuron collateral, producing inhibitory effect on neighboring motoneuron through an inhibitory interneuron (I), in this case known as a Renshaw cell.

4: Crossed and uncrossed pathways through interneurons, entering the motoneuron pool from extrinsic sources, such as brain or distant levels of spinal cord.

5: Direct extrinsic inputs to motoneurons from cerebral cortex, in primates.

6: Indirect ascending pathway conveying coded receptor signals to brain.

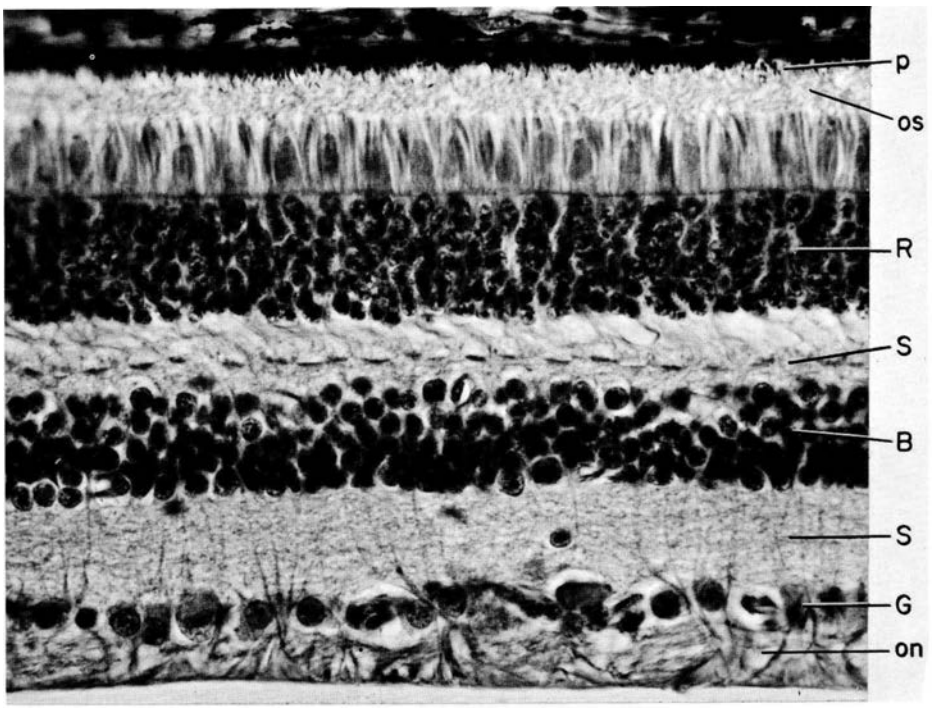


FIGURE 12 Light microscopic photograph of a thin section of the simian retina—an example of a laminated nervous structure with an unusual degree of segregation of neuronal cell bodies and synaptic neuropil. Cell bodies of receptor rod and cone cells (R), of interneurons (B), and of neurons with long axons of the optic nerve (G) are separated by zones of synaptic interaction (S). on—optic nerve fibers. os—outer segments of rods and cones. p—pigment layer. X450.

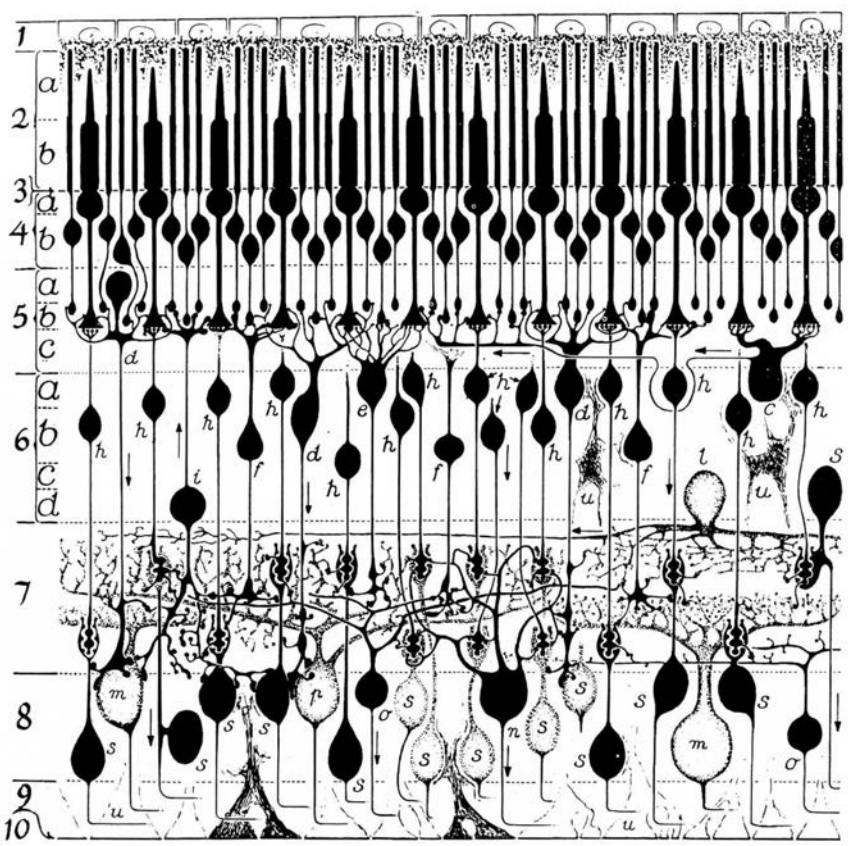


FIGURE 13 Diagram illustrating vertical arrangement of circuits from receptor to interneuron to efferent neurons. Within the synaptic layers (5 and 7) lateral interaction across the mosaic of vertically segregated fields can occur by way of special interneurons, the horizontal and amacrine cells. Only the efferent ganglion cells (layer 8) possess spike-conducting axons. Compare with Figure 12. (Courtesy of S. L. Polyak, The Vertebrate Visual System, University of Chicago Press, 1957)

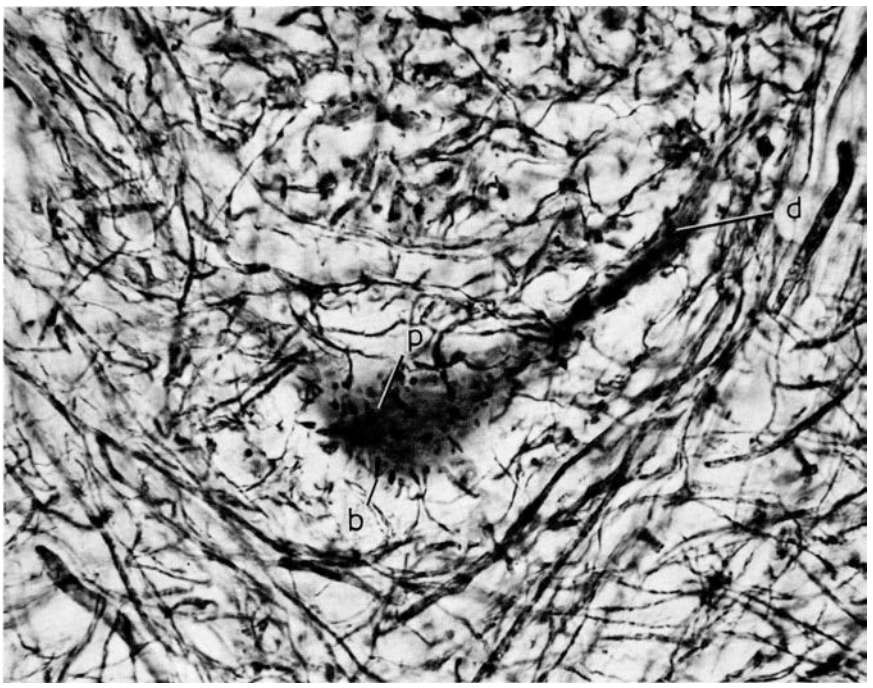


FIGURE 14A Cell body and two major dendrites of a motoneuron in the spinal cord of the cat. From the surrounding dense feltwork of axonic and dendritic branches, fine axonic telodendria pass to the receptive surface of cell body (p) and dendrites (d), and terminate as bulbous structures, the synaptic bulbs (b). Shrinkage of the neuropil away from the neuron surface makes possible the clear representation of the synaptic bulbs, and the latter appear to be less closely spaced because of the shrinkage. Although synaptic bulbs appear to be relatively homogeneous in such preparations, electron micrographs reveal at least five structural species of synaptic knobs, each with possibly different functional roles.[21] 15-micron section, Bodian silver stain. X750.

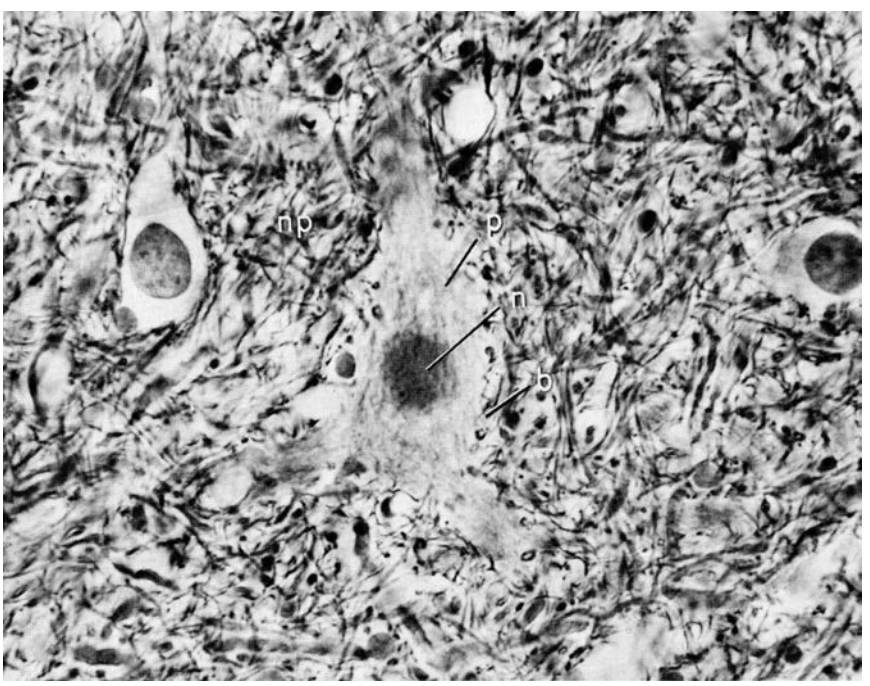


FIGURE 14B Region similar to that in 14a, but in monkey, in which shrinkage was avoided by superior preservation with glutaraldehyde. Synaptic bulbs (b) are obscured by the dense feltwork of very fine terminal axon arborizations which closely surround the motoneurons. p—perikaryon. n—nucleus. np—neuropil. 15-micron section, Bodian silver stain. X750.

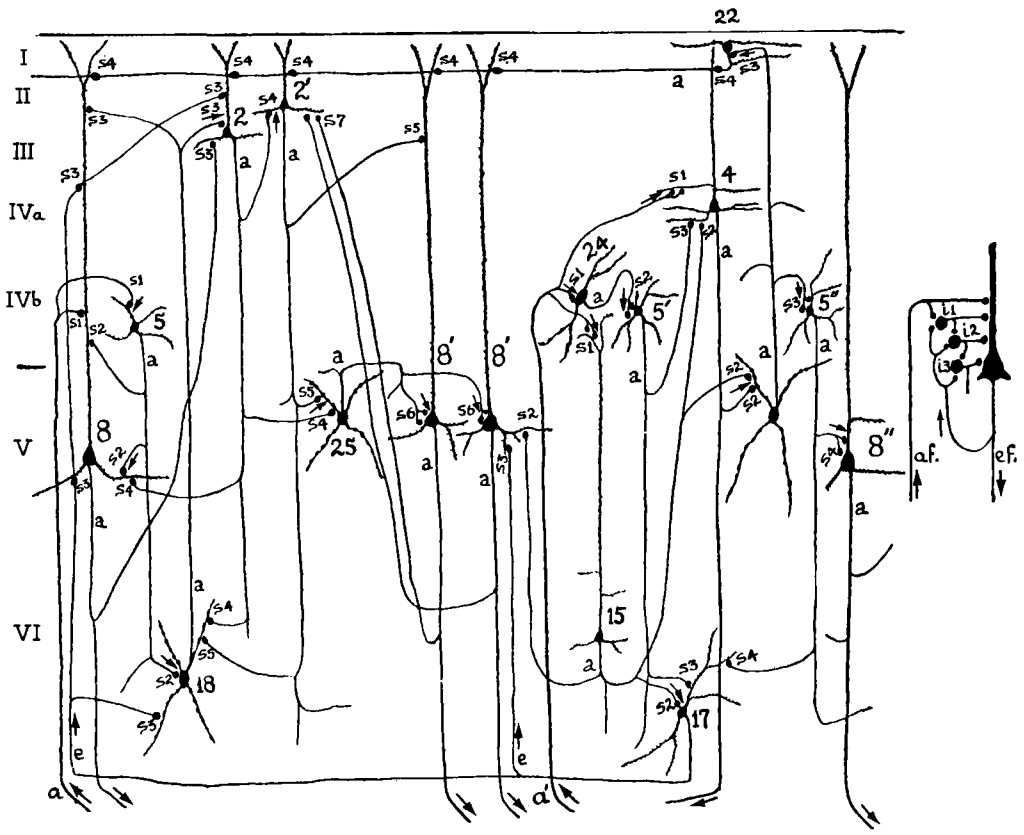


FIGURE 15 Diagram of some neuronal circuits in the cerebral cortex, according to Lorente de Nó.[24] Illustrated are convergence of input to receptor portions of neurons, dispersal of output of neurons by way of arborized axon collaterals and synaptic telodendria, recurrent or feedback circuits via axon branches to interneurons—both inhibitory and excitatory—and multiple pathways permitting both high speed and slower routes of transmission. According to Lorente de Nó, "This diagram exemplifies the broad plan upon which the central nervous system is organized."

The complexity of form and function that may exist in a single neuron, or in a functional pool of neurons, becomes trivial as one contemplates the patterns of connectivity in secondary sensory and motor centers, and especially in higher integrating centers, such as the cerebral cortex. Here, the laminar structure makes possible the overlapping of dendritic zones of successive tiers of vertically oriented or pyramidal neurons. The ingredients of complexity as shown in Figure 15, Lorente de Nó's diagram of some intracortical circuits, are the elements mentioned before: convergence of input to receptor portions of neurons, dispersal of output of neurons by way of arborized axon collaterals and synaptic telodendria, recurrent or feedback circuits via axon branches to interneurons —both inhibitory and excitatory—and multiple pathways permitting both high speed and slower routes of transmission.[24]

Of course, one must keep in mind that the diagram shows only the basic neuron types, and only about one per cent of the neurons in the plane of the diagram. That is because, for some unknown reason, the Golgi stain used affects only about that percentage of neurons in any given preparation. The complexity must also be measured by the existence of an estimated ten billions of neurons in the human cerebral cortex, each receiving patterned bits of information through several hundred to several thousand synaptic bulbs. Within the framework of complexity, there is evidence that a high degree of specificity still exists. Nevertheless, because the margin of safety, as well as complexity, increases as the number of neurons increases, destruction of a larger number and possibly a larger proportion of neurons in the cerebral cortex is required before a significant functional deficit appears. Rerouting by alternative pathways after injury is also more likely in the cerebral cortex and could explain relearning. Temporary interruption of circuits, by whatever means, could also produce switching from preferential to alternative lines of communication. There has been little tendency to postu-

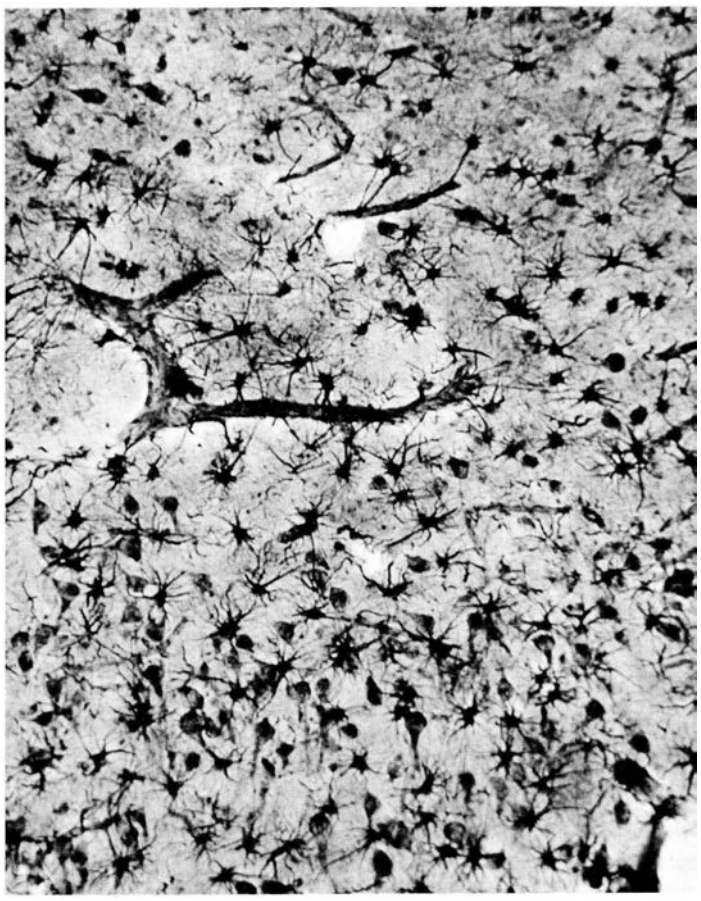


Figure 16 Photomicrograph of a section stained to reveal the complete outlines of astrocytes, or "skeletal," neuroglia. By means of overlapping foot-like terminals of radially arranged processes, the astrocytes form a complete membrane that covers the exterior surfaces of the central nervous system and the blood vessels that penetrate from the outer surface. Cajal gold-sublimate method. X150.

late anatomical plasticity and reorganization, because evidence for anatomical restitution of circuits, except by regrowth of axons outside the nervous system, is meager in adult or even immature mammals.

Neuroglia

The embryological development of neurons within a mass of closely packed cells, or epithelium, results in a rapidly increasing complexity in the geometry of neurons and their processes, according to a predetermined pattern. A large proportion of cells within the neural epithelium do not, however, develop as nerve cells but do develop complex geometries that result from their occupancy of space between neurons. The word neuroglia connotes the first conception of the neuroglia cells as the "neural glue" that separates and holds the neuron population together.[25] In vertebrates,two major types of neuroglia cells—the astrocytes and oligodendrocytes—can be clearly defined by means of a variety of light and electron-microscopic criteria.[26]

The major role in preserving the integrity of nervous tissue, even after nerve cells within it have been caused to degenerate, is filled with the so-called skeletal neuroglia, which contain an intracellular fibrillar skeleton. These cells form a complete membrane that covers the exterior surfaces and the blood vessels that penetrate into the central nervous system from the outer surface (Figure 16). Ultimately,the extensions of these fibrous neuroglia cells interweave among all other cellular components, so that neurons and their component parts, for example, are largely separated from each other by neuroglial processes. An important exception is the region of synapse, where synaptic telodendria make contact with receptive postsynaptic dendrites. Neuroglia never intervene between presynaptic and postsynaptic components of the synapse (despite a speculation by Ramón y Cajal that sleep was caused by such an interpositioning), but in certain regions they may circumscribe a synaptic zone containing a number of synaptic elements. They thus appear to insulate a functional synaptic zone from adjacent regions. Yet, onset of reflex behavior in monkey embryos occurs prior to differentiation of neuroglia or axon sheathing, so that synaptic function is not dependent on neuroglial function in at least formative functional stages.[27] However, the structural and insulating role of fibrous neuroglia cannot be separated from their

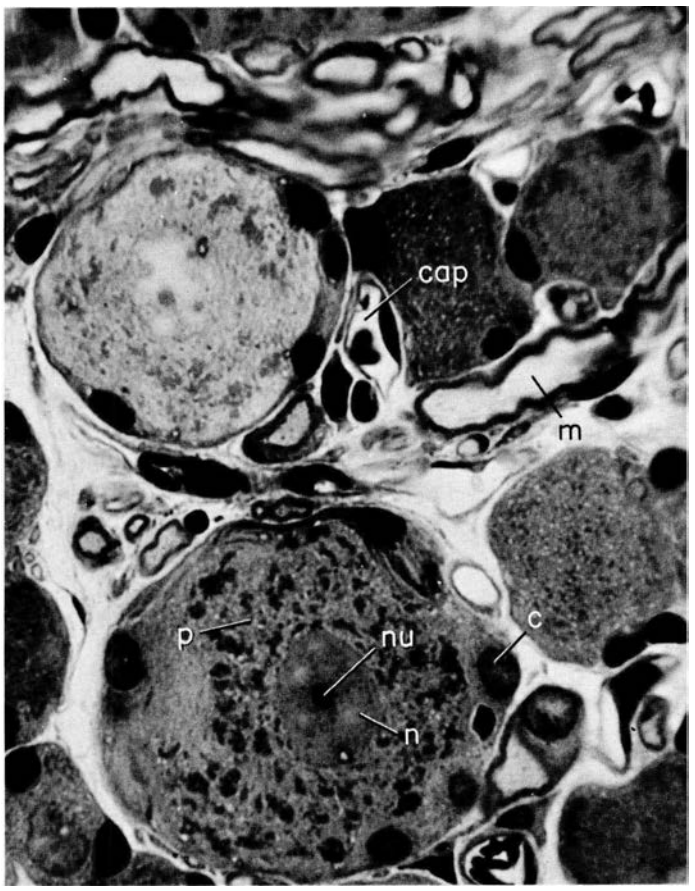


FIGURE 17 Section of spinal ganglion of monkey, showing a perikaryon (p) of a sensory neuron completely invested by capsule neuroglia cells (c). The exceedingly intimate apposition is characterized in electron micrographs by interlocking processes of the plasma membranes of neuron and neuroglia cells. Intimate juxtapositions of neuron and neuroglia are found primarily in non-synaptic areas. m—myelinated axon. cap—capillary blood vessel. nu—nucleolus. n—nucleus. Two-micron plastic section. Toluidin blue stain. X1000.

role in the transport of gas, water, and electrolytes from blood vessels to cells within the nervous tissue, and from their probable metabolic coupling with certain metabolic pathways of adjoining nerve cells. Microscopic data do not reveal the existence of a class of cells intermediate between neurons and neuroglia, although anaxonic neurons have been mistaken for such an intermediate class. Their fine structure, however, indicates their basic neuronal character.[28]

The second major class of neuroglia consists of cells that lack cytoplasmic fibrous bundles, but are abundantly equipped with cytoplasmic ribosomes and therefore are presumably involved in a high level of protein synthesis. It has been known for many years that these cells, the oligodendrocytes, are in intimate apposition with neuron cell bodies in the vertebrate nervous system, but the electron microscope has revealed a more general role—the formation of the sheaths of central nerve fibers, previously described. The rich content of ribonucleoprotein in such cells, and in the related Schwann cells in peripheral nerves, gives them a characteristic dark appearance in electron micrographs. Myelin-forming neuroglia appear to be specially sensitive to a variety of pathological conditions, including a variety of hereditary defects, so that many types of neurological diseases are characterized by degenerative changes of the myelin sheath—the so-called demyelinating diseases, such as multiple sclerosis. The unusual intimacy of the association of oligodendrocytes and Schwann cells to perikaryon and axons has in addition raised the suspicion that these neuroglia might develop a symbiotic relationship with their associated neurons in some vertebrate systems. For example, this intimacy is especially close in sensory ganglia (Figure 17). Dependency of each on the other for survival could be part of an as yet unknown, but important, functional relationship, but it is interesting that the greatest intimacy is found on sensory neuron perikarya, far from the receptor or transmitting parts of the neuron. It is also of interest that the oligodendrocytes in tissue culture can be identified by a characteristic, slow pulsatile activity, also seen in Schwann cells.[29]

A third type of neuroglia, the so-called microglia, occur irregularly in different parts of the central nervous system of various vertebrates and as yet have not been linked with any basic aspect of the normal functioning of neurons. They appear to invade the nervous system early in em-

bryonic development, along with primitive blood vessels, are motile in tissue culture,[29] and are thought to be active primarily in damaged nervous tissue as scavenger cells similar to those which enter tissues from the bloodstream in the adult.

Summary

Neurons are cells designed and organized to influence the probability of nerve impulse discharge in other neurons. The direct means of accomplishing this resides in special membrane properties and geometric design. The indirect means reside in the metabolism of the internal or cytoplasmic organelles, which are similar to those of many other secretory cells, except for the nature of the secretory product. Separating neurons from each other, except at critical contact points, are other specialized cells, the neuroglia, which play a structural supporting role, form insulating sheaths on nerve fibers, contribute to the ionic environment through electrolyte and water exchange, and probably have a symbiotic relationship of an undefined nature with at least some neurons.

The complexity of neuron populations is achieved by numbers, by qualitative diversity of component parts, and by geometric diversity. Segregation of function is combined with complex structural-functional modes of neuron interaction, involving inhibitory as well as excitatory neurons. While behavior, the focus of organic evolution, can be studied without respect to the neuron, one can hardly anticipate a satisfying scientific theory of behavior that does not encompass the neuronal level of organization. New and important structural-functional data at this level are emerging at an accelerated rate because of the recent advent of electron microscopy and microelectrode recordings of single neurons.

Principles of Cellular Organization In the Nervous System

SANFORD L. PALAY

ALTHOUGH THE CELLULAR COMPOSITION of the nervous system has long been recognized, the idea that it consists almost exclusively of cells with very little interstitial substance still surprises many investigators, including some neuroanatomists. It was Franz Nissl[1] who first understood that this concept was a necessary consequence of Cajal's Neuron Doctrine,[2] and he argued zealously against it. For a generation *das Nisslsche Grau* remained an obstacle to the final acceptance of the Neuron Doctrine, which required intimate contact between nerve cells at specialized junctions, termed synapses, as opposed to wide zones of interstitial substance in which the nerve cells and their processes were independently suspended and across which communication between nerve cells took place. In a different form, the argument continues today in discussions about the volume of the extracellular space in brain.

Nissl's arguments and the surprise of modern students are conditioned by the deficiencies of histological techniques at the light microscope level. All of these methods are selective. They display the cell bodies of neurons or their processes, or they stain a small proportion of the total number of cells; they reveal either the neurons or the neuroglial cells, rarely both together. In their selectivity lies their great merit, for the complexity of the nervous system does not permit one to visualize its constituent cells in the thick sections of light microscopy when all the elements are stained completely in a single section. It remained for electron microscopy, with its thin sections and its superior standards of tissue preservation, to demonstrate that between the nerve cells lie multitudes of terminal neuronal and neuroglial processes, all in intimate juxtaposition and filling the interstitial spaces of light microscopy almost completely. Overnight *das Nisslsche Grau* vanished. Its place was taken by the neuropil, an intricate

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tangle of axons, dendrites, and neuroglial processes, which had already been explored by Cajal and Herrick,[3] to name only two of the major investigators. The electron microscope disclosed what had been suggested much earlier by adherents of the Neuron Doctrine—that the neuropil is an elaborate and complicated fabric of neuronal processes in synaptic contact with one another. The unraveling of this fabric, the tracing of pathways through it, remains today the most compelling task of neuroanatomists, and the advent of satisfactory electron microscopic methods for dealing with this problem adds to its complexity as well as contributing to its solution.

Now, when we say that the nervous system consists almost exclusively of cells, we are really describing an epithelium. It is worth while pausing here to consider the concrete significance of this remark. An epithelium is essentially a sheet of cells of similar type and embryological origin that borders upon a lumen and rests upon a basal lamina, which separates it from the underlying connective tissue. The nervous system derives, both phylogenetically and embryologically, from the superficial epithelium of the organism. It contains a system of interconnected fluid-filled cavities, the ventricles of the brain and the central canal of the spinal cord, which derive from the lumen of the embryonic neural tube and which, at early stages, are continuous with the external environment. Finally, it is everywhere enclosed by a basal lamina, which effectively demarcates it from the other tissues surrounding it. As this neuroepithelium steadily becomes thicker during development, blood vessels penetrate into it, but they are always clearly labeled as invaders by their ensheathing connective tissue and the uninterrupted basal lamina. Even the smallest capillaries are always separated from the neural tissue proper by a basal lamina.[4]

In all epithelia the constituent cells are in immediate apposition to one another, like the pieces in a mosaic. The interstices between cells are only 200 to 400 Å deep and are continuous with one another as well as with the extracellular connective tissue space and the lumen. There are, however, junctional regions where the apposed surfaces of neighboring cells are modified or specialized to form a series of devices that are believed to serve the purpose of attachment.[5,6] There are three kinds of such devices, and usually they occur in series along the zone of attachment. The simplest is the tight junction, or *zonula occludens*. This is usually found close to the lumen at the junction of neighboring cells. The specialized region can be punctate or plaque-like or, most commonly, shaped like a ribbon running round the circumference of each cell. In this structure the two apposed surface membranes become straight and parallel, and their outermost leaflets appear to fuse together, obliterating the intercellular space and thus sealing off the lumen from the rest of the intercellular space. Although the completeness of this seal is still debatable, it has been proposed that the tight junction is a zone of decreased electrical resistance where ions and perhaps even larger particles might pass laterally from cell to cell.[7-11]

The most complex attachment device is the desmosome, or *macula adhaerens*. This surface specialization is disk-shaped and can be repeated several times around the perimeter of each cell. The interstitial space in these spots usually widens to 400 or 500 Å, and interstitial material condenses midway between the apposed surface membranes to form a plate bisecting the space. A highly ordered system of cytoplasmic filaments is arranged symmetrically on the cytoplasmic sides of the two plasmalemmas.

The third common device, the *zonula adhaerens*, resembles the last but is less complex. In simple columnar epithelia it occurs between the *zonula occludens* and the *macula adhaerens* along the lateral surfaces of neighboring cells. Like the *zonula occludens*, it can be shaped like a ribbon running round the perimeter of the cell. The interspace is widened only slightly, if at all, and a dense plate of interstitial material lies midway between the two plasmalemmas. Fluffy, dense patches of fine filaments are symmetrically arranged on the cytoplasmic sides of the membranes.

In the mammalian nervous system only two of these devices are found. The desmosome, or *macula adhaerens*, does not occur at all, and the *zonula occludens* and the *zonula adhaerens* occur in precisely specified places either together or separately. For example, both appear in series between neighboring ependymal cells lining the ventricles of the brain.[12] But in most other places they are found separately. Two examples may be cited: in the central nervous system the small, punctate *zonulae occludentes* between neighboring astroglial cells,[13] and in the peripheral nervous system the extensive *zonula occludens* between successive overlapping layers of the Schwann cell that compose the myelin sheath.[14] The *zonula adhaerens* is usually reduced in extent to a small spot, which, in keeping with the Latin terminology of these structures introduced by Farquhar and Palade,[6] I shall call the *punctum adhaerens*. These small attachment points occur widely throughout the central nervous system—for example, between neighboring dendrites,[15] between dendrites and cell bodies of neurons, between axons, and between terminals and dendrites or cell bodies at synaptic junctions.[16]

In most epithelia, the constituent cells all have a relatively simple shape—columnar, cuboidal, or squamous—corresponding to the polarity of the epithelium between the basement lamina and the lumen. The cells, more or

less alike and bound together by the junctional complexes just described, carry out similar functions, each unit repeating what its neighbors do with the monotonous regularity required by the function of the epithelium as an organ. All of the cells receive similar stimuli brought to them by the external environment, the blood, and the nerves. Communication between cells laterally is possible by means of the low resistance pathways represented by the *zonulae occludentes,* but this route does not appear to be polarized, and influences can pass from one cell to another in either direction.[9,10] When, however, we compare ordinary epithelia with neuroepithelium, we see that in the latter the cells have bizarre multipolar shapes and that they are oriented, not with respect to the axis between central lumen and basement lamina, but rather with respect to specific attachments to other cells, i.e., to the pattern of connectivity between cells in the epithelium. The junctional complexes between cells are adapted to additional functions, not merely to sticking neighbors together, but also to transferring activity functions from one cell to another. And finally, the junctions are polarized; consequently, the cells can transmit influences from one cell to another at specified points and in one direction only. Thus the cells of the neuroepithelium are organized into a communication system with each unit performing as a small computer that receives inputs, integrates them, and responds according to its immediate and remote history. The specialized sites where nerve cells come into functional contact with one another are termed synapses. As Gray and Guillery[17] have recently published a comprehensive review of synaptic morphology, this chapter will be confined to general considerations of definitions and correlations between function and structure.

Morphological recognition of the synapse depends upon the classic studies of Ramón y Cajal,[2,18] Held,[19,20] Auerbach,[21] Bartelmez and Hoerr,[22] and Bodian.[23,24] By describing nerve endings and their characteristic inclusions, they provided the criteria for identifying the tips of axons wherever they contact another nerve cell. In preparations for the optical microscope it can be seen that the preterminal portion of an axon expands into a button-like or bulbous ending containing mitochondria that are often arrayed in a palisade along the junctional interface. The details of the junction itself cannot be clearly made out in the optical microscope. But the higher resolution of the electron microscope and the more satisfactory preservation of fine structure in preparations for the electron microscope allow us to characterize the synapse in more detail than it was possible to do before.

Remembering the epithelial nature of the nervous system as discussed above, we are not surprised to see that the junction is, in most instances, a simple apposition of two cell membranes with an interstitial space, the synaptic cleft, continuous with that found elsewhere between cells.[17,25,26] This zone of apposition bears certain resemblances to the *zonula adhaerens* of other epithelia. First, the plasmalemma on one or both sides is furnished with tufts of fine cytoplasmic filaments, and the cleft itself is usually occupied by vague filamentous densities that may form a thin, dark plate midway between the apposed membranes. Second, the two apposed surfaces are strongly adherent, as is demonstrated by the fact that when nerve endings are separated from the rest of brain tissue by mechanical homogenization and differential centrifugation, the endings frequently bear an attached fragment of the postsynaptic membrane that is identifiable by means of its characteristic densities.[27]

It must be pointed out, however, that this structure is not precisely comparable to the *zonula adhaerens.* The cleft between the cells is widened from the usual 100–200 Å to 300–400 Å, and the cytoplasmic densities attached to the junctional membranes are typically disposed in an asymmetrical fashion. The dense filamentous material is usually thicker and more prominent on the postsynaptic side of the junction than on the presynaptic side, and in certain instances one or two additional layers of dense material lie beneath it, forming the so-called subsynaptic organelle.[28,29] Gray[30] has described small, tufted densities that lie in a hexagonal array on the cytoplasmic face of the presynaptic membrane. These asymmetrical and discontinuous densities strengthen the suggestion that the synapse lies at a modified *zonula adhaerens.*

When an extensive survey of synaptic morphology is made, it turns out that the junctional complex described in the previous paragraph is only one of a large number of variations on the structure of the *zonula adhaerens.* Gray[31] has attempted to divide synapses into two classes according to the appearance of the junctional complex. In Type I synapses, as just described, the synaptic cleft is a widened interstitial space and the postsynaptic density is a broad and continuous band, whereas the presynaptic density is narrow and may be discontinuous. In Type II synapses the synaptic cleft has the depth of the usual interstitial space and the pre- and postsynaptic densities are both thin, symmetrical, and often discontinuous. Type I synapses typically occur upon dendrites and dendritic spines, whereas Type II synapses occur on dendritic trunks and on the surfaces of perikarya. Early attempts to correlate these two types with function suggested that Type I synapses are excitatory and Type II synapses are inhibitory.[32-34] But a detailed comparison of junctional complexes in a wide variety of synapses shows that the situation is much more complicated than it appeared at first.

In the first place, although Type I and II synapses are

extremely common throughout the central nervous system and can be identified or recognized without any difficulty, there are numerous synapses that appear to be intermediate between the two, and others that are in some respects different from either. Small terminals have been encountered with two junctional interfaces, one upon a nerve cell body resembling Type II, and another upon a dendrite resembling Type I. Some of the larger terminals have junctional zones that fit the description of Type I and others that fit the description of Type II, both on the same postsynaptic dendrite.[16] There are, in addition, complex synapses in which a Type II junction is associated with numerous *puncta adhaerentia.* And, finally, there are large endings having a broad interface with a single postsynaptic surface characterized by a series of junctions, some Type I, some Type II, and some close junctions, or even tight junctions (Sotelo and Palay, unpublished observations). Variations such as these—and probably many more will be discovered as more areas in the central nervous system are investigated—make it difficult to construct a classification that includes all patterns.

In the second place, the attempt to correlate functional characteristics of a synapse with junctional fine structure met with failure as soon as it could be put to a test. The hypothesis was an optimistic one. Type I synapses should be excitatory, because they occur on dendritic spines and dendrites where electrical stimulation of afferents produces excitatory postsynaptic potentials and, under appropriate conditions, elicits firing of the postsynaptic axon. Type II synapses should be inhibitory, because they occur on cell bodies where stimulation of afferents produces inhibitory postsynaptic potentials and, under certain conditions, reduces the firing rate of the postsynaptic axon or silences it altogether. Where both morphological and electrophysiological tests can be performed on the same object, as on the hippocampal pyramidal cells[34,35] and the cerebellar Purkinje cells,[15,36] the correlation is excellent between Type I synapses and excitation, Type II synapses and inhibition. For example, in the cerebellar cortex the junctions between the parallel fibers and the Purkinje cell dendritic spines are always Type I and excitatory,[15,36] whereas the junctions between the basket fibers and the Purkinje cell body are always Type II and inhibitory.[32-34,36,37] It was soon discovered, however, that although the junctions between the climbing fiber and the Purkinje cell main stem dendrites are powerfully excitatory,[38] they are always Type II. This experience should make us cautious in drawing conclusions about the correlation between fine structure and function when we have, as yet, few samples on which to base interpretation.

One of the most important functional characteristics of the synapse is that it is polarized. Electrical activity in the preterminal fiber is transmitted across the synaptic cleft to the postsynaptic membrane by means of a chemical mediator released from the presynaptic ending. In the vertebrate central nervous system this mediator is in the vast majority of synapses an unidentified chemical, but it is assumed that it is a small molecule like acetylcholine or norepinephrine, which are known to be chemical transmitters in the peripheral nervous system, in autonomic ganglia, and at neuromuscular junctions. In only one instance in the central nervous system is the transmitter chemical known, and this is the cholinergic junction between the recurrent collateral of ventral horn cells and the Renshaw cells in the spinal cord. Carefully executed experiments by Katz and his collaborators[39,40] have shown, both in the neuromuscular junction and in the spinal cord, that the postsynaptic membrane behaves as if the transmitter impinged upon it in focalized bursts of several thousand molecules. The strong evidence for quantal release has given rise to the hypothesis that the transmitter is prepackaged in the nerve terminal in units of a small number of molecules waiting for release upon the appearance of the nerve impulse at the synaptic junction. At the same time as this evidence was accumulating, electron microscopists[25,26,41] discovered that presynaptic nerve terminals contain numerous small vesicles, often clustered against the presynaptic membrane at the location of the cytoplasmic densities discussed in the previous paragraphs. It was natural to suggest that these synaptic vesicles contain the quanta of transmitter required by the physiological evidence. Subsequent biochemical studies on synaptic vesicles separated by differential centrifugation have confirmed this suggestion.[42,43]

Synaptic vesicles, like chocolates, come in a variety of shapes and sizes, and are stuffed with different kinds of fillings. The most common synaptic vesicle is about 200 to 400 Å in diameter, is roughly spherical, and has a clear center. Such vesicles are found in axon terminals at neuromuscular junctions and in other known cholinergic endings, as well as in endings of undetermined function. Richardson[44] has provided the best morphological evidence that cholinergic endings contain this type of vesicle, and both Whittaker[43] and De Robertis[42] agree that acetylcholine is bound to these clear vesicles in fractions obtained from homogenates of brain. In terminals of autonomic nerve fibers in the intestine, vas deferens, and pineal body, a slightly larger vesicle is found containing a dense particle about 280 Å in diameter.[45-47] The particle is usually spheroidal, but it can also be rod-shaped, about 130 by 340 Å. Vesicles of this type have become associated with the transmitter norepinephrine because they occur in known adrenergic nerve fibers and because radioautography has shown that such endings take up and

localize exogenous norepinephrine labeled with tritium.[47] Combined morphological and biochemical studies on adrenergic nerve fibers have shown a good correlation between the number of synaptic vesicles containing a small dense granule and the quantity of catecholamine in the fibers under conditions of experimental depletion and forced uptake of exogenous norepinephrine.[48] Up to the present time, this type of vesicle has not been found in the central nervous system.

A third class of vesicle, larger than either of the two just described, appears in terminals in all parts of the central and peripheral nervous system. The vesicle itself is about 800 to 900 Å in diameter and contains a dense spheroidal droplet about 500 Å in diameter. This vesicle is the most puzzling of all. It occurs in large numbers in the presynaptic terminals of autonomic ganglia and in neuromuscular junctions in smooth muscle,[44-46] in the hypothalamus, and in small numbers in terminals on the cells of the various nuclei in the brain stem. It appears in the cerebellar cortex and in the basal ganglia. Numerous attempts have been made to correlate it with the distribution of catecholamines. The strange feature of this vesicle is that it occurs in company with the small, clear vesicle, often in very small numbers and usually located away from the presynaptic membrane. If each vesicle is the morphological counterpart of a specific transmitter chemical, the mixture of two classes of vesicle in a single ending suggests that more than one kind of transmitter can be stored and released at an ending. Although this suggestion has been made before on physiological grounds, it is not generally considered acceptable.

Finally, there is the class of vesicle that is characteristic of neurosecretory nerve endings in the neurohypophysis.[49] These are about 1200 to 1500 Å in diameter and contain a large, dense droplet that fills the vesicle almost entirely. They occur only in the perikarya, processes, and terminals of the cells belonging to the supraoptic and paraventricular nuclei. The droplets contain vasopressin and oxytocin, the polypeptide hormones produced by these cells, and they are depleted, like the hormones, by such stimuli as dehydration and suckling. It is interesting, in view of the comments made above concerning the 800 to 900 Å vesicles of the third class, that these neurosecretory vesicles share the terminals with swarms of small, clear vesicles of the first class. In this case the role of the larger vesicles seems evident, but the function of the small vesicles is puzzling.

Recently a number of authors have drawn attention to variations in the size and shape of the smaller vesicles (class I above). Some terminals, for example in the ventral cochlear nucleus, have vesicles of 400 Å, whereas others have vesicles of 450 Å diameter.[50] Some terminals, for example in the ventral horn of the spinal cord, have spherical vesicles, whereas others have elongated or discoid vesicles.[51-56] The authors have tried to correlate these types with the source of the afferent nerve fibers containing one or the other type of vesicle or with the physiological action, inhibitory or excitatory, of the terminals containing them. It must be pointed out that these differences in size and shape of the small vesicles did not become evident until glutaraldehyde came into general use as a fixative for the nervous system. Although this fixative has many merits, it should not lead us unhesitatingly to accept as valid small differences in size and shape, especially when accompanied by signs of inadequate preservation, such as swollen mitochondria, interrupted surface membranes, or enlarged intercellular spaces. In these interpretations it is assumed that the differences in shape, and perhaps in size, of vesicles indicate that they contain different transmitter chemicals, which have different effects upon the permeability characteristics of the postsynaptic membrane. But there is no very strong evidence to lead us to believe that the terminal is the exclusive determinant of excitation or inhibition in the postsynaptic membrane. Recent work on the ganglia of *Aplysia* shows that stimulation of the same axon can induce excitation of one cell and inhibition of another, and that the response may be a functional property of the postsynaptic cell and not its afferent.[57-59]

At the beginning of this discussion of synaptic morphology, I indicated that mitochondria are important for recognizing the preterminal part of the axon. These organelles have been somewhat overshadowed by the much more numerous and characteristic synaptic vesicles. Mitochondria are, however, concentrated in the terminals of axons more than elsewhere in the neuron.[19] (Recent studies by Sotelo[60] and Palay suggest that the terminals of dendrites can also be packed with mitochondria.) It is possible that this aggregation is simply accidental, a result of the centrifugal axoplasmic flow, which carries mitochondria to the farthest reaches of the axon like leaves floating on a stream. Certainly presynaptic bulbs that occur along the course of an axon, the so-called *terminaisons en passant,* usually do not display an aggregation of mitochondria, although one or two may be in the neighborhood. But that mitochondria are something special to the synapse is indicated by the fact that other cytoplasmic particulates like lysosomes and multivesicular bodies are rarely to be found in the terminal. Glycogen particles, which are found with increasing frequency, are probably produced locally. Although the role of the mitochondria in endings has not been investigated, it seems reasonable to suggest that they may be concerned with providing energy for maintaining the sodium pump, restoring the integrity of the surface membrane, sustaining various

enzymatic reactions in connection with synthesis of the transmitter, and perhaps constructing the synaptic vesicles.

Microtubules and neurofilaments both occur in axons, and both continue into the presynaptic terminals. In the *terminaisons en passant,* they simply course right on through, usually occupying the core of the terminal if it is ringed round with synaptic zones, or a region off to one side away from the synaptic zone if the terminal has a single synaptic interface. The microtubules seem to end just short of the terminal bulb at the tip of the axon. Neurofilaments, however, enter into the bulb in some instances and not in others. Gray and Guillery[61,62] have shown that to some extent this is a species difference; for example, endbulbs in the cat usually contain a loop or ring consisting of neurofilaments, whereas endbulbs in the rat usually contain only a few wisps of neurofilaments. In addition, there are regional differences within the same animal; neurofilaments are particularly prominent in the large nerve endings in the lateral vestibular nucleus but absent in the nerve endings of the cerebral cortex. In general, large nerve endings are more likely to have numerous neurofilaments than small nerve endings. In all cases, the neurofilaments and microtubules do not approach the presynaptic membrane, which seems to be the preserve of the synaptic vesicles.

In summary, then, we see that the synapse derives from the epithelial nature of the nervous system. It is a modified attachment plaque, which has become the site of further differentiation in the direction of polarity. At this point, where two cells are apposed, the surface membrane and subjacent structures of one cell have become modified to discharge (secrete) a sign of its activity, and the other cell has become modified to receive the signal. Morphologically, synaptic vesicles, synaptic cleft, and dense material adherent to the cytoplasmic sides of one or both cell membranes are essential features of the synapse. Nevertheless, it is impossible at present to make a completely satisfactory or adequate and all-inclusive definition of the synapse. When Sherrington[63] invented the term in 1897, he had in mind those points where nerve cells touch each other and transfer some kind of activity function from one cell to another. Identification of points where cells touch each other is a fairly simple matter, and as we find among these points some peculiarly characterized by the three features named above, it is reasonable to identify them with the synapse. But are these the only kinds of synapse? To this question we can now give a firm negative answer, and that answer opens the door to a great deal of confusion, which is likely to make it difficult to interpret morphological data for a long time to come.

One of the deviations from the pattern presented above is only apparent, and therefore I shall deal with it first. In the foregoing discussion we have been concerned with synapses that transmit their signals by means of a chemical mediator. There are well-documented examples of synapses that are capable of transmitting their activity by direct electrotonic spread of current from one cell to another. Some of these are rectifying, that is, the current can pass in only one direction (e.g.,the crayfish giant motor synapse[64] and the synapse between club endings and dendrites of the Mauthner cell in goldfish[65,66]), and some of them are nonrectifying, that is, the current can pass in either direction (e.g., the earthworm giant fiber septal synapse[67-69]). When these synapses are examined in the electron microscope, the apposed surface membranes of the cells or fibers involved are seen to approach each other very closely or to fuse together, giving rise to a *zonula occludens.*[70-72] Such junctions are similar to those seen in other epithelia,[6] for example, chick and squid embryos[9-11] and salivary glands of *Drosophila.*[8] These electrotonic synapses are, therefore, again modified attachment plaques between adjacent cells. Consequently, they are not really a deviation from the pattern presented above.

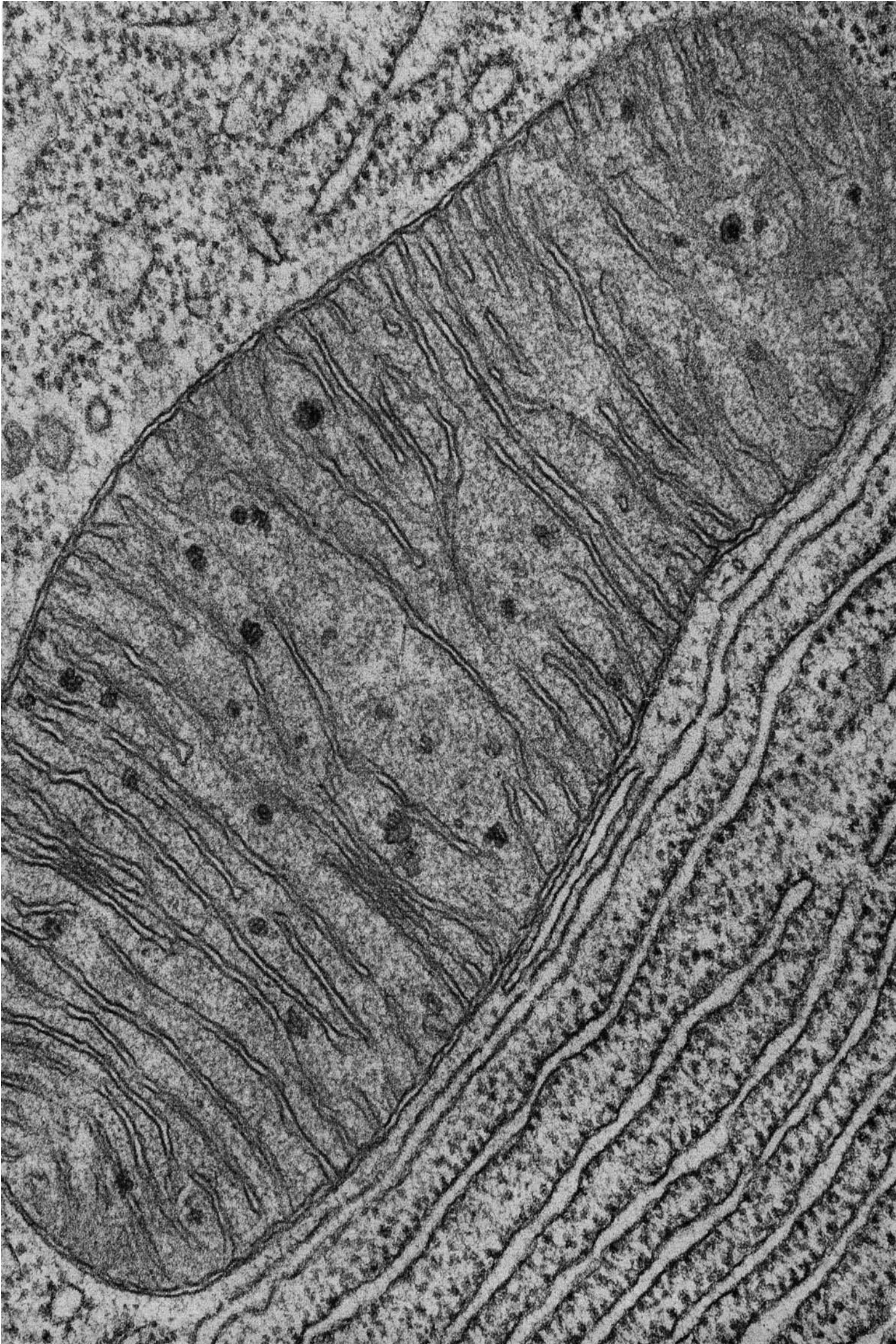
But there is a new kind of synapse that cannot be encompassed by this generalization. We now have two examples of this kind of synapse, and both of them appear to be inhibitory. These are the axon cap around the initial segment of the Mauthner cell axon[65,72] and the basket around the initial segment of the Purkinje cell axon in the cerebellar cortex.[32,36,37,73] In both of these structures afferent axons terminate in a maze of branching twigs filled with small vesicles and enclosing the axon of the postsynaptic cell in a field without making contact with it. Inhibition appears to be produced by a gross alteration in the ionic environment surrounding the postsynaptic membrane rather than by a precise microinjection of transmitter chemical onto a specific patch of membrane. The arrangement is located in an ideal site to produce inhibition of discharge from the initial segment in its center, because this is the region of the neuron where the action potential originates. Is this kind of junction a synapse? It lacks the important characteristic of contact or apposition between cells, but it is certainly a place where the activity of one cell specifically and critically affects the activity of another. Physiologically it must be called a synapse, and the morphological definition must be expanded to include it. But it must be recognized that in so doing, the morphological criteria for identifying a synapse become less compelling and less useful. They turn out to be characteristics of certain *kinds* of synapse, chemical or electrotonic. These are, fortunately for the investigator, the most common kinds, but they do not restrict our imagination.

If we turn now to the question of synaptic interrelations between neurons and the second great class of cells in the

nervous system, the neuroglial cells, we are confronted with even greater difficulties. For a long time neuroglial cells have been considered as purely supportive elements having nothing to do with the specifically neural task of the nervous system, i.e, the function of communication. Despite much speculation in recent times, precise work[74] in the past four years has shown that neuroglial cells do not participate in the electrical signaling activity of their neighboring nerve cells; they do not affect the action potential in nerve cells and are not themselves affected by it directly. Nevertheless, it must be admitted that only a few systems have been examined so analytically. Generalizations of this negative character are perhaps still premature. Junctions identified as possible synapses have been found between axons and specialized neuroglial cells in the neurohypophysis of the fish[75] and the monkey.[76] Although no physiological studies that bear on this question have yet been undertaken, these junctions must be examined very cautiously, for if they really are synapses, they may lead to a reappraisal of neuroglial function. The examples that have been published display thin condensations of dense material on either side of the apposed surface membranes involved or merely increased density of the membranes. The synaptic vesicles in the axonal member of the pair show no tendency to cluster at these points. The structure has enough resemblance to the simple *punctum adhaerens* in other regions to make one hesitate to call it a synapse. It must be recalled that attachment plaques between neuroglial cells and nerve cells are not generally seen, although *zonulae occludentes* and *zonulae adhaerentes* between neuroglial cells are extremely common throughout the nervous system. Other published examples[77] show contacts between ependymal cells and axonal terminals that display small collections of synaptic vesicles close to the axonal membrane, but there is no evidence of dense material beneath either the ependymal or axonal surface. Here again only part of the total synaptic complex is seen. In all of these equivocal junctions the axons in question arise from cells in the supraoptic nucleus (preoptic nucleus in the fish), which has special gland-like characteristics.[78] They are neurosecretory cells; they do not innervate another nerve cell or an effector organ, but are themselves the effector. It is possible that such cells retain their primeval attachments to the ependymal and neuroglial cells of the region without indicating functional interaction between them. In any case, our judgment has to be suspended until physiological records can be made of the responses of these pairs.

In the foregoing account I have tried to discuss some of the mechanisms which the cells of the nervous system, especially the neurons, use to communicate with one another. I have emphasized that these cells are arranged in an epithelium and are bound together by structural devices similar to those found in other epithelia. But it must be admitted that the nervous system differs considerably from other epithelia in important respects. Its constituent cells interact in a very precise way. This behavior is a consequence of the intrinsic polarity of each nerve cell, such that it possesses a receptive zone where impulses converge upon its surface, a conductile zone where the integrated response of the cell is conveyed to some distant point, and an efferent zone where that response is transmitted to the next cell, or cells, in line. Integration of the influences impinging upon its surface goes on in all parts of the neuron except probably the conductile zone. In the nervous system these polarized units are connected into chains of interacting entities and these chains are themselves assembled into hierarchies of increasing complexity,[79] with different degrees of priority in their effects upon the overall organization. The arrangement is extremely precise and orderly. The key to this organization and the most fundamental principle of structure in the nervous system is given by the pattern of connections between cells. This pattern can be analyzed in terms of the connections between groups of cells or between regions of the nervous system, a subject that has long occupied the attention of neuroanatomists and neurophysiologists. But there is a much more important level of analysis, at once more subtle and more fundamental: the detailed pattern of afferent terminals upon individual neurons and distribution of their efferents upon other cells. The tools for this kind of analysis, both anatomical[80-84] and neurophysiological,[79,85,86] are at hand and need only to be applied with some refinements and a great deal of patience in order to yield a completely new order of information about the organization and behavior of the nervous system. Physiological studies of mammalian brain and invertebrate ganglia have demonstrated that individual neurons have unique and characteristic connections with other neurons. Anatomical analyses of a few regions in the brain and spinal cord have shown that afferent terminals from specific sources are arranged in precise displays over the surfaces of the neurons. The effects of afferent impulses upon an individual neuron must, therefore, depend not only upon the frequency, timing, and type (excitatory or inhibitory) of those impulses, but also upon the spatial distribution of the synaptic terminals bringing these impulses, upon their number, size, and specific location on the neuron. Because the neural afferents to the nervous system represent topographically different, although overlapping, sites in the body, each neuron receives connections from a different complex of sources, and consequently each neuron is a unique entity. Its behavior is imposed upon it not only by its internal state and its indi-

vidual history, but also by its unique connections. Thus, modern neuroanatomy and neurophysiology disclose a common set of principles of organization that are fundamental to any discussion of the behavior of the nervous system. The nervous system is not a random net. Its units are not redundant. Its organization is highly specific, not merely in terms of the connections between particular neurons, but also in terms of the number, style, and location of terminals upon different parts of the same cell and the precise distribution of terminals arising from that cell. The central nervous system is organized as a series of interdependent hierarchies of analysis and control by means of a circuitry that provides for the reciprocal interactions necessary for modulation of both input and output. Finally, the cellular units of the nervous system, at whatever level considered, are organized by means of their connections and their intrinsic properties for integrative action.



MOLECULAR BIOLOGY

"We come now to the level of biological organization at which self-assembly and self-stabilization through simple molecular principles are not quite enough to account for the complete biogenesis of these much more elaborate subcellular structures." LEHNINGER, PAGE 42. *Such organelles are exemplified by the mitochondrion shown here in an electron micrograph,* PAGE 92.

INTRODUCTION

A. L. LEHNINGER

Molecular Biology: The Theme of Conformation

THIS PAPER INTRODUCES molecular biology, the subject of the following 170 pages in this book. The molecular approach to biology, particularly in the area of genetics, has in the last few years yielded enormous and undreamed-of progress. There can be no doubt that we now command penetrating and revolutionary new insight into cell biology from these developments. The spectacular success in molecular genetics is focusing attention on other areas of biology that might profitably be approached with the tools and the outlook of molecular science. In the past, the prospects of success in approaching the molecular basis of neurofunction and memory seemed so discouragingly remote that few biochemists or molecular scientists were attracted to the problem. But there is now considerable optimism and interest, and the challenges of neurobiology are being widely discussed.

A central and unifying theme in contemporary molecular biology, and one that is especially relevant to neurobiology, is the relationship between the *one-dimensional,* or linear, coding of information in the DNA molecule and the *three-dimensional* conformation of the protein molecules and the supramolecular assemblies that are the dynamic molecular effectors or instruments of the genotype

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of living cells. Biological information is coded in linear DNA molecules by triplets of nucleotides that contain specific sequences of the four different bases—adenine, guanine, thymine, and cytosine. Each triplet of nucleotides ultimately specifies a single amino acid residue in the protein molecule that is coded by the DNA, so that the information of the DNA is translated into the specific amino acid sequence of the protein molecule in a co-linear fashion.

But it is not the one-dimensional sequence of amino acids per se that is directly responsible for the biological activity of protein molecules; rather, the conformation or shape of the molecule in *three* dimensions endows it with its characteristic activity. Its specific conformation in three dimensions, for example, gives an enzyme molecule catalytic activity and specificity. When a native enzyme molecule is fully "unwound" by denaturation, it loses its catalytic activity, even though its amino acid sequence is still intact. The translation of one-dimensional into three-dimensional information is therefore a matter of fundamental concern in molecular biology. Just as the conformation of a macromolecule determines its biological activity and specificity, *changes* in conformation, and the *rate* of such conformational changes are basic elements in its dynamic function. Thus, during catalytic function, enzymes undergo changes of conformation, and these rapid changes are part of the catalytic process. But other types of conformational change may be extremely slow, and such slow reactions may also have a biological advantage. Conformation and conformational change are thus central elements in cell structure and function.

Information

First a few words must be said about the concept of information, as it will be such an important element in all the chapters in this volume. Modern thermodynamic theory holds that information has a real physical meaning and that it can be equated inversely with that state or quality of matter and energy that is termed entropy, the degree of randomness or disorder of a system. This conclusion has been drawn only relatively recently, following many decades of uncertainty.

The relationship between information and entropy is best illustrated by Maxwell's demon (Figure 1). The First Law of Thermodynamics, you will recall, is simply the principle of the conservation of energy. The Second Law can be stated in different ways, but a sufficient definition for present purposes is that all physical or chemical processes proceed in such a direction and extent as to maximize the entropy of the system and surroundings under a given set of conditions. For example, we know that if a gas confined in the left container seen in Figure 1 is allowed to escape through the orifice into the right container, it will do so until an equilibrium state is attained that has the maximum entropy, or randomness, of which the system is capable; the gas will distribute itself equally in both compartments and the temperature of the gas will be uniform throughout both compartments.

Clerk-Maxwell postulated his celebrated demon in 1876 in terms of this model. Suppose the orifice is controlled by a demon who, by means of a frictionless gate, allows the faster gas molecules of the population, i.e. the "hotter" ones, to enter the right compartment, but allows none of the slower, cooler molecules to pass. The demon would thus produce a situation in which the right compartment has a higher temperature than the left. Such a system would have *less* entropy than if the demon had not participated. The First Law will not have been violated because the total energy content of the system is the same; the frictionless gate makes no contribution. However, something will have happened to the system that we know from common experience never happens spontaneously—the separation of a population of gas molecules into zones of "hot" and "cold" molecules. Maxwell posed the paradox: since the demon does no work in operating the frictionless gate, such a separation of hot and cold molecules cannot violate the Second Law.

This paradox went unexplained until 1929, when Szilard provided the important clue: the demon must possess *information* as to which molecules are hot and cold in order to separate them, and the amount of information he uses to select the molecules must be equal to the difference in entropy between the system he produces and that which results when no information is used. Starting from this idea, modern thermodynamic theory states that there is an exact but inverse equivalence between entropy and the information content of a system. Information is thus orderliness, in the thermodynamic sense. While this much is clear, there are still many problems to be solved in applying thermodynamics to the quantitative evaluation of the informational content of biological systems.

The important lesson is that there is every thermodynamic reason to expect that the information-rich brain of a highly sophisticated, highly trained subject must possess more order than the brain of a naive, untrained subject that has never been exposed to experience. If this greater degree of order is not microscopically visible, then it must exist at the submicroscopic or molecular level.

Conformation of proteins

Now let us see how genetic information coded into linear sequences of amino acids determines the specific, informa-

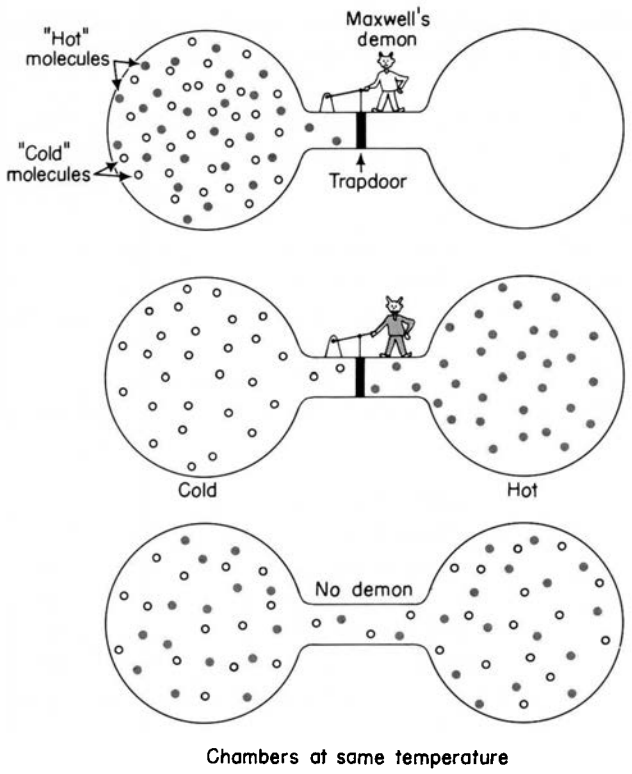


FIGURE 1 Maxwell's demon requires information to separate "hot" and "cold" molecules. (From A. L. Lehninger, 1965. Bioenergetics, New York, W. A. Benjamin, Inc., p. 226)

tion-rich, three-dimensional conformation of peptide chains. Modern physical methods, especially X-ray diffraction analysis, have revealed that protein molecules in their native state do not exist as long, stringy, floppy molecules, as might be suggested by their linear nature and the great length of their covalent-bond backbones. Rather they are coiled or folded (or both) into characteristic three-dimensional arrangements that are specific for each type of protein. Some of these arrangements may be beautifully symmetrical and regular, and some may appear rather irregular and amorphous. Whatever conformation a protein possesses in its native state, it is always reproducibly and precisely formed in nature; each myoglobin molecule, for example, is exactly like the next.

The specific three-dimensional conformation of protein molecules is self-organizing, because it is the conformation in which the macromolecule is thermodynamically most stable under biological conditions, such as those of hydrogen-ion concentration and temperature. This specific conformation of proteins is not dependent on the input of energy nor is it the result of covalent cross-linking. It is simply the automatic outcome of severe limitations on the freedom of rotation around single bonds in the peptide chain backbone, limitations that are produced in part by the occurrence of weak bonding between different functional groups of the molecule and in part by the geometrical rigidity of the peptide bond itself.

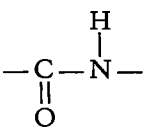
WEAK BONDS AND INTERACTIONS There are two sets of weak bonds or interaction forces that are important in determining the conformation and stability of protein molecules. One set is important in conferring geometrical specificity of three-dimensional conformation: these are hydrogen bonds and ionic bonds, both of which are expressions of the polarity or distribution of charges in the specific atoms participating in the bond. Ionic bonds have specificity of interaction, because a positively charged group such as $-NH_3^+$ will tend to repel other positively charged groups, but will attract a negatively charged group. Furthermore, because the attractive force between charges is inversely proportional to the square of the distance separating them, the strength of such an ionic bond will depend on the distance between the oppositely charged groups. Similarly, hydrogen bonds are geometrically specific. They can form only between a hydrogen atom bonded to an electronegative atom, such as nitrogen or oxygen, and some neighboring N or O atom with a partial electronegative charge; hydrogen bonds have characteristic interatomic distances and bond angles.

The other set of interactions is less specific in a geometrical sense, but confers thermodynamic stability on the conformation of macromolecules. These are the van der Waals forces and the so-called hydrophobic interactions. The forces involved in these interactions are not highly localized or directional, so they cannot strictly be called bonds; they are more appropriately called interactions. Van der Waals forces are very weak interactions resulting from mutual polarizability of nonpolar areas of interacting molecules; their strength is inversely proportional to the sixth power of the distance separating them. Hydrophobic interactions, which in aqueous systems are more important quantitatively than van der Waals forces, are actually the consequence of a property of the aqueous medium in which the macromolecule is dissolved. Hydrophobic structures, such as the nonpolar aliphatic or aromatic side-chains of leucine or phenylalanine, tend to cause some ordering of the surrounding water molecules and thus a decrease in the entropy of the water phase. Any process that causes a decrease in the entropy of a system cannot be spontaneous; for this reason, hydrophobic substances do not spontaneously dissolve in water to form true solutions. Hydrophobic groups are most stable in aqueous systems if they are allowed to come together in a tightly packed group to minimize the total hydrophobic area that is exposed to water molecules. Such an association of hydrophobic groups into a micelle maximizes the

entropy of the surrounding water. This tendency for hydrophobic groups to associate, driven by the propensity of the aqueous medium to maximize its entropy, is the major thermodynamic force in stabilizing the three-dimensional structure of biological macromolecules. To summarize, then, polar bonds confer geometric specificity; nonpolar or hydrophobic interactions confer thermodynamic stability.

DETERMINANTS OF PROTEIN CONFORMATION Because the backbone of the peptide chain consists of single bonds, it might appear that a long peptide chain can exist in an infinite number of conformations in space, since at each single bond there is normally complete freedom of rotation. Yet we know that protein molecules have only one or a very few specific, biologically active conformations. In principle, such unique conformations are the outcome of three determinants.

The first, discovered by Pauling and Corey, is that the peptide bond is a rigid co-planar structure. The peptide bond, as usually written,



would appear to allow free rotation between C and N. However, because of resonance stabilization, this "single" bond between C and N actually has about 40 per cent double-bond character, which is sufficient to prevent freedom of rotation around the C–N bond. All the peptide bonds in a long peptide chain can therefore be regarded as rigid planar structures. We see at once that the number of degrees of freedom of a peptide chain, and therefore the number of conformations possible, is thus greatly limited.

There is a second set of limitations to freedom of rotation around the remaining single bonds in the backbone. These are the size, shape, and polarity of the side chains, called R-groups, of the amino acid residues. Some of the R-groups are small, as in alanine and glycine, and thus do not get into each other's way if they are adjacent to each other in a chain. But other amino acids have bulky R-groups; if they are adjacent, they will offer a certain amount of hindrance to free rotation. Adjacent R-groups with the same electrical charge will repel each other; if they have opposite charges, they will attract each other. Such polar interactions also constrain freedom of rotation around the single bonds of the peptide chain backbone. Furthermore, highly hydrophobic R-groups, such as those of valine and isoleucine, will tend to associate together as closely as possible in hydrophobic interactions. Finally, it is clear that the ring structure of proline allows no freedom of rotation around the ring C–N bond; wherever proline occurs in a peptide chain there is a bend or kink in the chain.

The third type of constraint that limits freedom of rotation is the tendency for intrachain hydrogen bonds to form. Peptide chains with small or uncharged R-groups tend to form regular helical coils because of hydrogen bonding between peptide bonds in successive helical loops of peptide. The most prevalent coiled arrangements of peptide chains are the well-known α-helix and the collagen helix.

Because of these three limiting factors, most protein molecules have only one or two specific stable conformations.

TYPES OF CONFORMATION Two general types of conformation have been observed among proteins. The first is the purely helical configuration seen in fibrous proteins; the α-helix of α-keratin, the pleated sheet of β-keratin, and the coiled coil of collagen. The second type is found in the globular proteins, which are highly folded. Most globular proteins consist of short "runs" of pure α-helix, interrupted by irregular bending points caused by the bulky or polar nature of the R-groups of a few specific amino acids: proline, serine, threonine, and perhaps one or two others.

The availability of exact knowledge of amino acid sequences and of exact three-dimensional structures from X-ray methods for just a few proteins has made possible definition of the "ground-rules" by which an approximation of the three-dimensional structure of any protein may in principle be calculated, providing its amino acid sequence is known. In fact, Levinthal and his colleagues have devised a computer program for carrying out such sequential calculations. Figure 2 shows some different aspects of the backbone of myoglobin as displayed by a computer.

THE PRINCIPLE OF COOPERATIVE INTERACTIONS Denaturation, or unfolding, of native, globular protein molecules, without breaking the covalent backbone of the peptide chain, has traditionally been regarded as irreversible. However, irreversibility is perhaps only apparent. Renaturation or refolding of an unfolded chain to the original conformation should in principle proceed spontaneously if the native conformation is, in fact, the most stable one. However, a very long time may be required for unfolded molecules to refold to their specific native forms—in other words, to "try out" all possible conformations before they find the most stable one. Once some specific refolding has been spontaneously accomplished, the remainder of the process occurs more rapidly. This is because the interac-

tions involved in stabilization of conformation are *cooperative*. Whenever a series of weak bonds or interactions cooperate to stabilize a macromolecule in a specific conformation, it is characteristic that the rate of formation and the stability of each new weak bond formed is dependent on how many have already been formed. This is why refolding of a partially unfolded macromolecule can take place in only microseconds, but refolding of a completely extended molecule may take days or years. In part, these cooperative effects are a matter of the operation of thermodynamic factors related to entropy changes.

Oligomeric proteins

Many, if not most, protein molecules contain more than one peptide chain; these are called oligomeric proteins. There seems to be an upper limit to the length of single peptide chains; most are no longer than about 150 to 300

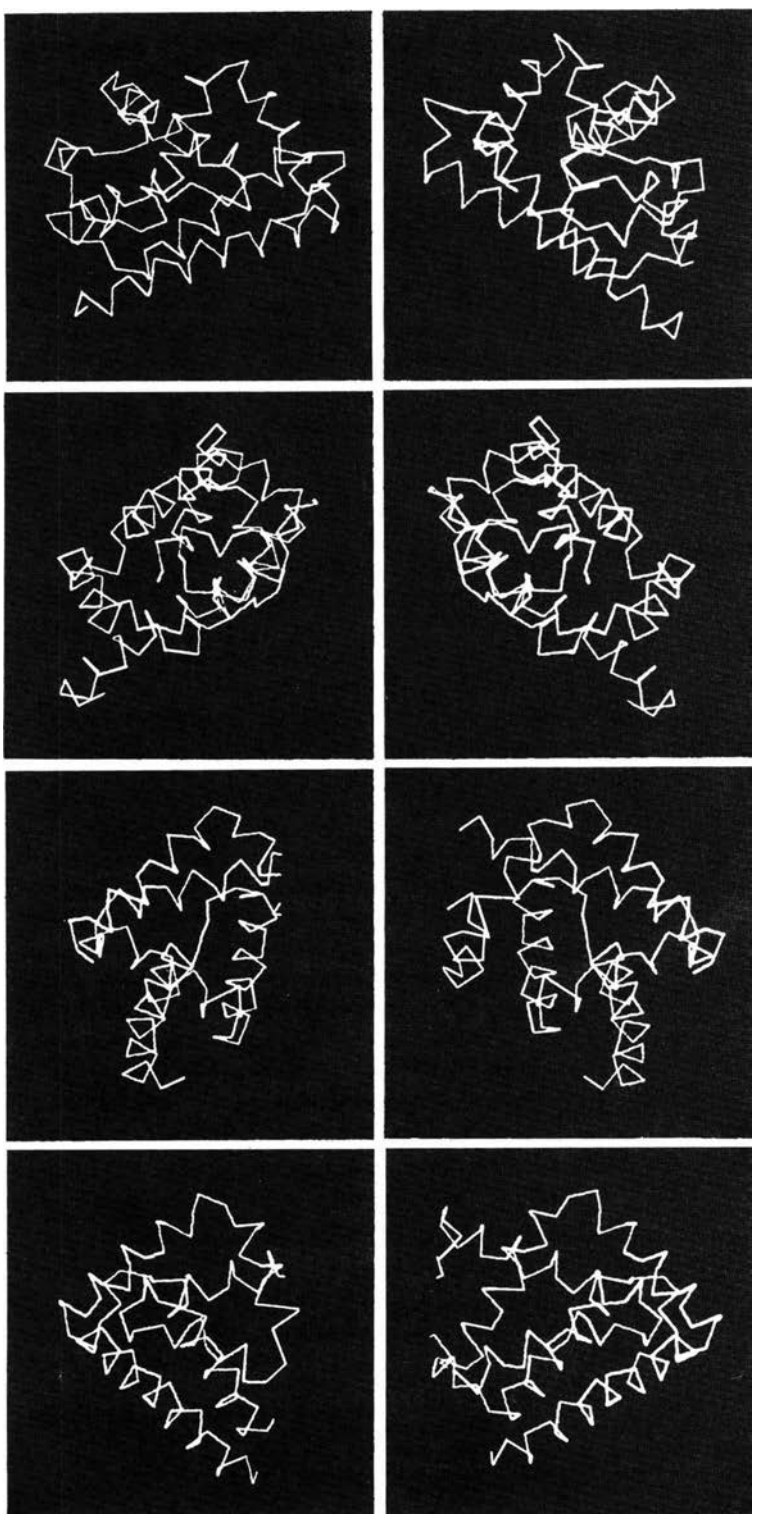


FIGURE 2 Computer representation of the backbone structure of myoglobin. In the successive photos (top to bottom, left to right), the molecule is rotated around the vertical axis. (From C. Levinthal, *Scientific American*, June 1966, p. 45)

amino acid residues. Yet some proteins have molecular weights exceeding one million. Examples are given in Table I. In such oligomeric proteins the separate peptide chains are not covalently bonded together; rather, they are held together through weak, cooperative interactions, particularly hydrophobic interactions. Thus human hemoglobin, which contains four separate peptide chains (two α-chains and two β-chains), does not dissociate readily under normal conditions. If dog hemoglobin and human hemoglobin are mixed in solution at *p*H 7.0, each is so stable that there is very little tendency for the four chains of each to dissociate and to reassemble into hybrid molecules. Hybrids can be made, but not by simple mixing of dilute solutions of the two hemoglobins.

It is therefore clear that something else is coded into certain peptide chains beside the three-dimensional conformation of the peptide chain itself. Also coded into the peptide chains of oligomeric proteins are the specific areas of the completely folded chain that fit a specific complementary area in the folded adjacent peptide chain so exactly and with such high stability that the two chains have very little tendency to dissociate. The specific conformation, or three-dimensional fit, of the separate subunits of oligomeric proteins is referred to as quaternary structure.

A number of oligomeric proteins have been successfully dissociated into their subunits; when mixed, they will spontaneously reassemble into complete molecules.[1] No work is required to put together the separate chains of oligomeric proteins, even in complex mixtures of proteins; they are thermodynamically self-assembling systems in which the specificity of fit is so high that the peptide chains that "belong" to each other will seek each other out from a complex mixture of proteins. Hydrophobic bonding is undoubtedly the major stabilizing interaction in such systems.

TABLE I

Subunits in proteins

Protein	Molecular weight	Number of peptide chains
Hemoglobin	65,000	4
Alkaline phosphatase (*E. coli*)	80,000	2
Enolase	82,000	2
Hexokinase	96,000	4
Aldolase	142,000	6
γ-globulins	150,000	4
Catalase	250,000	4
β-galactosidase	520,000	12 to 16
Glyceraldehyde phosphate dehydrogenase	145,000	4
Lactate dehydrogenase	126,000	4
Glutamate dehydrogenase (beef liver)	2,000,000	40

(From Sund and Weber, Note 1)

Supramolecular assemblies

ENZYME COMPLEXES The separate molecules of some multienzyme systems are known to associate into complexes or assemblies that are stabilized by weak but cooperative bonds and interactions.[2] For example, the fatty acid synthetase complex of yeast, which has been studied in great detail by Lynen and his colleagues, consists of seven individual protein molecules, each catalyzing an individual step in the sequential synthesis of palmityl CoA from malonyl CoA. Each of the seven enzyme proteins consists of three separate peptide subunits; the whole complex thus contains 21 peptide chains. This complex can be dissociated into the seven component proteins, but in separated form they have no enzymatic activity. When they are mixed together, they reassemble, and the assembly so formed can now catalyze all seven steps in fatty acid synthesis. Again we see the self-assembly principle operating. Evidently the amino acid sequence in each of the 21 chains codes the conformation of each subunit and the precise way in which the three subunits of each enzyme molecule fit together to form a complete enzyme molecule; it also codes the way all seven enzymes fit together in a stable, functionally active, geometrical array. The end product is actually more than the sum of the components, because singly the seven enzyme molecules are inactive. It therefore must be concluded that in the fitting together of the 21 chains into seven enzymes and these into the active multienzyme complex, subtle conformational changes have taken place that cause each of the inactive enzyme molecules to become active.

HIGHER-ORDER STRUCTURES The same principles of conformational stability and cooperative interactions that have been described in some detail for simple and oligomeric proteins and for enzyme complexes are also involved in the stabilization of the three-dimensional conformation of higher-order supramolecular structures. Tobacco mosaic virus, which consists of 2200 separate but identical peptide chains and a long RNA molecule, will reconstitute itself from its subunits spontaneously, with full regeneration of its infectivity. Recent work indicates that other virus molecules may also be at least in part self-assembling.

Ribosomes, whose function in protein synthesis is discussed in detail later in this volume, have an exceedingly complex subunit structure. They are composed of about 55 per cent protein and 45 per cent RNA. Each ribosome (diameter $\sim$220 Å) has a major and minor subunit and a

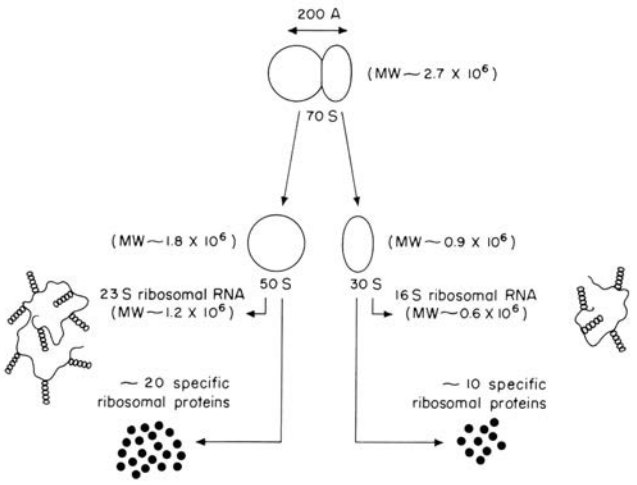


FIGURE 3 Subunit structure of the *E. coli* ribosome. (From J. D. Watson, 1965. The Molecular Biology of the Gene, New York. W. A. Benjamin, Inc., p. 326)

well-defined equatorial groove (Figure 3). Each subunit is in turn comprised of smaller subunits of RNA and protein molecules, some of which have been separated in pure form. Very little is known as yet of the possible enzymatic activity of protein subunits of ribosomes. In view of the exceedingly complex mechanism by which proteins are synthesized, it can be expected that the specific arrangement of subunits in ribosomes is so ordered genetically as to provide for the adsorption of messenger RNA (mRNA), presumably in or near the equatorial groove, then for the binding of amino acid derivatives of soluble RNA (sRNA) transferred to specific codon sites on the mRNA, and for the access of the peptide-forming enzymes.

MEMBRANES The structure, biosynthesis, and genetic differentiation of the different membrane systems of the cell represent the next great "sound-barrier" to be broken in molecular biology. In fact, we are already beginning to see a quiet revolution in this field, particularly with the advent of new techniques and knowledge concerning the structure, separation, and biosynthetic pathways of the complex lipids, which are universal components of membranes. Membranes also contain recurring protein monomers called structure proteins, making up about 50 to 60 per cent of their weight; most of the remainder is contributed by lipids. Membranes differ in lipid composition from one cell type to another; these differences seem to be genetically determined. For example, mitochondrial membranes characteristically contain large amounts of cardiolipin, whereas endoplasmic reticulum contains little or none.

From the physical and chemical point of view, biological membranes are extraordinary structures. They possess unusually high electrical resistance and capacitance and can sustain enormous electrical fields, and yet they allow water to pass through them freely. These physical properties are all the more striking because the lipid and protein components of membranes are not covalently bonded together to make these electrically tough structures; they are quite simply held together by cooperating weak bonds and interactions, and are stabilized by a high degree of hydrophobic bonding. Here again we see in action the principle of conformational fitting of molecular components and hydrophobic stabilization of the system to yield a flexible, highly resistant sheet that is but a few molecules thick.

The biogenesis of membranes is a most intriguing subject, for one can never see the end of a membrane sheet in the intact cell with the electron microscope; membranes are always continuous and must therefore be built by insertion of molecules. At least the phospholipid core of bilayer membranes appears to be self-assembling, according to recent work of Thompson and his colleagues,[3] who have studied the self-assembly of phospholipid bilayer membranes of great stability and high electrical resistance (Figure 4). Such membranes will make possible direct study of lipid system phase changes that can be initiated by electrical stresses and their possible relationship to conduction in nerve cells. Clearly it is the conformation of

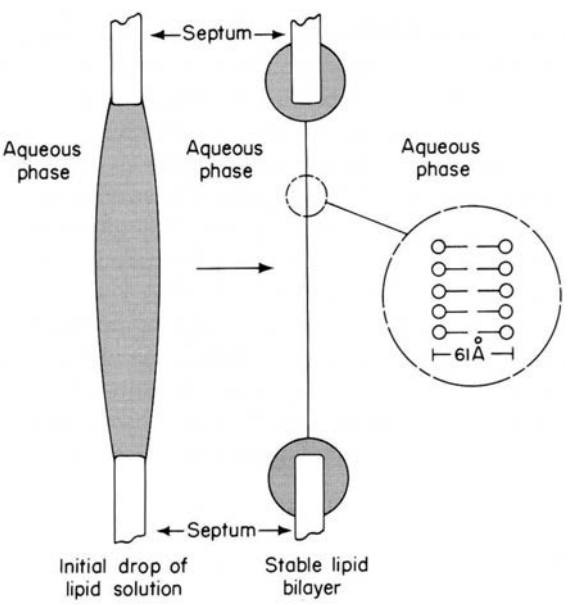


FIGURE 4 Self-assembly of lipid molecules into a bilayer system. (After C. Huang, L. Wheeldon, and T. E. Thompson, 1964, *J. Mol. Biol.* Vol. 8, p. 148)

lipid-protein-water systems that endows membranes with some of their most specialized and unique properties.

Membranes also offer interesting possibilities for two-dimensional coding of information, because the monomeric structure protein units comprising the protein layer(s) may conceivably be arranged in different mosaic-like patterns in the plane of the membrane.[3] Differences in two-dimensional patterns of protein could then be determinants of a complementary mosaic arrangement of different kinds of phospholipid molecules. From the physical-chemical point of view, it is conceivable that such changes in mosaic patterns can be induced by electrical or chemical stimuli, exerted either on the lipid or protein phase. Such coding of contact surfaces at nerve endings could be involved in induction of specific circuitry patterns in the nervous system. This type of two-dimensional surface coding has not been given sufficient attention.

Conformation of nucleic acids

Every biologist is familiar with the complementary double-helical arrangement of duplex DNA molecules, with its many important implications for molecular genetics. It is now clear, however, that this is but one of a number of different three-dimensional conformations that may be assumed by different nucleic acids, depending on their function. Circular forms of DNA molecules are found in some viruses, and such circular forms also may occur in a hypertwisted form. These conformational arrangements may be devices to control or limit the availability or accessibility of the DNA molecule for enzymatic replication or transcription. Messenger RNA is a single-stranded molecule, whereas soluble transfer RNA molecules are believed to have a more complex conformation that is only partially double helical, with large loops of the chain separating the paired helical regions. This may be an adaptation to make possible their specific biological function of serving as adaptors by means of the codon–anticodon interaction. The role of ribosomal RNA is still a great mystery, but presumably it participates in the binding of messenger RNA through some specific geometrical relationship to the ribosomal groove. The principles of cooperative weak bonding and hydrophobic stabilization developed above also underlie the conformational stability of nucleic acids.

Cell organelles

We come now to the level of biological organization at which self-assembly and self-stabilization through simple molecular principles are not quite enough to account for the complete biogenesis of these much more elaborate subcellular structures (Figure 5). Mitochondria, the powerplants of the cell, have been the most intensively studied organelles of the cytoplasm. They have dimensions of 10,000 to 30,000 Å, in comparison with the 200 Å diameter of a ribosome. A single liver mitochondrion contains about 15,000 complexes of respiratory chain enzymes, each of which contains a dozen or more separate enzymes. These enzyme complexes, which are regularly on the inner membrane of the mitochondrion, comprise about 25 per cent of the total mass of the membrane. The inner mitochondrial membrane is thus not merely an inert skin, but is in reality a sheet of multienzyme systems that catalyzes both respiration and phosphorylation, and active ion transport across the membrane. Recent work indicates that certain organelles such as mitochondria, kinetosomes, and centrosomes cannot form *de novo* from simple molecular precursors alone. Some pre-existing portion of mitochondrial structure is required to make growth and replication of these organelles possible. The recent discovery in mitochondria of DNA of a type that is different in size and base composition from nuclear DNA suggests that mitochondrial DNA must be passed on to the daughter mitochondria before their replication can be completed. Such a process may be the key to the mechanism of replication and biogenesis of mitochondria, which are now believed to undergo their own division cycle. But the basic theme developed in this introduction—the principle of self-organization through conformational interactions—also characterizes the assembly of microscopic regions of organelle ultrastructure, presumably coded by both nuclear and mitochondrial DNA.

Changes in the conformation of enzymes

Until now we have been considering the conformation of biological macromolecules and supramolecular assemblies in only a static sense. This is only one side of the coin. One of the major conclusions from recent work on enzyme action is that *changes* in conformation of macromolecules are often essential elements in the mechanism by which they carry out their specific dynamic biological functions. Conformational changes are involved not only in catalysis by enzymes, but also in the regulation of enzyme action by metabolic feedback inhibitors and activators. Let us briefly examine the nature of these changes.

Recent experimental studies have clearly shown that many enzymes change their conformation as they combine with their substrates, presumably to make a close fit between enzyme and substrate that in some way facilitates the subsequent catalytic process. This type of change is called the "induced fit" of the enzyme. One interpretation is that the enzyme molecule exists in two equilibrium

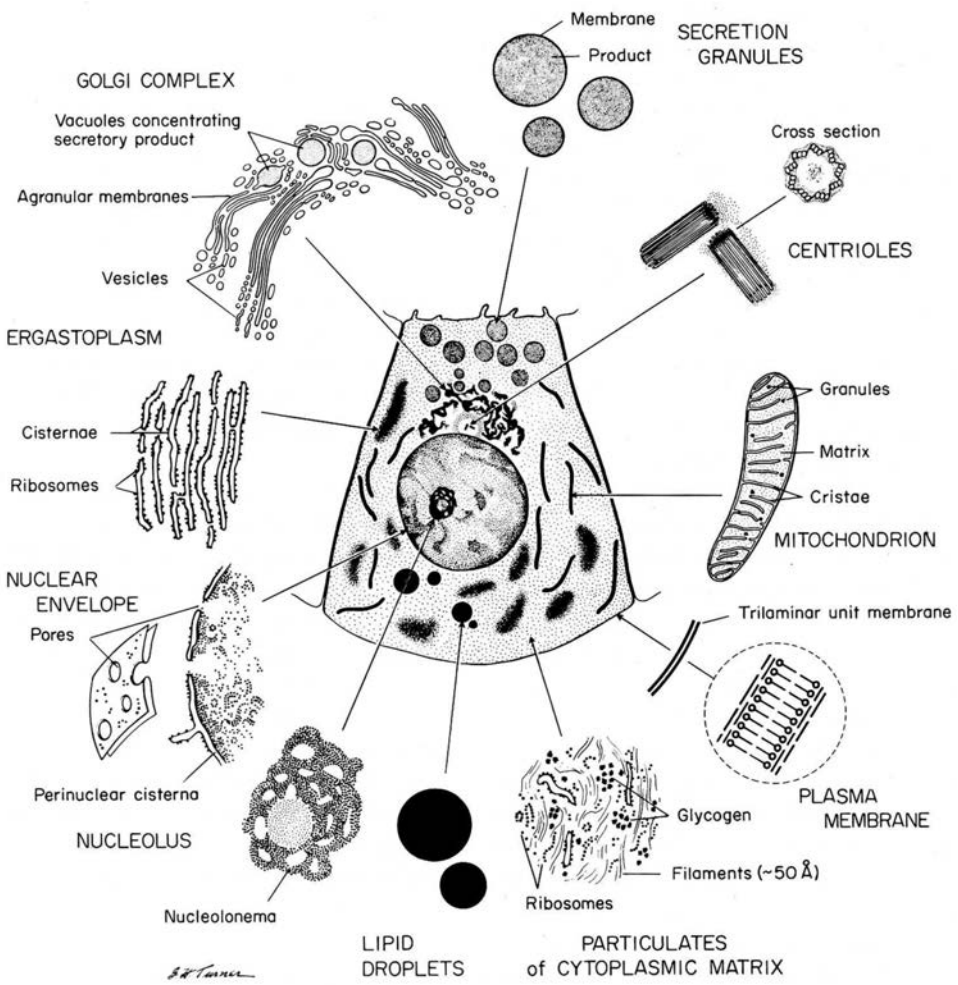


FIGURE 5 Ultrastructure of cell organelles. The drawing in the center illustrates a cell as it would be visualized by light microscopy. The enlargements around the periphery show the structures as revealed by the electron microscope. (From W. Bloom and D. W. Fawcett, 1962. A Textbook of Histology, Philadelphia, W. B. Saunders Co., p. 21)

forms differing in conformation, and that only one of these (E_2) can combine with substrate (S):

$$E_1 \rightleftharpoons E_2 \quad ,$$

$$E_2 + S \rightleftharpoons E_2 \cdot S \quad .$$

If a large excess of substrate is present, all of the enzyme molecules would be converted into form E_2 as the $E_2 \cdot S$ complex because of the reversibility of the reactions. This conformational change must, of course, take place at a very high rate, at least as great as the rate of the subsequent catalytic step.

Any process involving the making and breaking of large numbers of weak bonds that stabilize protein structure must be rather slow, and it is therefore unlikely that the "induced fit" involves anything approaching complete unfolding of the enzyme molecule E_1. From the principle of cooperative interactions, we know that whenever only a small fraction of the weak bonds stabilizing a protein are made and broken, such conformational transitions can take place rapidly. It is to be expected, then, that conformational changes during combination of enzyme and substrate must be relatively small in amplitude. This appears to be the case. In fact, such conformational changes are often only barely detectable with such commonly used

methods as optical rotation, optical rotatory dispersion, or the use of fluorescent probes for hydrophobic areas. It is rather interesting that most enzyme molecules have highly folded conformations with many bends and few α-helical runs. Such structures may be specially favorable for rapid and reversible changes in conformation. In general, the precise geometrical nature of the conformational changes occurring during enzyme action cannot be measured easily by absolute methods such as X-ray diffraction, although the mere occurrence of conformational changes can often be detected with extremely great sensitivity.

ALLOSTERIC CONFORMATIONAL TRANSITIONS In many linear multienzyme systems responsible for catalysis of sequential reactions in metabolism, the first enzyme of the system is often found to be inhibited by the product of the last reaction of the sequence. Such inhibition is not only specific for the last product (other intermediates in the sequence do not inhibit), but it is usually noncompetitive with the substrate of the first enzyme. That is, the product of the last enzyme inhibits the first enzyme in such a way that the inhibition cannot be reversed by increasing the concentration of the substrate of the first enzyme. As a corollary, usually there is absolutely no structural similarity between the inhibitory product of the last enzyme and the substrate of the first enzyme. Such enzyme systems are self-adjusting or self-regulating, and this property is genetically determined. The many complex enzymatic reaction sequences of intermediary metabolism are integrated by complex networks of such feedback controls.

The first, or controlled, enzyme in such sequences is called a regulatory, or allosteric, enzyme. The latter term, which implies "other space" or "other site," was coined by Monod and his colleagues to fit their hypothesis that such regulatory enzymes have two sites—one specific for its substrate (the catalytic site) and a second specific for the allosteric effector (the effector site). The effector may either activate or inhibit, depending on the enzyme. Many such allosteric or regulatory enzymes are now known,and some are multivalent, i.e., they can be activated or inhibited by more than one type of effector molecule, formed as end products of two or more diverging multienzyme sequences.

It has been postulated that allosteric enzyme molecules may exist in two forms in labile equilibrium. One of these is the catalytically active form E_s, the other is an inactive form E_i. Normally, in the absence of the allosteric inhibitor, all of the enzyme molecules are able to combine with substrate molecules and thus participate in catalysis. The two forms E_s and E_i are postulated to differ in conformation, probably only slightly. These relationships can be expressed as follows:

$$E_s \rightleftharpoons E_i \quad ,$$

$$E_s + S \rightleftharpoons E_s \cdot S \quad .$$

However, when the allosteric inhibitor I is present, it combines with form E_i and thus "traps" the enzyme in the inactive form:

$$E_s \rightleftharpoons E_i \quad ,$$

$$E_i + 1 \rightleftharpoons E_i \cdot I$$

The two forms have widely different affinities for S and I, presumably because of the conformational difference. Of special interest is the increasing evidence that enzyme molecules may have only two conformational states. This implies they engage in an on-off or flip-flop switching process characteristic of binary information-processing systems.

Similar conformational changes have been detected in the interaction of antigen and antibody and between actin and myosin of the myofibril. It is potentially of very great importance for the neurosciences that components of lipid-protein membrane systems may also undergo conformational changes, i.e., phase changes in either the lipid or protein phases, or both. Such changes in membrane structure may accompany or be responsible for the action potential and the associated changes in permeability to ions. The conformations of macromolecules or of supramolecular assemblies is therefore not rigid or fixed; they undergo rapid changes in the course of their activity. Such conformational changes are probably quite specific in their geometry.

Kinetics of cellular reactions

If conformational changes are, in fact, essential elements in biodynamic processes involving macromolecules, we must inquire into the rates of such changes and their relationships to the rates of cellular processes, specifically those associated with neurofunction.

In Table II are shown some data on the rates of various types of biochemical and chemical reactions.[4] The values given for the rates of protein and nucleic acid synthesis are for *Escherichia coli* cells having a division time of twenty minutes; they are therefore maximal rates. In any case, it is clear from comparison of these figures that the rate-limiting steps in macromolecule synthesis are the enzyme-catalyzed reactions by which covalent bonds are formed. It is also clear that the making and breaking of hydrogen and ionic bonds and of hydrophobic interactions can proceed at a much higher rate than can the over-all catalytic cycle of an enzyme. This means that conformational changes of macromolecules can occur at much higher rates

TABLE II

Rates of some biochemical and chemical processes

Protein synthesis (*E. coli*)	$\sim 10^2$ amino acids sec^{-1}
RNA synthesis (*E. coli*)	$\sim 10^4$ nucleotides sec^{-1}
DNA synthesis (*E. coli*)	$\sim 10^4$ nucleotides sec^{-1}
Enzyme turnover numbers	10^5 cycles sec^{-1}
Enzyme-substrate interaction	10^7 M^{-1} sec^{-1}
Antigen–antibody interaction	10^7 M^{-1} sec^{-1}
Helix → coil transition (denaturation or melting)	
Polypeptide ($n \geqq 100$)	10^6 sec^{-1}
Polynucleotide ($n \geqq 100$)	10^6 sec^{-1}
Hydrogen bonding	10^9 to 10^{10} M^{-1} sec^{-1}
Ionic interactions	10^9 to 10^{11} M^{-1} sec^{-1}
Hydrophobic interactions	10^8 M^{-1} sec^{-1}

(Extracted from Eigen and de Maeyer, Note 2)

than enzyme-catalyzed reactions, as is confirmed by studies of the rate of denaturation or melting of macromolecules. Weak bonds may thus play a special role in biology, because they may undergo making and breaking at much higher rates than do covalent bonds; conformational changes in macromolecules may be a molecular device selected during evolution to accommodate very fast biological processes that cannot be carried out via the usual covalent reactions of organic molecules.

We may now compare some of these values to the time constants for electrical changes in nerve cells, which are of the order of 10^{-3} per second, and for laying down the memory trace, which may occur in a matter of several seconds. The data in Table II suggest that, by some stretching of available information, the maximum rate of protein synthesis is such that it could just barely be compatible with the laying down of memory in the form of the covalently bonded primary backbone of protein molecules. But this may be stretching the data too far. Kinetically speaking, it is more probable that the memory mechanism involves changes in the conformation of macromolecules, or in conformational interactions among a group of macromolecules, whether this occurs on the membrane surface at specific nerve endings, in a specific circuitry, or within the cytoplasm of neurons.

Not all conformational changes in macromolecules are fast reactions; we have seen that renaturation of enzyme molecules after complete melting may be very slow. But even slow conformational changes may be biologically useful. In fact, Katchalsky and Oplatka have pointed out that the phenomenon of hysteresis in conformational transitions of macromolecules represents a form of molecular memory.[5]

Summary

From the simple examples developed in this introductory and orienting discussion, it is clear that the three-dimensional conformation of protein molecules, programmed into them by the one-dimensional genetic code of DNA, is a basic and central element in the structure of protein molecules. In addition, specific changes in conformation of macromolecules appear to be essential elements in their dynamic function. The kinetics of such conformational changes are thus crucial factors in their biological role. These considerations apply to the action of enzymes, to allosteric control systems, to enzymatic biosynthesis of polynucleotides, and in genetic coding.

As will be seen in the chapters that follow, such conformational changes are also central elements in the induction and repression of enzyme synthesis, in replication and transcription of DNA, and in genetic recombination. The antigen–antibody interactions in the immune response also involve such specific conformational interactions. Conformation and conformational changes represent the leitmotiv of modern molecular biology, and they can be expected to be central elements in the molecular organization of the neuron.

Weak Interactions and the Structure of Biological Macromolecules

NORMAN DAVIDSON

GENERALLY SPEAKING, the atoms in a biological macromolecule, like the atoms in any molecule, are connected with other atoms by covalent bonds. This pattern of connections, known as the primary structure, does not completely specify the three-dimensional configuration of the molecule because of the possibilities of rotation about many of the bonds, and because of the many ways in which solvent or other molecules in the surroundings can associate with the macromolecule of interest.

For a large molecule a large number of configurations are possible, given only the constraint of the pattern of primary covalent bonds. In the native state, for most biopolymers, only one or a few of the structures actually occur in significant amounts. There are several interactions between non-bonded atoms, which are individually weaker than typical covalent bonds, that operate to determine the preferred configurations of the molecule.

As a result of being weak, the interactions of interest are generally rather labile, so that mild changes in the environment (such as small temperature changes, changes of solvent, or the addition of small molecules to the solvent) can cause many configurational changes to occur, in vivo or in vitro, without changes being made in the primary structure. The denaturation and renaturation of proteins and nucleic acids, the binding of an antibiotic such as actinomycin to DNA, the association of a messenger RNA molecule with an sRNA molecule and a ribosome, the reversible association of a substrate with an enzyme, are all examples of such changes.[1]

The most important weak interactions that strictly fit the definition stated above are: (1) electrostatic forces, (2) van der Waals or London dispersion forces, and (3) hydrogen bonding. As we shall see, there is a certain special kind of combination of (2) and (3), known as a hydrophobic interaction, which is of unique importance and is designated as an interaction in its own right. In addition, we should mention, although we shall not discuss them in detail, (4) "coordinate bond formation" between a metal ion and a ligand, and (5) sulfur-sulfur bond formation.[2] These last two are examples of covalent bonding, but the bonds are often sufficiently labile so that they dissociate and re-form readily under mild changes in conditions and contribute to configurational changes.

A systematic, theoretical discussion of the nature of these forces, beginning with first principles, is beyond the scope of our discussion. We shall begin with brief dogmatic statements as to the nature of the several interactions. We shall then examine several cases, mainly ones of biological interest, in which these interactions, singly or working together, affect the structure of a molecule or a system. The modest object of our essay is thus to convey a sufficient understanding of the nature and magnitude of these weak interactions to permit the reader to appreciate their role in the various phenomena of molecular biology that are described in later chapters of this book.

The "small differences difficulty"

For many of the reactions and structural changes of interest, there is a characteristic difficulty in estimating the magnitudes and indeed the sign of the thermodynamic functions (ΔG, the change of Gibbs free energy; ΔH, the enthalpy change; and ΔS, the entropy change). Usually, they may be represented symbolically by the equation

$$A \sim B + C \sim D \leftrightharpoons A \sim D + B \sim C. \qquad (1)$$

The symbol $A \sim B$ means A interacting with B, and the equation asserts that in the change of interest, an AB and a CD interaction have been replaced by an AD and a BC interaction. We can then write, in an obvious notation,

$$\Delta G = G(A \sim D) + G(B \sim C) - G(A \sim B) - G(C \sim D). \qquad (2)$$

It may be possible at the present state of theoretical chemistry to estimate with moderate accuracy the strength of the AD interaction $G(A \sim D)$, and the strengths of the other interactions in Equation 2, but it is much more difficult, and at present often not possible, to calculate the small differences required for the evaluation of ΔG with sufficient accuracy.

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Electrostatic forces

The potential energy of interaction between two point charges or two spherical charge distributions of total charge Z_1 and Z_2, which are at a distance R and immersed in a solvent of dielectric constant D, is

$$\Phi = Z_1Z_2/DR. \qquad (3a)$$

This energy may be taken as an approximation for the free energy of interaction between two ions in a solvent of dielectric constant D, and Equation (3b) predicts free energies and equilibrium constants for ionic association reactions of the correct order of magnitude.

$$\Delta G = Z_1Z_2/DR. \qquad (3b)$$

The equations assert that the strength of the interaction increases with increasing ionic charge, and with decreasing distance of approach. Electrostatic interactions are stronger in a medium of low dielectric constant, such as the lipid medium of a membrane, than in a medium of high dielectric constant, such as water.

The electrostatic forces between adjacent Na^+ and Cl^- ions in NaCl crystals or between Mg^{++} and $O^=$ ions in MgO crystals are quite strong and are of the same order of magnitude as those between atoms in covalent bonds. As we shall see, the interactions between two ions in aqueous solution are much weaker, and are comparable to the other weak interactions to be discussed here.

The model of a solvent as a continuous medium with a dielectric constant D is not adequate for a molecular solvent, especially for a polar solvent such as water. Water molecules have a dipole moment. As a result of both electrostatic (ion-dipole) and covalent interactions, cations are usually strongly hydrated; in the case of Mg^{++}, for example, the hydrated cation is $Mg(H_2O)_6^{++}$. Because of electrostatic interactions and hydrogen bonding interactions, an anion such as $SO_4^=$ is also hydrated, although usually anion hydration energies are less and their hydrate structures less specific. An ion association reaction between a magnesium ion and an anion can then be written as

$$Mg(H_2O)_6^{+2} + SO_4(H_2O)_y^{-2} \rightarrow Mg \cdot SO_4(H_2O)_x + (6 + y - x)H_2O. \qquad (4)$$

In the above equation the ion pair is hydrated to the extent of xH_2O molecules, whereas the $SO_4^=$ anion has a hydration number of y. It is generally believed that $x < y < 6$, so that water molecules are released in reaction (4). From this point of view, in an ion-pair association a certain number of cation-to-water and anion-to-water bonds are broken and there is a favorable electrostatic association; thus the "small differences difficulty" applies.

Furthermore, in Equation (4), $6 + y - x$ bound water molecules are released per ion pair formed. This contributes a positive entropy change to reaction (4). From Equation (3b), and the general thermodynamic relation, $\Delta S = -\partial\Delta G/\partial T$, we obtain $\Delta S = Z_1Z_2(\partial D/\partial T)/D^2R$. Because $\partial D/\partial T$ is negative for a polar solvent such as water, the continuous medium point of view also predicts a positive entropy change when two ions of opposite charge interact. Positive entropy changes are, in fact, usually observed in association reactions in which the main interaction is electrostatic. In fact, it is often the case that $\Delta H > 0$, so that the equilibrium association constant *increases* with *increasing* temperature.

Dispersion forces and van der Waals forces

Any two electronic systems—for example, two xenon atoms, two benzene molecules, or a xenon atom and a benzene molecule—show a weak attractive interaction, caused by slight correlations between the motions of the electrons in the interacting molecules. The energy of this interaction, which is known as the London dispersion force, falls off as the sixth power of the distance between the interacting systems. The strength of the interaction increases with increasing number and looseness (polarizability) of the electrons in the systems.

Figure 1 represents the variation in melting and boiling points for the noble gases and for several series of hydrides of non-metallic elements belonging to the same column of the periodic table. Here the dispersion forces are the main source of the attractive intermolecular interaction. The regular increase in boiling point in the series He to Rn or CH_4 to SnH_4 means a corresponding increase in the strength of intermolecular forces.

In addition to the attractive interaction described above, there is a steep repulsive potential between two atoms or molecules when their interatomic distance decreases to the point at which the electron clouds interpenetrate. This combination of attractive and repulsive forces is known as the van der Waals forces, and they are the most universal source of weak intermolecular interactions.

Hydrogen bonds

As shown in Figure 1, the order of intermolecular forces for the hydrides of the sixth group elements H_2S, H_2Se, H_2Te increases as the atomic number increases, as would be expected for van der Waals interactions; but the intermolecular forces between H_2O molecules are much greater than those between any other sixth group hydrides. The same trends occur in the fifth and seventh groups. The anomalously large intermolecular forces for the substances NH_3, H_2O, and HF are caused by hydrogen bonding. The

hydrogen bond is a special interaction which may be schematically represented as B:···HA, where HA is a weak acid and B: is a basic atom with an unshared electron pair. The strength of the hydrogen bond increases with increasing acidity of HA and increasing basicity (proton-attracting power) of B. (When this trend proceeds too

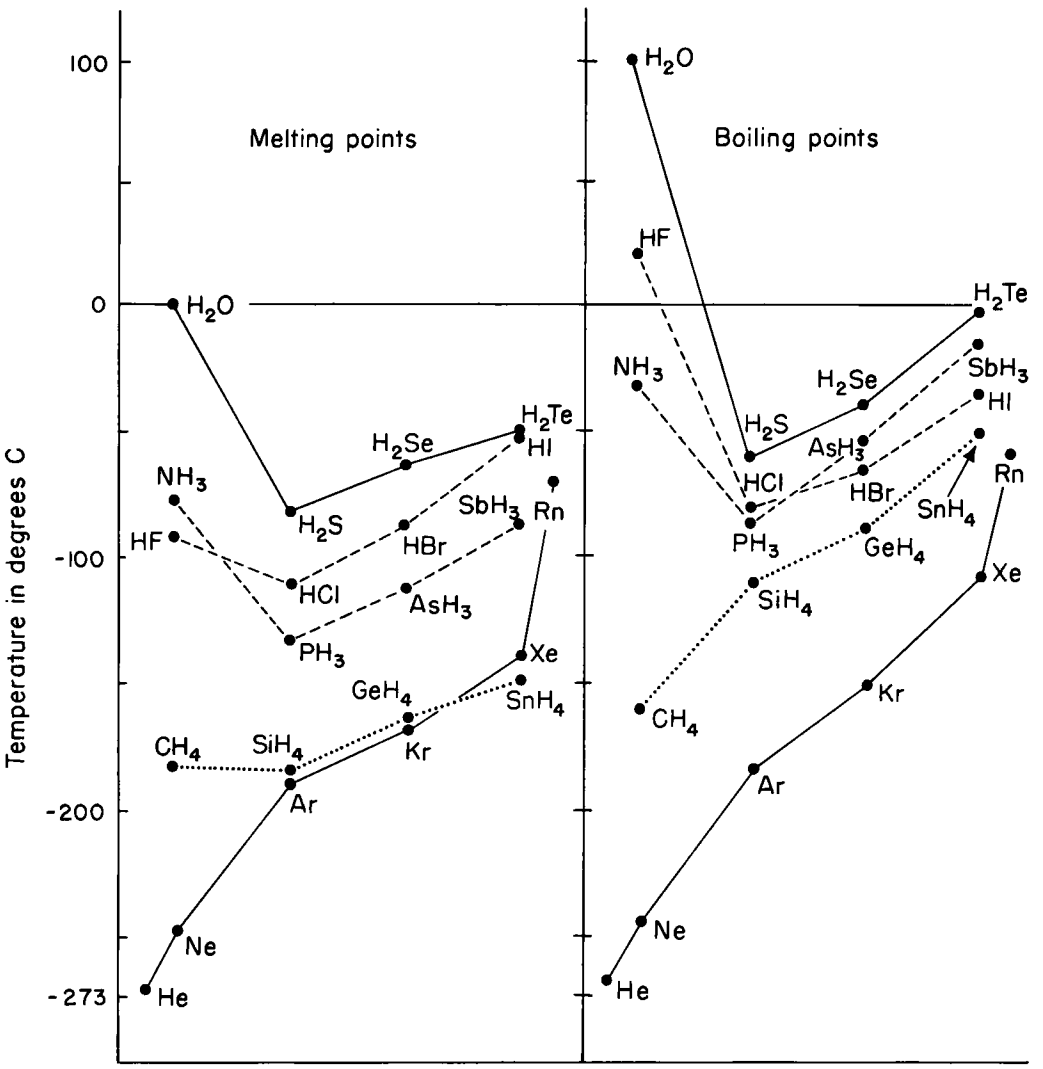


FIGURE 1 Melting points and boiling points of hydrides of nonmetallic elements, showing a general increase in intermolecular forces (London dispersion forces) with increasing atomic number in any one column of the periodic table, and showing abnormally high values for hydrogen fluoride, water, and ammonia caused by hydrogen bond formation. (From Pauling, Note 3)

far, one gets proton transfer to B, resulting in the structure $B — H^+ \cdots :A^-$, with the hydrogen bond now between the BH^+ ion and the $:A^-$ ion.)

A hydrogen bond is an interaction of a three-atom system, $B_1 : \cdots HA$. Usually the atoms are in a linear configuration. Ordinarily, one hydrogen atom can form only one hydrogen bond, and cannot simultaneously interact with a second group, B_2. Van der Waals forces do not show this same property; a solute molecule A immersed in a solvent of molecules B can attract and interact with as many B molecules as can fit around it; the strength of any one AB interaction is scarcely weakened by the occurrence of other AB interactions.

The inference can be drawn from Figure 1 that hydrogen bonds are weaker than ordinary covalent bonds but stronger than van der Waals forces. Several extensive discussions of hydrogen bonding have been published recently.[4,5]

Hydrophobic interactions

The term "hydrophobic bond" was coined by Kauzmann[6,7] to connote the tendency of the nonpolar groups, mainly the nonpolar side chains of certain amino acids, to adhere to one another in an aqueous environment. This is closely related to the tendency of the nonpolar hydrocarbon chains of soap molecules to associate in aqueous solution into micelles and to the tendency of nonpolar organic substances to be relatively immiscible with water—that is, to prefer to interact with other nonpolar molecules. There is no simple integral relation for the number of groups that interact with a given group; hence, we consider that the term hydrophobic interaction is preferable to hydrophobic bonding. These interactions are of critical importance for the structure of proteins and nucleic acids.

Abstractly, if R refers to a nonpolar group, the formation of a hydrophobic interaction can be represented by the equation

$$R \sim H_2O + R \sim H_2O \rightarrow R \sim R + H_2O \sim H_2O \qquad (5)$$

where $R \sim H_2O$ means an isolated R residue interacting with the solvent water molecules, etc. In general, reaction (5) has a favorable free energy. The prediction of the energetics of the reaction, however, suffers from the "small differences difficulty." The London dispersion forces contribute an attractive term to each interaction in Equation 5. However, water is known to be capable of forming special, organized, hydrogen-bonded structures around many hydrocarbon residues[8-11] and, most importantly, the liquid water itself is a highly structured liquid in which most of the protons are used for intermolecular hydrogen bonding. In fact, it appears that the strongest interaction that drives reaction (5) to the right is the H_2O–H_2O interaction; hydrophobic interactions occur mainly because water is hydrophilic.

Némethy, Scheraga, and coworkers[12,13] have developed a statistical mechanical theory of hydrophobic interactions. This theory includes detailed molecular models for the various structures involved, including those of liquid water, and it is not known, at present, whether the energies assigned to these various structures are correct. Nevertheless, the theory does seem to predict correctly some of the main qualitative features of hydrophobic interactions. Figure 2 illustrates some of the structures proposed for nonpolar amino acid side chains in contact with each other. Table I is illustrative of the thermodynamic predictions of the theory. The main points in the table are: (1) at low temperatures, positive entropy change causes the negative free energy of a hydrophobic interaction between aliphatic-type side chains of amino acid residues; (2) at low temperatures, the enthalpy change is positive—that is, it opposes the hydrophobic interaction, (3) the enthalpy and entropy both decrease markedly with temperature, and (4) the strength of the interaction increases with increasing temperature, at least over a certain temperature range. These observations had previously been made by Kauzmann[6,7] on the basis of thermodynamic data on the solubility of nonpolar compounds in water.

Sinanoğlu and Abdulnur[14] have also developed a detailed model for a hydrophobic interaction for the specific case of the interaction between the planar aromatic purine and pyrimidine ring systems of DNA in its Watson-Crick structure, as compared to the denatured state in which the organic rings are individually immersed in water. Their result is that the main stabilizing contribution is the positive free energy required to create a cavity in water (caused

TABLE I

Theoretically calculated thermodynamic parameters for the formation of a leucine-isoleucine hydrophobic bond of maximum bond strength[13]

t °C	ΔF° kcal/mole	ΔH° kcal/mole	ΔS° e.u.
1	-0.8_4	2.3	11.6
10	-0.9_5	2.0	10.4
20	-1.0_5	1.6	9.2
25	-1.0_9	1.4_5	8.5
30	-1.1_3	1.2	7.8
40	-1.2_1	0.8	6.5
50	-1.2_6	0.4	5.1
60	-1.3_1	−0.1	3.6
70	-1.3_4	−0.6	2.1

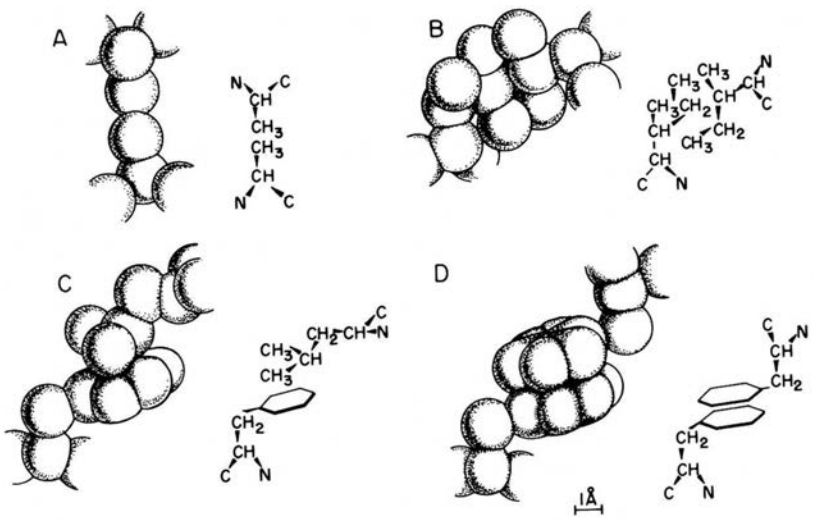


FIGURE 2 Illustrative examples of hydrophobic bonds between pairs of isolated side chains. The hydrogens are not indicated individually. Atoms are drawn to scale, but with the van der Waals radii reduced by 20 per cent for the sake of clarity. The structural formulas to the right of each space-filling drawing indicate the arrangement of the atoms. A: alanine-alanine bond (minimum strength). B: isoleucine-isoleucine bond (maximum strength). C: phenylalanine-leucine bond (max. strength). D: phenylalanine-phenylaline bond (maximum strength). (From Némethy and Scheraga, Note 12)

essentially by its high surface tension) and this is largely enthalpic, not entropic, in contrast to the results of the Némethy and Scheraga studies.

The next few sections are devoted to a description of phenomena in which the interactions previously discussed play an important role. These examples are by no means exhaustive as to all the major effects; it is hoped that they do impart some general appreciation of the operation of weak forces and are at the same time interesting per se. Furthermore, an effort has been made to select some examples that differ from those cited in a number of other reviews of these subjects.

Ion association

In dilute aqueous solution, cations and anions can associate to form ion-pair complexes even in cases where there is little or no contribution from covalent bond formation.[15-17] For example, the equilibrium constants of the association reactions of the cations, M^{n+} with the sulfate anion,

$$M^{n+} + SO_4^{-2} \rightleftharpoons M \cdot SO_4^{(n-2)} \qquad (6)$$

are 5.0, 10.0, 170, 170 liter mole^{+1} for the ions Na^+, K^+, Mg^{++}, and Ca^{++} (these particular constants are for 25°C and extrapolated to zero ionic strength).[15]

We cite an additional case of direct biological interest. The association constants of the adenosine triphosphate anion, ATP^{-4}, with the ions Na^+, K^+, Mg^{++}, Ca^{++} are 15, 14, 25,000, and 21,000 liter mole^{-1} (at 30°C, in a supporting electrolyte medium of 0.1 M N-ethylmorpholine buffer).[18] The importance of the examples cited so far is that the magnitudes of the association constants are such that 10^{-4} to 10^{-1} M concentrations of cations are needed for significant complexing of anions of charge -2 to -4, and that the binding is stronger the higher the charge on the ions. Many such labile equilibria occur under physiological conditions. Furthermore, and as already mentioned, ion association reactions occur more readily in media of low dielectric constant.

The effects of ionic radius, predicted by Equation 3, are not apparent in the results quoted so far, in that there are no significant differences between the smaller ions, Na^+ and Mg^{++}, and the larger ions of the same charge types, K^+ and Mg^{++} respectively. This is essentially because of the small differences situation, in that water molecules tightly bound to the cations are being displaced by the anions in the ion association reactions. However, the effect of ionic size can be seen clearly in the kinetics of formation and dissociation of certain ion-pair complexes, even though they are not apparent in the equilibrium constants.[19] For example, the formation rate constants for the $Mg^{++} - ATP^{-4}$ and $Ca^{++} - ATP^{-4}$ complexes at 25°C are 1.2×10^7 and $\geq 1 \times 10^9$ liter mole^{-1} second^{-1}, respectively, and the dissociation rate constants are 1.2×10^3 and $\geq 2 \times 10^5$ second^{-1} respectively. Thus, there is a large difference in rates and no significant difference in equilibrium constants. It is probable that the main reaction path for the formation reaction is a pre-equilibrium in which a water molecule is dissociated from the hydration sphere of the Mg^{++} and Ca^{++} ions; in this dissociation step, as well as in the dissociation of the cation-adenosine triphosphate (ATP) complexes, the effects of ionic size are marked. Hence, the binding of water molecules and the binding of the ATP anion are stronger for the smaller cation (Mg^{++}) than for the larger cation (Ca^{++}).

Histone-nucleic acid interactions

The association of histones and DNA in the nucleohistone complexes is an interesting example of an interaction of biological macromolecules in which electrostatic forces play a major, but not an exclusive, role.

Histones are positively charged proteins, rich in the basic amino acids lysine and arginine, with molecular weights of about 10,000 to 20,000. Nuclear DNA in higher organisms occurs as a nucleoprotein complex in which the main protein constituents are the histones. The combining ratios are such that for each negative phosphate group of the nucleic acid there is approximately one positively charged amino acid residue of a histone. The complexes are of great biological interest because they may be important for the regulation of gene expression.[20]

There are several kinds of histone molecules, and they have been classified into fractions on the basis of chromatographic separations.[21] (It is by no means certain that each fraction for any one species is a homogeneous protein, but the degree of heterogeneity is not too great.[22]) Some characteristics of the several fractions are given in Table II. The most obvious is that the lysine-plus-arginine content is fairly constant, but their ratio varies greatly. The nucleo-histone complex is dissociated by increasing the salt concentration, and there have been several recent quantitative studies dealing with this dissociation.[23,24] These results are summarized in Table III.

Several important points may be noted. First, increasing the concentration of added inert salt causes dissociation of the complexes. The salt weakens the electrostatic interactions by shielding. This effect is the principal evidence that there are major electrostatic contributions to the binding.

Second, a given histone fraction dissociates over a rather narrow range of salt concentration. This indicates that the binding is cooperative. That is, a bound histone molecule is either completely dissociated or it is completely associated by being attached to the nucleic acid in such a way that all of its positively charged residues interact with different negatively charged phosphate groups of the nucleic acid.

TABLE II

Properties of histone fractions

Fraction[21]	Lysine content mole %	Arginine content mole %	Average charge/ residue[a]	Percent of total calf thymus histone
I	25.8	2.9	0.23	30
II	13.5	7.9	0.10	50
III–IV	9.9	12.8	0.13	20

a) Calculated from the amino acid composition.[21]

TABLE III

Salt dissociation results for nucleohistones

Histone fraction	Salt concentration range over which histone fraction dissociates for NaCl (molar)	$NaClO_4$ (molar)
I	0.4–0.5	0.15–0.25
II	0.8–1.0	0.36–0.45
III–IV	0.8–1.0[b]	~0.45
	0.9–1.6	>0.45

a) These results were obtained by gel electrophoresis or column chromatography analysis of the protein in the supernatant (histone) and pellet (nucleohistone) fractions after sedimenting native nucleohistone through a solution with a given salt concentration.
b) By disc electrophoresis, the histone III–IV fraction consists of two fractions, each about 10% of the total histones.[23,24] The amino acid differences between these fractions are not yet known, but they show different salt dissociation properties.

Third, different histone fractions dissociate at different salt concentrations, and the ease of dissociation does not correlate with the over-all charge density of the histone molecule (cf., Table II). Precise conclusions cannot be derived from this observation because the detailed structure of the nucleohistone complexes is not known. However, it is plausible that the peptide chain of the histone is stretched along the nucleic acid chain, with positive histone and negative nucleic acid charges in close juxtaposition. Other interactions, hydrophobic or hydrogen bonding, may well contribute some of the binding force and be mainly responsible for the differences between histone fractions.

Fourth, on a concentration basis, $NaClO_4$ is about twice as effective as NaCl in causing dissociation. This shows that even for these simple salts the ions are not acting solely as point charges. It is likely that the perchlorate anion itself interacts rather strongly with the histone protein, perhaps by a hydrogen bonding interaction with the positively charged amino and guanidino groups of lysine and arginine.

Other positively charged proteins, such as ribonuclease and cytochrome-C, bind to nucleic acids. The binding is weaker, however, and dissociation can be effected at salt concentrations about tenfold lower.[25]

In summary, then, the histone-DNA interaction is cooperative and is largely the result of an electrostatic interaction between positively charged sites on the histones and negatively charged sites on the nucleic acid. Hydrogen bonding or hydrophobic interactions are also significant,

and are probably responsible for the differences in binding affinity between different histone fractions.

Proteins

Several recent reviews have dealt in detail with protein structure and the factors affecting it,[26,27] and we shall not give an extensive discussion here. Furthermore, we shall not attempt to describe the several ordered repeating structures known to occur in natural polypeptides—the α-helix, the parallel and antiparallel pleated sheets, and the collagen fold.[28] It should be emphasized that these satisfy the criteria originally proposed by Pauling, Corey, and Branson[29,30] for acceptable structures—that there should be the maximum number of linear hydrogen bonds, and that the bond angles and lengths should be those known to be energetically favorable, based on structural studies with small molecules.

An examination of several features of the known molecular structure of myoglobin conveys a realistic appreciation of hydrophobic interactions and of several other aspects of protein structure.[27,31,32] Myoglobin has a high α-helix content, as indicated in Figure 3. Let us examine, as an example, the environment of some of the amino acid residues in the E α-helical segment. We start with E-7, a histidine for which the imidazole ring is engaged in a special interaction with the heme group at the oxygen binding site. In the figure the residues from E-8 through E-20 have been classified as to whether the side chains point out toward the aqueous environment outside or toward the inside of the protein. Those that point in all have nonpolar hydrophobic side chains (glycine, valine, leucine). All the polar residues (in this case, two examples of threonine and one of lysine) point out, as do several smaller nonpolar amino acids (glycine and alanine).

These features hold in general.[31] Almost all of the polar groups (except for those performing some special function, such as the histidines interacting with the heme group) are on the surface. The interior is made up of nonpolar residues in van der Waals contact with neighbors. The structure is compact, with little empty space inside. In addition, there are few water molecules inside, while most of the polar groups on the surface have oriented bound water molecules attached. Otherwise, there is no "iceberg-like" structure of oriented water molecules around the protein.

In myoglobin, the heme group is not covalently attached to the polypeptide chain. It is held in place largely by hydrophobic interactions with amino acid residues. It is also held in place by a coordinate bond from the imidazole of histidine F-8 to the iron of the heme group. In some proteins, the heme groups are covalently attached to the amino acid backbone. This is the case in cytochrome-C, for example, where there are two covalent bonds from the heme to cysteine sulfurs. The iron atom is coordinated to four heme nitrogens, one imidazole of a histidine, and one methionine sulfur.[33] The coordinate bonds to the iron probably play a significant role in stabilizing the native structure of such molecules.

It is of interest that if hydrated myoglobin crystals are exposed to xenon gas, a 1:1 compound is formed in which the xenon is located in a pocket close to the imidazole ring of F-8, one pyrrole ring of the heme group, and nonpolar residues of the nonpolar amino acids F-4, F-5, G-5, H-14, and H-18. Thus, the anesthetic gas, xenon, is bound to myoglobin in what is essentially a hydrophobic pocket.[34]

Effect of Urea on Protein Denaturation and Hydrophobic Interactions In the denaturation of a globular protein in aqueous solution, the peptide chain unfolds, the hydrogen bonds between amino acid residues are broken, and the various hydrogen bonding groups now donate or accept hydrogen bonds to the water (or the urea dissolved therein). The nonpolar residues are immersed in water; that is, the hydrophobic interactions are broken. The change between internal hydrogen bonding and hydrogen bonding to the solvent may not contribute much to the energetics of the reaction, because the total number of such bonds is unchanged. The main driving force stabilizing the native structure are then the hydrophobic interactions. (This is not to say that the hydrogen bonds are not important in determining the native structure—a structure without good hydrogen bonds is energetically unfavorable.)

At one time, it was thought that urea is a denaturant because of its ability to disrupt intramolecular hydrogen bonds.[35] It now appears that the main effect of urea is to decrease the strength of hydrophobic interactions. There is clear evidence that urea weakens hydrophobic interactions,[36] as seen in Table IV. The essential point is that nonpolar amino acids, such as leucine and phenylalanine, are more soluble in 8 M urea than in water, whereas the reference amino acid, glycine, with no side chain, and threonine and histidine, with polar side chains, are slightly less soluble. These data have been used for a systematic, theoretical discussion of the denaturation of proteins in urea.[37]

Nucleic acids

A general discussion of the structure and function of the nucleic acids is not appropriate here. I wish only to point out some of the evidence illustrating the contributions of the several interactions that contribute to the stability of the two-stranded Watson-Crick structure of native DNA.

Electrostatic, hydrogen bonding, and hydrophobic interactions are all significant.

Figure 4 shows the hydrogen bonding, base-pairing scheme for the complementary adenine-thymine (AT) and guanine-cytosine (GC) base pairs of DNA.[38] Figure 5 is a drawing of the Watson-Crick structure.[39] One can see that the planar base-paired purine and pyrimidine ring systems are stacked one upon another in a compact way. If a native DNA molecule in solution is heated under suitable conditions, the hydrogen bonds are broken, the two strands separate, and each assumes a random coil configuration in which the individual purine and pyrimidine residues are individually immersed in the aqueous solvent and hydrogen bond with the solvent. This is the phenomenon of denaturation. It is a cooperative phenomenon and takes place over a narrow temperature range, so one can speak of the denaturation or melting temperature.

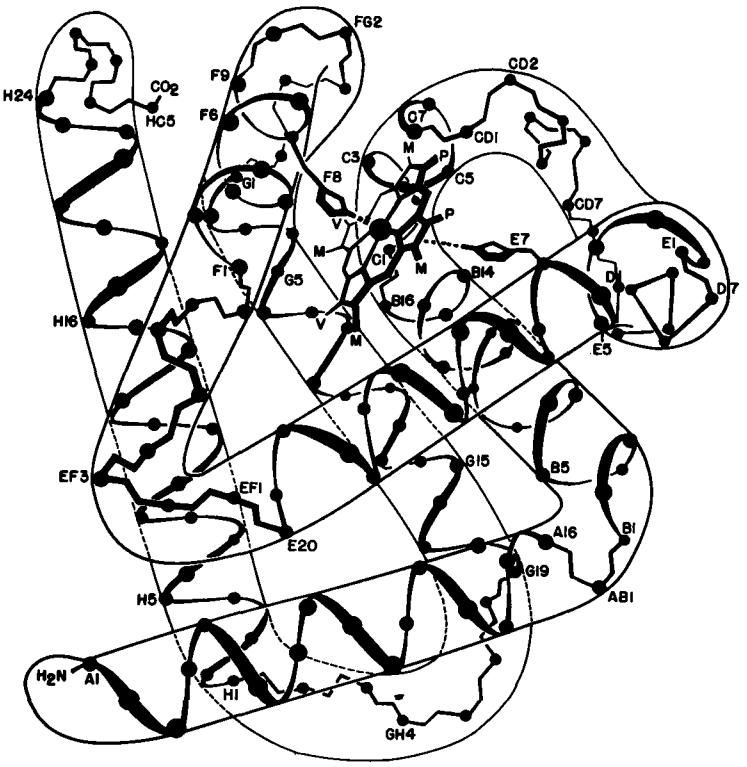
We first note that electrostatic forces contribute an unfavorable term to the free energy of the native structure. The individual strands are negatively charged—because of the phosphate groups—and there is an electrostatic repulsion between them. These electrostatic interactions are diminished by added supporting electrolyte. In the presence of a 1:1 supporting electrolyte such as NaCl, the de-

TABLE IV

Solubilities at 25.1°

Solute	Solubilities at urea concentration of:				
	0	2*M*	4*M*	6*M*	8*M*
	g/100 g solvent				
Glycine	25.1	22.7	20.4	17.5	15.0
Alanine	16.7	15.3	13.7	12.1	10.6
Leucine	2.16	2.37	2.34	2.29	2.25
Phenylalanine	2.80	3.42	3.94	4.33	4.67
Tryptophan	1.38	1.98	2.65	3.31	3.95
Methionine	5.59	6.19	6.74	7.00	6.99
Threonine	9.80	9.56	9.07	8.31	7.41
Tyrosine	0.0451	0.0600	0.0732	0.0870	0.0986
Histidine	4.33	4.66	4.70	4.46	4.23
Glutamine	4.30	4.49	4.49	4.30	4.02
Asparagine	2.51	2.89	3.08	3.22	3.32
Diglycine	23.3	21.3	19.5	17.2	15.5
Triglycine	6.30	7.76	8.00	8.60	7.84
Carbobenzoxyglycine[a]	0.456	0.879	1.47	2.38	3.65
Carbobenzoxydiglycine[a]	0.075	0.150	0.260	0.395	0.595

a) Recent studies with these derivatives indicate that they may exist in an alternative crystalline modification (with lower solubility) in urea solutions. It is believed, however, that the data given here all represent equilibrium with the original crystalline form. (From Nozaki and Tanford, Note 36)



	Amino acid	Environment		Amino acid	Environment
E7	His	Heme	E14	Ala	out
E8	Gly	in	E15	Leu	in
E9	Val	in	E16	Gly	(?)
E10	Thr	out	E17	Ala	out
E11	Val	in	E18	Ileu	in
E12	Leu	in	E19	Leu	in
E13	Thr	out	E20	Lys	out

FIGURE 3 α-Carbon diagram of myoglobin molecules obtained from 2 Å analysis. Large dots represent α-carbon positions. For detailed explanation of the labeling and identification of the amino acid residues, see Notes 31 and 32. Stretches of α-helix are represented by smooth helix with exaggerated perspective and are given letter-number labels (except for helix D1–D7, nearly normal to plane of paper, which is shown by single straight lines connecting α-carbons). Nonhelical regions are designated by letter-letter-number symbols and represented by three-segment zigzag lines between α-carbons. Fainter parallel lines outline high density region as revealed by 6-Å analysis. Heme group framework is sketched in forced perspective, with side groups identified by M = methyl, V = vinyl, P = propionic acid. Five-membered rings at F8 and E7 represent histidines associated with heme group.

The identity and environment of some of the amino acid residues in the E α-helical segment are indicated below the diagram. (From Dickerson, Note 32)

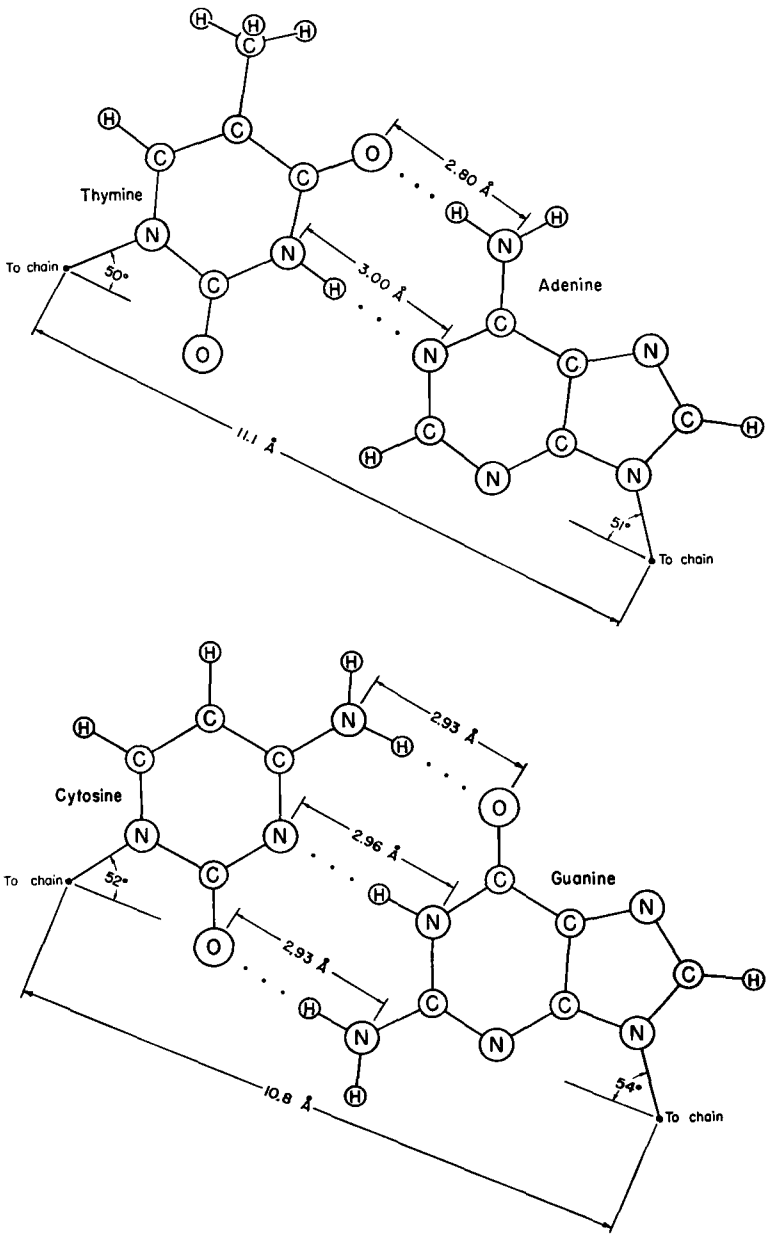


FIGURE 4 Representation of the complementary hydrogen bonding scheme for an adenine-thymine and a guanine-cytosine base pair. (From Pauling, Note 38)

naturation temperature decreases with decreasing salt concentration (there is an 18°C decrease in the denaturation temperature for a factor-of-10 decrease in sodium-ion concentration in the range 10^{-4} M < Na^+ < 10^{-4} M).[40-42] Small concentrations of divalent cations, such as Mg^{++}, or positively charged polymers, such as the histones, markedly raise the denaturation temperature. (The cations are the important ions for this effect; NaCl and Na_2SO_4 at equal sodium-ion concentration confer equal stability; $MgCl_2$ is much more stabilizing than NaCl at equal chloride-ion concentrations.). A theoretical analysis suggests that the electrostatic repulsion contributes an unfavorable free energy of about 0.16 kilocalories per mole of nucleotide pairs in 0.010 M NaCl and 0.04 kilocalories in 0.1 M NaCl.[42] These contributions are small compared to those caused by hydrogen bonding and hydrophobic interactions, but they are responsible for the observed change in melting temperature with salt concentration.

Hydrophobic interactions are important in the stabilization of nucleic acid structures. As already noted, in native DNA each base is hydrogen-bonded to its complement in the other strand, and is engaged in a stacking or hydrophobic interaction with the bases just above and below it. Both hydrogen bonding and stacking interactions are broken on denaturation.

Some conformational changes, however, appear to be caused purely by stacking interactions, with no contribution from hydrogen bonding. One such example occurs for short or long oligomers of riboadenylic acid, at *p*H 7 (there is a hydrogen-bonded, base-paired structure at low *p*H). Several reliable criteria for ordered structures in polynucleotides, notably optical rotation and hypochromism,[43-46] indicate that the molecules have a somewhat ordered structure in aqueous solution at low temperatures. This becomes a disordered structure upon heating. X-ray evidence and the non-cooperative nature of the transition suggest that the ordered structure is single-stranded and does not involve hydrogen bonds.[47] Figure 6 shows the change in absorbance with temperature for polyadenylic acid (poly-A) samples of various chain lengths. The increase in absorbance with increasing temperature is the result of the disruption of the vertically stacked structure. The figure also shows a schematic representation of the transition from a stacked to an unstacked structure.

The quantitative analysis of data like this is somewhat uncertain, and different authors have interpreted their data to give a ΔH for the stacking interaction between −6500 and −13,000 kilocalories per mole of interactions. However, two qualitative points are very clear. First, and most important, there is a strong vertical hydrophobic or stacking interaction. This must also occur in the two-stranded nucleic acids. Second, the degree of order decreases with increasing temperature. Therefore, the enthalpy of the interaction is negative and the main driving force is enthalpic, not entropic. This differs from the hydrophobic interactions of the aliphatic side chains of amino acids in proteins, in which the strength of the interaction increases with increasing temperature. The enthalpic driving force may be characteristic of planar pi-electron systems, as in the purines and pyrimidines. Many

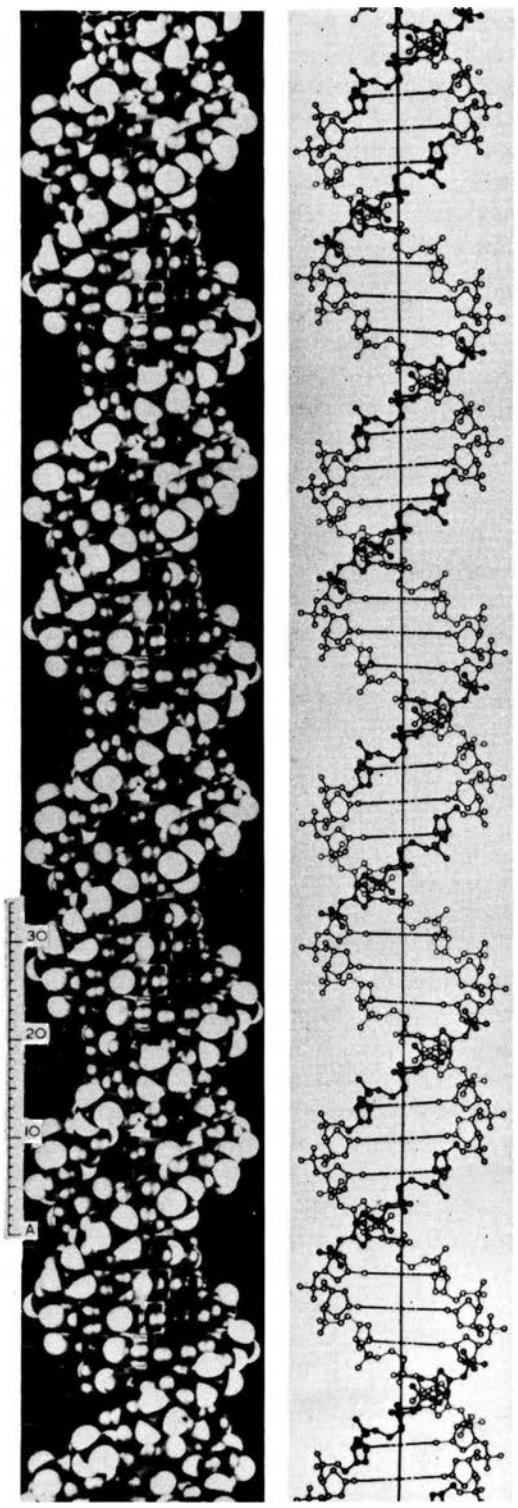


FIGURE 5 Molecular model of DNA structure.

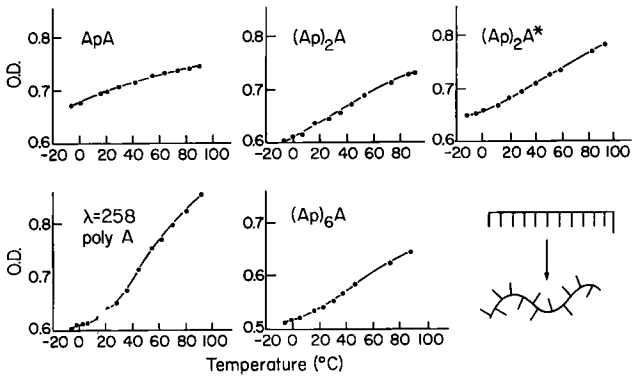


FIGURE 6 Absorbance at 258 mμ of poly A and oligoadenylates as a function of temperature. Solvent is 0.1 M-LiCl-0.01 M-cacodylate buffer, except $(Ap)_2A$*, for which the solvent is 2 M-LiCl-0.01 M-cacodylate (*p*H 7). The increase in absorbance with increasing temperature is a measure of decreased vertical stacking and increased disorder with increasing temperature. A schematic representation of the transition from a stacked to an unstacked structure is at lower right. (From Harbury, Note 33)

other lines of evidence indicate the importance of vertical stacking interactions.[48]

The free energy contribution of hydrogen bonding can be roughly estimated from the observations that base-paired homopolymer systems with three hydrogen bonds per base pair denature 25°–50° higher than do systems with two hydrogen bonds per base pair. Thus, the difference in denaturation temperature of a DNA containing only GC base pairs and one containing only AT base pairs (cf. Figure 4) is 41°.[49] The complex dI:dC (two hydrogen bonds) melts about 55° below the polymer dG:dC (three hydrogen bonds).[50] The complex rA:rU (two H bonds) melts 25° below r2-amino-A:rU (three H bonds).[51] (In the preceding statements, dI stands for polydeoxyinosine, rA for poly-riboadenosine, r2-amino-A for polyribo-2-amino adenosine, etc.)

Analysis of these data in terms of the statistical mechanical theory of denaturation suggests that each hydrogen bond contributes about 1 kilocalorie to the free energy of helix formation; an AT and a GC base pair contribute two and three kilocalories, respectively. Analysis of other experiments on the width of the denaturation transition suggests that the stacking interactions contribute about seven kilocalories per nucleotide pair.[52]

The estimates of the various thermodynamic quantities are not certain at present, but the prevailing attitude among physical chemists who work on these problems is that the hydrogen bonds are less important, in terms of energy, than are the hydrophobic interactions. However,

TABLE V

Shifts in the resonant frequency of the N_1 proton of guanosine and the N_3 proton of uridine upon the addition of other nucleosides

Guanosine	
Nucleoside added	Shift in cycles/sec of N_1 proton
Adenosine	−0.1
Guanosine	−7.1
Cytidine	−134.7
Uridine	−1.2
Uridine	
Nucleoside added	Shift in cycles/sec of N_3 proton
Adenosine	−8.2
Guanosine	0
Cytidine	−0.6
Uridine	0

The solvent was an equal volume mixture of dimethyl sulfoxide and benzene. The chemical shifts were measured at 60 Mc/s and −4°C. The dimethyl sulfoxide resonance was used as an internal standard. The N_1 proton of guanosine and the N_3 proton of uridine were measured at a base concentration of 0.05 M. Then additional nucleosides were added to bring the total molarity to 0.2 M and the proton frequency measured again. The table gives the added compound and the net shift between the two measurements. Because of broadening due to the ^{14}N quadrupole moment, the accuracy of the relative shift measurement was limited to about ±1.0 c/s. (From Note 53)

the hydrophobic interactions are relatively nonspecific, and the hydrogen bonding pattern of the complementary base pairs is entirely responsible for the information content of the nucleic acids, and thus for their central importance in molecular biology.

Furthermore, there have been some recent indications that there may be some special stability, not yet completely understood, associated with the Watson-Crick pattern of hydrogen bonds. These are investigations by nuclear magnetic resonance (n.m.r.) and by infrared spectroscopy of base pairing of suitably substituted, monomeric derivatives of the several important purine and pyrimidine ring systems. These studies have been carried out in organic solvents. As the association is between monomers and the reactions are taking place in organic solvents, there are no significant vertical stacking interactions. An examination of the structures in Figure 4 will reveal that without the constraints of fitting into a helical polymeric structure, a large number of additional base pairing schemes are possible with at least two hydrogen bonds per base pair (for example, A to G, C to U, U to U, A to A, and G to G). The data in Table V show that, by an n.m.r. criterion, the N_1 proton of guanosine (guanine linked to ribose) shows much greater evidence for hydrogen bonding with added C than with itself, with G, with A, or with U.[53,54] Similarly, the N_1 proton of U shows the greatest interaction with adenine derivatives in solution. Similar infrared experiments in which it is possible to actually measure the equilibrium constants for the association also show that A interacts preferentially with U and G with C.[55,56]

These results suggest the possibility that the hydrogen bonding, in addition to being responsible for all of the information content of the nucleic acids, actually contributes a greater amount to the energetics of the structure than is commonly believed.

Conclusion

The most important conclusion is that the known weak interactions, mainly electrostatic, van der Waals, and hydrogen bonding, are sufficiently varied in their expression so they can be and are responsible for the rich diversity of conformations and conformational changes observed for biological macromolecules.

From time to time it has been suggested that new kinds of interactions, not present in small molecules, might be important for biological macromolecules. As Kauzmann wrote in 1959,[7] "There is, however, every indication that the behavior of large molecules (especially synthetic polymers and polypeptides) can be accounted for completely in terms of the same forces as those that occur in ordinary chemical processes. It seems fruitless, therefore, to speculate about these possibilities at this time, even though we should be careful not to close our eyes to them."

Conformations of Proteins

ELKAN R. BLOUT

PROTEINS ARE by far the most important organic constituent of animals, because they comprise approximately three-fourths of the dry weight of such organisms. It has long been recognized that proteins occupy an important position in the architecture and functioning of living matter, and that they are intimately connected with all phases of chemical and physical activity of the cell. It is now clear that proteins represent the direct expression of the genetic material in the cell—the nucleic acids—and that they serve many different functions, some still undefined. Among the known protein functions are serving as structural elements (of which collagen is the most prevalent type) and participating in muscular contraction processes. Many proteins, such as enzymes and hormones, have regulatory functions, and some proteins are concerned with immunological defense mechanisms.

One of the main objectives of modern protein chemistry and biochemistry is to explain the special functions of these large, complex molecules in terms of their structure. In this article I briefly review the known periodic structures found in proteins, describe some of the transformations these structures undergo under a variety of conditions, and then indicate some of the directions that current research is taking in the attempts to answer questions on the relation of the structure of these molecules to their function.

Fundamentally, any protein can be considered to be a polymer in which the monomer unit is one of approximately twenty α-amino acids, and in which each amino acid residue is joined end-to-end in a defined sequence by peptide bonds. Proteins range in molecular weight from about 10,000 to approximately 1,000,000. This means that those proteins with the lowest molecular weight have about 100 amino acid residues, whereas the higher molecular weight proteins may have as many as 10,000 residues.

It is interesting that the low-molecular-weight proteins —those, say, in the range of 10,000 to 35,000—usually consist of a single polypeptide chain. They are all truly one molecule in a chemical sense, and all the atoms in the molecule are joined by at least one covalent bond. In contrast, proteins with higher molecular weights—approximately 50,000 and up—are often aggregates of two, four, or more single polypeptide chains, and do not show their biological activity until they are aggregated. Thus, these aggregates may be considered compounds in a physiological or biochemical sense, even though they are actually many molecules in a chemical sense; biologically they act as an entity.

The essential unit of the polypeptide chain is the repeating sequence shown in Figure 1. Bonds that make up the peptide unit in a polypeptide chain are the $-\overset{\overset{O}{\|}}{C}-C_\alpha$ bond, the $N-C_\alpha$ bond, and the $\overset{\overset{O}{\|}}{C}-N$ bond. The first two show normal interatomic distances, and freedom of rotation around these bonds should be comparable to that found in ordinary organic compounds. The $\overset{\overset{O}{\|}}{C}-N$ bond

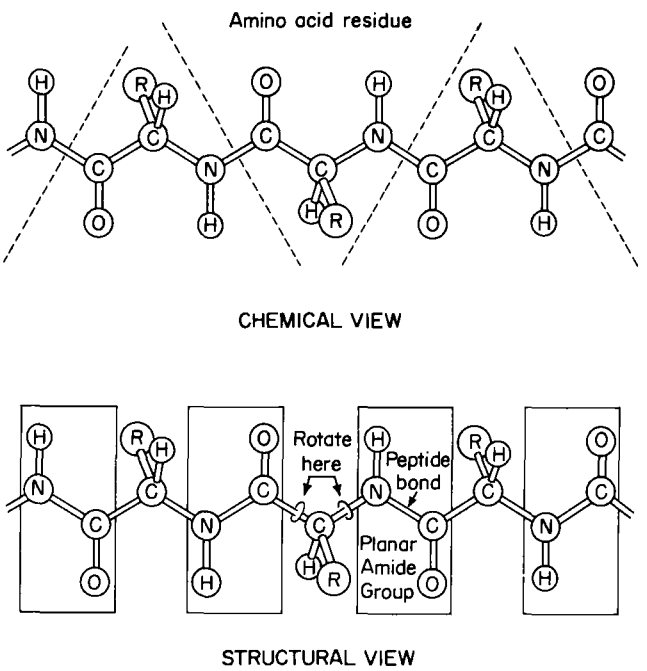


FIGURE 1 Diagramatic representation of a polypeptide chain. (From Corey and Pauling, Note 34)

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of the peptide group has a large amount of double-bond character,[1] and this has two important effects. First, it constrains the atoms in this bond to a planar or nearly planar configuration. Theoretically, peptide bonds may have a cis-arrangement of the carbonyl oxygen and the hydrogen attached to the nitrogen, but, as far as is now known, all protein peptide bonds are trans-. Second, the distribution of electrical charge in the peptide group is such that there is excess negative charge on the oxygen of the carbonyl group and, therefore, a deficiency of electron density in the nitrogen–hydrogen bond. As a result, the carbonyl group has a very strong attraction for hydrogen-bonding donor groups, and the N–H groups have a strong attraction for nucleophilic groups. Thus, in a polypeptide chain there is a good "built-in" arrangement for hydrogen bond formation.

If we consider a polypeptide chain made up of 100 amino acid residues, each of which has three covalent bonds, and consider that free rotation is theoretically possible around 200 of the 300 bonds, one can see immediately that the number of shapes, or *conformations*, such a molecule can assume is very large—practically infinite. However, in the working biological macromolecule the possible number of shapes that are assumed by a protein chain is not infinite. In fact, there is increasing evidence that these chains in their native state assume a favored conformation.[2] It has been known for many decades, however, that proteins can be "denatured" by many means, including varying temperature, *p*H, salt concentration, solvent, etc. Thus, we can define the process of denaturation—or, better, the denatured states—as those in which the conformation of the polypeptide chain is altered from that of the "native" state. A consequence of this definition is that there is not just a single denatured state for proteins; there is a large number of such states.

Now, let us consider briefly the conformations of protein molecules in their native or biologically active states. Over thirty years ago W. T. Astbury showed that the X-ray diffraction patterns of several proteins were distinctive, and concluded that they indicated a periodic order in the structure of many proteins. As a result of X-ray investigations on simple peptides, Pauling and his collaborators suggested in 1951 that a fundamental unit of protein structure was helical in nature—the now well-known α-helix.[3,4] Since that time many other periodic repeating structures have been found in proteins and synthetic polypeptides, principally as a result of X-ray studies on synthetic polypeptides and proteins, and from model-building experiments. Diagramatic representations of some of these structures are shown in Figure 2. Such polypeptide structures are usually favored by energy considerations and may be formed as a result of *intra*molecular and *inter*molecular hydrogen-bonding as a result of steric factors, and sometimes because of hydrophobic interactions, as discussed by Dr. Davidson in the preceding chapter.[5] It should be noted that each of the three periodic structures in Figure 2 exists in two senses; that is, right- and left-handed helices or, in the case of the extended structures, those in which both chains run parallel and those in which a pair of chains has an antiparallel relationship. A periodic structure is one in which successive peptide bonds are related one to another by a repeating translation in three-dimensional space. Three such periodic structures are shown in Figure 2; that is, the α-helix, the

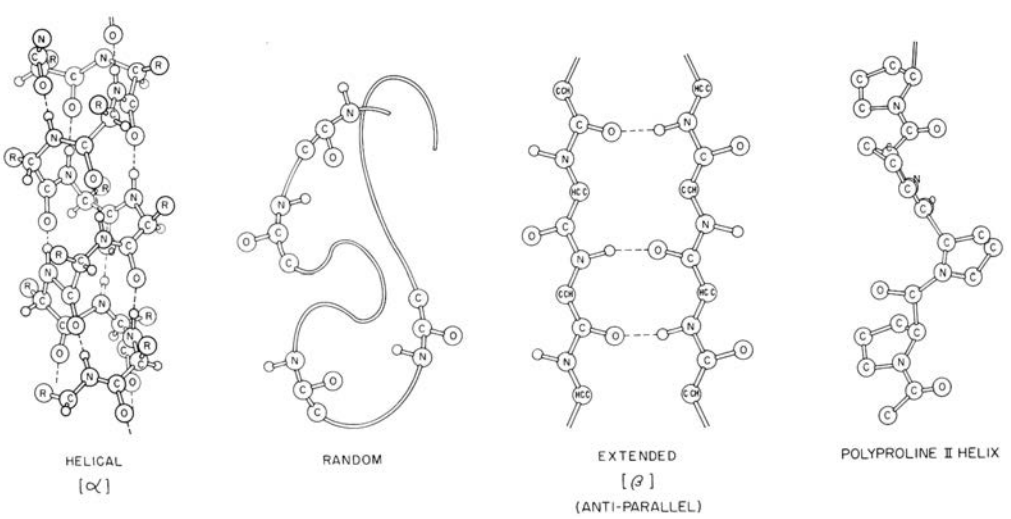

FIGURE 2 Diagramatic representation of some conformations of polypeptide and protein chains.

TABLE I

Dimensions of some L-polypeptide periodic structures (and the collagen triple helix)

Conformation	Rise (A)/Residue	Residues/Turn	Pitch = Rise (A)/Turn
α-Helix	1.5	3.6	5.4
β-Structures			
Parallel	3.25	2.0	6.5
Antiparallel	3.5	2.0	7.0
Polyproline I	1.90	3.33	6.3
Polyproline II	3.1	3.0	9.3
Collagen	2.86	3.33	9.5

β-form, and the polyproline II helix. Both of the optical conformers of the three periodic structures in Figure 2 have been found in synthetic polypeptides, but not all have yet been found in proteins. In Table I some data on the dimensions of various periodic L-polypeptide structures are summarized.

Having defined some polypeptide structures, we can now ask two logical questions: (1) are such structures found in proteins (or only in the synthetic polypeptide analogs)?; and (2) do such structures, found as a result of solid-state X-ray diffraction studies, also exist in solutions of polypeptides and proteins?

The elegant work of Kendrew and Perutz on myoglobin and hemoglobin,[6,7] the oxygen-transporting proteins of mammalian organisms, provided the definitively affirmative answer to the existence of the right-handed α-helix in proteins. A diagramatic representation of the polypeptide chain of myoglobin is shown in Figure 3 of the preceding chapter. For clarity, only the backbone of the chain is shown. It is immediately apparent that this molecule consists of relatively short stretches of α-helix joined by nonhelical regions. About 70 to 75 per cent of the peptide residues in myoglobin are in the (periodic) α-helical conformation. Myoglobin is a "complex" protein in that it consists of a polypeptide chain and a nonprotein moiety—the heme group that contains a porphyrin ring. The heme group and the polypeptide chain are in intimate contact as a result of many hydrophobic interactions. If the heme is removed from myoglobin, the protein remaining is called apomyoglobin.

In addition to the helical regions and the porphyrin ring, it should be noted that about 25 to 30 per cent of the peptide bonds in myoglobin are in fixed and definite, but nonperiodic, structures. The determination of the conformations of these fixed, but non-ordered protein regions is one of the main problems in protein structure analysis today. Finally, we should note something that is not evident in the two-dimensional representation of myoglobin; that is, that the molecule is highly compact; the amino acid side chains fill much of the space between the turns of the main peptide chain.

The existence of periodic peptide structures other than the α-helix has been verified by recent work on collagen. Collagen is a protein of high molecular weight, approximately 300,000, and consists of three polypeptide chains, each having a molecular weight of about 100,000. The biological unit is the three-chain superhelix. Investigations of this important protein by Ramachandran and collaborators[8-10] and by Rich and Crick,[11,12] have shown that its structure is essentially that of three left-handed polyproline II helices coiled about one another in a right-handed superhelix (Figure 3).

Finally, it should be noted that other recent work[13-16] indicates that the milk protein β-lactoglobulin contains a large amount of β (extended) conformation in its native structure. Thus it has been shown that at least three of these periodic structures, proposed for peptide chains as a result of X-ray diffraction work on model polypeptides, exist in proteins.

I have already emphasized that much of the solid-state structure work on polypeptides and proteins has utilized X-ray diffraction methods. When this technique can be applied, it provides the most fundamental quantitative information on protein structure. However, it now appears that for various technical reasons the structures of all proteins may be difficult to determine by this approach, and it has not yet been proved that the solid-state structure is identical with the conformation in solution—the normal environment for many proteins.

As a consequence, there has been considerable effort in the past ten years to apply other physical–chemical methods to determine solution conformations. Some of these methods are listed in Table II. Almost all of them are optical. It would obviously be impossible even to attempt to discuss them all; instead, I will limit myself to a rather arbitrary choice of recent results. I should also note that most of the methods, except for X-ray diffraction and, in part, infrared spectroscopy, are not absolute; that is, the information obtained must be calibrated with model compounds of known structure and the results then translated to the unknown protein structure.

First, however, it may be worthwhile to consider some of the optical properties of a polypeptide chain. One of the most striking physical properties of synthetic polypeptides and proteins is their ability to rotate the plane of polarized radiation. This property is called "optical activity" and is a consequence of amino acids containing

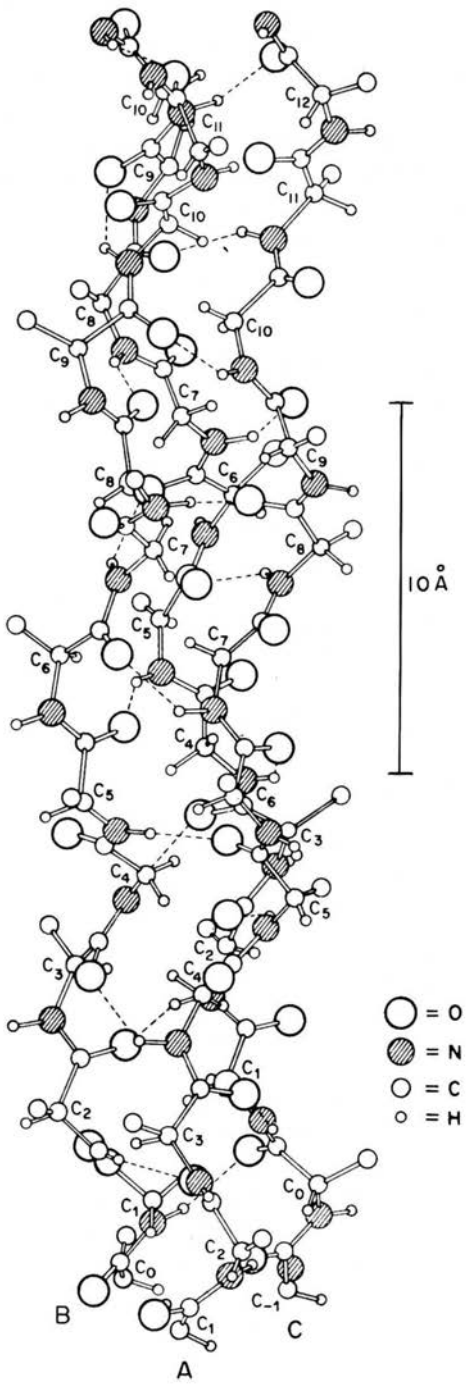

FIGURE 3 Perspective drawing of the collagen structure. The backbone and the β-carbon atoms of the supercoiled triple helix are shown. Only the α-carbon atoms are marked. (From Ramachandran, Note 10)

TABLE II

Some methods for determination of protein conformations

A. Solid State
   1. X-Ray diffraction
   2. Infrared spectroscopy

B. Solution
   1. Absorption spectroscopy (ultraviolet and infrared)
   2. Optical rotatory dispersion and circular dichroism
   3. Dye binding
   4. Fluorescence
   5. Chemical modifications

asymmetric carbon atoms, but even more important, the periodic structures previously referred to confer on the polypeptide and protein further dissymmetry, which is reflected in much enhanced optical rotatory properties. Although these properties can be observed in various regions of the electromagnetic spectrum, they are most obvious and maximal in the absorption bands of the compounds. A diagramatic representation showing the relationship between optical rotation and absorption of an optically active compound is shown in Figure 4. As a result of many investigations in several laboratories over the past ten years, it is now clear that each of the periodic structures exhibited by polypeptides and proteins shows distinctive optical rotatory dispersion and circular dichroism curves in solution. Some representative circular dichroism and optical rotatory dispersion data for various conformations of polypeptides are in Figures 5, 6, and 7, and a summary of some of the rotatory parameters characterizing these conformations is shown in Table III.

TABLE III

Rotatory dispersion characteristics of some L-polypeptide conformations and collagen

Conformation	Trough ([R']<O)	Crossover ([R']=O)	Peak ([R']>O)
	238		
Random	205	198	190
α-Helix	233	224	199
β-Structure (Antiparallel?)	230	~220	205
Polyproline I	208	217	223
Polyproline II	217	205	195
Collagen	207	197	<190

(All values are in mμ; [R'] is the reduced residue rotation.)

With this background, we now turn to a consideration of conformations and conformational changes in proteins, and ask the following questions: (1) Are protein conformations in solution grossly similar to those found in the solid state? (2) Can protein conformations be reversibly changed in solution? (3) Is all of a protein molecule necessary to determine its precise conformation? (4) What are the methods available for determining the structure of the nonperiodic portions of protein molecules?

To attempt to determine the answer to question one, it was natural to turn to a study of the solution properties of myoglobin, the protein whose solid-state structure is known with greatest certainty. From rotatory dispersion investigations of myoglobin in the visible and far ultraviolet regions,[17-19] it appears that the α-helix content of this molecule in solution is very similar to, if not identical with, that observed in the solid state. Two methods were employed to reach this conclusion: an analysis of the near ultraviolet and visible rotatory dispersion data, and a determination of the magnitudes of the rotation in the peptide Cotton effect region. The wavelength region below 240 millimicrons is the portion of the electromagnetic spectrum where Cotton effects, whose origin lies in optically active peptide absorption bands, have been observed. Figure 8 shows the far ultraviolet rotatory dispersion data for ferrimyoglobin and apomyoglobin. The

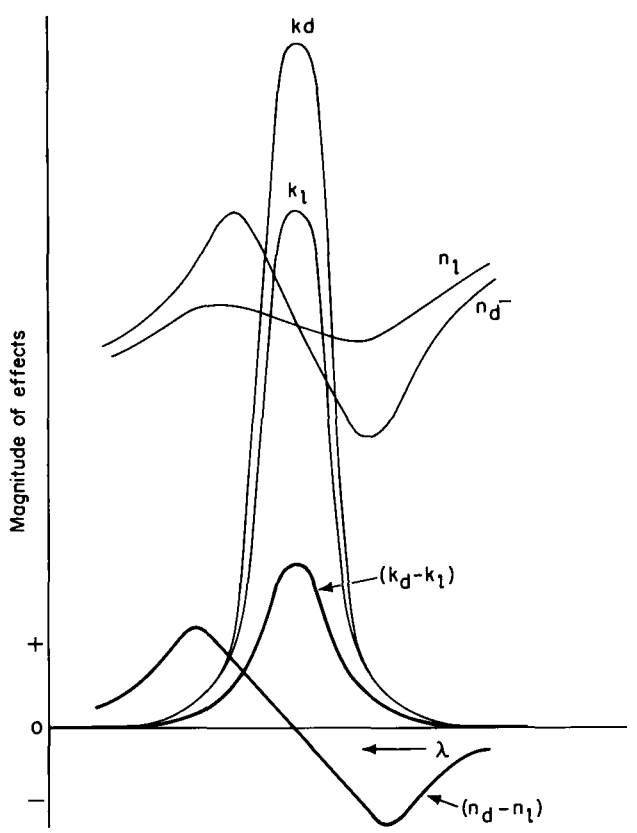

FIGURE 4 Diagram showing the relationships of refractive indices (n_l and n_d) and the absorptions (k_l and k_d) for right and left circularly polarized light in the region of an optically active absorption band. The characteristic "Cotton effect" observed by measuring optical rotatory dispersion is n_d–n_l, and the circular dichroism band is k_d–k_l. (From Heller and Fitts, Note 35)

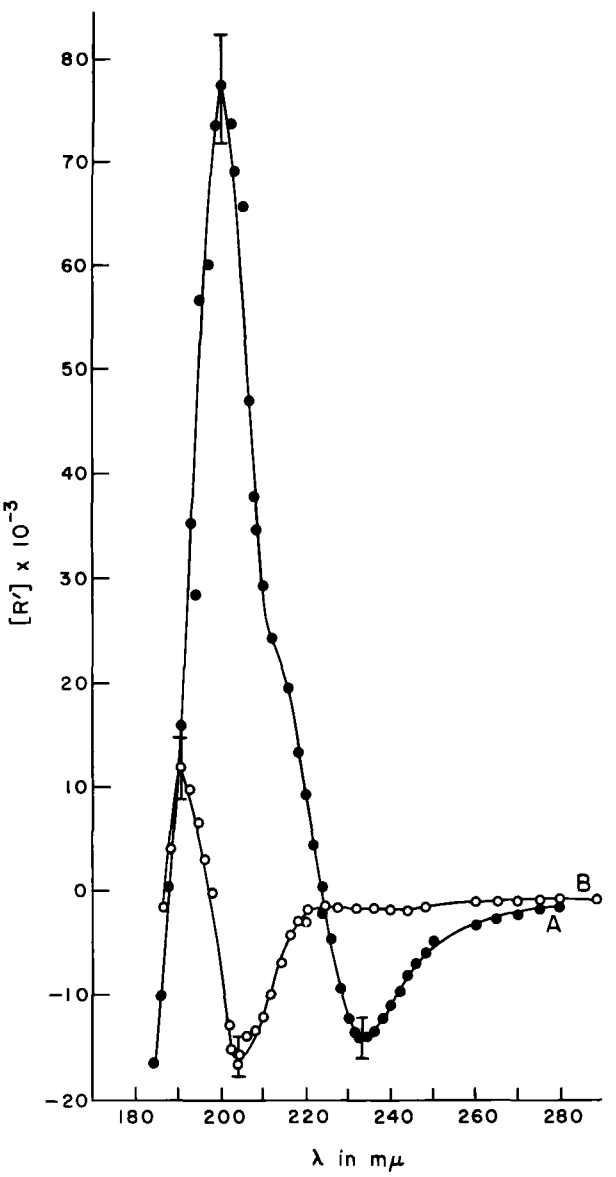

FIGURE 5 The far ultraviolet optical rotatory dispersion of the α-helical (A,●–●–●) and random forms (B, O–O–O) of poly-α-L-glutamic acid. (From Blout, et al., Note 36)

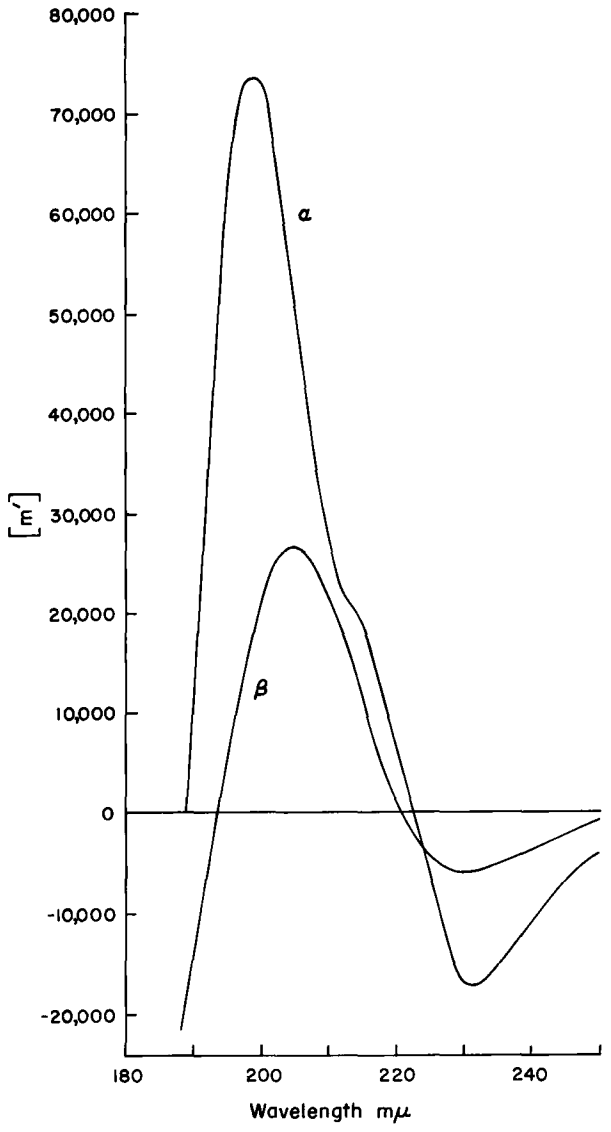

FIGURE 6 The optical rotatory dispersion of the α-helical and β-conformations of poly-L-lysine. (From Davidson, et al., Note 37)

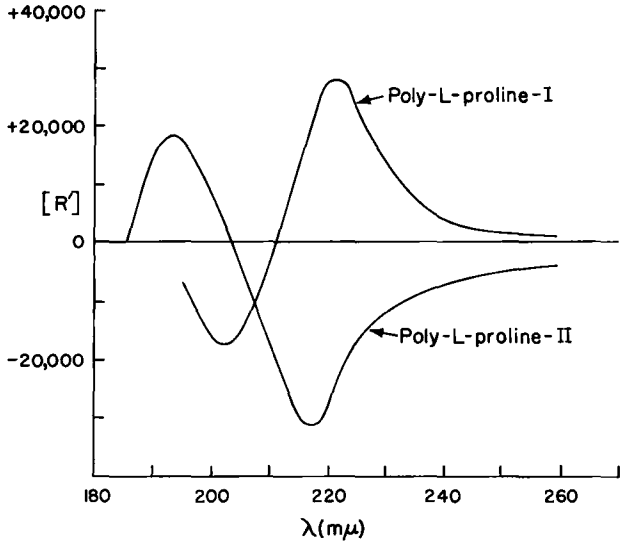

FIGURE 7 The optical rotatory dispersion of poly-L-proline-I as an axially-oriented film and poly-L-proline-II in aqueous solution. (Redrawn from Blout and Shechter, Notes 38; Blout, et al., Note 39)

trough at 233 millimicrons, which seems to be a characteristic of the α-helical conformation, is large enough to permit the calculation that the helix content in myoglobin is about two-thirds of that found in a fully α-helical model polypeptide. Thus, as a result of such studies, one can conclude that myoglobin in solution has approximately the same helix content as that determined by X-ray diffraction in the solid state. It is not certain, but is inferred, that the portions of the molecule that are helical in the solid state are identical to the portions of the molecule that are helical in solution.

Question two, which asks if protein conformations can be reversibly changed, is closely related to the question of whether there is a thermodynamically favored state for protein molecules. Synthetic polypeptides have been highly useful model compounds for studying changes in conformation. In particular, investigations with synthetic polypeptides composed of only one type of amino acid residue in the polypeptide chain (homopolypeptides) have shown unequivocally that the conformations of these macromolecules can be altered reversibly under a variety of physical conditions.[20] One of the most frequently used compounds in these studies is poly-α-L-glutamic acid. If the *p*H of solutions of polyglutamic acid is changed from four to six, there are marked changes in the hydrodynamic and optical properties.[21-23] Figure 9 illustrates one such change. These observed rotation changes are a direct consequence of changes in the structure of the molecule from the α-helical form at *p*H 4 to a random conformation at *p*H 6 and above. Reducing the *p*H from 6 to 4 results in the reformation of the α-helix and in the observed reversible change in rotation. Similar changes in gross conformation can be effected by variation in solvents (with other synthetic polypeptides), by temperature changes, and by changes in ionic strength. In other words, all the reagents used to "denature" proteins can be used to study

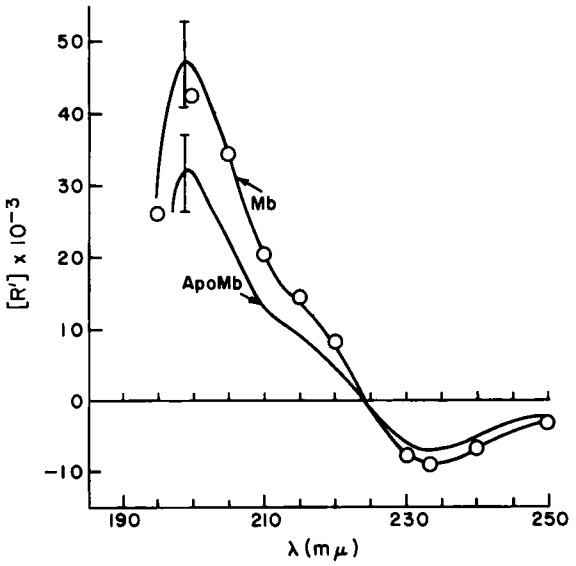

FIGURE 8 Optical rotatory dispersion, plotted as reduced mean residue action, [R'], of myoglobin (Mb) at 30°, apomyoglobin (apoMb) at 5°, and reconstituted Mb at 30°, all in water. The solid curves show results for Mb and for apoMb. The open circles indicate [R'] values for reconstituted Mb. (From Harrison and Blout, Note 29)

reversible conformational changes in their synthetic protein analogs and, thus, to define the physical parameters of the polypeptide structures.

One particularly interesting series of experiments involving a reversible conformational change of synthetic polypeptides was carried out by the late Professor Henri Lenormant when he worked in our laboratory several years ago.[24,25] Professor Lenormant was examining the infrared spectra of oriented films of polyglutamic acid and polylysine and made the provocative observation that the infrared spectra changed markedly some hours after the films were prepared. Further investigation of this phenomenon showed that (a) the change was caused by changes in relative humidity surrounding the film, (b) that the change involved a conformational change from the α-helix conformation to an extended (β) structure, and (c) that the change was reversible. The results of the infrared spectral measurements that proved these points are shown in Figures 10, 11, and 12. It should be noted that these results have recently been confirmed by X-ray diffraction methods.[26] The over-all process and the effect on the conformation of the amount of water surrounding this polypeptide can be represented diagramatically in the manner shown in Figure 13. These results are provocative because they illustrate the relative ease with which conformational changes can be effected in protein-like molecules and suggest that simply by varying the relative amount of water surrounding a protein molecule, it is possible to alter its conformation markedly and hence, by inference, its biological function.

Reverting now to proteins and examining the question of reversible changes, mention should be made of the work of Anfinsen and his colleagues on ribonuclease.[27,28] It has been shown that ribonuclease can be reversibly reduced and denatured by treatment with disulfide bond-breaking reagents and urea; this gives a denatured protein with no biological activity. If the urea is removed and the disulfide bonds reformed by oxidation, a "renatured" enzyme is obtained that shows practically full biological activity. In other words, proteins can undergo reversible conformational changes similar to those observed in synthetic polypeptides and with a concomitant reformation of biological activity. This leads to the implicit conclusion that the original conformation is reformed.

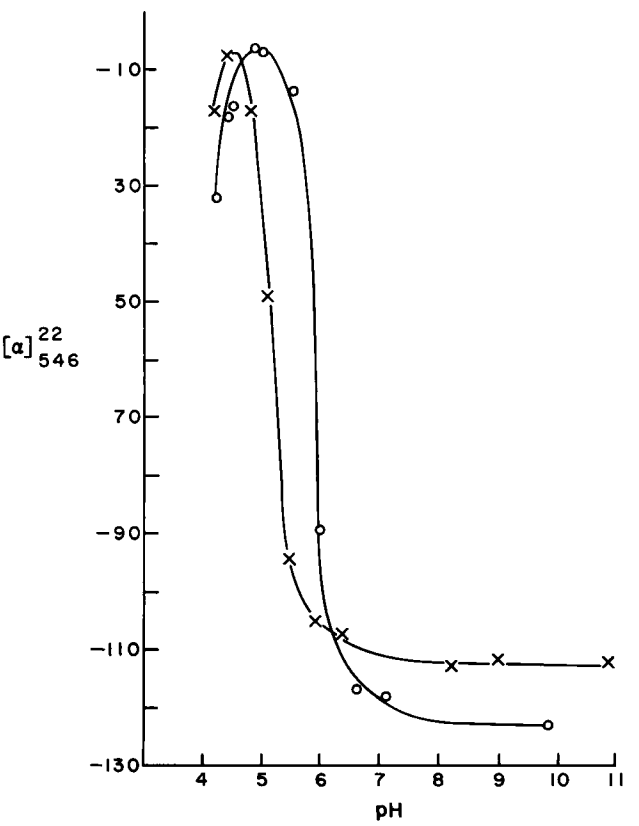

FIGURE 9 The optical rotation at 546 mμ of poly-α-L-glutamic acid as a function of *p*H. (-O-O-O-), in water and (-XXX-), in 0.2M NaCl. (From Idelson and Blout, Note 22)

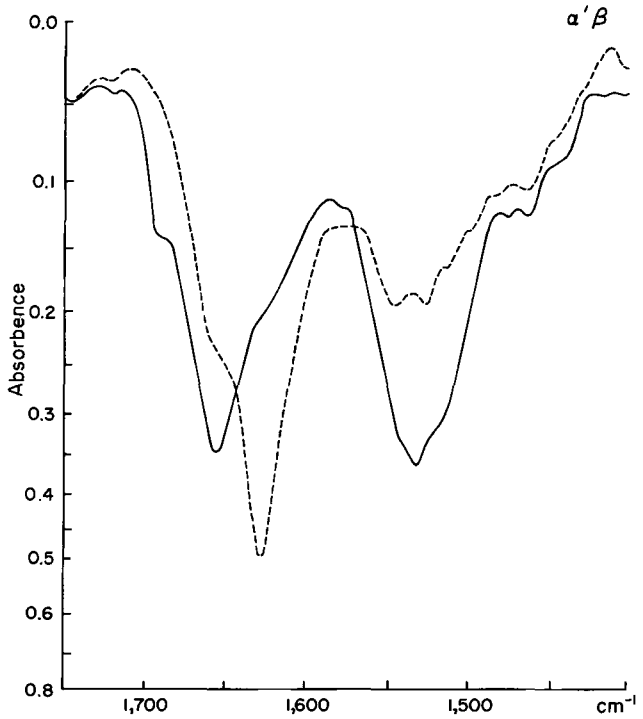

FIGURE 10 The infrared spectra of an oriented film of poly-α-L-lysine·hydrochloride. Note C = O amide band characteristic of α-helix at 1655 cm^{-1} (parallel dichroism) and C = O amide band characteristic of β-conformation at 1625 cm^{-1} (perpendicular dichroism). (From Blout and Lenormant, Note 24)

Recent studies of similar nature have been performed with myoglobin.[29] The heme portion of myoglobin can be removed by acid treatment, and the resulting apomyoglobin can then be examined in solution. Measurements of the far ultraviolet rotatory dispersion and calculation of the helix content indicate that apomyoglobin has a helical content about 10 to 15 per cent lower than does native myoglobin, which contains the heme group (Figure 8). Other experiments showed that the apomyoglobin could be denatured to an essentially random conformation by treatment with 8-molar urea. If the urea is removed from the solution by dialysis, the apomyoglobin spontaneously refolds to a conformation that has the same helix content as the native apomyoglobin. Furthermore, if heme is added to this renatured apomyoglobin solution, a regenerated myoglobin results, with a helix content and other properties apparently identical with the native molecule (Figure 8).

Thus, as a result of several lines of investigation with both synthetic polypeptides and proteins, it appears that we can answer the second question affirmatively. It is possible to change conformations of proteins reversibly under suitable conditions.

The experiments with myoglobin also provide a partial answer to the third question, and indicate the necessity for the whole protein molecule to be present for the native conformation. The removal of even the nonprotein moiety, the heme group, obviously changes the conformation of this molecule, and its reinsertion changes the molecule back to its original helix content.

We now turn to question four—how one may determine the structure of the nonperiodic, but structured, regions of proteins, and to the further question: is it reasonable to expect that the entire conformation of proteins in solution can be determined? It has been indicated here that methods are now available to determine the amount of the ordered regions of polypeptides and proteins in solution. However, it is obvious, even on superficial examination of the solid-state structure of myoglobin, that there are many regions of the molecule that are structured, but not in a periodic manner. Investigations now under way aim at determining the conformations of the structured, but non-periodic, regions of protein molecules. The methods that are being used can be

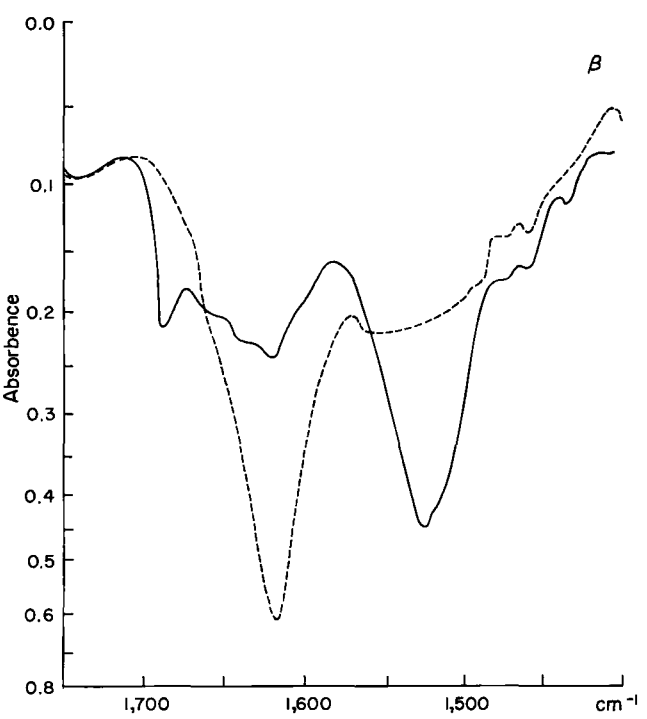

FIGURE 11 The infrared spectra of the same film as in Figure 10 after exposure to about 65% relative humidity for 5 hours. Note presence of C = O amide band of β-form at 1625 cm^{-1} (perpendicular dichroism) and loss of α-helical C = O amide band. (From Blout and Lenormant, Note 24)

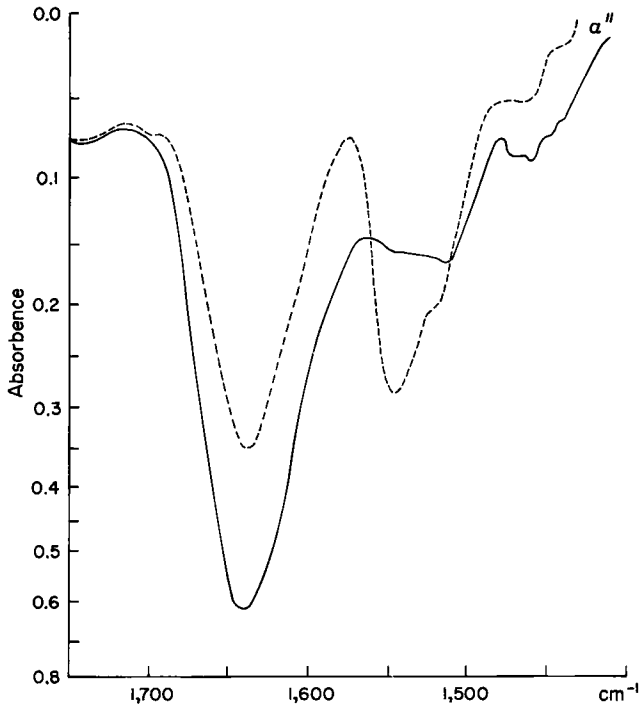

FIGURE 12 Infrared spectra of the same film of poly-L-lysine · hydrochloride as in Figure 11 after exposure to 100% relative humidity for 3 hours. Note C = O amide band of α-helical conformation at approximately 1650 cm^{-1} (parallel dichroism) and essentially complete loss of characteristic 1625 cm^{-1} C = O band of β-conformation. (From Blout and Lenormant, Note 24)

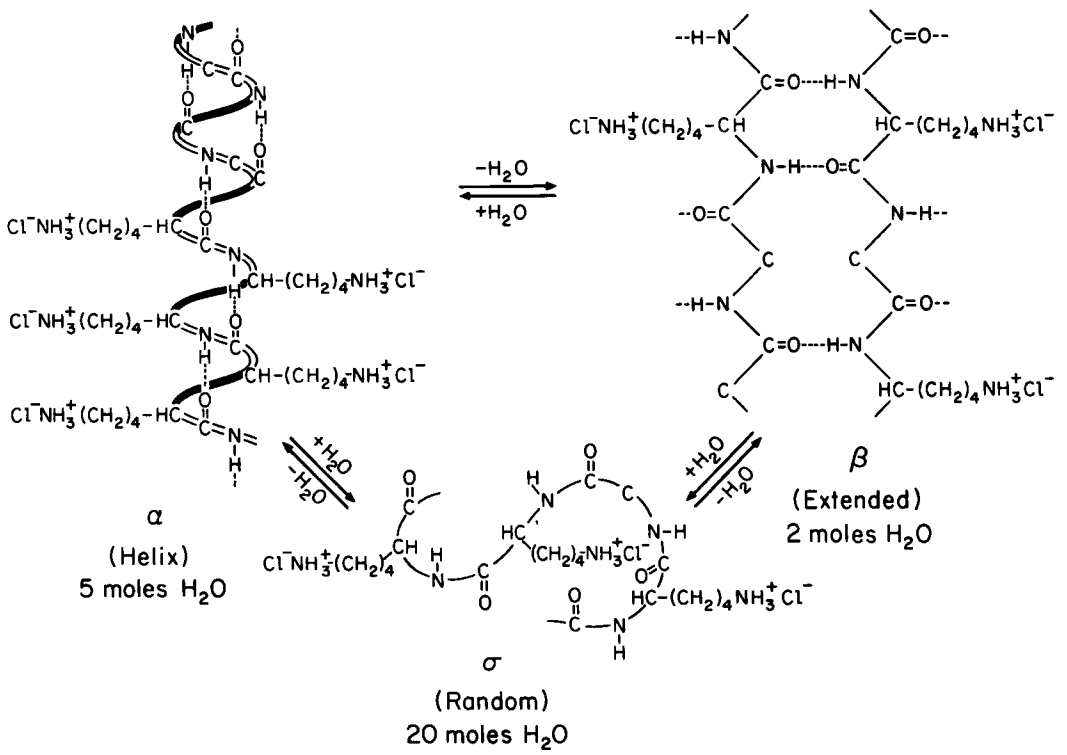

FIGURE 13 Diagramatic representation of conformations of poly-L-lysine · hydrochloride.

characterized as "optical probe techniques." Such optical probes may utilize either the distinctive properties of one amino acid residue in the protein, e.g., a particular tyrosine residue in a particular environment, or the addition of an extrinsic probe, e.g., a light-absorbing molecule, such as a dye, that binds specifically to one portion of the protein molecule. Examples of the intrinsic optical probe are provided by the observed Cotton effects of particular aromatic amino acid residues in proteins. A recent example of such work is that of Glazer and Simmons,[30] shown in Figure 14, where correlation is made of Cotton effects of ribonuclease around 280 millimicrons with certain tyrosine residues in the molecule. If the structure of the molecule is changed, in this case by addition of 1.5 per cent of sodium dodecyl sulfate to the solution, the Cotton effects disappear.

Extrinsic optical probes include dyes that bind at specific portions of protein molecules, and which, upon binding, change some of their optical properties, such as their absorption, their rotatory properties, or their fluorescence. Glazer has described an example of such phenomena[31] in work indicating that proflavin binds in a one-to-one mole ratio to chymotrypsin and also inhibits its proteolytic action. Presumably, this means that the proflavin is binding at the active site or at the region associated with the proteolytic activity of this enzyme. Upon binding of proflavin to chymotrypsin, changes in adsorption can be observed,[31] and very recently Joseph Parrish[32] in our laboratory has observed that proflavin–chymotrypsin complexes show Cotton effects in the absorption region of proflavin, although proflavin itself is not optically active. This means, of course, that proflavin is being bound in an asymmetric manner to the chymotrypsin molecule, presumably at the active site. It is hoped that with such studies, and related studies with model systems[33] in which the conformations are known, we will be able to delineate additional regions of protein structure in solution.

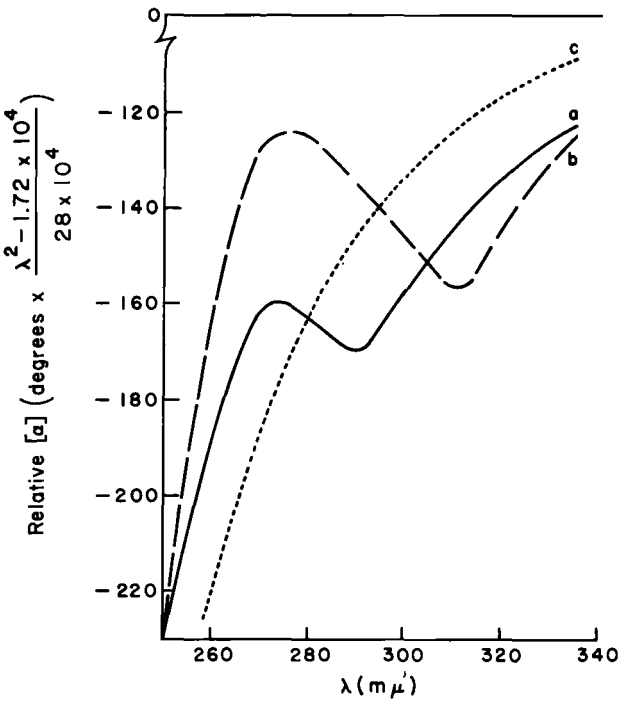

FIGURE 14 Rotatory dispersion of ribonuclease in the region of the tyrosine Cotton effect. Ribonuclease (0.48%, in 0.5 cm. cell) (a) in 0.15M phosphate buffer at *p*H 6.2; (b) in 0.15M glycine-NaOH buffer at *p*H 11.5; (c) in 1.5% sodium dodecyl sulfate. (From Glazer and Simmons, Note 30)

Summary

The following can be concluded from conformational studies of synthetic polypeptides and proteins: (1) proteins show unique structures in the solid state; (2) many of the specific periodic structures that proteins exhibit, such as the α-helix and the polyproline-type helix, have been shown to persist in solutions of proteins; (3) the conformations of synthetic polypeptides and some proteins can be reversibly altered under mild physical and chemical environmental changes; (4) little is known about the detailed conformations of the nonperiodic, but structured, regions of proteins. Undoubtedly, the peptide chain conformations in these regions are a direct result of the amino acid sequence and the interactions between and among side chains and main polypeptide chains, but the underlying principles and the magnitudes of the forces involved are not yet definitely understood. Finally, some possible methods for the determination of conformations in these nonperiodic regions of protein have been suggested.

Structure and Structural Transformations of Nucleic Acids

D. M. CROTHERS

ONE OF THE central problems in the neurosciences is to elucidate the means by which information is stored in the nervous system. It would seem safe to say that specific chemical substances, probably macromolecules, are somehow involved in the storage mechanism. In any general consideration of the classes of substances that might play such a role, it is natural to pay special attention to nucleic acids, whose involvement in the encoding and processing of genetic information is well-established. It can hardly be supposed that the mechanism of information storage in the brain is the same as that for genetic information. However, a close examination of the properties of nucleic acids can disclose characteristics of these materials that could be adapted to the very different purposes of information storage in the nervous system.

A DNA molecule, as the ultimate repository of genetic information, has a specific structure elegantly adapted to its function. In addition, it is important to emphasize that, when the message is transcribed, the part played by the DNA molecule is dynamic rather than static. In order that the base-pairing characteristics of its component nucleotides be recognized, the structure must almost certainly be substantially altered, although this may involve only a small region of the molecule at any one time. In this respect, one might contrast nucleic acids with a printed page, which is essentially unaltered by reading. Thus, in examining the details of information processing, it is important to consider both static and dynamic features of nucleic acid structure.

Nucleic acid structure

The static aspect of nucleic acid structure has naturally received first attention. The original suggestion of a double helical structure for DNA by Watson and Crick [1] was one of the most fruitful single hypotheses of modern biology. The transfer of genetic information from DNA to messenger-RNA, and hence to amino acid sequence, rests in large measure on the specific base-pairing requirements proposed by those authors. However, for the problems of the present, which are more concerned with understanding these processes on a detailed molecular level, the dynamic aspects of the structure are of equal importance. As the study of structural changes of nucleic acids has been somewhat neglected, especially the kinetic parameters, the emphasis here is on this aspect of the problem.

The original Watson–Crick structure for double-helical DNA, although fully vindicated in principle, has undergone considerable refinement in detail.[2] In addition, substantial variation in structure is observed for the wide variety of synthetic polynucleotides now available. Double and triple helical species are found, and the parameters of the structure, such as the angle of the plane of the base pairs with respect to the helix axis, differ from one material to another. At present, it is difficult to make generalizations concerning these structures; systematic examination of the homopolymers and copolymers now available should eventually provide a knowledge of the factors that determine the detailed structure.

The transfer RNAs present an important structural problem of another kind. These polynucleotides, with molecular weights falling within the range shown by globular proteins, resemble these proteins in structure, in that they seem to contain helical regions, and possibly also a specific three-dimensional conformation. Physical measurements such as ultraviolet hypochromism,[3] hydrodynamic changes on heating,[4] and the kinetics of tritium exchange[5] indicate considerable order, presumably helix formation with complementary base pairing. However, the base sequence of an alanine-specific transfer RNA (tRNA) determined by Holley and his associates[6] indicates quite clearly that the structure cannot be a perfect helix in the Watson–Crick sense. Local base pairing is possible, and Figure 1 shows some of the structures that can be postulated. The actual structure is that of minimum free energy, as activity is regained after heating and cooling, but it is not at all obvious how this criterion is related to other features, such as the degree of hydrogen bonding. Unambiguous determination of the structure will remain difficult, if not impossible, until a tRNA can be crystallized for

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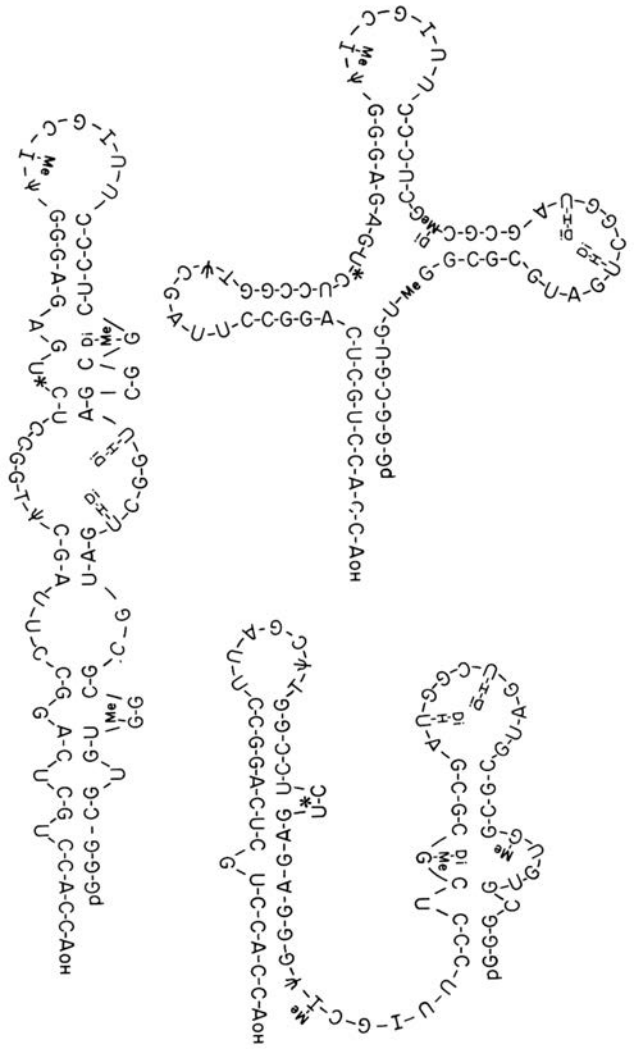

FIGURE 1 Possible helical structures, with complementary base pairing, postulated for the alanine-specific transfer RNA. (From Holley, et al., Note 6)

X-ray diffraction studies.

When isolated in solution, the other common ribonucleic acids, messenger and ribosomal, are probably present primarily as the single-stranded, or coil, form. Studies on synthetic RNAs[7-9] indicate that there is some local structure, particularly stacking of adjacent bases on each other, in these coil forms. There is also the possibility of helix formation resulting from local base-pair complementarity in the naturally occurring single-stranded materials. Double-helical RNA is known to occur in the replicative form of the RNA from RNA viruses, where it acts as a replacement for DNA.

Structural changes

Evidence for structural changes of nucleic acids is available from a number of sources. One of the most common physical measurements on DNA is the so-called "melting curve," illustrated in Figure 2,[10] in which the variation of the ultraviolet absorbance of the solution is determined at various temperatures. Other physical properties, such as viscosity, sedimentation coefficient, optical rotation, banding density in CsCl, etc., also change markedly over a narrow temperature range. These changes reflect the disorganization of the double helical structure, with the formation of two random-coil polymer chains, a process that is called the helix–coil transition.

Less drastic structural changes result from the binding of small molecules to DNA. One of the best studied of such systems is the binding of acridine dyes, which are thought to insert or intercalate between the planar base pairs in the DNA structure, a hypothesis first advanced by Lerman.[11] Figure 3 shows how one aspect of the X-ray diffraction pattern varies when certain dyes are bound. The layer line spacing corresponding to the pitch of the helix increases as the degree of fiber hydration increases. This can be interpreted[12] as indicating external binding of the dye at low humidity, with insertion and consequent length increase at higher water content.

Even when the helix form of DNA is stable, local structural fluctuations do occur. This is shown by the fact that the H-bonding protons are exchanged with the solvent, although at a very slow rate, as shown in Figure 4.[13] Very little is known at present about the dynamics of these fluc-

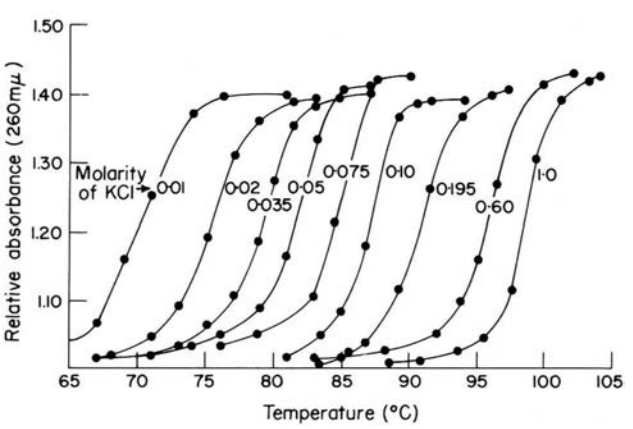

FIGURE 2 "Melting curves" for DNA samples at varying salt concentrations, as measured by Marmur and Doty.[10] The ratio of the absorbance at 260 mμ at temperature T to that at room temperature is plotted as a function of T.

tuations, although they may have relevance to such important processes as transcription of the genetic message and genetic recombination.

The helix-coil transformation

The purpose of detailed studies of the helix–coil transformation is to gain information about static and dynamic aspects of the interactions that stabilize the helical form and thereby contribute to the specificity necessary for information storage and processing. The transition is represented schematically in Figure 5, where cross lines represent hydrogen-bonded base pairs. The intermediate states between all-helix and all-coil forms contain alternating helix and coil regions in the same molecule. If there are interactions in the system that make these intermediate states unfavorable, the system will tend to go abruptly from all-helix to all-coil over a narrow region of external conditions. That this is the case may be seen from the titration curve shown in Figure 6. The ionization of the guanine and thymine bases is coupled to the helix–coil transition, so that instead of occurring over about 2 *p*H units as expected for an acid–base titration, the entire reaction occupies only about 0.1 *p*H unit.[14] Such a transformation is said to be cooperative.

To analyze the factors that make the "melting" of DNA cooperative, the interactions in the system are divided into two classes (Figure 7). First, there is the head-to-head interaction between two hydrogen-bonded bases, resulting both from the hydrogen bond and from the interactions such as dipole–dipole, etc., between the two bases themselves. The second interaction is that between neighboring H-bonded base pairs, referred to as the "stacking" interaction. The free energy of stacking is known to be highly favorable (about −7 kilocalories per mole of base pairs)[15]; and this is responsible for the suppression of intermediate states in the transition. Because each alternation between helix and coil results in the loss of the stacking free energy, the intermediate states, which by definition have alternating helix and coil regions, are greatly disfavored. In those intermediate states that are present, there tend to be very few alternations, so that each helix–coil alternation region is very long. This length can be referred to as the cooperative length; our current estimate is that it may be of the order of 1000 to 2000 base pairs for DNA.[16] The cooperative nature of the transition and the long cooperative length are direct consequences of the large negative free energy of stacking.

The total heat of the transition has been measured[17-20] and is about 8 kilocalories per mole of base pairs (heat absorbed on melting); most of this is associated with the stacking interaction. The total energy of the hydrogen-

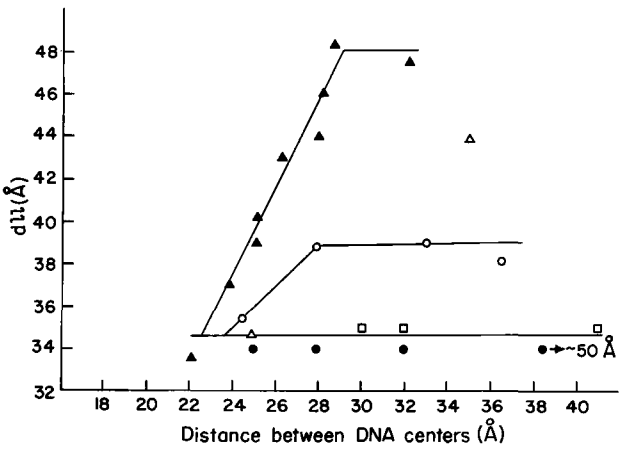

FIGURE 3 Effect of hydration on the structure of sodium–DNA and dye–DNA complexes is illustrated by plotting the layer line spacing *versus* the distance between neighboring DNA molecules, the latter being a measure of degree of hydration. —▲—▲— Proflavine–DNA, D/p (≡ dye molecules per phosphate) = 0.17; —○—○— Proflavine–DNA, D/p = 0.10; —△—△— acridine orange–DNA, D/p = 0.17; —□—□— p-rosaniline–DNA; —●—●— sodium–DNA. (From Neville and Davies, Note 12)

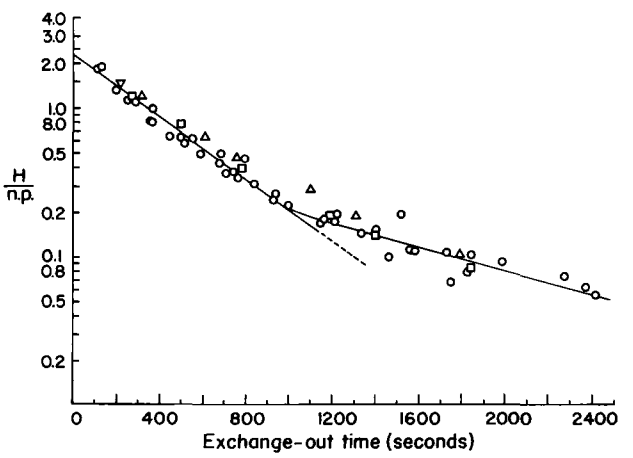

FIGURE 4 Hydrogen–tritium exchange of native calf thymus DNA at 3.5°C (○) DNA, sonicated, (□) DNA, sonicated and EDTA dialyzed; (▽) DNA, unsonicated; △ DNA, sonicated. (From Printz and Von Hippel, Note 13)

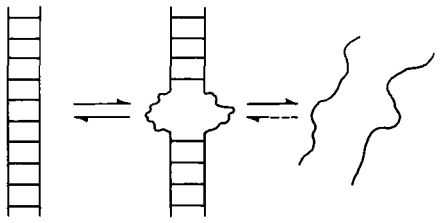

FIGURE 5 Schematic view of the helix–coil transition.

bonding interaction is close to zero, because H bonds to water must be broken in order to form the base-to-base H bonds. The H bonds serve largely a discriminatory function, because a pairing in which proper internal H bonds could not be formed would be energetically quite unfavorable.

The stacking interaction, which the above observations implicate as the major source of helix stabilization, results both from the electronic interaction energy between the two stacked base pairs, and also from the tendency of the solvent (water) to force the bases together. This latter is the so-called "hydrophobic" interaction (see the article by Davidson in this book), and one of the current problems is to determine its exact nature. It is perhaps worth pointing out that the large negative stacking free energy results mostly from a favorable heat,[7-9] whereas the most common conception of hydrophobic bonding in terms of the increased order of the solvent around the solute[21] would ascribe the effect primarily to a favorable entropy. The contribution of the solvent to the free energy of stacking is not yet fully understood.

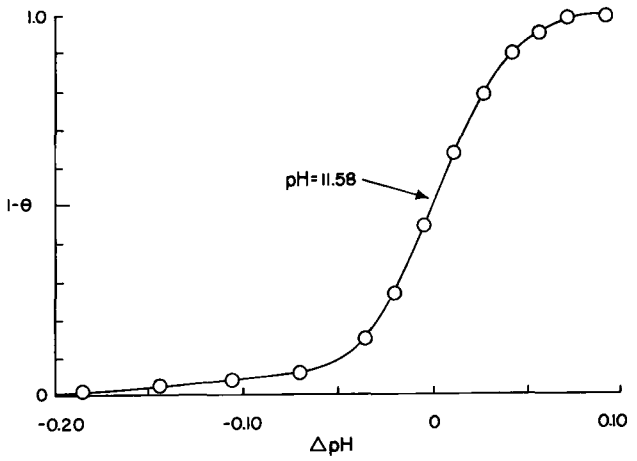

FIGURE 6 Helix-coil transition curve in the basic *p*H range at room temperature, showing 1—⊖, the fraction of bases in the coil state, as a function of the change in *p*H.

The stability of the coil form at high temperature results from its high entropy relative to the helix. Freedom of rotation around many bonds is gained in the disorganization of the helix, and at sufficiently high temperature the resulting entropy compensates the unfavorable energy in the transition.

The helix–coil transition is amenable to description in terms of an order–disorder transition on a linear lattice. Each unit (base pair) can exist in one of two states, bonded (l) or unbonded (O), and a sequence of bonded and unbonded units defines a particular configuration of the molecule. The probability (P_i) of configuration i depends on its free energy (f_i) according to the basic equation

$$P_i = \exp(-f_i/kT)/Q, \tag{1}$$

where

$$Q = \sum_i \exp(-f_i/kT). \tag{2}$$

With a knowledge of the stacking free energy and the total free energy of helix formation (which varies with temperature, being zero at the "melting" temperature), the free energy f_i can be calculated for each configuration. Equations (1) and (2) are then used to determine the prob-

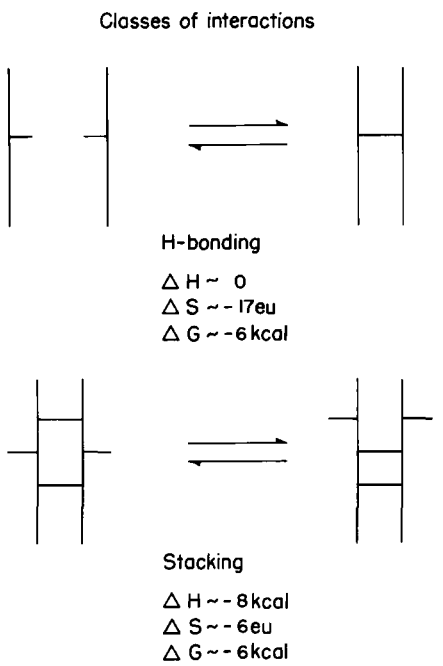

FIGURE 7 Two major classes of interactions which stabilize helical polynucleotides, with rough estimates of the thermodynamic parameters for each.

ability of each configuration, and hence the average properties of the system.

As indicated above, the sharpness of the transition from helix to coil is directly related to the stacking free energy, but an additional complication must be considered in calculating the breadth of the transition of DNA molecules. This arises because the two kinds of base pairs, A–T and G–C, have different intrinsic stabilities, as demonstrated by the observation that the "melting" temperature of a DNA sample increases linearly with its G–C content.[10] Consequently, regions of the molecule that are rich in G–C are more stable and hence melt at a higher temperature than regions rich in A–T, local composition fluctuations being an inevitable result of a nonregular sequence. The net result is to broaden the transition substantially, and also to make the calculations considerably more difficult. Figure 8 shows a transition curve, calculated from an approximate theory using quantities estimated from the properties of synthetic (homogeneous) polynucleotides, for a DNA assumed to contain a random sequence of bases. This is compared with an experimental curve for bacteriophage T2 DNA, with reasonable agreement. The transition that would be predicted if the heterogeneity of base-pair stability were neglected is very abrupt on this scale.

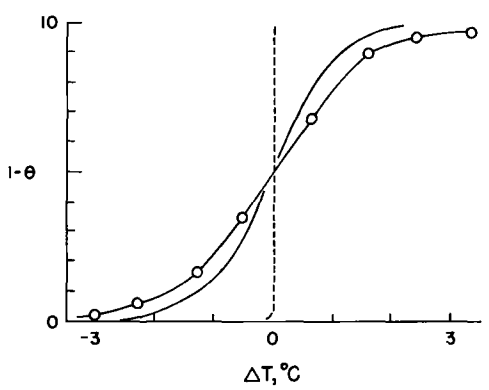

FIGURE 8 An experimental helix–coil transition curve for bacteriophage T2 DNA (open circles) compared with a theoretically predicted curve (solid line). The transition curve that would be predicted if heterogeneity of base pair stability were neglected is shown for comparison (dashed line).

Kinetics of helix–coil transitions

The helix–coil transition in DNA is a complicated process, in which unwinding of the helix and breakage of the hydrogen bonds between the pairs must both occur, although not necessarily in exact parallel. In addition, the cooperative character complicates the mechanism. Therefore, it is not surprising that the relation between the overall rate of the transition and the rates of the elementary steps is not obvious. Yet it is essential that this relation be established, as the major interest lies in the dynamics of the elementary steps. In general, the rate of the process is much slower than that of any of the component steps. Figure 9

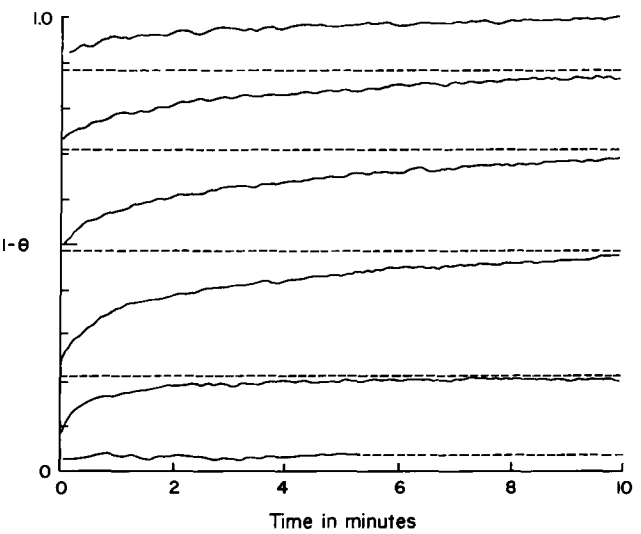

FIGURE 9 The time variation of the absorbence at 270 mμ of a T2 DNA sample after the addition of small increments of alkali in the basic *p*H transition zone. The equilibrium absorbence after each addition is indicated by a dashed line. The absorbency change is normalized to give the fraction of bases in the coil state.

shows the kinetics of denaturation of bacteriophage T2 DNA, which occupies several minutes, even though the opening and closing of a single H-bonded base pair can occur in less than 10^{-7} seconds.

The major dilemma in interpreting the rate of this process is in deciding on the nature of the rate-limiting step. The transition requires both untwisting of the double helix and breakage of hydrogen bonds, and either of these features could be rate determining. The only way to distinguish between the two is to examine the consequences of the assumption that each, in turn, is rate limiting, and to compare the predictions with experiment.

The assumption that the rate of bond breakage is limiting can be treated with a model that is the kinetic analog of that used for the equilibrium statistical-mechanical theory. The basic assumption is that the rate of transformation of a unit from coil to helix depends on the states of only its nearest neighbors. Thus, using 0 for coil and 1 for helix, there are three types of reactions to consider:

$$\text{(a)}\ 100 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 110 \qquad \text{growth}$$

$$\text{(b)}\ 000 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 010 \qquad \text{helix nucleation}$$

$$\text{(c)}\ 101 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} 111 \qquad \text{coil nucleation}$$

where each k is the rate constant for the indicated reaction.

The equilibrium constant for reaction (a) is usually denoted by s, with $s = k_1/k_{-1}$. Because the nucleation of a helical region requires the formation of a base pair that is not adjacent to an existing pair, the stacking free energy gained in reaction (a) is absent in reaction (b). Therefore, the equilibrium constant for reaction (b) is much smaller than for (a), and is usually given the symbol σs. σ is about 10^{-4}, a result found from the equilibrium theory.[15] The σs factor is actually modified by weighting functions for closure of rings of the two backbone chains, but this need not concern us here. Since

$$\sigma s = k_2/k_{-2}, \tag{3}$$

the nucleation reaction (b) must be characterized either by a forward rate constant k_2 which is much smaller than k_1, or by a backward rate constant k_{-2} larger than k_{-1}, or some combination of these. It is not yet known which alternative holds. Similar arguments can be made concerning reaction (b).

On the basis of reactions (a), (b), and (c), it is simple to write down a kinetic equation for θ, the fraction of units in helix form:

$$\frac{d\theta}{dt} = k_1(f_{100} + f_{001}) - k_{-1}(f_{110} + f_{011}) + k_2 f_{000} - k_{-2} f_{010} + k_3 f_{101} - k_{-3} f_{111}, \tag{4}$$

where f_{ijk} is the frequency of occurrence of each of the eight possible states of the triplet.

It has not yet been possible to solve equation (4) in closed form. The major difficulty is that the way the f_{ijk} vary with time depends on the frequency of occurrence of quadruplets, which depend on quintuplets, etc. There is one condition under which the f_{ijk} are known, however. This is at equilibrium, because they can be calculated from the statistical theory. Thus, if the reaction starts from equilibrium, the *initial* rate can always be written in terms of a simple equation involving the rate constants for the elementary steps. This is a very useful feature for a simple characterization of the model.

One can distinguish two approaches to measurement of the kinetics of the helix–coil transition. Under the conditions of relaxation kinetics, one would bring the system to a point within the transition zone and then make a very small perturbation in the external conditions so that only a small change in θ results. Or, alternatively, one could make a large perturbation so that a major portion of the transformation occurs in a single step. The theoretical approaches to these two situations are of necessity different, because the former permits certain linearizing assumptions that are invalid for large perturbations.

Schwarz[22] has developed an approximate theory for the relaxation kinetics of this model. His treatment introduced the above-mentioned observation, that the initial rate is related by a simple equation to the basic rate constants. Several important features of cooperative reactions emerge from his calculations. As a cooperative reaction involves many sequential steps, the over-all rate is considerably slower than the individual steps. In addition, a less obvious result is that the relaxation rate is slowest at the midpoint of the transition.

More recently, we have developed[23] an approximate method for generating complete kinetic curves for both small and large perturbations of this same model. One example of such a curve is shown in Figure 10. It should be noted that for this cooperative reaction ($\sigma = 10^{-4}$) the over-all rate is several orders of magnitude slower than the elementary step, the time for which would be of the order of $1/k_{-1}$. Also, even though the cooperative reaction involves transformations among many states, the total reaction is remarkably well described by a single exponential decay, as shown by the linearity of the logarithmic plot, Figure 11. In addition, in all cases we find that the initial rate is well defined, so that this should be a suitable measure of the over-all rate for a system that satisfies the assumptions of the model.

Using the initial rate as a measure of the kinetics, we can summarize the dynamic characteristics of the cooperative

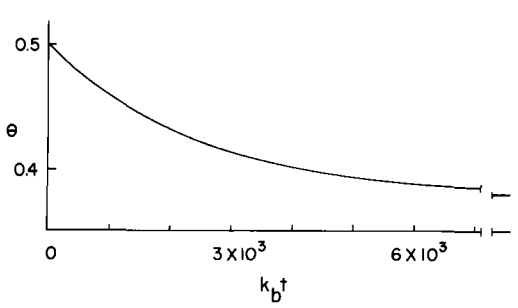

FIGURE 10 A typical kinetic curve generated for a cooperative reaction as described in the text. For this particular system, in which $\sigma = 10^{-4}$, the rate of the transition is about 3 orders of magnitude slower than the elementary step. The tone axis is $k_b t$, where t is time and k_b is identical with k_{-1} defined in text.

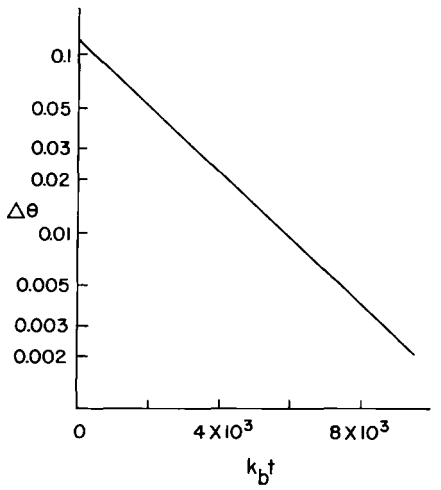

FIGURE 11 A logarithmic plot of the decay curve shown in Figure 10, demonstrating that the reaction is quite well described by a single exponential term, even though the reaction involves many steps.

reaction. Near the middle of the transition the rates of helix formation (k_1) and breakage (k_{-1}) are nearly equal. The relaxation kinetics under these conditions can be regarded as involving the diffusive motion of the boundary between helix and coil (Figure 12). The time required for relaxation is approximately that required for the "diffusion" of the boundary over the average length d of helix or coil. The time is proportional to d^2, and as d^2 is proportional to $\sqrt{\sigma}$ (from equilibrium theory), the rate is proportional to σ. For a large perturbation, k_{-1} is substantially larger than k_1, so a linear movement of the boundary results, at a rate proportional to $k_{-1} - k_1$. The time required is proportional to d, so the rate is proportional to $\sigma^{\frac{1}{2}}$. In all cases the rate depends on both the initial and final conditions, and is

1. Relaxation, $k_f \approx k_p$
   . . . 00001111 . . .
   $\leftrightarrow$
   "diffusion" of boundary
   time $\sim d^2$, $d^2 \sim \sigma^{-1}$

2. Large perturbation, $k_b \rangle k_f$
   . . . 00001111 . . .
   $\rightarrow$
   linear motion of boundary
   time $\sim d$, $d \sim \sigma^{-1/2}$

FIGURE 12 Summary of the qualitative behavior expected for the kinetics of a cooperative process under relaxation conditions, and when the perturbation is large.

much faster for a large perturbation than in the relaxation zone.

If the rate-limiting factor in DNA denaturation is the resistance to untwisting the molecule, the rate should depend on the length of the chain, as the viscous resistance would increase with length. One model[14] is to regard the process as the diffusion of the "twist" of the two strands about each other from the center to the ends of the molecule. In this case, the time required should depend on the square of the length of the diffusion path. As shown in Figure 13, the relaxation time for the denaturation of a series of T2 DNA samples of varying molecular weight M varies with M^2 for M below 20×10^6. This relation fails above $M = 20 \times 10^6$, the relaxation time becoming much less dependent on M. It was originally proposed[14] that this phenomenon was the result of single-strand breaks in the

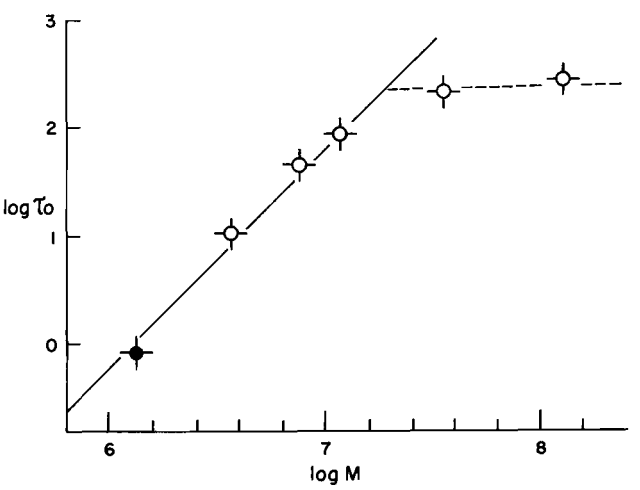

FIGURE 13 Variation of the log of the time required for denaturation of a DNA sample as a function of its molecular weight M. Times are measured in seconds.

DNA, which would act as points of free rotation. Recent work of Elson, Spatz, and Baldwin[24] eliminates this hypothesis. At the present time the reasons for the complicated behavior of this system are obscure, and more experimental evidence is needed before further hypotheses are justified.

One system in which the question of the rate-limiting factor can be asked directly is the helix–coil transition in the dAT:dAT alternating copolymer. This polynucleotide has the ability to form hairpin helices with a single strand, as shown in Figure 14. In denaturation, the rate could be limited by twisting of the hairpin or by the rate of unpairing. The latter is independent of the length of the

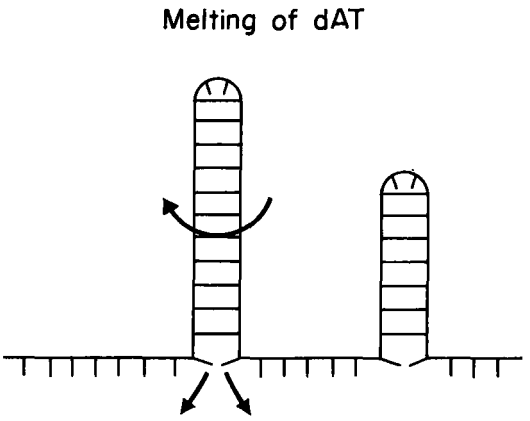

FIGURE 14 A simplified model for the melting of the dAT copolymer, illustrating two possible rate limiting factors: rotation of the hairpin helices, and breakage of the hydrogen-bonded base pairs.

helix, whereas the former becomes slower for longer helices. Thus, the two assumptions lead to different consequences, and comparison with the experimental measurements of Spatz and Baldwin[25] on this system can distinguish between the two. We have made such calculations, with the conclusion that the major rate-limiting factor is resistance to rotation, and that the apparent frictional coefficient is roughly what one would expect when a DNA helix is twisted in solution.

One important question is the rate of the elementary step of helix growth, reaction (a). In systems found to be limited by resistance to rotation, it is possible to set only a lower limit on the rate of this step. From our calculations on the rate of melting of the dAT copolymer, we estimate that k_1 is larger than 10^7. This is consistent with recent work of Pörschke,[26] Eigen, and Müller, on oligomers of adenylic acid (which form helices in the acid range), where it was concluded that the time required for helix growth is around 10^{-7} to 10^{-8} seconds. The rate of the nucleation steps may be quite different. For example, the opening of a base pair in the middle of a helical region is probably considerably slower than the helix-growth reaction, perhaps of the order of 10^{-2} to 10^{-4} seconds.

Binding of actinomycin

Structural changes in DNA are induced by the binding of small molecules. This is shown, for example, by changes in the viscosity of DNA solutions in the presence of Hg^{++},[27] proflavin,[11] actinomycin,[28] etc. In collaboration with Dr. Müller, we have recently carried out a study of the properties of the complex of actinomycin with DNA.[29] The actinomycins are chromopeptides of fairly complex structure[30] that bind strongly to DNA and block the synthesis of messenger RNA.[31] The kinetics of formation of the complex is complicated, with several slow first-order steps indicating structural rearrangements of the initially formed intermediate complex. The nature of the sequential steps is not yet certain, but studies on analogous compounds show that the peptidic parts of the molecule, while not essential for binding, are necessary for the slow first-order parts of the reaction. Therefore, the structural change involves a mutual adaptation of DNA and oligopeptide, a system we regard as a simple model for a DNA–protein interaction.

The dissociation of actinomycin from DNA involves the reverse of the structural change, and is remarkably slow, with an average dissociation time of about 10 minutes. This feature is probably essential to its biological action, because a rapidly dissociating molecule would not be an effective block for the progression of RNA polymerase along the DNA template. In this regard it is noteworthy that actinomycin analogs lacking the peptidic portions, and therefore showing rapid dissociation reactions, are much less effective in blocking RNA polymerase, even though their binding constants are about the same as for actinomycin.

This system, therefore, exemplifies the use of a conformational change to control the time scale of response of a chemical system (the dissociation of the complex). It is quite possible to imagine the cyclic coupling of conformational changes—of a very different kind, to be sure—to produce periodic phenomena on almost any time scale. The periodic electrical activity of the brain may be cited as an example.

Summary

Conformational changes in nucleic acids are common. They occur to a lesser or greater extent in all interactions in which these substances take part, from genetic control to transcription and translation of the message, because the nucleic acid must of necessity be altered by the interaction. The stabilization of the double helical form by highly cooperative interactions rigidly maintains the integrity of the whole molecule while permitting the local structural fluctuations necessary for recognition processes. The individual steps in base-pairing reactions are very rapid, as must be the case for an efficient information processing system. In addition, we may anticipate the importance of mutual conformation changes in the interaction of DNA with the substances, probably proteins, that exert control over genetic function.

Thermodynamics and Some Molecular Aspects of Biology

LARS ONSAGER

A GREAT VARIETY OF BIOLOGICAL PROCESSES invite thermodynamic analysis. Organs and organelles perform remarkable feats of concentrating useful substances and eliminating metabolic end-products, and the organism utilizes a respectable fraction of the energy obtainable from the conversion for maintenance, activity, and growth.

On the operational definition of thermodynamic functions

As in other kinds of bookkeeping, the trickiest questions that arise in the application of thermodynamics deal with the proper identification and classification of the entries; the arithmetic is straightforward. It behooves us to inquire how and to what extent the concepts of thermodynamics may be related to the observable properties of a biological system.

The first law of thermodynamics guarantees the conservation of energy, so that the operational definition of that quantity is always clear.

The second law of thermodynamics forbids perpetual motion of the second kind and implies the existence of a definable entropy for any system in a state that can be reached by a succession of reversible processes. These "thermodynamic" states are typically defined as states of "equilibrium" under specified restraints on composition, energy, and external boundary conditions, in the sense that no spontaneous change can occur in the system as long as the constraints remain fixed. For such thermodynamic states the condition for mechanical equilibrium

$$0 = \delta E = \Sigma X_i \, \delta x_i,$$

in terms of displacements x_i and corresponding forces X_i, can be readily generalized in the form:

$$0 = \delta E = \Sigma X_i \, \delta x_i + T\delta S + \Sigma \mu_j \, \delta m_j, \qquad (1)$$

which fixes the definition of temperature (T), entropy (S), and chemical potentials (μ), except for a trivial choice of units.

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The third law of thermodynamics even allows us to define an absolute entropy by the relation:

$$S(T) = S(0) + \int_0^T dQ/T, \qquad (2)$$

where $S(0)$ is zero for an ideal crystal.

In practice, one usually ignores whatever disorder arises from the mixing of isotopes and from the variable orientations of nuclear spins; these tend to remain so nearly constant in ordinary processes that no harm is done by these assumptions. Moreover, while many crystals do indeed present a practically perfect arrangement of atoms at low temperatures, there are many others that are not so nearly ideal, and for those we must recognize a positive residual entropy [$S(0) > 0$]. For example, among the known modifications of solid water only one (Ice II) is ideally ordered.

We may take the point of view that crystals which remain disordered at low temperatures are not, strictly speaking, thermodynamic systems. For glasses we have no other choice; these are prepared from the liquid phase by deep undercooling and a measure of thermal hysteresis appears on reheating. In such cases, we can still recognize lower and upper limits for the entropy, and the second law of thermodynamics still forbids transformations inconsistent with the lower limit. Moreover, to the extent that heating and cooling within the solid range may be performed reversibly, the corresponding changes of the entropy are well defined.

In systems in which chemical reactions take place, we can certainly recognize the ultimate chemical equilibrium as a thermodynamic state, but chemists ordinarily extend such recognition to arbitrary mixtures of reactants and products, at least as long as they can keep track of the changing composition. Similarly, we are inclined to recognize distributions and gradients of temperature and chemical potentials in systems in which transport processes occur. The underlying idea is always that as long as the gradients are not too steep and the irreversible processes not too fast, we may still assume that the state of the system is well enough defined in terms of the "instantaneous" and, if appropriate, local, thermodynamic constraints.

The question of how far we may thus extend the concepts of thermodynamics, possibly enriched by some additional variables, is properly a matter of kinetic lore and theory. In biological systems, we encounter rather large numbers of different thermodynamic components and other constraints. The experimental study of all possible interactions would be an enormous task; we can and must attack it with the aid of such general rules as we may discover empirically and, ideally, understand it in the light of molecular theory.

Molecular information theory

Once we attribute the pressure of a gas to random molecular motion and the equalization of temperatures to statistical exchange of energy, the laws of both Boyle and Gay-Lussac are deduced by very simple analysis, as are Henry's law and the law of mass action. On this basis, Avogadro's conjecture requires equipartition of kinetic energy (of translation). To justify the conjecture on kinetic grounds, one first has to compute the entire Maxwellian distribution of molecular velocities—or do this concurrently. Once its kinetic basis was clarified, that long-suspect hypothesis of Avogadro, promoted to the status of "law" and enriched by the generalizations of van't Hoff and Arrhenius, came into its own as a cornerstone of the nascent physical chemistry. The predictions derived from the second law of thermodynamics became statistical rather than absolute; the temperature and the chemical potentials were reinterpreted as parameters of statistical equilibrium distributions, and the entropy itself as a measure of disorder according to Boltzmann's formula

$$S = k \ln W, \qquad (3)$$

where Boltzmann's constant k is simply the gas constant per molecule and W measures the volume in the many-dimensional phase space or, on the basis of quantum theory, simply the number of different states compatible with the thermodynamic constraints. The temperature then measures the price of free energy in terms of information, the unit of which is the bit; currently, the summer rate in Boulder is about 5×10^{20} bits per foot-pound.

We have ample evidence that this is, indeed, a realistic interpretation of thermodynamics. Microscopists are well acquainted with the Brownian motion and know that meaningful averages pertaining to that phenomenon can be predicted quantitatively according to Einstein's prescription. Similarly, instrument builders refrain from increasing the sensitivity of their devices beyond the point at which an energy of the order kT will produce a signal; or, for sport, they may build an overly sensitive instrument and gather enough data to determine Boltzmann's constant, although hardly with the accuracy obtained by the best alternative procedure.

Boltzmann's formula (3) allows us—at least in principle—to compute the thermodynamic functions of a system whenever we can obtain a substantially complete catalog of its molecular states. For many small molecules the needed information can, in fact, be inferred from spectroscopic observations. Whenever feasible, this is considered the best way to determine the entropy of a gas; it is generally more accurate than the calorimetric method, and the question of a residual entropy is automatically settled.

Our knowledge of the thermodynamic properties of other solid or fluid systems of one or several components still rests essentially on calorimetric and equilibrium studies.

It is not out of the question that a computation from "first principles," based on our present ideas about the fundamental laws of molecular mechanics, might yield more accurate results; but as yet the available mathematical techniques are inadequate for all but the simplest tasks in this field.

In other cases, our understanding of molecular mechanics and statistics plays a somewhat more modest, but still quite indispensable, role: it helps us to characterize valid analogies and to devise appropriate mathematical descriptions of the thermodynamic functions, so that we can interpolate and even extrapolate the results of actual measurements. It is a rather forbidding task to explore in full detail a system of, say, 37 different components; for a practical approach we study simpler systems containing two, three, or perhaps four at a time, and apply such general laws of mixtures as may be appropriate.

A body of sophisticated theory helps us to understand the interactions of charged entities—small, simple ions as well as macromolecules, micelles, and charged surfaces—in solutions of dielectrics. More often than not, the mathematical analysis is performed on a level intermediate between the extreme "microscopic" (individual molecules) and the "macroscopic" (the entire system). A general result of "classical" statistical mechanics (valid when quantum-mechanical effects related to the uncertainty principle may be disregarded) makes the following approach possible: The *average forces* acting upon individual particles in a specified configuration of two or more types possess a potential, w, and the relative probabilities of possible configurations can be described as an ordinary "Boltzmann distribution" in terms of the "potential of the average force":

$$f = (\text{constant})\, e^{-w/kT}$$

On this basis, we expect, for example, that the effective

Coulomb interaction between two ions in a liquid will be determined by the dielectric constant, which we can measure on the macroscopic scale, and we need not worry about the detailed statistics of the solvent molecules except, perhaps, when we consider a pair of ions very close together. In some sense, the potential w is a generalization of the thermodynamic free energy, which plays the same role in Einstein's theory of density fluctuations and light scattering.

Even the autocorrelation of positions and other dynamic variables at different times may be analyzed to good advantage; such considerations pervade the modern theory of transport processes. Einstein's theory of Brownian motion pioneered in this field; on the near-macroscopic level it was shown some time later that microscopic symmetry in past and future implies reciprocal relations for the coupling of macroscopic transport processes.

In these theories, as in Einstein's theory of Brownian motion, it is assumed that at a given time only the spatial distribution of components and energy is relevant to the statistics of the subsequent redistribution. The validity of similar reasoning applied to other irreversible processes such as dielectric or elastic relaxation generally hinges on analogous assumptions. For example, studies of conduction and dielectric relaxation in ice have revealed an interesting coupling between the dielectric relaxation and the migration of ions; for practical purposes the only mobile ions in ice are excess protons (OH_3^+) and proton vacancies (OH^-). Substantially equivalent descriptions were formulated by Onsager and Dupuis in kinetic terms and by Jaccard in terms of "irreversible thermodynamics." The only storage of information recognized in these theories is determined by the average orientation of the water molecules. This is justified on the grounds that the electrically active defects are few and far apart so that their interactions may be disregarded, even though they are all of the long-range Coulomb type.

In this context, we may note that more likely than not the coupling of dielectric relaxation and ionic migration is a general phenomenon pertinent to the dielectric dispersion of concentrated solutions of electrolytes in all polar liquids. The typical decrease of the dielectric constant with increasing concentration need not by itself imply rigid solvation.

Biological systems

Typically, a biological system contains a much greater number of components than are found in other objects of thermodynamic study; by and large we must deal with these as we deal with other many-component systems, except that we must always remain on the alert for molecular interactions of exceptional specificity. Moreover, the common concept of a homogeneous volume phase implies dimensions that are large compared to the molecules and small compared to the moon; in the structured interior of a small cell, that concept may be just marginally valid. An unsymmetrical membrane with incomplete equilibration between its two faces presents a pair of interacting surface phases, a contingency that was not foreseen even by Gibbs.

Whether or not we may count on a strictly homogeneous phase, a statistical equilibrium can be defined for an isolated system under any fixed set of permanent constraints, and under these conditions the temperature, the chemical potentials of neutral molecular components, and the electrochemical potentials of ionic species are precisely defined.

Biological activity involves transport processes as well as chemical reactions, so that we must expect variation of at least the chemical potentials within some of the phases. The local potentials should be reasonably well defined, at least for small, mobile molecules, considering that a solute whose coefficient of diffusion is 10^{-5} will spread to a distance of 10^{-4} centimeters in 10^{-4} seconds and to 10^{-5} centimeters in 10^{-6} seconds. It should take more time for the biological processes to produce a large amount of any major component; after all, the reacting species usually must also find each other by diffusion. Admittedly, a substantial concentration difference may still exist across a membraneous barrier; but then the solute concentration in the barrier layer must, perforce, be very low, and the potentials remain defined in the main reservoirs.

As regards macromolecules, the biopolymers are typically synthesized in well-defined sequences, unlike the random copolymers produced in ton lots by chemical factories. This gives a slight advantage to the application of thermodynamics, as does the tendency to assume a characteristic conformation in preference to that of a random coil. The need for a molecule to select a particular amino acid among 20 or a particular nucleic acid among 4 at every step adds significantly to the free energy required, although it is a minor item in the budget, worth at most 4.3 bits $\sim 3k \sim$ 1.8 kilocalories per mole per monomer for the protein and 2 bits $\sim 1.4\,k \sim$ 0.84 kilocalories per mole for the nucleic acid.

The mechanism of muscular contraction certainly invites speculations about the kinetics and statistics of the conformational changes that may be involved; but it would appear that we need more information about the characteristic proteins before we can develop a good theory.

The various types of selective transport through membranes present interesting problems of kinetics more or less closely related to thermodynamics. It would appear

that the "irreversible thermodynamics" could be safely applied wherever the differentials of chemical potential are small ($\ll kT$); but this is about the only quantitative result that does not depend on a reasonably detailed understanding of the kinetic mechanism.

It is not difficult to understand that an essentially paraffinic layer is impermeable to many ions, because it takes too much energy to transfer an ion from water to a liquid environment of low dielectric constant. How, then, do the smallest ions get into the layer and through it?

An anionic carrier could facilitate passive trading of one monovalent cation for another, driven by a decrease of free energy over-all; the traveling assembly can be neutral. Nonpolar side chains could facilitate the penetration, or the carrier could be attached to some normal constituent of the membrane, lipid, protein, or whatnot. Active transport trading—say Na^+ for K^+ against the potential drops at the expense of ATP—would seem to require a convertible carrier that is alternately phosphorylated and hydrolized on opposite sides of the membrane (Lehninger, this volume).

Electrical conduction through a membrane entails the entry of uncompensated charges, and the observations on nerve axons indicate that different ions—Na^+ and K^+—move in different channels. Moreover, the permeability for Na^+ *increases* when the membrane is depolarized; this *reduces* the driving force on the ion. It has been observed that, in general, small ions can pass through cell membranes more readily than large ones, and certain proteins have been found to facilitate the passage through artificial lipid membranes.

Some of the lessons learned from the study of protonic semiconductors may be pertinent in this context. In ice, as in KH_2PO_4, two kinds of electrically active, structured defects participate in the conduction; ions (H_3O^+, OH, H_3PO_3, $HOP_4^=$) mediate the shuttling of protons from one molecule or group to another while, according to Bjerrum, doubly occupied and vacant H bonds facilitate the rotation of molecules or groups. Effective charges may be ascribed to defects of both species. In ice, each kind carries about half an elementary charge, but in KH_2PO_4 the ions carry much less than the bonding defects, which are both outnumbered and outrun in this crystal. Whatever the division, the defects exert Coulomb forces determined by their effective charges and by the *high-frequency* dielectric constant; relatively small statistical effects associated with the polarization complicate the picture slightly and serve to define finite, if large, low-frequency dielectric constants.

Hypothetical ion channel

Against this background it is not out of the question that an ion channel might be formed by a bundle of protein helices with nonpolar side chains on the outside (Figure 1), stretched between the faces of the membrane and containing at least one reversible chain of proton-bonded polar groups in the interior, together with enough other polar groups to provide a somewhat flexible, local solvation for

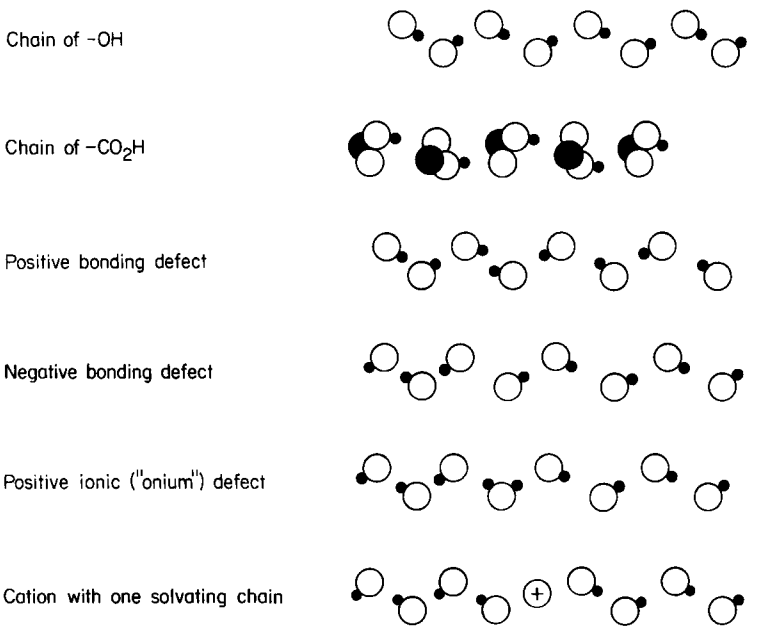

FIGURE 1 H-bond chains and electrically active defects.

the ion. A few molecules, or even a thin column of water, may also be present. As a reversed stretch of chain lengthens behind the advancing ion, the polarization charge travels with it and compensates a reasonable fraction of its own charge, to keep the electrostatic energy within tolerable bounds. Only the net effective charge passes with the ion itself; but the subsequent polarization current transported by one or two defects brings the total up to a full elementary charge and readies the channel for forward passage of the next ion.

This mechanism produces different effects according to the availability of mobile defects. Simple transport of ions results if the solvating defects serve exclusively for repolarization as well. If mobile protons or proton vacancies, as well as Bjerrum defects, are available, the channel can transmit protons. Finally, if an anionic defect moves with, say, potassium while Bjerrum defects repolarize—or vice versa—the channel will trade K^+ for H^+ and carry no net electric current.

The mechanism regulating the transmission of Na^+ in the propagation of action potentials would seem to require a specialized structure connected with the channel, but not immediately involved in the transport. The observed sensitivity of the regulation to changes in the electric field across the membrane indicates that this field must act with a considerable leverage—hardly less than corresponds to the transfer of a full elementary charge through the whole potential drop—but again, for speed that charge must be divided. A controlling device answering to these specifications could depend on a set of reversible chains of hydrogen bonds, quite possibly somewhat biased and unsymmetrical, linked to the structure of the channel so as to regulate either the admission of ions or their mobility in the channel.

Enzyme Complexes

LESTER J. REED

A SUBSTANTIAL PORTION of the enzyme complement of most cells is normally found to be firmly bound to, or incorporated within, membranes. Until recently, it has been tacitly assumed that those enzymes that are not so bound are distributed at random through the liquid phase of the cell. It is now becoming clear, however, that many of these "soluble" enzymes are not randomly distributed in the intact cell, but rather are organized in specific ways into functionally significant assemblages.[1] These enzyme assemblages may be present as discrete multienzyme complexes, such as the α-keto acid dehydrogenase and certain fatty acid synthetase complexes, which may, in a functional sense, be regarded as a type of subcellular organelle. Other "soluble" enzyme systems, such as the glycolytic system of enzymes, appear to be more or less loosely attached to membranes,[2] possibly to "foundation" macromolecules, such as structural proteins, which are, in turn, an integral part of the membranes. The net result of either type of organization is localization, or compartmentation, of enzymes. The implications of such organization of enzymes are far-reaching and will be considered in detail later in this paper. Suffice it to say for the present that interactions among the components of organized enzyme systems may give rise to increased efficiency, or to new three-dimensional arrangements and thereby new modes of activity, and may also provide the basis for elaborate control mechanisms.

First, I would like to direct attention to examples of discrete multienzyme complexes. These complexes have a definite morphology, and each of them catalyzes a coordinated sequence of reactions. The architecture, assembly, and function of these complexes are interesting in themselves. Moreover, it is reasonable to suppose that holding together enzymes with related activities serves ends that are similar for relatively simple particles and for the more elaborate arrays associated with the larger structural elements of the cell. Attention to such systems may be repaid by insight into the general advantages a cell obtains from the specific ordering of its enzymes. Second, the important roles played by structural protein and phospholipid in the organization and function of the electron transport chain are discussed. Finally, the possible consequences of the intracellular organization of enzymes are considered in some detail.

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α-Keto acid dehydrogenase complexes

Several enzymes participate in the oxidative decarboxylation of the α-keto acids pyruvate and α-ketoglutarate which is shown in the reaction:

$$RCOCO_2H + CoA\text{-}SH + DPN^+ \rightarrow RCO\text{-}S\text{-}CoA + CO_2 + DPNH + H^+$$

This represents the main pathway of α-keto acid oxidation in animal tissues, and it occurs widely among microorganisms as well. Enzyme systems that catalyze this reaction have been isolated as multienzyme complexes with molecular weights of several million from pigeon breast muscle, pig heart muscle, beef kidney mitochondria, and *E. coli*. Two classes of complexes have been obtained, one specific for pyruvate, the other for α-ketoglutarate.

The available evidence indicates that the oxidative decarboxylation of pyruvate and α-ketoglutarate proceeds by way of the sequence shown in Figure 1,[3,4] where the brackets indicate enzyme-bound intermediates. The *E. coli* pyruvate dehydrogenase complex (molecular weight about 3.8 million) has been separated into three enzymes: pyruvate decarboxylase, dihydrolipoyl transacetylase, and a flavoprotein, dihydrolipoyl dehydrogenase.[5,6] The complex has been reconstituted from the isolated enzymes. The *E. coli* α-ketoglutarate dehydrogenase complex (mol wt about 2.3 million) also has been separated into three enzymes, analogous to those obtained from the pyruvate dehydrogenase complex and it, too, has been reassembled from the isolated enzymes.[7] The three enzymes are α-ketoglutarate decarboxylase, dihydrolipoyl transsuccinylase, and dihydrolipoyl dehydrogenase.

RESOLUTION AND RECONSTITUTION OF THE *E. coli* PYRUVATE DEHYDROGENASE COMPLEX The individual enzymes are linked in the two complexes by noncovalent bonds. The flavoprotein and decarboxylase molecules can be selectively dissociated from the pyruvate dehydrogenase complex without significant loss of enzymatic activity. The molecular weights of the decarboxylase, flavoprotein and transacetylase are approximately 183,000, 112,000, and 1 million, respectively. The transacetylase has in turn been dissociated into disordered, inactive subunits in the presence of dilute acetic acid. Rapid dilution of the acid solution into suitable buffers results in restoration of enzymatic activity and the characteristic structure of the native transacetylase unit. The transacetylase is apparently a self-assembling system.

The decarboxylase and the flavoprotein do not combine with each other, but each of these components does combine with native or with reconstituted transacetylase. Apparently, the transacetylase possesses specific binding sites for the decarboxylase and for the flavoprotein. When the three components are mixed at neutral *p*H, a large unit spontaneously forms; this resembles the native pyruvate dehydrogenase complex in composition, enzymatic activities, and appearance in the electron microscope.

The composition of the pyruvate dehydrogenase complex, based on enzymatic, chemical, and physical data, is summarized in Table 1. It consists of 12 molecules of the

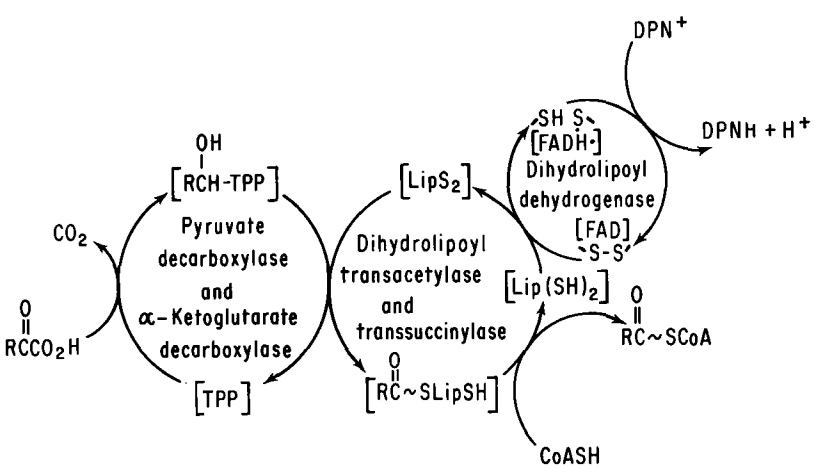


FIGURE 1 Reaction sequence in pyruvate and α-ketoglutarate oxidation. The abbreviations used are: TPP (thiamine pyrophosphate); $LipS_2$ and $Lip(SH)_2$ (lipoyl moiety and its reduced form); CoASH (coenzyme A); FAD (flavin adenine dinucleotide); DPN^+ and DPNH (diphosphopyridine nucleotide and its reduced form).

TABLE I

Composition of E. coli *pyruvate dehydrogenase complex*

Component	Mol wt	No. of molecules	No. of chains per molecule*
Decarboxylase	183,000	12	4 ($\alpha_2\beta_2$)
Flavoprotein	112,000	6	2 (γ_2)
Transacetylase	1,000,000	1	24

*Tentative data.

decarboxylase, 6 molecules of the flavoprotein, and about 24 identical polypeptide chains comprising the transacetylase aggregate. Preliminary chemical data indicate that both the flavoprotein and the decarboxylase contain two or more polypeptide chains.

MACROMOLECULAR ORGANIZATION OF *E. coli* PYRUVATE DEHYDROGENASE COMPLEX Interactions among the prosthetic groups of the three separate enzymes of the pyruvate dehydrogenase complex occur within a unit in which the movement of the individual enzymes is restricted and from which intermediates do not dissociate. Highly favorable positioning of the three enzymatic components and, by inference, of the prosthetic groups of these components must be assumed in order to account for the occurrence of the over-all reaction. Granting this assumption, it still appears impossible for the prosthetic groups of the three separate enzymes to be close enough to each other to interact. Although there is as yet no experimental evidence, the possibility has been considered that one or more of the component enzymes of the complex undergoes a conformational change during the coordinated reaction sequence, bringing the bound prosthetic groups into juxtaposition. An alternative possibility is that the lipoyl moiety, which is bound covalently to the ϵ-amino group of a lysine residue in the transacetylase (Figure 2),[8] rotates between the prosthetic groups of the other two enzymes (Figure 3).

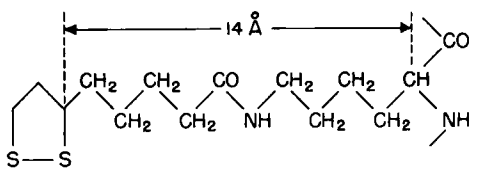


FIGURE 2 Functional form of lipoic acid in *E. coli* pyruvate and α-ketoglutarate dehydrogenase complexes. The carboxyl group of lipoic acid is bound in amide linkage to the ϵ-amino group of a lysine residue, providing a flexible arm of approximately 14 Å for the reactive dithiolane ring.

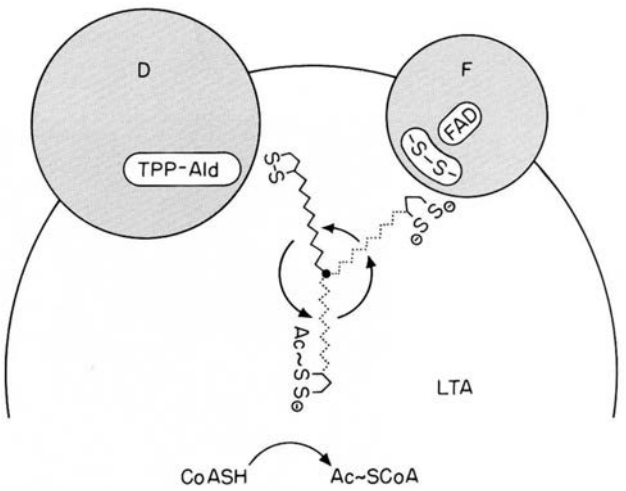


FIGURE 3 A schematic representation of the possible rotation of a lipoyllysyl moiety between "aldehyde-thiamine pyrophosphate" bound to pyruvate decarboxylase (D), the site for acyl transfer to CoA, and the reactive disulfide of the flavoprotein (F). The lipoyllysyl moiety is an integral part of dihydrolipoyl transacetylase (LTA). The net charge on the lipoyl moiety during its cycle of transformations may be 0, minus 1, or minus 2. This change in net charge may provide the driving force for displacement of the lipoyl moiety from one site to the next within the complex.

These biochemical studies indicate that the pyruvate dehydrogenase complex is an organized mosaic of enzymes in which each of the component enzymes is so located as to permit efficient coupling of the individual reactions catalyzed by these enzymes. This concept has been confirmed and extended by electron microscope studies.[1,9] Electron micrographs of the pyruvate dehydrogenase complex negatively stained with phosphotungstate show a polyhedral structure with a diameter about 300 Å (Figure 4A). There appears to be a tetramer of structural elements in the center of the polyhedron, surrounded by an array of subunits. Electron micrographs of the isolated transacetylase (Figure 4B) show particles, 120 to 140 Å on a side, that closely resemble the core of the pyruvate dehydrogenase complex. Some of the images seen in Figure 4B have the appearance of two parallel rows of subunits, with a length of 170 to 200 Å. The two types of images are enlarged in Figure 4C and 4D. The appearance of the transacetylase particle suggests that it is composed of subunits situated at the eight vertices of a cube. In the model shown in Figure 4E and 4F, the subunits are represented as spheres. (It is recognized that in the actual transacetylase molecule the subunits are in all probability not uniform spheres. Furthermore, each subunit, i.e., sphere, apparently consists of three identical poly-

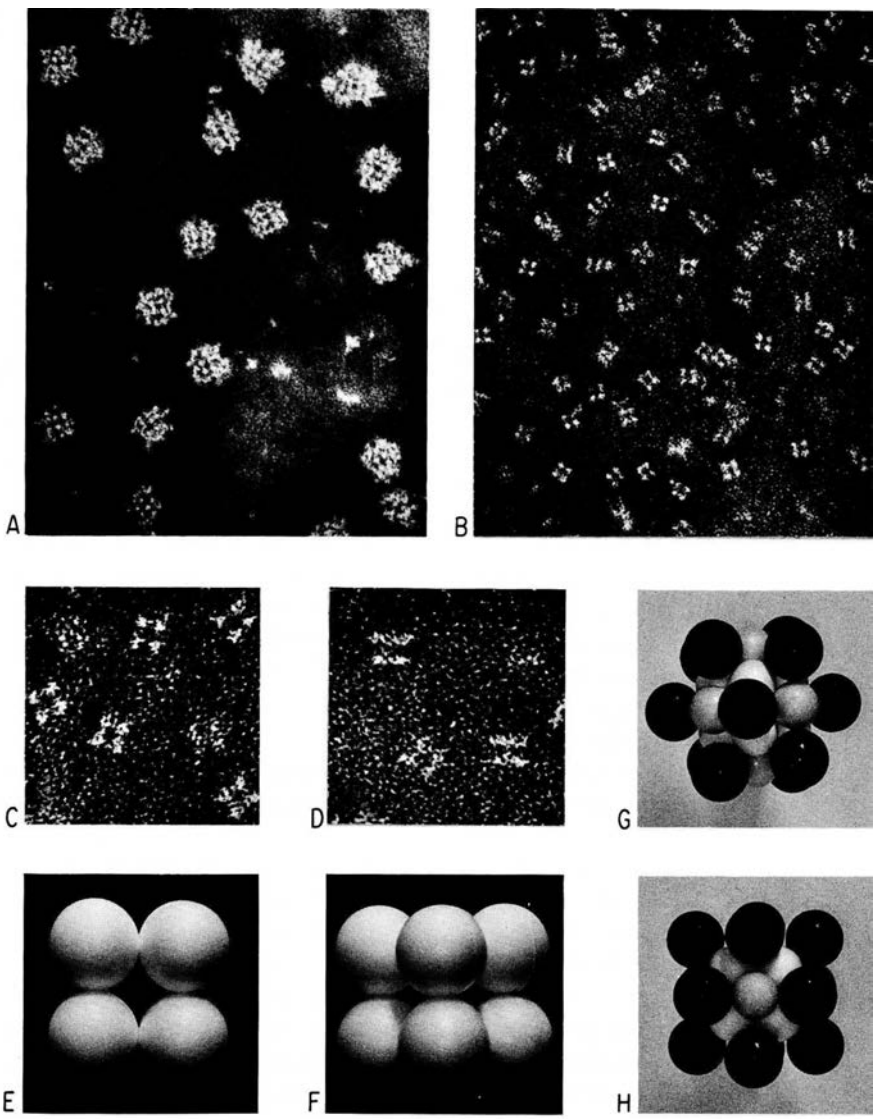


FIGURE 4 A: Electron micrograph of the *E. coli* pyruvate dehydrogenase complex negatively stained with phosphotungstate, *p*H 7.0. X 200,000. B: Electron micrograph of dihydrolipoyl transacetylase isolated from the *E. coli* pyruvate dehydrogenase complex. X 200,000. C, D: Selected images of the transacetylase particle showing tetramers (C) and two parallel rows of subunits (D). X 400,000. E, F: Tentative model of the transacetylase consisting of eight spheres at the vertices of a cube, viewed down a fourfold axis (E) and a twofold axis (F). These views of the model correspond to the orientations of the transacetylase particle shown in (C) and (D). G, H: Tentative model of the *E. coli* pyruvate dehydrogenase complex. The 12 molecules of pyruvate decarboxylase (large black spheres) and 6 molecules of flavoprotein (medium-size spheres) are aligned, respectively, on the 12 edges and in the 6 faces of the transacetylase cube.

peptide chains.) In these photographs the model is viewed down a fourfold axis (4E) and a twofold axis (4F).

The structure of the transacetylase apparently determines the structure of the pyruvate dehydrogenase complex. The molecules of pyruvate decarboxylase and of the flavoprotein appear to be aligned on the edges and on the faces of the cube. The experimental finding that the native pyruvate dehydrogenase complex contains approximately 12 molecules of decarboxylase and 6 molecules of flavoprotein suggests that the decarboxylase molecules are located on the 12 edges and the flavoprotein molecules on the 6 faces of the cube. A model of the pyruvate dehydrogenase complex, assembled on this basis, is shown in Figure 4G and 4H.

E. coli α-KETOGLUTARATE DEHYDROGENASE COMPLEX When mixed, the three enzymes of the *E. coli* α-ketoglutarate dehydrogenase complex combine spontaneously to produce a large unit that resembles the native α-ketoglutarate dehydrogenase complex. Electron micrographs of the isolated transsuccinylase show images (Figure 5A) that are strikingly similar to those seen with the transacetylase (cf. Figure 4B). It has not yet been possible to obtain satisfactory electron micrographs of the intact α-ketoglutarate dehydrogenase complex, since it apparently dissociates during negative staining of the specimen with phosphotungstate. However, various data strongly indicate that the macromolecular organization of the α-ketoglutarate dehydrogenase complex is similar to that of the pyruvate dehydrogenase complex.

Combinations of the enzymes comprising the pyruvate dehydrogenase complex with those of the α-ketoglutarate dehydrogenase complex were examined. The two flavoproteins are interchangeable with respect both to complex formation and function, and enzymatic, chemical, physical, and immunological data obtained thus far indicate that the two flavoproteins are very similar, if not identical. However, the data indicate that the two decarboxylases and the two transacylases are not functionally interchangeable, nor do these components form hybrid complexes; although functionally similar, these enzymes are chemically different.

BIOSYNTHESIS OF THE *E. coli* α-KETO ACID DEHYDROGENASE COMPLEXES The studies of Henning and coworkers[10,11] with acetate mutants of *E. coli* K12 indicate that the structure and interactions among the subunits of the pyruvate dehydrogenase complex are under direct genetic control. The structural genes for the decarboxylase and for the transacetylase are closely linked. The location of the structural gene for the flavoprotein is not yet known. Under certain conditions of growth, *E. coli* K12 has been observed to produce the three components of the pyruvate dehydrogenase complex in precisely the proportions required for the construction of the complex. The mechanism by which the cell coordinates the rates of synthesis of the components of the aggregate is thus far unknown.

EVOLUTION OF THE *E. coli* PYRUVATE DEHYDROGENASE COMPLEX The component enzymes of the pyruvate dehydrogenase complex can utilize free lipoamide (or lipoate) in place of the protein-bound lipoyl moiety, although at reduced rates.[3,4] This observation suggests that in a primitive organism the three enzymes, now joined to form the pyruvate dehydrogenase complex, may have existed as separate entities, with lipoate (or a structurally related compound) serving as a "mobile" coenzyme, shuttling among the three enzymes. In the course of evolution, the three enzymes may have acquired the capacity of combining with each other. An arrangement of this kind may have been more efficient and more easily controlled than a series of three separate enzymes. Subsequently, the ability to attach the lipoyl moiety covalently to the transacetylase may have developed, fixing the coenzyme in a position close to the active sites requiring its presence.

MAMMALIAN α-KETO ACID DEHYDROGENASE COMPLEXES The basic similarity in mechanism of α-keto acid oxidation observed with both bacterial and mammalian α-keto acid dehydrogenase complexes suggests similarities in composition and macromolecular organization. This indeed appears to be the case. Pyruvate and α-ketoglutarate dehydrogenase complexes isolated from beef kidney mitochondria and from pig heart muscle appear to be composed of three enzymes, analogous to those comprising the corresponding bacterial complexes.[1] The macromolecular organization of the three enzymes in the mammalian α-ketoglutarate dehydrogenase complex closely resembles that of the two bacterial complexes.[12] The three-dimensional structure of the mammalian pyruvate dehydrogenase complex differs somewhat from that of the other three complexes.[12] It appears that the principles of macromolecular organization of both the bacterial and mammalian α-keto acid dehydrogenase complexes are similar and may well be elaborations of the principles of self-assembly observed with the coat proteins of "spherical" viruses.[13]

Electron micrographs of the mammalian α-ketogutarate dehydrogenase complex and the transsuccinylase component isolated from it are shown in Figure 5. The appearance of the transsuccinylase particle (Figure 5B) is strikingly similar to that seen in the bacterial transacylases

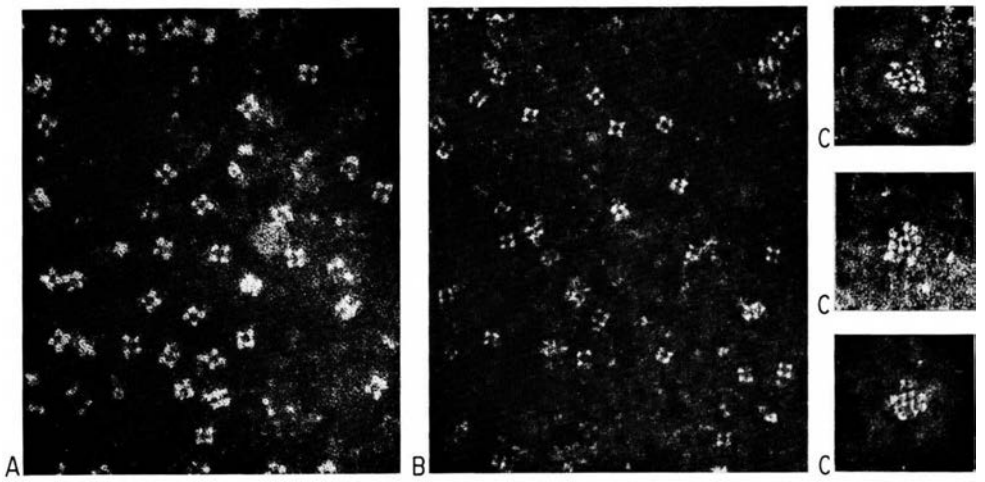
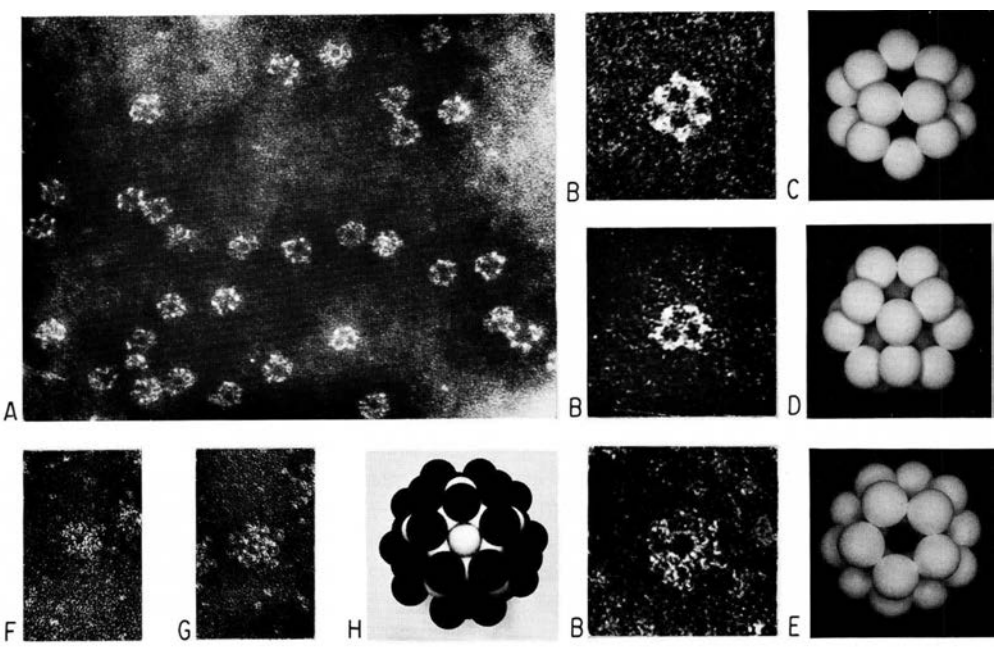


FIGURE 5 A: Electron micrograph of dihydrolipoyl transsuccinylase isolated from the *E. coli* α-ketoglutarate dehydrogenase complex; negatively stained with phosphotungstate. X 200,000. B: Electron micrograph of dihydrolipoyl transsuccinylase isolated from the mammalian α-ketoglutarate dehydrogenase complex. X 200,000. C: Electron micrographs of the mammalian α-ketoglutarate dehydrogenase complex. X 200,000.

FIGURE 6 A: Electron micrograph of dihydrolipoyl transacetylase isolated from the mammalian pyruvate dehydrogenase complex; negatively stained with phosphotungstate. X 200,000. B: Selected individual images of the transacetylase particle. X 400,000. C–E: Tentative model of mammalian transacetylase consisting of twenty spheres at the vertices of a pentagonal dodecahedron, viewed down a twofold axis (C), a threefold axis (D), and a fivefold axis (E). These views of the model correspond to the orientations of the transacetylase particle shown in (B). F, G: Electron micrographs of the mammalian pyruvate dehydrogenase complex. X 200,000. (Refer to text for interpretation.) H: Tentative model of the mammalian pyruvate dehydrogenase complex. The 30 molecules of pyruvate decarboxylase (large black spheres) and 12 molecules of flavoprotein (medium-size spheres) are aligned, respectively, on the 30 edges and in the 12 faces of the transacetylase dodecahedron.



(cf. Figure 4B and 5A), and the gross appearance of the mammalian α-ketoglutarate dehydrogenase complex (Figure 5C) resembles that of the bacterial α-keto acid dehydrogenase complexes (cf. Figure 4A).

Electron micrographs of the mammalian pyruvate dehydrogenase complex and the transacetylase component of this complex are shown in Figure 6. The diameter of the transacetylase particle (Figure 6A and 6B) is approximately 210 Å. Its appearance suggests that it is composed of subunits situated at the vertices of a pentagonal dodecahedron. In the model shown in Figure 6C through 6E, the subunits are represented as spheres; in these photographs the model is viewed down a twofold axis (6C), a threefold axis (6D), and a fivefold axis (6E). Although the mammalian pyruvate dehydrogenase complex tends to dissociate under the negative staining conditions used, some images (Figure 6F and 6G) were seen, with the appearance of a symmetrical mulberry, in which small particles, presumably molecules of pyruvate decarboxylase and flavoprotein, appeared to be attached to and to surround the transacetylase particle. The molecules of decarboxylase and flavoprotein are probably aligned, respectively, along the thirty edges and in the twelve faces of the transacetylase dodecahedron. A model of the mammalian pyruvate dehydrogenase complex, assembled on this basis, is shown in Figure 6H.

NATURE OF THE α-KETO ACID DEHYDROGENASE COMPLEXES *in situ* The isolated mammalian pyruvate and α-ketoglutarate dehydrogenase complexes, like their bacterial counterparts, are relatively large units with distinct three-dimensional structures. It remains to ask whether the molecular architecture of these complexes, as they occur in the bacterial cell or in the animal mitochondrion, is accurately represented by the aggregates that have been isolated, and if so, where these complexes are located. There is as yet little, if any, direct evidence bearing on these questions. Particles like the ones described may very well be present as such, either free or membrane-bound. On the other hand, it is conceivable that these enzyme systems might be collected into smaller aggregates bound to, or localized within, "repeating units" of membranes, as proposed by Green.[14] If these subcomplexes were detached from the membranes in the course of isolation, they could aggregate to produce large complexes that are not normal components of the cell. However, aggregation of subcomplexes released from membranes during the rupture of bacterial cells or animal mitochondria would be likely to produce complexes of several different sizes. No such subcomplexes have been detected by sucrose density gradient centrifugation of bacterial or mitochondrial extracts. Essentially all of the pyruvate and α-ketoglutarate dehydrogenase activities in the extracts are associated with large units that possess the same sedimentation characteristics as the isolated complexes. The evidence available, although not conclusive, suggests that the large α-keto acid dehydrogenase complexes, and not smaller subcomplexes thereof, are present in the intact bacterial cell or animal mitochondrion. It is of interest to note in this context that a single *E. coli* cell or beef kidney mitochondrion contains at most a few hundred molecules of the α-keto acid dehydrogenase complexes.

Fatty acid synthetases

The synthesis *de novo* of long-chain saturated fatty acids from malonyl coenzyme A and acetyl coenzyme A is catalyzed by multienzyme systems referred to as "fatty acid synthetases."[15] The over-all reaction may be represented by the following general equation:

$$\text{Acetyl CoA} + 7\ \text{malonyl CoA} + 14\text{TPNH} + 14\text{H}^+ \rightarrow \text{palmitate} + 14\text{TPN}^+ + 8\text{CoA} + 7\text{CO}_2 + 6\text{H}_2\text{O}$$

The fatty acid synthetases of yeast, pigeons, and mammals occur in the cytoplasm as multienzyme complexes that have resisted fractionation into enzymatically active components. The fatty acid synthetases of bacteria and plants, on the other hand, can be obtained readily as separate enzymes.

Lynen and co-workers[16] have isolated a fatty acid synthetase system from baker's yeast as an organized unit with a molecular weight of approximately 2.3 million. The complex catalyzes a coordinated sequence of seven distinguishable chemical reactions. The intermediates are protein-bound, apparently in thiolester linkage with specific thiol groups. Lynen and co-workers have reported that there are seven different N-terminal amino acids and approximately three moles of each per mole of yeast synthetase. His interpretation of these data is that the synthetase consists of seven different proteins and that each contains three subunits whose average molecular weight is 100,000. Supporting this suggestion is the observation that subunits with an apparent average molecular weight of 110,000 have been obtained by treating the complex with urea or with deoxycholate at *p*H 9. The possibility has also been considered that the synthetase consists of three identical subcomplexes (molecular weight about 700,000), each an assemblage of seven proteins. Electron micrographs of the yeast synthetase negatively stained with phosphotungstate show particles with a diameter of 200 to 250 Å, exhibiting a high degree of structural order (Figure 7). Substructure is apparent in the images, possibly corresponding to subunits.

Attempts to separate the yeast synthetase into enzy-

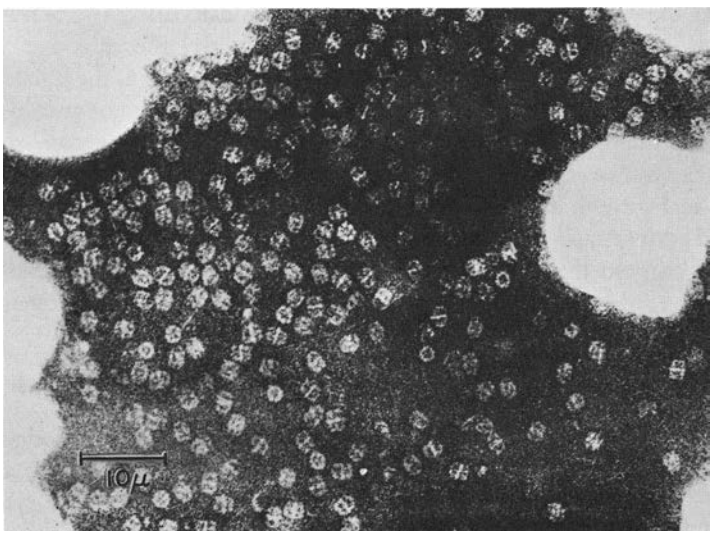


FIGURE 7 Electron micrograph of the yeast fatty acid synthetase negatively stained with phosphotungstate. (From F. Lynen, private communication)

matically active components have been unsuccessful. Lynen suggests that the synthetase subunits are stabilized by their participation in the complex, but become very labile as soon as they are separated. A related possibility is that mutual conformational (allosteric) interactions among the individual components are necessary to produce a catalytically active system. An equally plausible explanation is that the reagents (e.g., urea, deoxycholate) used to dissociate the synthetase also disrupt the otherwise stable conformations of the component enzymes.

The fatty acid synthetase system of avian liver has been isolated in a homogeneous state as an organized unit with a molecular weight of approximately 500,000.[17] It is rather labile, dissociating, for example, in buffers of low ionic strength. The avian fatty acid synthetase is significantly smaller than the yeast synthetase complex although, presumably, the number and sequence of reactions catalyzed by the two synthetases are similar.

In contrast to the fatty acid synthetase complexes of yeast and pigeon liver, the enzyme system catalyzing fatty acid synthesis in *E. coli* can be readily separated into several distinct, enzymatically active fractions.[15] Five different enzymes and an acyl-carrier protein have been obtained in a partially or highly purified state. The reactions of fatty acid synthesis in *E. coli* occur with the substrates bound as thiolesters to the prosthetic group, 4′-phosphopantetheine, of a unique protein, acyl-carrier protein (ACP), with a molecular weight of 9500.[18] A protein similar to ACP is apparently involved in fatty acid synthesis in plants and in mammals and birds as well.

Wakil and co-workers[19] reported recently that phosphorylated sugars, particularly fructose 1,6-diphosphate, stimulate fatty acid synthesis by purified fatty acid synthetase systems of pigeon liver and of *E. coli*. Kinetic studies indicate that fructose 1,6-diphosphate behaves as an allosteric effector in these enzyme systems.

It would appear that, in a multienzyme system in which intermediates are covalently bound to one of the protein components, greater efficiency would be achieved if the enzymes were arranged in a definite pattern, by attachment to one another or to a membrane. This reasoning, together with the isolation of yeast and avian fatty acid synthetases as organized units, leads us to suspect that the *E. coli* synthetase system might well exist in the intact cell as an organized unit or as a membrane-bound system. It should be emphasized in this connection that the procedures used in extracting a multienzyme system from intact cells or organelles may well determine whether or not an organized unit of enzymes is obtained, particularly in cases where the cohesive forces binding the component enzymes to one another (or to a membrane) are weak.

Tryptophan synthetase

The tryptophan synthetase of *E. coli* is composed of two nonidentical and readily separable protein components, designated the A and B proteins (or α and β subunits).[20] The A protein (or α subunit) is a single polypeptide chain with a molecular weight of approximately 30,000. The B protein is believed to be composed of two identical

polypeptide chains, designated the β_2 subunit (mol wt 108,000). The tryptophan synthetase complex consists of one β_2 and two α subunits, $\alpha_2\beta_2$. The complex catalyzes the following reactions:

$$\text{1) Indole} + \text{L-serine} \xrightarrow{\text{pyridoxal-P}} \text{L-tryptophan}$$

$$\text{2) Indoleglycerol-P} \rightleftharpoons \text{indole} + \text{glyceraldehyde-3-P}$$

$$\text{3) Indoleglycerol-P} + \text{L-serine} \xrightarrow{\text{pyridoxal-P}} \text{L-tryptophan} + \text{glyceraldehyde-3-P}$$

Reaction 3 probably represents the physiological function of the enzyme. It is believed to be a specific reaction, not merely the sum of reactions 1 and 2. A unique property of the tryptophan synthetase complex is that each subunit exhibits trace catalytic activity in one of the other reactions. Thus the α subunit can catalyze reaction 2 and the β_2 subunit can carry out reaction 1. However, for maximum activity in reactions 1 or 2 under normal conditions, and for any activity in reaction 3 the two subunits must be in physical contact. The β_2 subunit is also capable of deaminating serine, an activity that is absent not only from the α subunit, but also from the intact tryptophan synthetase. Enzymatic studies with mutant proteins have indicated that mutant α subunits can activate the normal β_2 subunit in reaction 1, and most β_2 subunits can activate the normal α subunit in reaction 2, but none of the mutant tryptophan synthetases can catalyze reaction 3.

The modification of the single-component activities, which occurs on aggregation of the α and β_2 subunits, appears to be a consequence of the aggregation itself rather than of the juxtaposition of two independently functional active centers. The change in catalytic properties that accompanies the aggregation of the two subunits could be caused by the mutual effects of each subunit on the conformation of the other or the improvement of the active center of each subunit by the introduction of amino acid side chains contributed by the other.

The fully associated enzyme ($\alpha_2\beta_2$) is in relatively rapid equilibrium with the free subunits.[21] Pyridoxal phosphate and serine together markedly increase the association of the two subunits; the cofactor pyridoxal phosphate seems to play the greater role. It should be recalled that this coenzyme is also involved in the association of the subunits of glycogen phosphorylase *a*. In other cases as well, cofactors apparently influence the association of enzyme subunits. Pyridoxal phosphate and serine may well be functioning as allosteric effectors, but it is not yet known whether these two substances are bound to tryptophan synthetase at sites other than the active site of the enzyme complex.

It has been suggested that a cell could control the rate of production of tryptophan by altering the state of aggregation of tryptophan synthetase. However, approximate calculations, based on the association constants measured in vitro and on estimates of the concentration of the subunits in a bacterial cell, indicate that the enzyme in vivo would be at least 60 per cent associated, even in the complete absence of pyridoxal phosphate and serine. According to this line of reasoning, the production of tryptophan could not be very effectively controlled by shifting the association equilibrium within the limits available. Moreover, since tryptophan synthetase catalyzes the terminal reaction in the tryptophan biosynthetic sequence, feedback inhibition of the enzyme catalyzing the initial reaction or repression would be more economical. Measurements of the association of the tryptophan synthetase subunits in vivo must be made before a final decision is reached concerning the physiological significance of the effect of pyridoxal phosphate and serine on complex formation.

At least in the case of tryptophan synthetase, the formation of a complex has consequences that go beyond the increased efficiency that might result from assembling the enzymes of a metabolic sequence into an organized unit. It would not be surprising if close examination of the more elaborate multienzyme complexes should reveal similar effects.

Organization of enzyme systems in membranes

The respiratory chain of mitochondria is generally conceived to be an organized assemblage of enzymes capable of carrying out all the functions of electron transport and related processes, including ATP production, active ion transport, and contractile activities.[22] Some 15 or more enzymes are involved in these integrated processes. Whether this assemblage of enzymes is attached to the surface of mitochondrial membranes at recurring intervals, or is an integral part of the membrane itself, is not yet clear. It is becoming clear, however, that structural protein and phospholipid play important roles in the organization and function of this system of enzymes.

Green and co-workers have demonstrated that beef heart mitochondria contain a protein fraction, designated structural protein, which comprises about 50 per cent of the total mitochondrial protein.[23] This protein is extremely insoluble at neutral *p*H, but dissolves at extremes of *p*H or by use of detergents such as sodium dodecyl sulfate. The monomeric form has a molecular weight of about 22,500 and shows a pronounced tendency to aggregate spontaneously. The structural protein of beef heart mitochondria combines with phospholipid and reacts with cytochromes *b*, *a*, c_1, *c*, and with myoglobin to form

specific stoichiometric complexes. Moreover, the functional properties of cytochrome *b* and of myoglobin are altered by this association with structural protein. Structural proteins have also been isolated from membranes of the endoplasmic reticulum, erythrocytes, and chloroplasts. These and other observations have led to the proposal that structural protein and phospholipid form a network system that makes up the matrix of the membrane layer and that the electron transport system, and other enzymes and enzyme systems as well, are attached to this network. It does not appear that any order existing in the arrangement of cytochromes along mitochondrial membranes can reside in the structural protein per se, since Criddle and co-workers[24] have reported recently that a single region on mitochondrial structural protein seems to be involved in complex formation with all of the heme proteins that can be bound to it. That mitochondrial structural protein does play a critical role in the organization and assembly of the electron transport chain is pointed up by the recent work of Woodward and Munkres.[25] These investigators have observed that mitochondrial structural protein from two maternally inherited, respiratory-deficient mutants of *Neurospora crassa* differ from wild type with respect to single amino acid replacements. The structural protein from one mutant (*mi-1*) has one less tryptophan residue per mole and one more cysteine residue than does wild type structural protein. A second mutant (*mi-3*) has one less tryptophan residue than wild type, but not an extra cysteine residue. The amino acid replacing tryptophan is not yet known. The mutant structural protein differs from wild type structural protein in its ability to bind small molecules, such as ATP and reduced diphosphopyridine nucleotide, and the enzyme malate dehydrogenase as well. In the mutants *mi-1* and *mi-3*, as compared to wild type *Neurospora crassa*, cytochrome *c* occurs in 16-fold excess, riboflavin in 2-fold excess, and long-chain unsaturated fatty acid in 15-fold excess. In addition, deficiencies in cytochrome *a*, cytochrome *b*, and cytochrome oxidase have been observed in mutant *mi-1*. Thus, structural alterations in mitochondrial structural protein (resulting apparently from alterations in mitochondrial DNA) appear to disrupt the organization and assembly of the electron transport chain in *N. crassa*.

In further work, Munkres and Woodward[26] have shown that in *N. crassa* mutants the conformational and functional properties of malate dehydrogenase (a mitochondrial enzyme controlled by a nuclear gene) are greatly influenced by attachment to mitochondrial membranes or to mitochondrial structural protein. These observations suggest that genetically determined alterations at a "locational site" on malate dehydrogenase produce irregular binding of the enzyme to mitochondrial membranes and hence malfunction of the enzyme in vivo. Thus it appears that genetically determined structural stereospecificity of *both* enzyme and structural protein is important in determining the conformational and functional properties of enzyme-structural protein complexes.

Another example in which the in vivo function of a system of enzymes is altered by an apparent defect in a "foundation" macromolecule is furnished by Wagner and co-workers.[27] A group of four enzymes is responsible for the synthesis of valine and isoleucine from pyruvate and α-ketobutyrate in *N. crassa*. Some isoleucine-valine requiring mutants apparently possess these four enzymes, yet cannot synthesize the two amino acids at a rate sufficient for growth. Biochemical and genetic evidence indicate that these mutants lack the ability to integrate the four enzymes into a catalytically active unit.

That phospholipid plays an important role in the organization and function of the electron transport chain in beef heart mitochondria is indicated in that components of the chain lose the capacity for electron transfer when lipid is removed, and regain this capacity when lipid is reintroduced. That phospholipid per se is not primarily responsible for the organization of the chain is indicated by the finding that most of the phospholipid can be extracted from mitochondria without affecting the structural integrity of the mitochondrion as judged by electron microscopy.[28]

Several interesting and provocative proposals have been advanced concerning the macromolecular organization of the electron transport chain,[22,29,30] but as yet no single proposal is generally accepted. It appears that structural protein and phospholipid play important roles in the organization and function of the system, but neither bears primary responsibility for the organization. Perhaps the specificity of the organization is provided by protein–protein interactions among the component enzymes of the system, with phospholipid influencing the conformation of the proteins. Stability of the complex may in turn be provided by attachment to a network of structural protein. There can be little doubt that potentialities for elaborate conformational changes, and hence cooperative effects related to metabolic control, exist in this complex system.

Consequences and implications of the organization of enzymes

It is appropriate to ask if there are any obvious advantages for a cell that result from its assembling the enzymes concerned with a given metabolic sequence in a definite

pattern, by attachment to one another or to a membrane. Advantages of two general types may be suggested. First, the aggregation of the component polypeptide chains may produce particles that have catalytic properties unlike those of any of the separate chains; that is, aggregation may itself produce, enhance, or modify the desired activity. Second, if the enzymes that operate in sequence on a given substrate are maintained in proximity to each other, the over-all efficiency of the pathway may be altered, even if the catalytic activities of the individual enzymes are not changed by aggregation.

In the first category is the possibility that the enzymatic activity of a component appears only if it is maintained in a conformation that would be thermodynamically unstable if aggregation did not occur. This is a very difficult thing to prove. Except in a few cases, the procedures required to dissociate aggregates of proteins are variants—gentler ones if possible—of the methods used to disrupt the conformation of a single polypeptide chain. Catalytic activity may be lost on disaggregation because conditions cannot be found that will dissociate the particle without at the same time disrupting the otherwise stable conformation of the component chains. It may in fact be necessary to denature one or more of the chains in order to drive the dissociation process. The idea that the formation of a protein aggregate or membrane-bound array may confer new three-dimensional arrangements and thereby new modes of activity on the components of the complex is an attractive one. This is particularly so in view of the increasing interest in, and evidence for, the "allosterism" concept,[31,32] which rests on the proposition that the three-dimensional structures of proteins are relatively easily perturbed by interaction with specific low molecular-weight compounds or with other proteins.

The collection of several proteins into a complex or a membrane-bound array might also produce an active site involving amino acid side-chains contributed by more than one of the components. In this case as well, aggregation could modify the catalytic activities of the separate components. Tryptophan synthetase of *E. coli* appears to be an example of this phenomenon. Another example may be the pyruvate dehydrogenase complex of *E. coli*. The lipoyl residues that are bound—covalently, as it happens—to dihydrolipoyl transacetylase operate as coenzymes in the reactions carried out by the other members of the complex. In this case, the coenzyme binding site and the rest of the active center are found on different subunits.

The assembly into a complex or a membrane-bound array of a series of enzymes that operate sequentially on a given substrate may very well increase the efficiency of the over-all process, even if the intrinsic catalytic activity of the components is not altered by their association. The increased efficiency of a complex as compared with that of separate enzymes dispersed at random should be most obvious when all intermediate products are strongly bound to the complex. This is true with the α-keto acid dehydrogenase complexes and with the yeast fatty acid synthetase. The turnover number of the *E. coli* pyruvate dehydrogenase complex is high; approximately 80,000 molecules of substrate can be oxidized per minute per molecule of complex. In such a case, the encounter of an intermediate produced by one component with the next catalytic site in the sequence should be very much more probable in an organized complex than in a mixed solution of separate enzymes. By the same token, an intermediate product bound to an aggregate would be more likely to continue through the complete reaction sequence carried on by the complex than to be diverted into a competing process catalyzed by enzymes that are not present in the complex.

The inherent advantages of a multienzyme complex or a membrane-bound array of enzymes as regards efficiency are not so clear if the intermediate products are not strongly bound. If free diffusion is primarily responsible for transferring an intermediate from one active site to the next, then there might be some advantage in placing the two sites close together. The quantitative importance of this advantage is difficult to estimate, however, primarily because free diffusion from one catalytic site to the next may not be a realistic model of the transfer of a substrate from one to another of a sequence of enzymes in the cell. Convective mixing or simple mechanical stirring by the active movement of the cell or its contents could easily produce a distribution of an intermediate quite different from that predicted by a diffusion model. Perhaps the high degree of compartmentation observed in cells of higher organisms serves to offset this effect. In this context, it may be important to build up a high local concentration of an intermediate for efficient functioning of a membrane-bound enzyme with a high Michaelis constant (*Km*).

If the rate of output of a given sequence of enzymes can be affected by assembling them in an ordered array, then this feature of the system could conceivably be used for regulatory purposes. The over-all activity of the pathway would be controlled if the components of the complex could be joined or separated at need. Something of the sort is observed at a simpler level with acetyl CoA carboxylase; activation of the enzyme by citrate is accompanied by formation of an aggregate.[33] However, little evidence exists as yet that control mechanisms of this kind are present in the more complicated multienzyme systems.

There are indications in the literature that enzymes other than those discussed in detail here exist in the cell as multienzyme complexes. These include three of the enzymes in the tryptophan biosynthetic pathway of *N. crassa*,[34] the sulphite reductases of *Salmonella typhimurium* and *N. crassa*,[35] and the nitrate reductase of *N. crassa*.[36] Nevertheless, there are as yet few well-documented examples of multienzyme complexes, and it is difficult to judge how widely this mode of organization may be distributed in nature. It is possible to suggest a few ways in which multienzyme complexes or membrane-bound arrays of enzymes that do exist might have evaded observation. The chief trend of classical enzymology has been toward breaking down metabolic processes into sequences of reactions, each reaction catalyzed by a single enzyme. A major preoccupation of enzymologists has been the separation and purification of the enzymes involved at each stage of each pathway. To be described as "pure," an enzyme must generally be shown to possess the highest possible catalytic activity in one reaction and no detectable activity in any other. Judged by this criterion, a multienzyme complex is an "impure" enzyme. Confronted with such an object in the context of an analytic effort, a worker would proceed, if he could, to remove the "contaminants."

Procedures frequently employed to resolve mixtures of enzymes cannot be assumed to preserve the integrity of protein aggregates. For example, mixtures of proteins are often exposed to concentrated salt solutions in the course of fractionation; the subunits of hemoglobin have been shown to dissociate in concentrated solutions of some salts. The *p*H of a protein solution is frequently varied during a separation procedure; the state of aggregation of tobacco mosaic virus protein changes markedly with *p*H. The dilution of the contents of the cell inevitably accompanies the extraction and isolation of an enzyme; at low concentration, a complex of marginal stability might dissociate as a simple consequence of mass action. More important, the dissociation of an aggregate could be favored if specific low molecular weight compounds present in the cell are diluted or removed by dialysis, gel filtration, or fractional precipitation of the protein components. For example, the binary tryptophan synthetase complex can be resolved very cleanly by sucrose density gradient centrifugation if pyridoxal phosphate and serine are absent. On the other hand, if may be noted that the *presence* of guanosine triphosphate appears to favor the dissociation of glutamic dehydrogenase at high protein concentrations.

The sense of the foregoing argument is that efforts to remove "contaminating" activities from a protein aggregate might very well succeed. With an increasing awareness that many of the classical methods of disrupting cells and of extracting and fractionating enzymes may preclude detection and isolation of organized multienzyme systems, it is entirely possible that more of these complexes will be found.

Summary

It is becoming clear that enzymes are not randomly distributed in the intact cell, but rather are organized in specific ways into functionally significant assemblages. A substantial portion of the enzyme complement of most cells is normally found to be firmly bound to or incorporated within membranes. Some "soluble" enzyme systems are present as discrete multienzyme complexes, such as the α-keto acid dehydrogenase and certain fatty acid synthetase complexes. Other "soluble" systems of enzymes, such as the glycolytic system, appear to be more or less loosely attached to membranes. The net result of either type of organization is localization or compartmentation of enzymes. Attention to such supramolecular systems may be repaid by insight into the general advantages that cells, including nerve cells, obtain from the specific ordering of their components.

Cell Organelles: The Mitochondrion

ALBERT L. LEHNINGER

THE ASCENT FROM simple self-assembling systems such as oligomeric proteins and enzyme complexes to the level of supramolecular organization of subcellular organelles is a very steep one. In making it we cross a boundary to a level of organization at which the self-assembly principle doubtless operates in microscopic regions, but cannot account for the biogenesis of the entire organelle. It is an important result of recent research on simple forms such as *Paramecia* that biogenesis of cytoplasmic organelles cannot proceed without some pre-existing organelle structure that can be replicated or at least built upon by accretion.[1] We therefore come into the relatively uncharted but extremely important domain of cytoplasmic inheritance—the transmission of cytoplasmic characteristics to progeny in a non-Mendelian manner that does not appear to involve the cell nucleus. It is in this level of the structural hierarchy of the cell that we also meet the most fundamental problems of cell development and differentiation, problems of crucial importance in attacking the cell biology of the nervous system.

This chapter will consider in some detail the rapidly increasing knowledge of the biology and molecular biology of the mitochondrion, perhaps the best understood of the various cytoplasmic organelles.[2–8] A little over fifteen years ago, mitochondria were identified as the exclusive site of Krebs tricarboxylic acid cycle activity, fatty acid oxidation, electron transport, and oxidative phosphorylation. In the years since, the mitochondria have been known to generations of students as the "power plants" of the cell. But just in the past two or three years a wholly new aspect of mitochondrial structure and function has emerged; these organelles contain a specific type of DNA different from that in the cell nucleus, they participate in protein synthesis, and they may be elements in cytoplasmic inheritance and differentiation.

Mitochondria were first observed in muscle cells by Kolliker almost a hundred years ago.[2] In the last years of the nineteenth century they became a favorite study object of cytologists. It was not until the late 1940's, however, that newly perfected differential centrifugation methods made isolated mitochondria available for direct biochemical study, and the early 1950's before thin-sectioning methods allowed electron microscopy of mitochondria.

Figure 1 shows an electron micrograph of a mitochondrion in an acinar cell of bat pancreas and, next to it, a diagramatic representation of a "generalized" mitochondrion, showing its two membranes and the infoldings of the inner membrane, called cristae. Figure 2 is a schematic diagram of the major organized enzymatic systems of all mitochondria—the Krebs tricarboxylic acid cycle, the respiratory chain, and the energy-coupling mechanisms for phosphorylation.

The Krebs cycle is ultimately responsible for oxidation of glucose, fatty acids, and amino acids to carbon dioxide and water, via the metabolic intermediate acetyl-coenzyme A; the acetic acid moiety of the latter is the immediate fuel for oxidation. Electrons or reducing equivalents extracted from these acetic acid residues by the operation of the Krebs cycle are then transported down the respiratory chain, a linear sequence of enzymes having prosthetic groups that can be reversibly reduced and oxidized by gaining and losing electrons. These electron carriers have successively increasing oxidation-reduction potentials and thus carry electrons from the cycle dehydrogenases, via the flavoproteins, to the series of cytochromes ending in cytochrome a–a_3, where they reduce oxygen. A large decline of free energy occurs at three of the steps in this sequence, where there is some form of energy-coupling or conservation, not yet identified as to its molecular basis, that permits formation of adenosine triphosphate (ATP) and phosphate at the expense of oxido-reduction energy. This is the process of oxidative phosphorylation, or respiratory chain phosphorylation.[2,6,7] Only the mitochondria possess the enzymatic equipment for oxidative phosphorylation: over 90 per cent of the ATP of the cell is thus regenerated in the mitochondria from its energy-discharged form adenosine diphosphate (ADP).

Although ATP formation is the most conspicuous and most familiar way in which energy production from biological oxidations is used by the cell, we will see later that at least two other modalities of oxidative energy coupling occur in mitochondria: ion transport and mechanochemical changes. These three modalities are evident in mitochondria from many different cell types.

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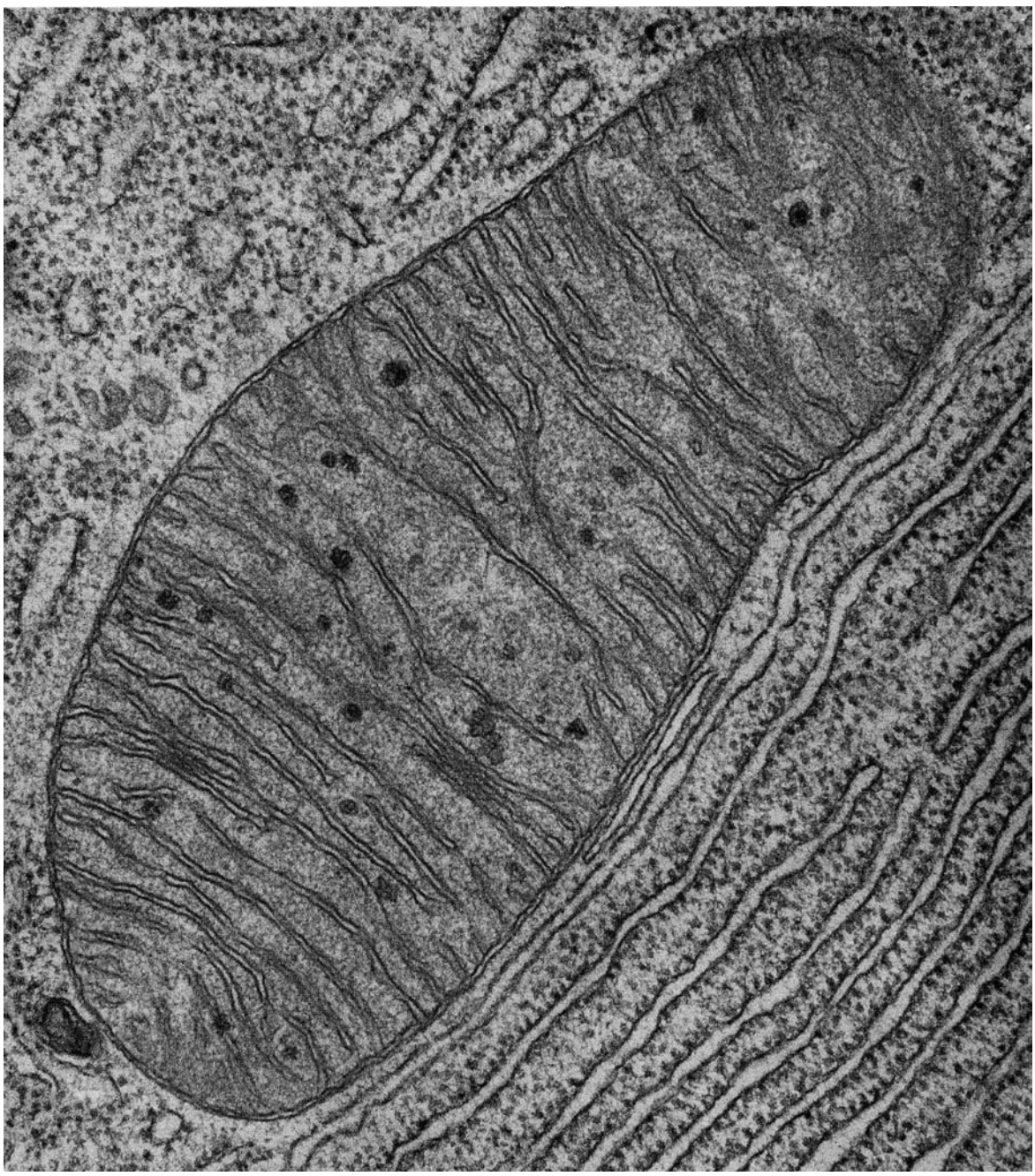

Number and location in the cell

Mitochondria are found in the cytoplasm of all aerobic, respiring cells excepting the bacteria. There is, however, wide variation in the number of mitochondria per cell. The mature mammalian sperm cell has relatively only a few—from 16 to 24 per cell, depending on species. Similarly, yeast cells may contain relatively few mitochondria. In rat hepatocytes, which have been most studied, there are about 1000 in each cell, and they comprise from 15 to 20 per cent of its total volume. Kidney epithelial cells contain about 300 mitochondria. Oöcytes contain the largest number, in some cases as many as 300,000 per cell. In green plant cells mitochondria are fewer in number and sometimes almost lacking, but in such cells chloroplasts take over some of the mitochondrial functions. Mitochondria

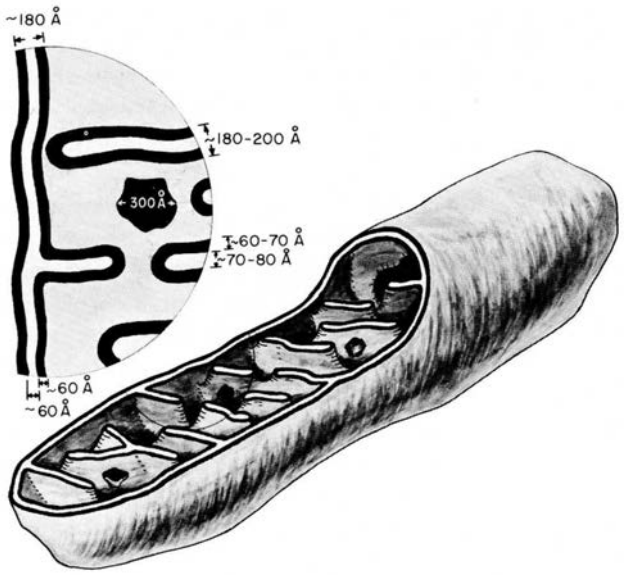

FIGURE 1 At the left is an electron micrograph of a mitochondrion in an acinar cell of bat pancreas (courtesy Dr. Keith Porter). Above is a diagram of a "generalized" mitochondrion, showing the two membranes and inner matrix (courtesy Dr. George Palade).

are also found in higher micro-organisms such as molds, yeasts, and protozoa, but not, as has been mentioned, in bacteria. Actually, bacteria and the mitochondria of higher cells are about the same size. The bacterial plasma membrane serves the same biochemical function as the mitochondrial membrane in higher cells, in that it contains the enzymes of respiration and phosphorylation. We will return to this similarity.

There is a growing impression, but relatively few critical data, that the number of mitochondria per cell is relatively constant for any given cell type. There is evidence for a few types of cells that the number of mitochondria per cell is held constant by a process in which each daughter cell receives half the parent mitochondria, which then divide after cell division has taken place.[2] In fact, it is possible to see individual mitochondria apparently about to divide in electron micrographs of livers of thyroxine-poisoned rats.[9]

The location of mitochondria in cells is of interest.[2] They are frequently found near sources of fuel; for example, in liver cells of fasting rats mitochondria will often be found wrapped around lipid droplets, a source of fatty acids. More often, however, they are found near structures that require their end-product, the energy-carrying ATP molecules. In muscle cells, mitochondria are often found regularly aligned along the sarcomeres of the ATP-utilizing myofibril. In the sperm cell, they are wound helically around the midpiece, presumably to furnish ATP to the mechanochemical "engine" in the tail fibers, with a diffusion pathway of minimum length. In the pancreas acinar cell, which is a factory for synthesizing digestive enzyme proteins, the mitochondria are located in and near the ribosome-covered endoplasmic reticulum. We have already seen how mitochondria are found in axon endings, near the site where vesicles are formed and transmitter substances are synthesized. In renal tubule cells the mitochondria are oriented with their long axis in the direction of active transport processes across epithelial cell barriers. In fact, it was first recognized by Cowdry many years ago that in many different types of epithelial cells mitochondria are often oriented in the direction of secretory activity. This orientation suggests that mitochondria participate in active transport processes.[2] Later we will see that mitochondria are in fact able to accumulate certain cations in a process that is linked to respiration. Their specific orientation in epithelial cells also has another possible meaning; it suggests that there is some kind of ultrastructural or biochemical gradient along the long axis. Actually, some mitochondria appear to have "sticky" ends: end-to-end associations are frequent, but side-to-side infrequent. The molecular basis for such chemotactic phenomena will be of considerable interest.

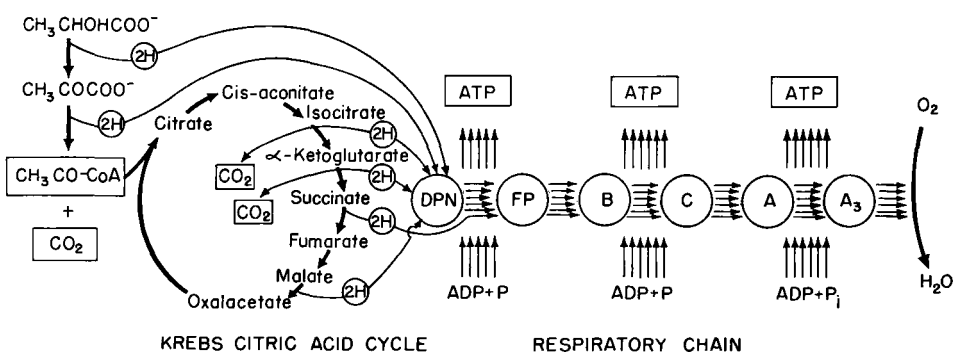

FIGURE 2 Schematic representation of Krebs tricarboxylic acid cycle, electron transport, and oxidative phosphorylation.

Mitochondrial structure and compartmentation

Mitochondria from different cell types have some structural characteristics in common. All possess an outer and an inner membrane; the outer is smooth, the inner puckered or infolded, sometimes in an extremely complex manner. In most, the inner membrane has a much larger area than the outer. There are thus two geometric compartments, one between the two membranes, the other within the inner membrane; the latter is filled with a granular matrix. It is now certain that the two membranes are quite different in structure and in biochemical and physical properties.

In liver mitochondria, those most often pictured in textbooks, the cristae are transverse and relatively few in number; they resemble pleats of an accordion (Figure 3). In kidney cortex mitochondria, there are many transverse cristae that appear to be independent of the inner membrane. In reality, they are continuous with it, but only through a very narrow neck. In muscle mitochondria the cristae are very numerous and completely fill the body of the mitochondrion. Cristae are not always septumlike. In the mitochondria of some neurons the cristae are viliform and often anastomose. In other types of neurons they are longitudinal rather than transverse.

The most remarkable variations in the mitochondrial inner membrane are seen in tissues having intense activity, particularly of a high frequency. Flight muscle mitochondria of insects have been explored by Smith.[10] He has found that in blowfly muscle they possess densely packed cristae punctured by regular fenestrations that are in exact register. It has been speculated that the fenestrations provide diffusion pathways for substrates, ADP, and ATP. Mitochondria of the cricothyroid muscle in the larynx of the bat show very regular prismatic folding of the cristae.[11] The mitochondria from the heart of the canary, which has a heart rate of about 1000 per minute, has a most extraordinarily regular lattice-work of inner membrane, which is so perfect it appears quasi-crystalline (Figure 4).[12]

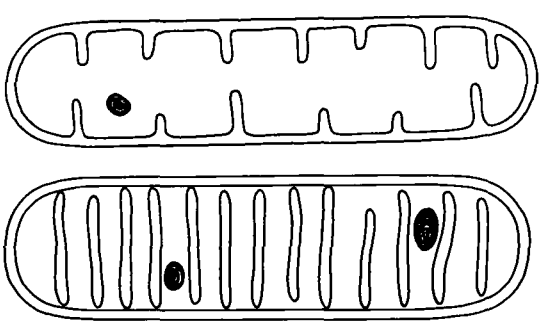

FIGURE 3 Cristae in liver and kidney mitochondria. In liver mitochondria (above) the cristae are not profuse, but in kidney mitochondria they are numerous and regularly arranged. In both cases they are continuous with inner membrane. (From A. L. Lehninger, The Mitochondrion, W. A. Benjamin, Inc., New York, 1964).

The most important conclusion from such comparative morphological studies is that the number and area of the cristae are directly related to the respiratory activity of the cell. The inner membrane of liver cell mitochondria, which have a moderate rate of respiration, has an area of about 40 square meters per gram of protein[13]; muscle mitochondria may have up to 400 square meters per gram of protein. The total area of the inner membranes of the 1000 mitochondria in a liver cell is several-fold greater than the entire plasma membrane of the intact liver cell. The generalization has been made that there is a positive correlation between the rate of respiration of a cell and the area of the inner membrane of its mitochondria. We will now see the reasons for this correlation.

Enzymatic studies in the past few years have shown that the electron-carrier enzymes, namely the flavoproteins and cytochromes, are all located in the inner mitochondria membrane, together with the enzymes required to regenerate ATP from ADP and phosphate at the expense of the energy yielded from electron transport. On the basis of membrane fragmentation studies, it has been postulated that groups or clusters of the electron-carrier enzymes, called respiratory assemblies, are studded in or on the inner mitochondrial membrane at regular intervals. Spectrophotometric data show that the electron-carrier molecules occur in simple molar ratios to each other. A single liver mitochondrion can be calculated to contain about 15,000 such respiratory assemblies in the inner membrane; a heart mitochondrion, 50,000. It can also be calculated that these assemblies are on the average about 200 Å apart from each other.

Recently, fragmented outer and inner membranes of rat liver mitochondria have been successfully separated by density-gradient centrifugation.[14-17] The inner membrane contains all the respiratory electron carriers and ion transport mechanisms, as well as many of the dehydrogenases, as expected from earlier work. The outer membrane, interestingly, contains all the monoamine oxidase of the mitochondrion, which thus serves as an identifying marker.[15] There are other important biochemical differences. For example, the two membranes differ in lipid composition: all the cholesterol of mitochondria is in the outer membrane, but all the cardiolipin, a glycerol phosphatide, is in the inner membrane.[17]

The two membranes also differ ultrastructurally, as revealed by negative contrast electron microscopy. The in-

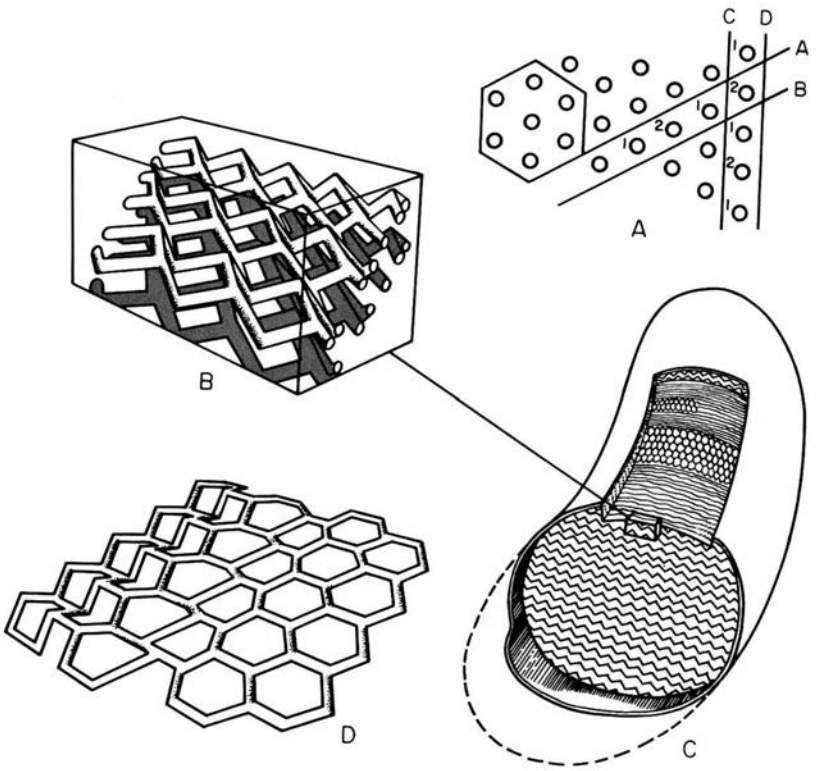

FIGURE 4 Diagramatic representation of the conformation of cristae in mitochondria of canary heart.
A. Cross-section of a field of zigzag cristae.
B. Two tiers of cristae shown in three-dimensional view of zigzag array.
C. Disposition of zigzag and retiform arrays within a mitochondrion.
D. Schematic representation of the development of a hexagonal pattern from zigzag cristae.
(From D. Slautterback, 1965. *J. Cell Biol.*, 24, 1)

ner membrane contains on its inner surface knoblike structures, ~90 Å in diameter, attached to the core by stalks. These have been called "elementary particles" by Fernández-Morán, their discoverer.[18] Although they were first postulated to be assemblies of electron-carrier molecules,[19] more recent evidence suggests that they may represent molecules of the coupling enzymes that are necessary to form ATP at the expense of electron transport.[20] The outer membrane does not contain such knobs.

Another important difference is in the permeability. The outer membrane is freely permeable to all simple electrolytes, H_2O, sucrose, and even some polysaccharides. The inner membrane, however, is normally impermeable to H^+, OH^-, Na^+, Cl^-, K^+, and Mg^{++} ions, as well as to sucrose and other sugars. Permeability of the inner membrane to intermediates of the Krebs tricarboxylic acid cycle appears to be determined by a series of specific carrier molecules in the inner membrane.[21] Similarly, the passage of ADP and ATP through the inner membrane is determined by a specific carrier that is inhibited by atractyloside, a glycoside from a toxic plant of North Africa.[7,22]

It is now known that fuel molecules such as pyruvate, fatty acids, and glycerophosphate must first penetrate from the surrounding cytoplasm into the inner mitochondrial compartment before they can be oxidized. In addition, both phosphate and ADP must also penetrate into this compartment and the ATP formed must then leave the compartment to get to the exterior cytoplasm. Specific exchange-diffusion carriers for transport of certain Krebs cycle intermediates and of adenine nucleotides represent control mechanisms. Rat liver mitochondria contain such transport mechanisms, but mitochondria of flight muscles of insects are "tight," and contain no membrane carriers for exit or entry of Krebs cycle intermediates.[21] This is presumably an evolutionary adaptation to allow the mitochondria to keep a high internal concentration of these metabolites and thus maintain a high rate of respiration.

Since mitochondria of the liver cell may make up as much as 20 per cent of the volume of the cell, the fraction of the total ADP and ATP of the cytoplasm that is segregated within mitochondria is considerable. There is some evidence that the ATP of the cytoplasm is not functionally homogeneous; presumably geometric compartmentation of some of the ATP within the mitochondria is one of the ways in which functional heterogeneity of ATP is made possible.

Size and shape changes

Mitochondria in many cell types may change their shape and volume with time, sometimes very rapidly. These changes are not simply the result of local hydrodynamic stresses; they are respiration-linked and can be influenced by respiratory or phosphorylation inhibitors. Actually, volume changes of isolated mitochondria can easily be measured in vitro by following changes in light scattering. Two general types of light-scattering change have been recognized. One is the so-called low-amplitude swelling and contraction, of which all mitochondria are capable. The amplitude of these changes is usually very small, corresponding to only a few per cent volume change. In fact, it is not completely certain that there is a volume change at all. Low-amplitude light-scattering changes may correspond to energy-dependent reorganization of the refractile substances of the mitochondrion, particularly the inner membrane and matrix, as recently shown by Hackenbrock.[23] The swelling change is induced when the ratio of external ADP:ATP becomes low and no ADP is left to phosphorylate. When ADP is now added, respiration speeds up quickly, the mitochondria shrink, and the inner membranes change conformation as ADP is phosphorylated. Such changes may be very rapid.

In large-amplitude swelling and contraction, which is exhibited by liver and kidney, but not by brain, mitochondria, swelling proceeds to three to five times the normal volume in the absence of ADP. Restoration of respiration, or addition of ATP, causes them to contract again to the original volume. These changes are slower than low-amplitude changes. During large amplitude cycles there is a definite change in permeability of the membranes; they are more permeable in the swollen state. These findings have suggested that the respiration-linked mechanochemical changes in the inner membrane represent the activity of a structural cybernetic system, in which the permeability and transport properties of the mitochondrion membrane are elements in the self-adjustment of respiratory rate and ATP formation to the ambient supply of substrates, ADP, and phosphate. Similar energy-linked membrane changes, in either conformation or volume, are also seen in chloroplasts, kinetosomes, and bacterial protoplasts.

Energy-linked ion transport in mitochondria

Although respiration and oxidative phosphorylation are the most conspicuous functions of mitochondria, it has recently been found that accumulation of certain cations may replace oxidative phosphorylation of ADP as an energy-conserving mechanism.[8,24] Such accumulation of cations by mitochondria shows a strict stoichiometry with electron transport, just as does ATP formation. Furthermore, very recent work in this area has suggested the possibility that it is ion transport that is the most fundamental energy-conserving mechanism of electron transport in mitochondria and that ATP formation is ultimately driven by an ion transport process.

Although it was first known as early as 1952 that isolated mitochondria can retain or accumulate cations, it was not until 10 years later that Vasington and Murphy made the key observations that opened this area to more quantitative study.[25] They found that Ca^{++} ions were accumulated in very large amounts by isolated mitochondria, exceeding the normal Ca^{++} content by several hundred-fold, in a respiration-linked process. Backleakage of the accumulated Ca^{++} took place only slowly. The remarkable thing about this finding is that Ca^{++} had traditionally been regarded as an uncoupling agent and a toxic substance for mitochondria. It was quickly found that phosphate also was accumulated from the medium with the Ca^{++}; the molar accumulation ratio Ca^{++}:P was about 1.7, or approximately that of hydroxyapatite or bone crystal. Accumulation of Ca^{++} is inhibited by respiratory inhibitors such as cyanide or antimycin A, and also by 2,4-dinitrophenol, but not by oligomycin. The amount of Ca^{++} and phosphate that can be accumulated is enormous—up to 25 per cent of the dry weight of the mitochondrion. Under these conditions the calcium phosphate actually precipitates in microcrystalline, electron-dense deposits readily visible in electron microscopy.[2,8]

One of the most important conclusions from recent work is that the accumulation of Ca^{++} (as well as of Sr^{++} and Mn^{++}, which are accumulated by a similar mechanism) is stoichiometric with electron transport,[26] as is seen in Figure 5. In oxidative phosphorylation it is now well known that three molecules of ATP are generated from ADP and phosphate as each pair of electrons goes down the respiratory chain to oxygen. However, when the mitochondria are presented with Ca^{++}, they no longer phosphorylate ADP to ATP; they accumulate Ca^{++} and phosphate instead. For each pair of electrons passing each energy-conserving site, about 1.7 Ca^{++} ions and 1.0 phosphate ion are accumulated, or a total of about 5 Ca^{++} ions and 3 phosphate ions for all three sites of the chain. Because the mitochondria have a higher affinity for Ca^{++} than for ADP, Ca^{++} uncouples phosphorylation because it uses the energy of electron transport as it is accumulated. Oxidative phosphorylation and cation accumulation are alternative, respiration-linked processes. The ability to accumulate Ca^{++} is as universal a mitochondrial property as is oxidative phosphorylation itself. A number of cations can be accumulated by mitochondria (see below), but

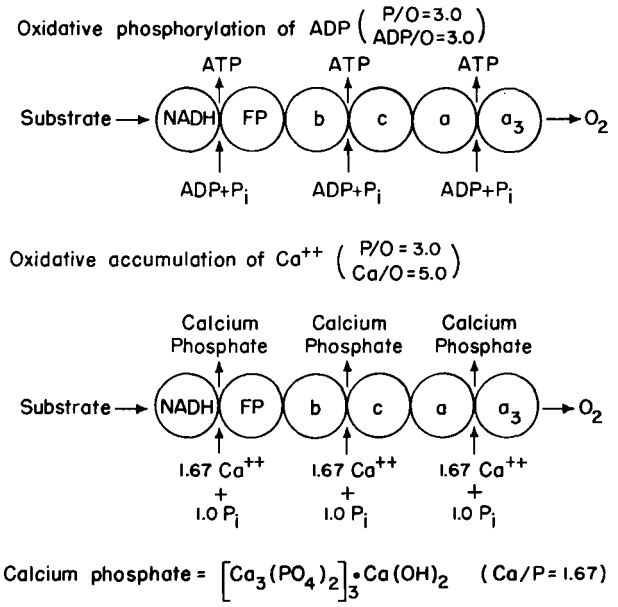

FIGURE 5 Stoichiometry of oxidative phosphorylation of ADP and oxidative ion transport in mitochondria.

Ca^{++} accumulation appears to be of most significance in the intact cell. Because the external concentration of Ca^{++} can be reduced to less than 1×10^{-6} moles per liter by respiring mitochondria,[8] they could serve as very effective systems for temporarily sequestering free ionic Ca^{++} during excitation and relaxation phenomena.

Mitochondria accumulate Ca^{++}, Mn^{++}, and Sr^{++} by very similar processes; in each case phosphate, when present in the medium, is also accumulated in such a ratio as to match the composition of the insoluble phosphates of those cations.[8] The accumulation of the phosphate is a purely passive process. However, other anions, such as chloride, are not accumulated. Only a few anions are permeant through the membrane (phosphate, acetate, arsenate) and others such as chloride, bromide, nitrate, and sulfate are impermeant and thus cannot accompany Ca^{++}.[8,27]

Mitochondria will also accumulate other cations that are normally impermeant, such as K^+, Na^+, Mg^{++}, and others, but only in the presence of certain inducing agents that specifically increase the permeability of the membrane to specific cations (Table I).[8] K^+, but not Na^+, will be pumped into mitochondria during respiration if the cyclic peptide antibiotic valinomycin is present.[28] Both K^+ and Na^+, as well as Li^+, Rb^+, and Cs^+, will be pumped during respiration, if the peptide antibiotic gramicidin is present.[29] Certain poisons, such as Zn^{++} ions or Hg derivatives, will permit Mg^{++} ions to be pumped by mitochondria. It is especially significant that parathyroid hormone preparations enable mitochondria to accumulate both Mg^{++} and K^+.[30] It is assumed that these agents produce flaws in the mitochondrial membrane that allow only certain ions to enter. The antibiotics mentioned above are toxic because they cause the mitochondria to divert their energy from the normal formation of ATP into continuous pumping of K^+ or other ions, with accompanying back-efflux. The available data indicate that there is a definite stoichiometry of about two divalent cations or four monovalent cations accumulated per pair of electrons per energy-conserving site in the respiratory chain.[8]

TABLE I

Energy-linked cation accumulation in isolated mitochondria

Cation	Inducing agent	Stoichiometry Number of ions accumulated per pair of electrons per site
Ca^{++}	None required	2.0
Sr^{++}	None required	2.0
Mn^{++}	None required	2.0
K^+	Valinomycin; gramicidin	4–7
Na^+	Gramicidin	—
Li^+, etc.	Gramicidin	—
Mg^{++}	Parathyroid hormone; Zn^{++}	≤ 2.0

The mechanism of mitochondrial ion transport is a matter of intense interest at the moment. One school of thought has postulated that electron transport leads to generation of high energy intermediates (designated $X \sim I$) that can be utilized to form either ATP or to transport ions by means of a sequence of chemical reactions having common intermediates. This is the traditional chemical coupling hypothesis. However, another point of view has recently developed, the chemiosmotic coupling hypothesis.[13] In this mechanism, energy coupling is not brought about by chemical reactions with a common intermediate, but by a coupling of independent flows. The hypothesis postulates that the individual reactions of electron transport lead to a separation of H^+ and OH^- ions across the mitochondrial membrane by a vectorial arrangement of the electron-carrier molecules of the respiratory chain enzymes in the plane of the membrane, so that the H^+-utilizing reactions extract H^+ from the inside of the membrane and the H^+-generating reactions extrude H^+ to the outside. Electron transport is thus visualized as generating a *p*H gradient across the H^+-impermeable mitochondrial membrane. Such gradients have now

been observed and measured; about 6 H+ are extracted from the inside and transferred to the surrounding medium for each electron passing the full length of the respiratory chains, under certain conditions.[13,31] Mitchell has proposed that this hydrogen-ion gradient then drives (or rather "pulls") either ATP formation or cation uptake through secondary mechanisms. For example, by exchange diffusion mechanisms, K+, Ca++, or Na+ may exchange for H+, and anions such as phosphate may exchange for OH−. Mitchell proposes that the *p*H gradient also can form ATP by the action of a reversible adenosine triphosphatase in the membrane, which is polarized in the opposite direction from the electron carriers in the membrane, as follows:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad \text{to outside} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \text{ADP} + \text{Phosphate} \rightarrow \text{ATP} + \text{H+} + \text{OH–+} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad \text{to inside} \end{array}$$

The reaction of ATP formation is pulled to the right because H+ and OH− react with the "sinks" of excess OH− and H+ generated on the inside and outside of the membrane respectively during electron transport. The thermodynamic driving force is the very strong tendency for H+ and OH− ions to combine to form water, expressed by the ion product

$$[\text{H+}]\,[\text{OH–}] = 1 \times 10^{-14}\ (\text{at } 25°)$$

The chemiosmotic coupling hypothesis has not yet been successfully put to a critical test, but the outcome of such tests will have wide implications in cell biology, since mitochondria, chloroplasts, and the plasma membrane of bacteria have energy-conserving mechanisms that appear to be identical in principle.

Biogenesis of mitochondria

Interesting and exciting as much of the current work on mitochondrial energy conversions is, there is another area that has some unexpected fascinations of its own. Just in the past two to three years evidence has come to light that mitochondria are bearers of biological, i.e. genetic, information and that they may be extremely important elements in cytoplasmic inheritance and in cytoplasmic development. Mitochondria have recently been found to possess their own DNA, unmistakably different from that of the cell nucleus in molecular properties. Accompanying this development has been the demonstration that isolated mitochondria can synthesize proteins from amino acids[32] and, furthermore, that mitochondria may undergo division during or after cell division. These recent developments are very interesting in the light of past history, since early cytologists had speculated very broadly on a possible genetic role of these structures. In fact, in 1890 Altmann postulated that mitochondria are self-duplicating, cell-like structures to which he gave the name "bioblasts."[2]

Protein synthesis in mitochondria

Although there have been many reports of protein synthesis in isolated mitochondria dating back to 1952, there are a number of puzzling discrepancies in the observations.[33] One major difficulty is that bacterial and ribosomal contamination of isolated mitochondria can cause spurious amino-acid incorporation that is hard to distinguish from a true mitochondrial process. Recently it has been established that bacterial contamination has indeed been a complicating factor in many past studies of mitochondrial protein synthesis. By means of a density gradient separation method that removes labeled bacteria, Wheeldon has been able to demonstrate incorporation of amino acids into mitochondrial material unambiguously.[34] From a number of recent studies it is now clear that mitochondria are completely self-contained units with respect to protein synthesis (Figure 6). It is necessary to supplement them only with ATP and an ATP-regenerating system (such as phosphopyruvate and pyruvate kinase), inorganic phosphate, and Mg++; respiration is not required as long as a steady supply of ATP is available.[33] All other components and catalysts required by protein synthesis, such as mRNA, transfer RNA, and activating enzymes, are presumably already present in the mitochondria. In fact, some of these have been found in mitochondrial extracts. One of the big mysteries, however, is whether mitochondria contain their own ribosomes; most electron micrographs of mitochon-

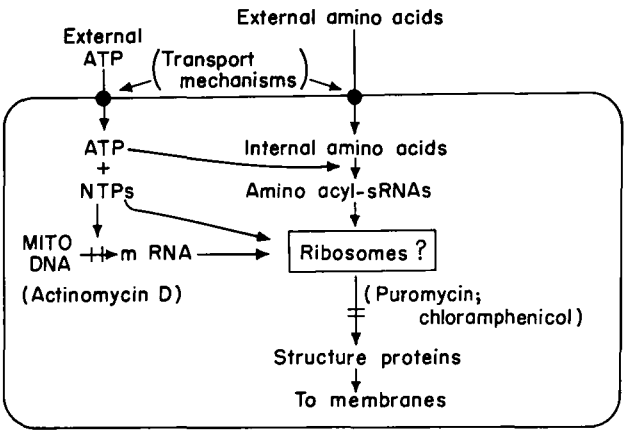

FIGURE 6 Protein synthesis in mitochondria.

dria show no granular elements that appear to be identical with ribosomes. However, it is possible that there are only a relatively few ribosomes in each mitochondrion of a mature nondividing cell. Mitochondria from rapidly dividing cells may conceivably have a larger and more visible complement of ribosomes.

There are three relatively unique properties of the protein-synthesizing reactions in mitochondria that also contributed to the somewhat conflicting observations on this process. The incorporation of a given labeled amino acid may be greatly inhibited by other amino acids in the medium. Part of the reason for such inhibitory effects is that an energy-linked amino-acid transport process, presumably across the mitochondrial membrane, precedes the incorporation reaction. The anomalous inhibitions by amino acids may be the result of competitive effects in the transport process, rather than in the incorporation reaction.[33]

The second characteristic property of protein synthesis in mitochondria is that it is inhibited by chloramphenicol. This agent is known to inhibit protein synthesis in bacteria, but not in ribosomal preparations isolated from animal tissues. This makes it possible to distinguish true mitochondrial protein synthesis from that occurring in extramitochondrial ribosomes.[33] There may, however, be a more fundamental meaning to the chloramphenicol sensitivity, since Linnane and his colleagues have found that chloramphenicol inhibits the normal formation of mitochondria that occurs when glucose-repressed yeast cells are derepressed under aerobic conditions.[35]

The third characteristic of mitochondrial protein sythesis, and a most revealing one, is the finding that actinomycin D inhibits protein synthesis in mitochondrial preparations, providing their permeability is first altered to allow the antibiotic to enter. This finding has great significance, since actinomycin D is a rather specific inhibitor for transcription of DNA into messenger RNA via the DNA-directed RNA polymerase. Evidently mRNA is relatively unstable in mitochondria and must be continuously synthesized by a polymerase, from precursor ribonucleoside triphosphates in the presence of template DNA.[36]

Very recently, several laboratories have succeeded in isolating mitochondrial DNA in pure homogeneous form (for references, see Reich and Luck[37]). This DNA is quite different in size, base composition, and thus buoyant density, from the DNA of the nucleus of the same cell. Sedimentation measurements show this DNA to be quite homogeneous. Van Bruggen and his colleagues have very recently published some physical studies on highly purified DNA from chicken liver mitochondria.[38] Such specimens showed remarkable ability to reanneal after they had been melted by heating. This is a very sensitive test for homogeneity of DNA molecules. Other physical measurements indicate that the molecular weight is about 10,000,000. Direct electron-microscopic examination showed mitochondrial DNA to be circular in conformation and hypertwisted, and in these respects mitochondrial DNA resembles the DNA of certain viruses.[38]

It has been calculated that each chicken liver mitochondrion contains about 40,000,000 daltons of DNA; possibly the DNA is in the form of four identical circular molecules of about 10,000,000 molecular weight. Each of these molecules of DNA can theoretically code about 60 peptide chains having a molecular weight of 17,000, if there is no redundancy.

The occurrence in mitochondria of a distinctive type of DNA, different from that of the nucleus, raises a number of very important and fundamental questions about the nature of cytoplasmic inheritance, the interplay between nuclear and mitochondrial DNA in cell development, and specifically which proteins, if any, are coded by mitochondrial DNA. Only a few tentative statements can be made at this time. There is some genetic evidence that the cytochromes in yeast cells are genetically determined by the nucleus; presumably they are synthesized not in the mitochondria, but rather in the extramitochondrial ribosomes, on mRNA manufactured from nuclear DNA. Very recently a number of studies have been carried out on the identification of the proteins of mitochondria that become labeled during mitochondrial protein synthesis in vitro. Roodyn has established that neither malic dehydrogenase nor cytochrome c of mitochondria becomes labeled.[39] On the other hand, he, as well as others, have shown that mitochondrial structure protein, presumably the sheath protein of the mitochondrial membranes, is the only protein fraction that becomes labeled.[32,33,39] We are therefore left with the possibility that most of the mitochondrial proteins may be genetically coded by nuclear DNA, and only a few by mitochondrial DNA. If this is the case, much of the mitochondrial DNA may be redundant or, alternatively, may be repressed.

Replication of mitochondria

There is now great interest in the mechanism of the biogenesis of mitochondria. There have been two poles of thought. Either mitochondria are built *de novo* from simple building-block molecules or they arise by fission of parent mitochondria and then grow by accretion. Some direct information has recently come from some studies on *Neurospora* mitochondria by Luck.[40,41] After labeling the mitochondria (and other cell components) of a cholineless mutant of *Neurospora* with radioactive choline, Luck fol-

lowed the appearance of radioactivity in the mitochondria of second and third generation cells by means of radioautography. In brief, he found that *all* mitochondria of the progeny were labeled in each generation. The mitochondria in each daughter cell were found to have about half the radioactivity of the mitochondria of the parent cell, a finding consistent with a mechanism in which the mitochondria of the parent cell go to both daughter cells, divide, and grow in size by addition or insertion of building-block molecules. Although these experiments may have some other interpretations, they come as close as now seems possible to defining the manner of mitochondrial replication, at least in this particular mold.

Linnane and his colleagues have found that mitochondrial biogenesis in glucose-repressed yeast cells may occur by a somewhat different process. In this case, mitochondria are formed from simpler, membraneous precursors.[42] In both cases, however, there is evidence that the mitochondria must arise from an organized precursor of some kind.

Very recently, Tatum and his colleagues[43] have reported some extremely important experiments that demonstrate by direct means that mitochondria can carry genetic information. There is a slow-growing mutant of *Neurospora* that is unable to make inositol. Direct examination has shown that the mitochondria of this mutant respire very slowly. Mitochondria were isolated from such mutants and by micromanipulative methods injected into the cytoplasm of wild-type *Neurospora* cells. The injected cells, after a few generations, were transformed into a slow-growing form resembling the original mutant. These experiments therefore indicate that the injected mutant mitochondria carried the information, presumably in their DNA, for the biogenesis of slowly respiring mitochondria in later generations of the wild-type cells, possibly via a division process.

These recent developments are reminiscent of the old theory that the ancestors of mitochondria are bacteria, which in the course of evolution had infected large host cells and ultimately developed a symbiotic metabolic relationship with them.[2] In the light of very recently acquired information, this is an intriguing hypothesis. Mitochondrial DNA may be the evolutionary remnant of what was once a bacterial DNA. Furthermore, the characteristic chemical differences between the inner and outer mitochondrial membranes may correspond to vestiges of the original bacterial cell membrane and wall respectively. Cardiolipin is a characteristic lipid of the inner mitochondrial membrane; a very similar class of lipids, the glycerol phosphatides, is widely distributed in bacterial membranes.[17] Similar lines of evidence are now developing that chloroplasts of higher green plant cells also contain their own DNA; they may also have arisen in evolutionary history from parasitizing chlorophyll-containing bacteria.

Mitochondria of neurons and glial cells may therefore play a vital role not only as power plants, but also as carriers of the cytoplasmic genome. In fact, brain mitochondria have recently been found to carry out protein synthesis,[44,45] and their possible participation in the specialization of these cells for neurofunction demands the closest experimental attention.

The Ribosome — A Biological Information Translator

ALEXANDER RICH

THE MAJOR MOLECULAR pathways of biological information transfer are now reasonably well understood. For many years it has been recognized that the proteins, linear polymers of amino acids, provide much of the functional machinery of the cell. This includes both the enzymes that have catalytic activity and a wide variety of structural proteins found in membranes and fibers—in short, most of the stable molecular architecture of living systems. Even though the proteins are assembled out of only 20 amino acids, they have exceedingly diverse chemical and biological properties. Only recently have we begun to understand the manner in which the chemical and biological properties of proteins are related to their three-dimensional structure.

The uniqueness of all proteins lies in the sequence of the amino acids found within the molecule. This sequence apparently determines the folding of the polypeptide chain and the attendant formation of secondary and tertiary structures that are ultimately responsible for the chemical properties of the molecule. However, only in recent years have we come to understand the manner in which the cell derives the information for assembling the linear amino acid polymers. As recently as a decade and a half ago the nucleic acids were regarded as relatively uninteresting chemical constituents of cells. Today we recognize them as the class of polymer molecules that contain most of the genetic information stored in the cell. In many respects, the central problem of molecular biology is an attempt to understand the manner in which genetic information stored in the nucleotide sequence of DNA is eventually transferred into the amino acid sequence of proteins.

The linear polymer of DNA is composed of four different nucleotides; the linear polypeptide chains of proteins are composed of 20 different amino acids. The process of protein synthesis involves the translation of polynucleotide information into a polyamino acid structure. This is carried out in a remarkably interesting micro-organelle of the cell called the ribosome. The ribosome functions as a translating instrument, and in this chapter I discuss various aspects of its structure and function. The importance of the ribosome cannot be overstated: ribosomes are coexistent with life. Every living cell contains these particles, which carry out the process of information translation from the nucleic acids to the proteins.

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Protein synthesis

A general outline of the flow of information in biological systems is presented in schematic form in Figure 1. A messenger-RNA molecule is formed by the action of RNA polymerase on DNA. By working on one strand of DNA, a polyribonucleotide is formed which has a sequence identical to that of the opposite DNA strand. Information in the messenger-RNA strand is encoded in groups of three nucleotides called codons. Each amino acid is specified by one or more codons. However, the amino acid used during polymerization is one that has been activated by becoming attached to a transfer-RNA molecule by the action of an activating enzyme. Each amino acid has its own activating enzyme, which catalyzes the formation of a covalent bond with a particular amino acid. It is believed that during the activation of the amino acid a small portion of the polynucleotide strand of the transfer RNA is "read" by the activating enzyme. The transfer RNA has about 70 nucleotides, but it is likely that only a small number are involved in this reading process. In real sense, this process represents the molecular junction between the proteins and the nucleic acids, because it is believed that the additional steps involved in protein synthesis are carried out largely by means of the highly specific interactions between the purines and pyrimidines found in the codon triplets of the messenger RNA and the complementary anticodons on the transfer RNA. As shown in Figure 1, the ribosome acts as an assembly point for both the messenger RNA and the activated transfer-RNA molecules, forming the complex that is active in protein synthesis.[1]

It is useful to compare the molecular size of the various components active in protein synthesis. Let us consider for the moment the synthesis of hemoglobin. This protein is made of polypeptide chains containing approxi-

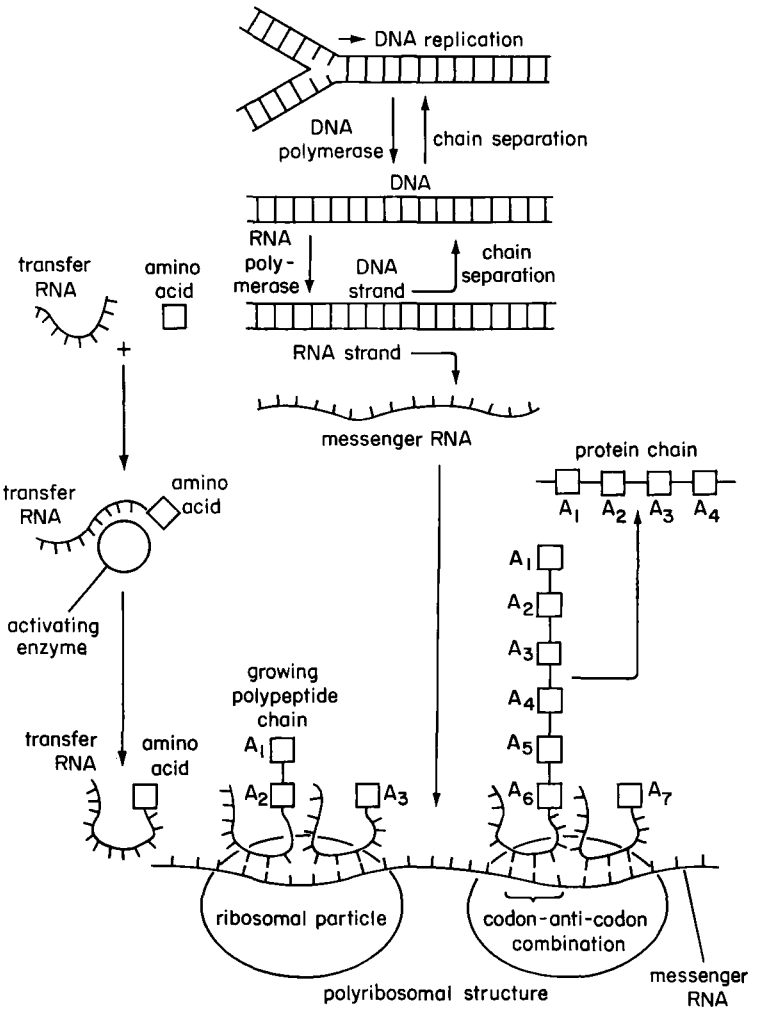


FIGURE 1 Schematic outline of biological information transfer.

mately 150 amino acids. Accordingly, one would estimate that the total information needed to specify these amino acids would be found in a messenger RNA containing some 450 nucleotides, if we ignore for the moment any special requirements needed for the initiation, termination, or control of polypeptide chain synthesis. In its stable configuration, a polynucleotide has the planar purine and pyrimidine bases stacked on top of each other with a spacing of 3.4 Å between the bases. This suggests that a messenger RNA for hemoglobin would have a total contour length of 1500 Å. Ribosomes are readily seen in the electron microscope as roughly spherical particles with a diameter of 200 to 220 Å. Thus, if this lengthy messenger-RNA molecule is utilized fully, one would expect it could accommodate several ribosomes at the same time. This is just what is seen in the study of protein synthesis. If immature red blood cells, reticulocytes, synthesizing hemoglobin are exposed briefly to C^{14} amino acids, lysed, and then sedimented on a sucrose density gradient, they produce the sedimentation pattern shown in Figure 2.[2] The solid line representing the optical density shows two peaks caused by ribosomes. Sedimentation is to the left, and the sharper peak, labeled 76S, is composed of single ribosomes, while the broader peak sedimenting more rapidly arises from the polyribosomal or polysomal clusters that are active in protein synthesis. This is shown by the large peak of radioactivity (dashed curve) found in that part of the gradient. The dotted lines show the distribution of various-sized polyribosomes and the numbers indicate how many ribosomes are seen in each cluster, as determined by an electron-microscope study. The major cluster is a ribosomal pentamer. Addi-

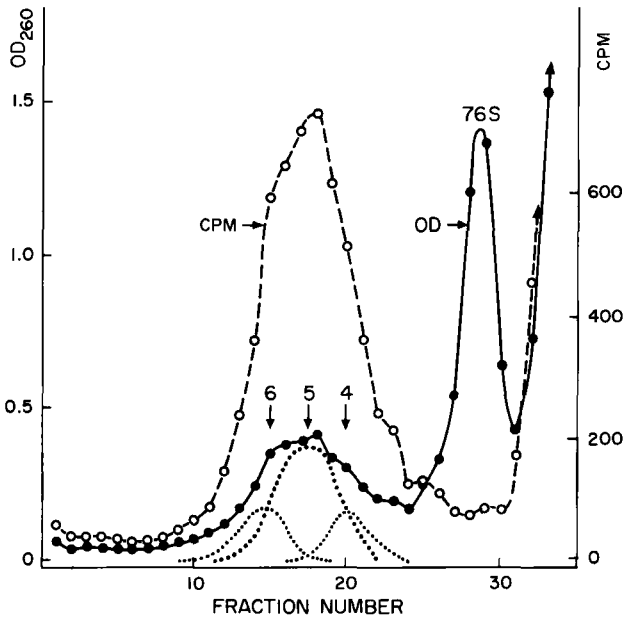


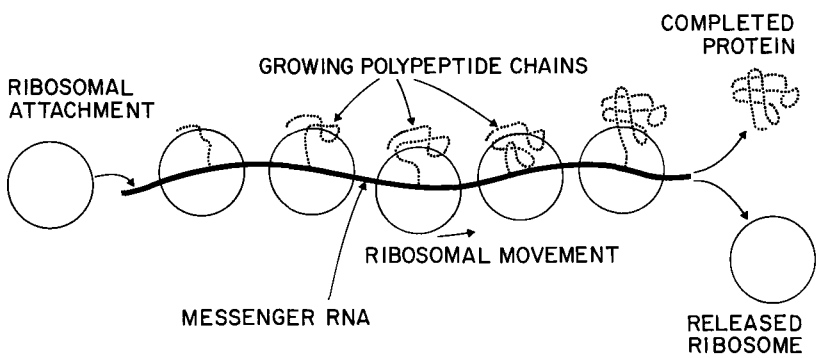
FIGURE 2 Sucrose density gradient analysis of reticulocyte lysate. Sedimentation is to the left. OD = optical density; CPM = counts per minute. The dotted lines show the distribution of polysomal tetramers, pentamers, hexamers.

tion of a small amount of ribonuclease converts this peak entirely to single ribosomes, which still retain the radioactivity of the partially synthesized and nascent chains.

Considerable effort has gone into analyzing the protein synthetic mechanism, and a current view of the process is shown diagramatically in Figure 3. Five ribosomes are shown attached to the messenger-RNA strand and they contain nascent or growing polypeptide chains of various lengths. In this diagram, ribosomal attachment occurs at the left and the ribosomes move along the messenger in association with the addition of activated transfer-RNA molecules; this leads to a gradual elongation of the polypeptide chain. On the right side of the diagram the ribosomes and completed polypeptide chains are released. The polypeptide chain is illustrated as folding as it elongates. Evidence for that process has been obtained from analyzing the synthesis of enzymatically active proteins, as discussed below.

The movement of ribosomes along the messenger strand is not a cooperative process; they do not move in register, but at random. Thus, attachment of free ribosomes at one end of the messenger strand before the ribosomes have detached at the other end would give rise to a transient population of hexamers. Alternatively, detachment of ribosomes at the right end of the messenger strand before attachment has occurred at the other end would give rise to a transient population of tetramers. This is undoubtedly the explanation for the observed distribution of polysomes in the lysate from the reticulocyte cell where hemoglobin is being synthesized (Figure 2).

One can learn a great deal about the details of ribosome and polysome organization by electron-microscope studies. Figure 4A, 4B, and 4C are various electron micrographs of polysomes synthesizing hemoglobin in reticulocytes. Figure 4A is of material obtained from the middle of the polysome distribution of Figure 2. The ribosomes have been shadowed with platinum, and they can be seen to occur in tightly packed clusters containing predominately five ribosomes. Figure 4B is of a thin slice of a guinea pig erythroblast in which polysomal clusters are scattered throughout the cytoplasm of the cell. They appear rather similar to those in Figure 4A, except that the size of the cluster is somewhat smaller because a very thin slice is prepared for electron microscopy and, in general, the polysomes have lost one or more ribosomes. Figure 4C shows polyribosomes obtained from the rabbit reticulocyte and stained with uranyl acetate, which makes visible a thin, positively staining strand running between



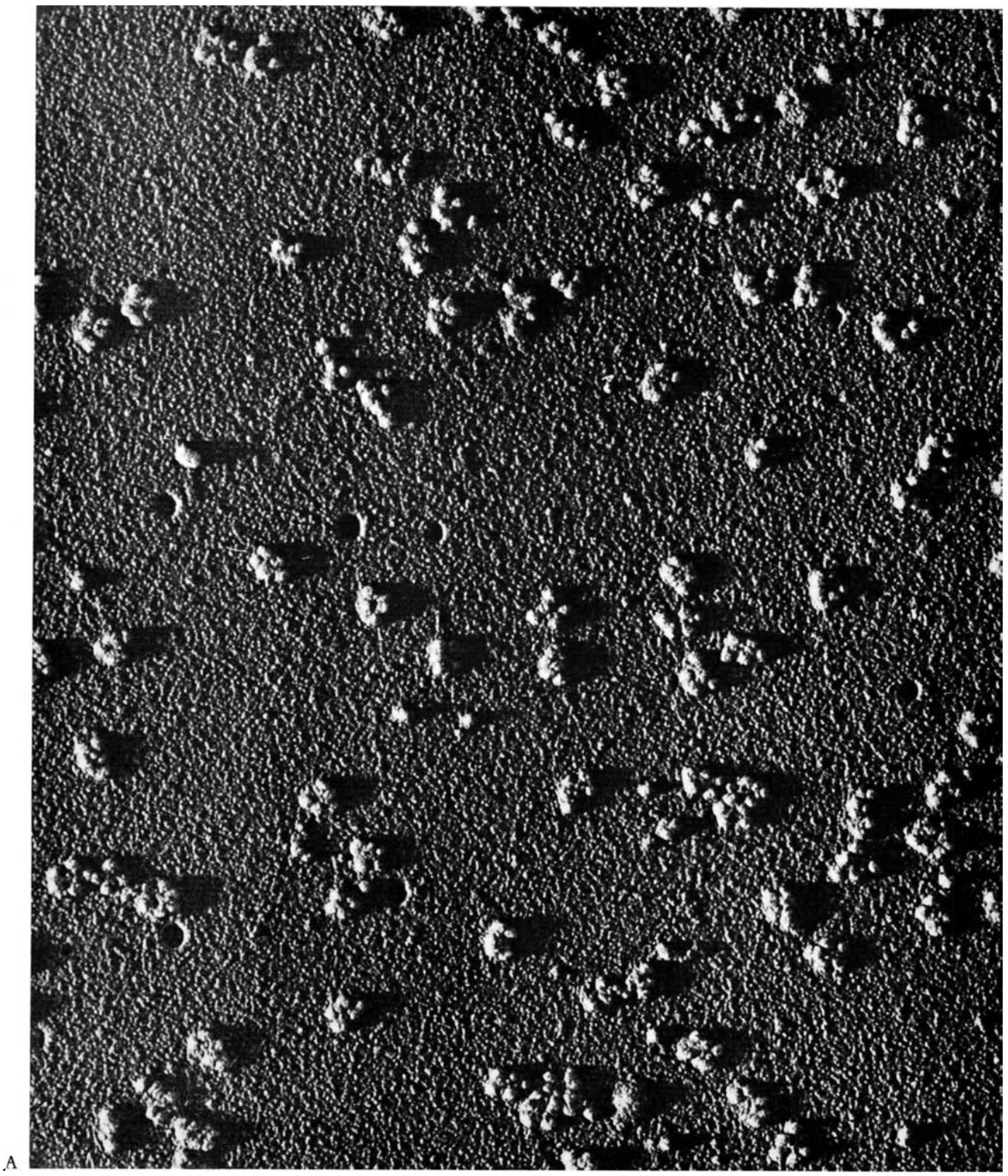
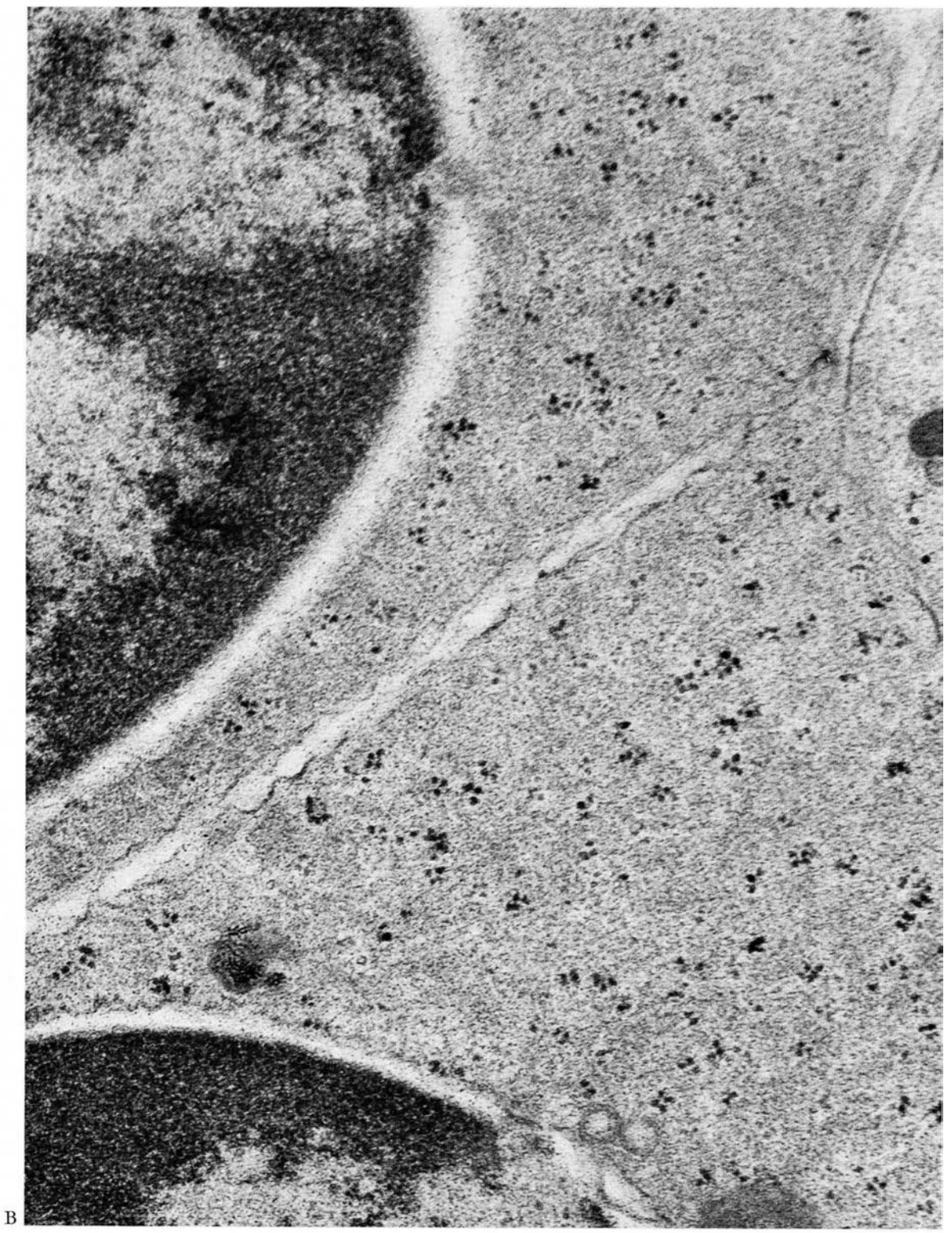
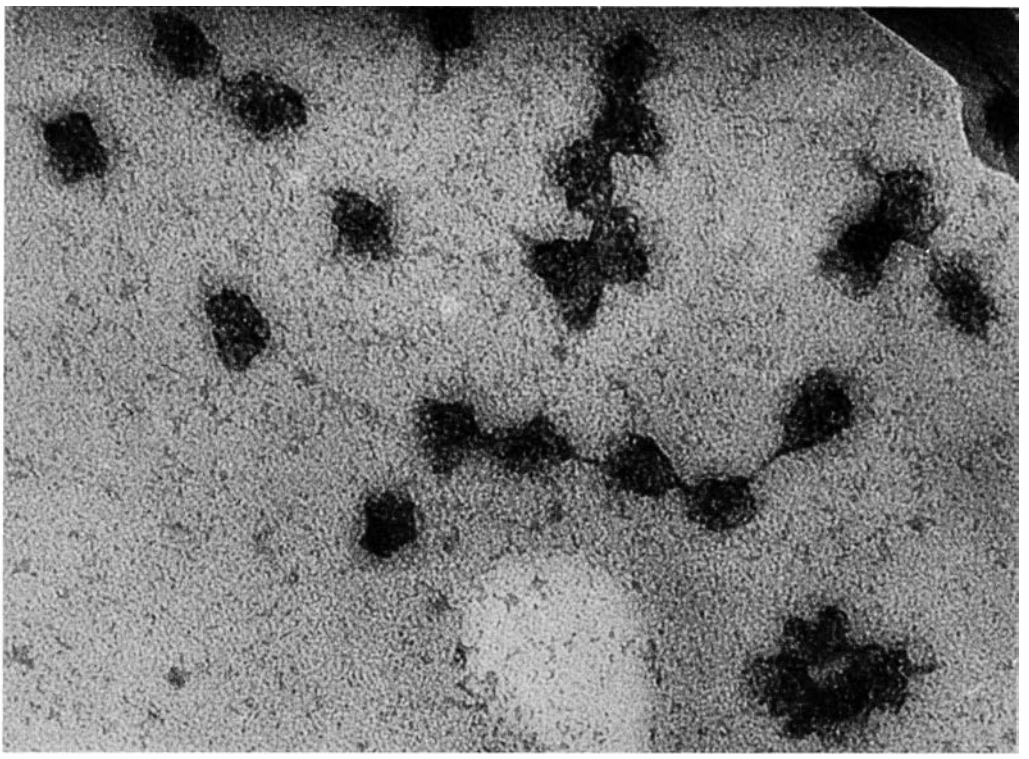


FIGURE 4 Electron micrographs of polysomes from cells synthesizing hemoglobin. A. Platinum shadowed polysomes from rabbit reticulocytes. Clusters of 5 ribosomes occur frequently.



B

FIGURE 4B Thin slice of guinea pig erythroblasts which show clusters of polysomes scattered through the cytoplasm. Parts of two darker staining nuclei are present. (Photo courtesy of D. Fawcett)



C

Figure 4C Reticulocyte polysomes stained with uranyl acetate.

the ribosomes. It has a diameter of approximately 15 Å, which is consistent with its being a single strand of messenger RNA. Because the strand is not completely covered with ribosomes, we would expect it to be highly sensitive to ribonuclease, as indeed it is. The interribosomal gap is 50 to 150 Å. Hence, the over-all contour length of the reticulocyte polysome is near the 1500 Å estimated to be required to contain all the information needed for assembling the protein.

Proteins in general vary considerably in the length of their polypeptide chains. This means that the sizes of the messenger RNA and polysomes also vary. Figures 5A and 5B are electron micrographs of larger isolated polyribosomes obtained from *E. coli* and from chick embryos. The bacterial polyribosomes contain approximately 50 ribosomes and have a more or less extended configuration. The chick embryo polysome is roughly the same size, but can be seen to exist in a more or less coiled configuration. The shapes of these polysomes are not especially relevant, as they have been obtained from isolated specimens. In order to obtain a better estimate of the over-all shape of a polysomal structure, it is necessary to resort to electron microscopy of tissue slices. This allows one to visualize polysomes in a great variety of patterns.[3] In tissues, polysomes are generally found in two different positions, either freely suspended in the cytoplasm, as in Figure 4B, or attached to the endoplasmic reticulum. The latter is especially useful in cells that are producing proteins for export, such as in the pancreas or liver. Frequently, these cells are highly differentiated. In this regard, it is interesting to note that nature has organized the protein synthetic system in such a way that if a large number of proteins must be utilized at a particular place, the process is effected not by sending proteins to that place but rather by setting up the assembly factories in that area. Thus, only the information, the messenger RNA, is specifically transported to a particular site. Ribosomes probably remain attached for a considerable time to the endoplasmic reticulum or, in the nervous system, to the membranes at the end of a neuron. There they probably accept different kinds of messenger-RNA molecules at different times, depending upon metabolic requirements.

Ribosomal architecture

In the previous discussion, the ribosome has been illustrated as an open, structureless circle or—in the electron microscope—as a roughly spherical structure. However,

recently we have begun to accumulate considerable chemical information concerning its organization. All living cells contain ribosomes, but they do not contain the same type of ribosomes. Two main groups appear to exist among living organisms. The major division seems to be between the protocaryotic organisms, such as bacteria or blue-green algae, and the eucaryotic organisms, which include yeast, protozoa, and all higher forms.[4] The former organisms have ribosomes with a sedimentation constant near 70S, while that of the latter group is closer to 80S. It is not clear why ribosomes exist only in these two different categories. The bacteria and blue-green algae are devoid of a nuclear membrane and have no endoplasmic reticulum or intracellular organelles such as mitochondria or protoplasts. Why these characteristics should also be associated with substantial differences in ribosomal organization is unknown. Most of the information we have concerning ribosomal structure comes from the study of bacterial ribosomes, predominately those found in the *E. coli*. Table I lists various characteristics of bacterial ribosomes and, where the information is available, the analogous information from mammalian ribosomes. The *E. coli* ribosome has a total particle weight of nearly three million and is made up of subunits of unequal size—50S and 30S. Each of these contains RNA that accounts for almost two-thirds of their total mass. The additional mass is made up of a large number of smaller proteins, the characterization of which is incomplete at the present. Associated with the 50S subunit are two molecules of RNA—a very large one with a sedimentation constant of 23S and a smaller one with a sedimentation constant of 5S. The 5S subunit is a polynucleotide containing slightly more than 100 nucleotides and is distinct from the transfer RNA, which contains approximately 70 nucleotides. In contrast, the 23S RNA contains over 4000 nucleotides, and is believed to exist as a single polynucleotide strand with considerable secondary structure. The 30S ribosomal subunit contains an RNA molecule with approximately 2000 nucleotides. Ribosomal RNA accounts for over 80 per cent of the total RNA of the cell, yet, remarkably enough, we do not have any understanding of its function at the present time. Why RNA must be used to build a structure designed to translate information found in a messenger-RNA strand is as yet an unsolved problem. The function of the messenger RNA seems abundantly clear, and the function of the transfer RNA can be understood in terms of its acting as an adaptor to facilitate the proper sequential assembly of individual amino acids on a messenger strand. However, we do not understand the particular benefits that derive from using the same kind of polymer to make what appears to be a structural framework for the ribosome. It is widely held that the highly coiled ribosomal RNA serves as a matrix to which are attached a variety of smaller proteins, but we do not have enough information to evaluate such a proposal.

The analysis of ribosomal proteins has been considerably clarified by studying them on polyacrylamide gels where the denatured material produces a series of sharply defined bands.[5] The 50S subunit appears to have 15 to 20 proteins, while the 30S unit may have 10 to 15. The exact number of these is not known with certainty, but it has been shown recently that it is possible to disassociate some of the ribosomal proteins both from the 50S and the 30S particles by suspending them in very high concentrations of cesium chloride.[6] Some ribosomal functions are lost by removing these proteins, but they can be regained if the protein subunits are added.[7]

The bracketed figures in Table I represent the analogous information concerning mammalian ribosomes. These are considerably larger than those in *E. coli;* they have a sedi-

TABLE I

Composition of E. coli *ribosomes*

	Sedimentation constant	Particle weight (daltons)	RNA Component		Protein Component	
			Sed. Const.	Mol. Wt.	Total Amt.	No. of Proteins
Ribosome	70s	2.8×10^6			(daltons)	
	[80s]	[4.1×10^6]				
Ribosomal units	50s	1.9×10^6	23s	1.2×10^6	700,000	15–20
	[60s]		[28s]			
			5s	35,000		
			[5s]			
	30s	0.9×10^6	16s	6×10^5	300,000	10–15
	[40s]		[18s]			

Figures in brackets apply to mammalian ribosomes.

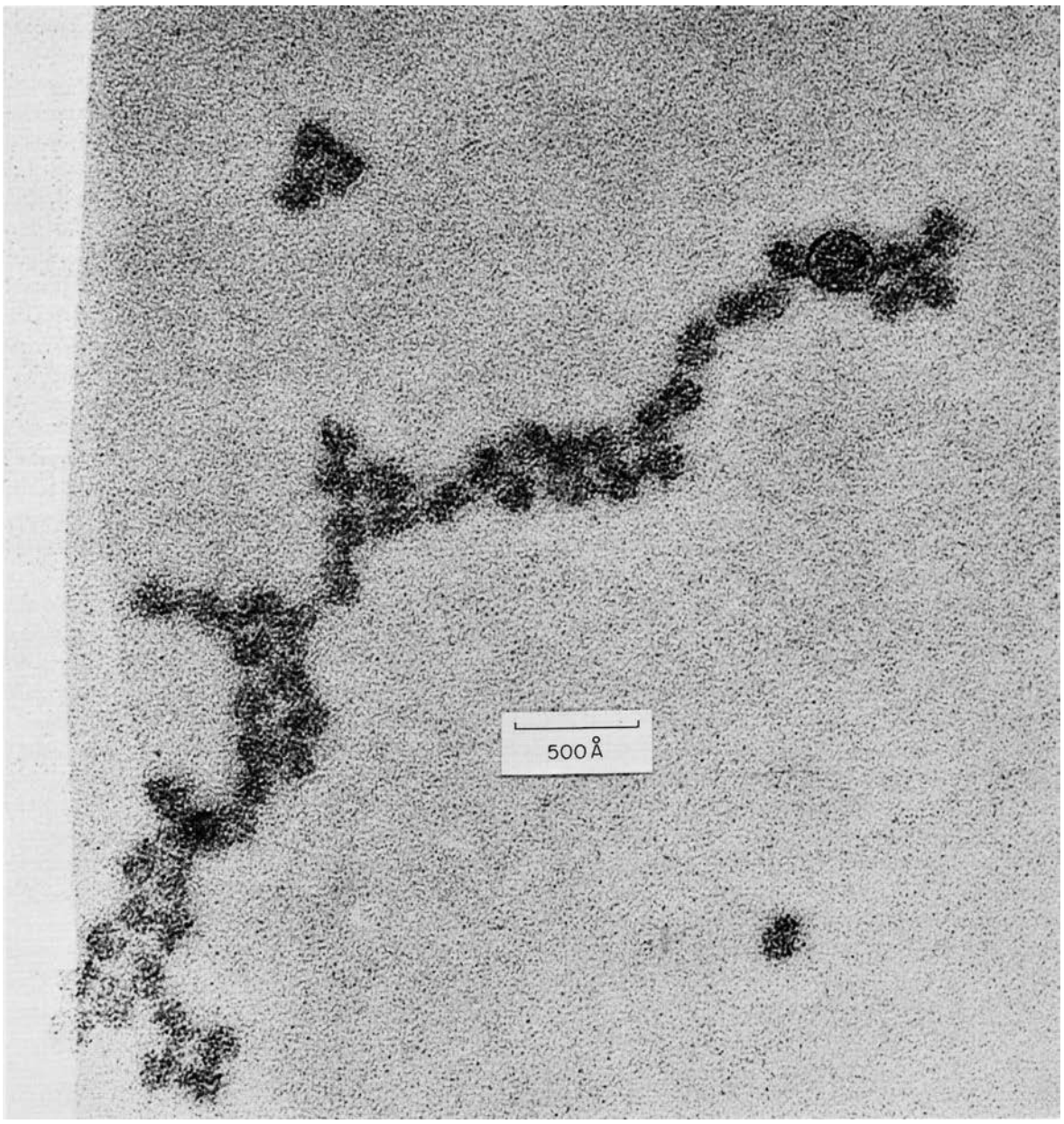


FIGURE 5A Electron micrograph of large polysomes from *E. coli*.

mentation constant of 80S and a molecular weight near four million. Although the general organization of the ribosome is parallel to that in bacteria, there are considerable differences, because the mammalian ribosomes have more protein than the bacterial type. It remains to be seen whether this incremental protein will be associated with more complicated regulatory phenomena in higher organisms.

Ribosomal function

Ribosomal function can be demonstrated by carrying out protein synthesis in a defined system in vitro. For example, the addition of polyuridylic acid to active ribosomal systems results in the polymerization of phenylalanine.[8] Another function of ribosomes is binding transfer-RNA molecules, including those that have been activated

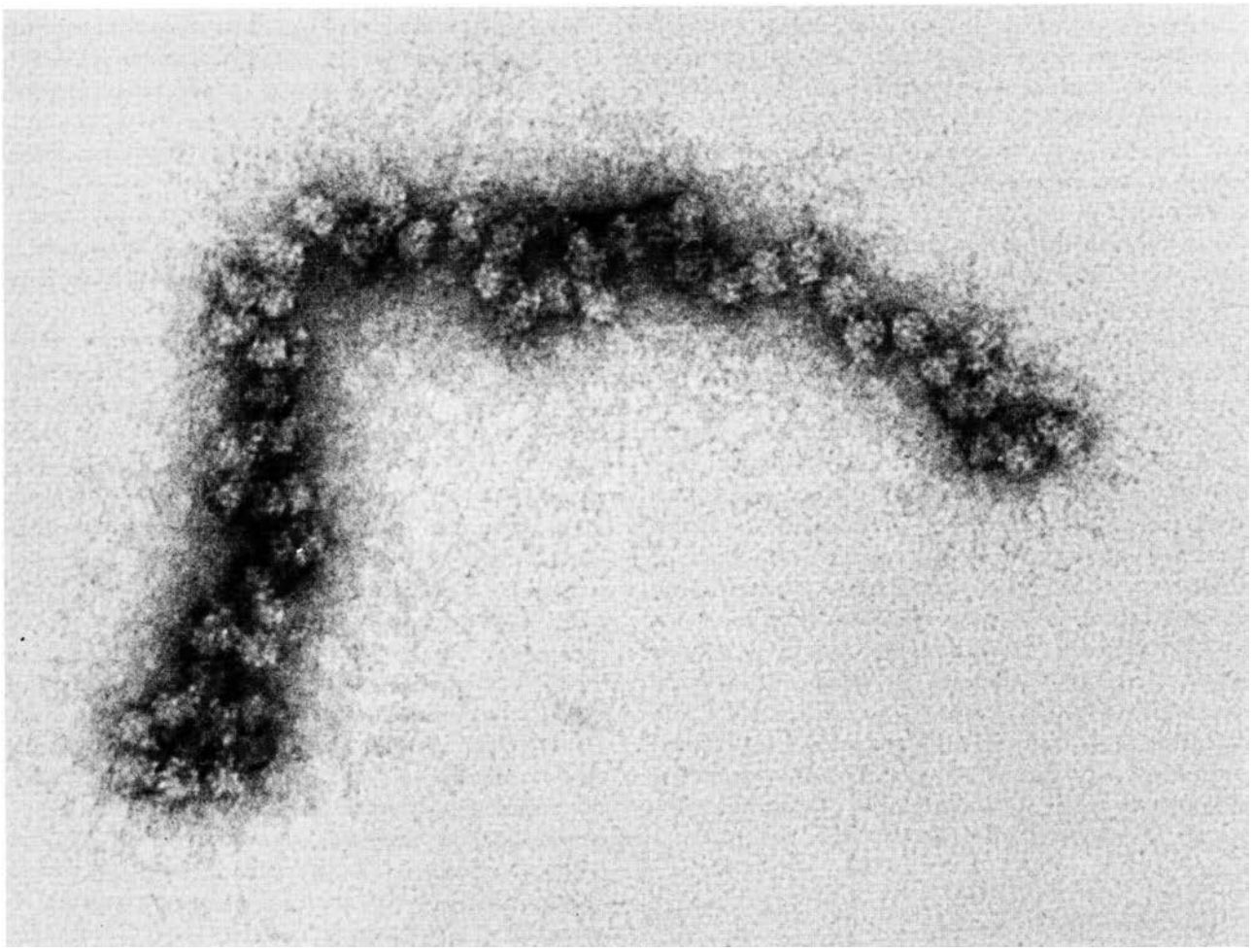


FIGURE 5B Electron micrograph of large polysomes from chick embryo.

through the addition of amino acids, as well as those that have not been activated.[9] It has been shown that the ribosomal subunits have differing functions. The 30S subunit can bind onto synthetic messenger RNA, while the 50S subunit cannot.[10] Alternatively, the 50S subunit can bind to it a transfer-RNA molecule.[9]

The transfer-RNA molecules have a special role to play in protein synthesis. Not only do they serve to bring in individual activated amino acids, they also hold the nascent growing polypeptide chain to the ribosomal structure.[11] All transfer-RNA molecules have a common sequence at their 3′OH terminus—cytidylic acid, cytidylic acid, adenylic acid. The amino acid or growing polypeptide chain is attached to the terminal adenylic molecule. Transfer RNA will not bind to the ribosome unless this terminal trinucleotide sequence is present. The antibiotic puromycin disrupts ribosomal function because it has a structure analogous to the terminal adenine with its attached amino acid.[12] Thus, it appears to function by being held in the ribosome, where it serves as an acceptor molecule for the nascent or growing polypeptide chain. Because it lacks the rest of the transfer-RNA molecule, it is released directly into the surrounding medium together with the nascent chain. In this way, protein synthesis is aborted.

Ribosomes can bind transfer RNA, but the actual amount held depends on whether the ribosome is active or inactive in protein synthesis. For example, if the transfer-RNA molecules in the reticulocyte cell are selectively labeled and the cell contents are analyzed on a sucrose density gradient similar to that shown in Figure 2, the single ribosome peak, inactive in protein synthesis, contains approximately one transfer RNA per ribosome. However, the ribosomes active in protein synthesis found in the polysome peak contain two transfer-RNA mole-

cules per ribosome.[13] Enzymatic work carried out with the reticulocyte system suggests that there is a two-step process in the formation of a peptide bond, one of which is consistent with the loading of a transfer-RNA molecule onto one ribosomal site while the other involves conversion of the transfer-RNA molecule into a peptidyl transfer RNA.[14]

It is clear that this process must be accompanied by a movement of the ribosome along the messenger-RNA strand. The process can be understood by the schematic representation shown in Figure 6. Here the ribosomal surface has two sites associated with it—an amino-acyl transfer-RNA site (A) and a peptidyl transfer-RNA site (P). When both sites are loaded, peptide bond formation (1) results in the transfer of the polypeptide chain from site P to site A, accompanied by the release of the transfer RNA, which formerly held the peptide chain on site P. This allows the movement of the transfer RNA (2) and of the messenger RNA (3) from site A to site P; site A is then freed so it can be filled again with a new activated transfer-RNA molecule. This is an iterative or cyclic process involving the translation of the transfer-RNA—messenger-RNA complex by some 10 Å relative to the rest of the ribosomal structure. It is likely that this transfer process is energy requiring, and the consumption of guanosine triphosphate, which is associated with peptide bond formation, may be involved in the process.

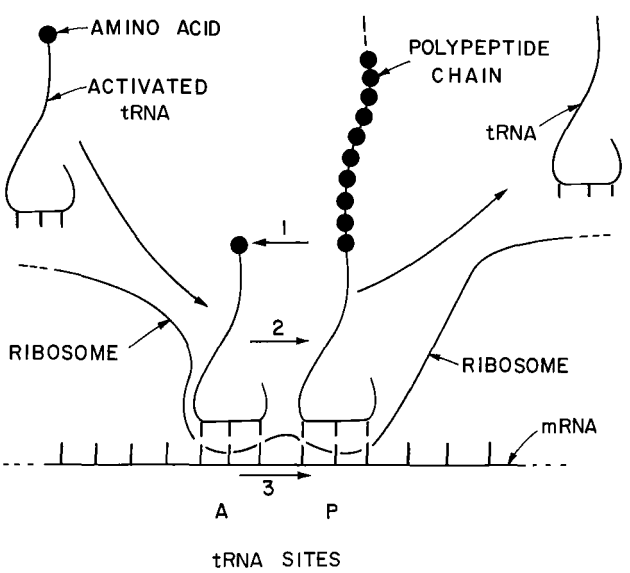


FIGURE 6 Diagram illustrating the action of the two transfer RNA (tRNA) sites during protein synthesis: (1) represents formation of the peptide bond; (2) and (3) represent the movement of tRNA and messenger RNA from site A to site P on the ribosomal surface.

It would be erroneous to regard the ribosome as having a purely passive role in the assembly of amino acids. Recent work on the mode of streptomycin action suggests that ribosomal structure may be profoundly implicated in the translation of the genetic code.[15] Ribosomes from streptomycin-sensitive organisms appear to bind the streptomycin molecule in such a way that faulty translation is introduced and the incorrect amino acid is inserted; thus it is clear that the ribosome participates in some manner yet to be elucidated.

The "reading site" of the ribosome is that point at which the messenger RNA and activated transfer RNA are held together, as shown in Figure 6. Attached to the end of one transfer-RNA molecule is the nascent or growing polypeptide chain, which ultimately emerges from the ribosome as protein synthesis continues. It has been possible to make an estimate of the length of a growing polypeptide chain buried within or shielded by the ribosome. If polysomes are labeled only in the nascent chain and are then subjected to proteolytic digestion, only those portions of the nascent chain external to the ribosome are digested, while the ribosome itself is not attacked.[16] By this type of experiment it has been possible to isolate the proteolysis-resistant fragment of the polypeptide chain and to demonstrate that the resistance derives from ribosome shielding. The fragment contains 30 to 35 amino acids. In a fully extended polypeptide chain, amino acids occupy 3.6 Å per residue. Accordingly, if this portion is extended, there is enough material in the proteolysis-resistant fragment to account for a length of approximately 100 Å. The transfer RNA must be located at the end of this fragment, but at the present time we have no information concerning its configuration. However, it is likely that the reading site is buried deep within the ribosomal structure, possibly between the two ribosomal subunits.

Folding of polypeptide chains

There is good evidence that the folding of a polypeptide chain is determined by the sequence of its amino acids. Extensive experiments with ribonuclease and other protein enzymes have shown that it is possible to denature and subsequently to refold the protein to regain the original catalytic activity without the use of any other cellular structure. Accordingly, it is not unreasonable to anticipate that the folding process begins as the polypeptide chain emerges from the ribosome. In the case of very long polypeptide chains that are almost completed, the molecule probably will have folded up into a form that is substantially the same as that of the completed molecule. As an example, we can consider the enzyme β-galactosidase, which is made of four identical subunits, each of which

has a molecular weight of 135,000 and contains approximately 1100 amino acids. When 1000 or so of these amino acids have been polymerized, the nascent protein, although still attached to the ribosome, likely will have folded up to make a protein subunit somewhat similar to that found in the native molecule. This is believed to be why enzymatically active β-galactosidase molecules can be found even though they are attached to ribosomes.[17] It has been shown that these molecules are nascent and that the enzymatic activity is associated with the tetrameric form of the molecule.[18] Even though the nascent chain is not quite complete, it probably serves as a condensing site for accepting three other subunits, which produce an enzymatically active complex. This can be detected on the polyribosomal structure.

Beta-galactosidase is an inducible enzyme, so it is possible to compare the induced polyribosomal pattern of *E. coli* with that of a noninduced culture. Figure 7 shows a sucrose density gradient analysis of polyribosomes from *E. coli*.[19] The profile is shown both before and after an inducer has been added to the system. Before the addition of the inducer, no enzymatic activity can be seen to be associated with the polyribosome. A short time after adding inducer, a substantial amount of polysome-bound β-galactosidase can be detected. The amount that is bound to polysomes increases substantially as the time of induction increases. The enzymatic activity is attached to *E. coli* polysomes, which contain about 50 ribosomes. The behavior of the polysome-bound β-galactosidase activity is very similar to the behavior of radioactive nascent chains. For example, if the polysomal material is treated with ribonuclease, it promptly breaks down into single ribosomes that still retain the enzymatic activity attached to them, in much the same way as the nascent-chain radioactivity remains attached to ribosomes after polysomes are disrupted.

Polycistronic and monocistronic messenger RNA

The β-galactosidase polysome is very large. Induction of β-galactosidase is also associated with induction of two other proteins.[20] However, studies with bacterial deletion mutants that are unable to make the additional proteins, show that they have smaller polyribosomes than those produced in the normal wild type.[21] That is because the messenger RNA contains information for making more than one protein. This is called a polycistronic messenger RNA in contrast to monocistronic systems such as the mRNA for hemoglobin, which codes for only one protein. A number of these systems have been analyzed, but it is not known whether polycistronicity is a common occurrence or whether single cistrons are more frequent.

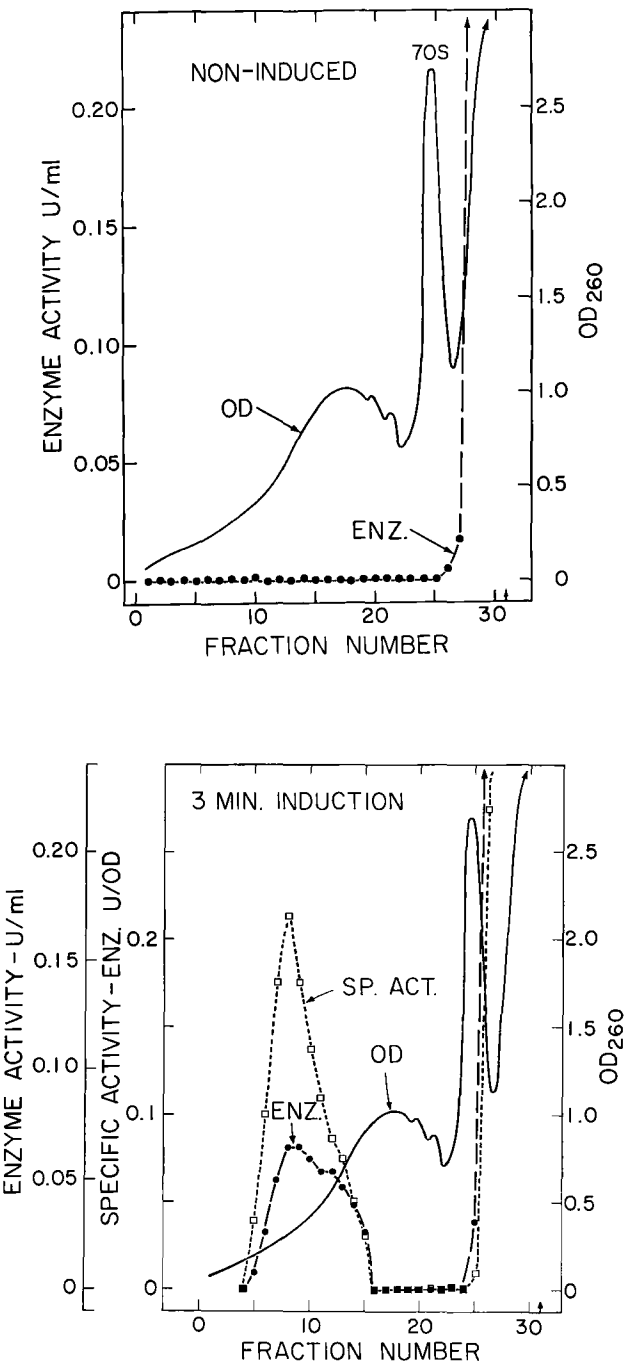


FIGURE 7 Sucrose gradients of *E. coli* lysates, which were assayed for β-galactosidase activity. a) Non-induced culture shows activity only at the top of the gradient. b) After 3 minutes of induction, enzyme activity is seen on the larger polysomes. Specific activity reaches a peak near polysomes containing 50 ribosomes.

Many examples of polycistronic systems are believed to occur in bacteria, especially those associated with bacterial induction. However, the frequency with which it occurs in mammalian cells is still an open question and will require further investigation.

There are several advantages to the organism in a system of coordinate induction of more than one protein molecule and the synthesis of these proteins through the use of a polycistronic messenger. Several components of a system can be supplied under control of a single genetic function such as in the operon, which is a familiar component of bacterial genetics.[20] In mammalian cells the situation is not so clear. Many proteins contain more than one type of subunit, and it is frequently interesting to ascertain whether or not the messenger RNA for these different subunit proteins are connected. An example is in the synthesis of gamma globulins, which make up the antibodies produced in the lymph nodes and spleens of immunized animals. The gamma globulin molecule is made of a combination of two heavy and two light polypeptide chains with molecular weights near 55,000 and 23,000 respectively.

Antibodies are extremely diverse and can react with a very large number of antigenic molecules. The molecular basis for this diversity is not understood at the present time. However, it may be that one aspect of antibody diversity arises because of different combinations of heavy and light chains. If this is so, it would require a separate messenger RNA for the light and heavy polypeptide chains, because if they were linked together in one polycistronic messenger, there would probably be an obligatory coupling of a particular light chain with a particular heavy chain. An analysis of the polyribosomes of antibody-producing tissue demonstrates that they have a biphasic polysome profile with one peak of polysomes occurring in the region of 7 to 8 ribosomes and another in the region of 16 to 20 ribosomes[22] (Figure 8). In hemoglobin synthesis, a polysome containing 5 ribosomes synthesizes a polypeptide chain of molecular weight 17,000. Hence, the two polysome peaks found in the antibody protein-synthesizing systems are producing chains of molecular weights near 25,000 and 50,000 to 60,000, respectively. This is consistent with the production of heavy and light gamma globulin chains on separate messenger-RNA molecules. Accordingly, it is possible that part of antibody heterogeneity is related to different combinations of the two types of subunits.

Our knowledge of ribosomal structure is rudimentary at the present time. We understand the general nature of its functions in the translation of polynucleotide sequence information into polypeptide sequence, but the molecular details underlying this activity are unknown. We have a reasonable idea of the mechanics of amino acid addition and peptide chain assembly, but again the information is usually of a general, rather than a highly specific nature. However, the rapid pace of work in this area makes it likely that in the near future we will have a much fuller knowledge of the three-dimensional structure of the ribosome and will thereby understand some of the fine details of information translation.

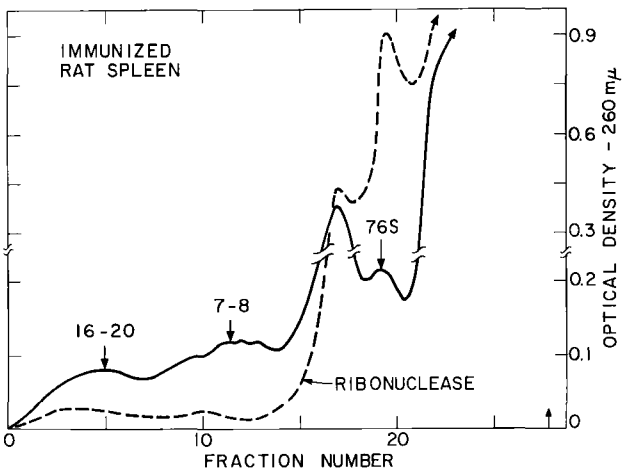


FIGURE 8 Spleen lysate analyzed on a sucrose density gradient. The spleen of an immunized animal shown here has two peaks in the polysome region, the spleen of a non-immunized animal does not show many polysomes and does not have a biphasic distribution. 76S represents single ribosomes, the peak to the left of it is a dimer peak, which is unrelated to protein synthesis.

Summary

Molecular information in biological systems is stored as a sequence of nucleotides in polynucleotide structures. Ultimately, this information is translated into the sequence of amino acids in protein molecules. This takes place on the ribosome, a macromolecular, cellular constituent built out of both proteins and nucleic acids. We have some information concerning the role of the ribosome in this process, but only limited information regarding the structure of ribosomes. During the translation of genetic information from messenger RNA, many ribosomes act at the same time on individual messenger-RNA molecules. However, we do not know how the ribosome carries out its important functions.

Metabolic Regulation and Information Storage in Bacteria

BERNARD D. DAVIS

THE FIRST PORTION of this chapter considers the physiological significance of allosteric regulation of enzyme action, and of regulation of specific enzyme formation; the mechanisms of these processes are discussed in detail in other chapters in this volume. Also reviewed briefly are the mechanisms of a third set of processes concerned with regulating the over-all rate of synthesis of the various major classes of macromolecules. The remainder of the paper considers additional aspects of bacterial physiology and genetics that also seem relevant to the interests of students of the neurosciences, particularly with reference to the problem of understanding information storage.

Regulatory mechanisms

METABOLIC PATHWAYS Before gene expression and its regulation could be studied in bacteria, it was necessary to determine the sequential steps of some metabolic pathways and to learn to measure the levels of the specific enzymes involved. This development received its greatest impetus in 1940, when Beadle and Tatum[1] introduced the use of mutants of the mold *Neurospora crassa* that were blocked in various biosynthetic reactions. Since then most of the steps in the syntheses of the 20 amino acids have been defined, largely through work on *Neurospora* and on the bacterium *Escherichia coli*. The advantages of studying such microbes include their homogeneous cell populations, their rapid growth and biosynthesis, and, above all, the possibility of selecting mutant strains that are sharply blocked in the formation of a single enzyme. This last feature recurs constantly in modern studies on bacterial physiology: the key to one discovery after another, especially in regulatory mechanisms, has been the isolation of a particular mutant. Other powerful tools have been provided by the sophisticated use of isotopic tracers and by modern methods of cell fractionation, enzyme purification, and chromatographic analysis.

The results of such studies have revealed the general pattern of biosynthesis outlined in Figure 1. Omitting the detailed steps, this figure shows that the various pathways of biosynthesis branch off from a variety of specific intermediates in two central, energy-yielding pathways: glycolysis and the Krebs tricarboxylic acid (TCA) cycle. Thus, these central pathways, which had been discovered earlier as the major routes of catabolic breakdown of fuels, also serve an anabolic function; hence, they may be more precisely designated as amphibolic pathways.[2]

Bacteria and molds were found to contain essentially identical biosynthetic pathways (except for lysine and tryptophan); and the same pathways have been found, wherever sought, in higher plants and in animals. (Mammals, however, have economized in genes and enzymes by dropping those 11 amino acid pathways that each contain from 5 to 15 enzymes, while retaining the short pathways [1 to 3 enzymes each] to the other 9 amino acids.) The pathways summarized in Figure 1 are thus for the most part directly relevant to the nervous system, although its cells must also possess many specialized pathways. Of even greater relevance for present purposes, however, is the underlying principle: to understand regulation you've got to know the territory being regulated.

REGULATION OF SYNTHESIS OF SPECIFIC PROTEINS A common mechanism is used to regulate the formation of two distinguishable classes of enzymes. The first class includes those that convert optional or alternative foodstuffs to members of the amphibolic pathways; these enzymes are called *adaptive* or *inducible*, because they are formed only in the presence of the substrate (or a structurally related inducer). (For example, lactose is converted to glucose plus galactose by one enzyme, and the latter is converted to glucose phosphate by three enzymes.) The second class includes the enzymes of biosynthetic pathways: these do not require induction, but, on the other hand, they are regularly found to be *repressible*, either partly or completely, by an excess of the end product of the pathway. In his chapter in this volume, Stent[3] describes the brilliant explanation of both these types of regulation by Jacob and Monod[4] in terms of a regulator gene, whose product, when in the proper configuration, acts on a

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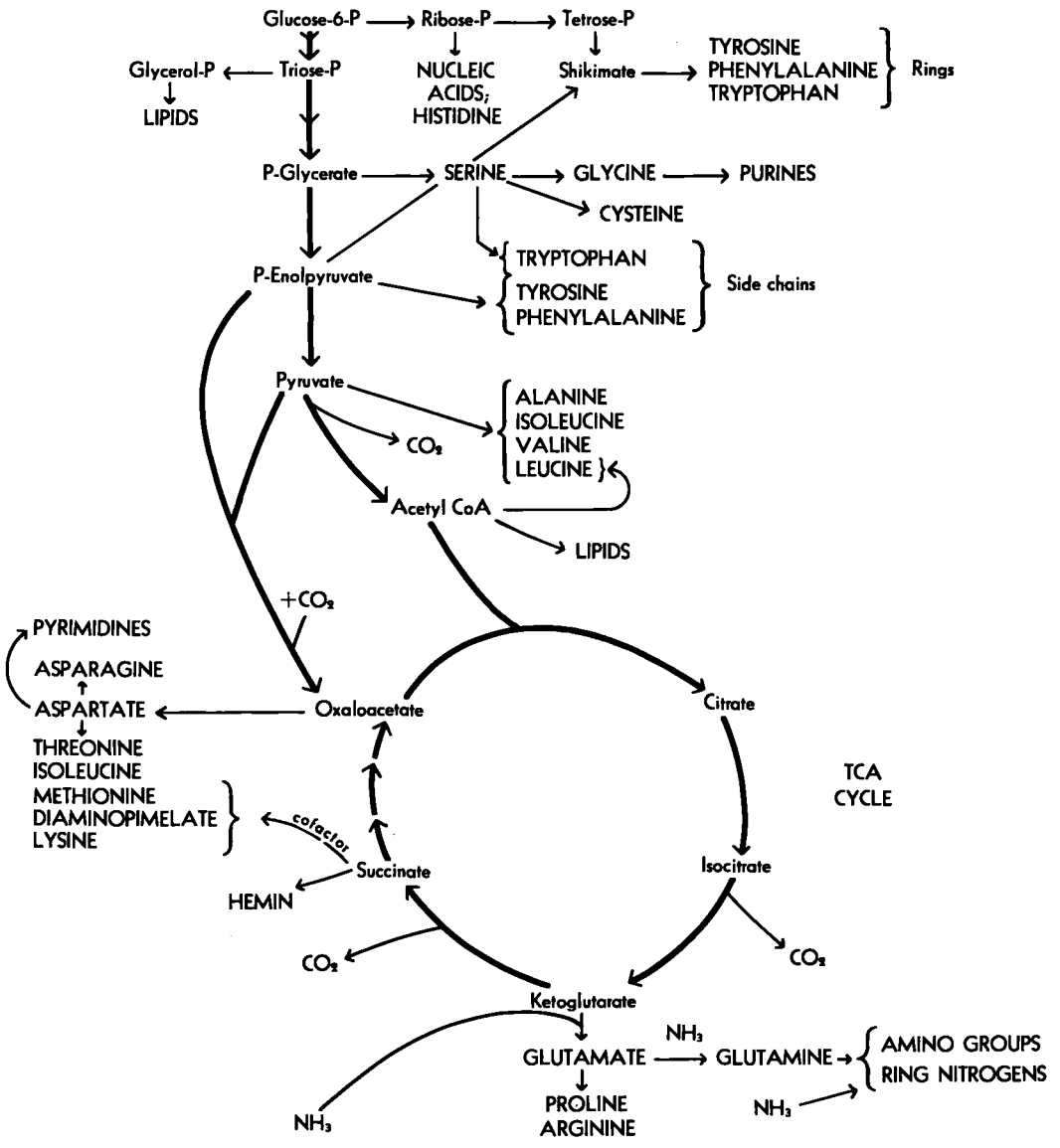

FIGURE 1 Relation of amphibolic to anabolic pathways. Amphibolic pathways have heavy arrows.

specific operator region and thus represses expression of one or more genes adjacent to that region.

I would like to make only a few points about the physiological significance of this mechanism. First, it seems to function primarily to provide economy in protein synthesis, for synthesis of the end product of a pathway is much more delicately and rapidly regulated by the feedback inhibitory effect of the end product on the activity of an enzyme of its own pathway (see next section). Second, the response was discovered through an interaction of the foreign and the domestic economy of the cell, i.e., the exogenous supply of a substance spares the formation of the enzymes involved in the synthesis of that substance. However, end-product repression has an even more fundamental role in the purely domestic cellular economy. Thus, it is responsible, at least in large part, for governing the widely differing rates of expression of the innumerable genes in a cell that are competing for a common supply of precursors; and the need for such a mechanism is illustrated by the finding that any one fully induced (or derepressed) gene contributes so much protein (around 5 per cent of the total cell protein) that genes

must generally be strongly braked. Furthermore, this pattern of cruising at a rate far below capacity provides the cell with the possibility of adjusting with great speed to an altered environment.

This reserve capacity was revealed by a finding of Gorini and Maas[5]: repression of an arginine biosynthetic enzyme by an excess of added arginine led to exponential dilution of the pre-existing enzyme; but restoration of the enzyme, after elimination of the exogenous arginine, occurred at an explosive rate—approximately 25 times the normal steady-state rate of synthesis of the enzyme (relative to total protein synthesis), as seen in Figure 2.

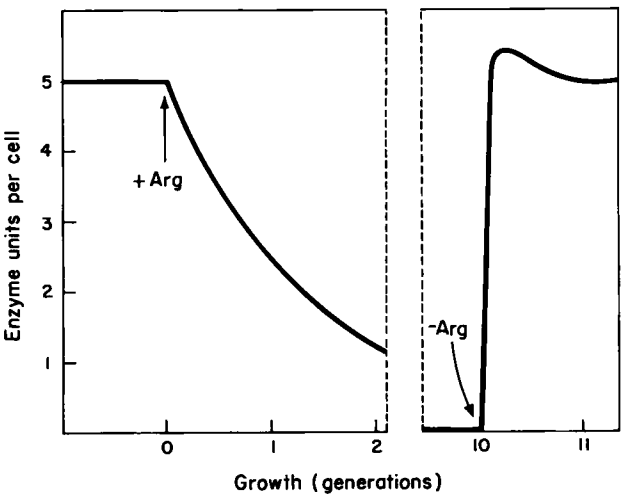

FIGURE 2 Formation of an enzyme of arginine biosynthesis (ornithine transcarbamylase) by *E. coli:* repression on addition of excess arginine (first arrow) and derepression on eliminating the arginine (second arrow). (Based on Gorini and Maas, Note 5)

REGULATION OF ENZYME ACTIVITY The synthesis of low-molecular-weight metabolites is regulated largely through the instantaneous feedback inhibitory effect of these substances on the action of branch-point enzymes in their sequences. The development of this field is reviewed in this book by Atkinson.[6] The importance of this process in bacteria, which are in general selected in evolution for maximum growth rate, is illustrated by its widespread use: every biosynthetic pathway studied has shown some degree of feedback inhibition of the initial enzyme of that pathway. Moreover, in branched pathways, such as the one that converts aspartate to diaminopimelate, lysine, methionine, and threonine, the enzymes at each branch point show this special property; and in the common pathways that precede such branches the initial reaction is usually catalyzed by two or more parallel enzymes, each carrying out precisely the same chemical transformation, but each subject to a different feedback regulation.

Feedback inhibition of enzyme action has much the same relation to foreign and domestic economy that was noted above for feedback repression of enzyme formation: it was discovered through the economy in biosynthesis effected by the exogenous addition of various metabolites, but its major role in the cell is concerned with regulating the internal economy. This regulation ensures that the rate of activity of each pathway is varied to maintain its end product at a relatively constant intracellular level, specified by the setting of the internal "chemostat."

The most cogent evidence for this proposition has been provided by the properties of mutants that have lost the capacity for feedback inhibition in some pathway. Moyed[7] found that certain amino acid analogs (e.g., 2-thiazolylalanine as an analog of histidine) inhibit growth by mimicking the inhibitory effect of the normal end product on the regulatory enzyme of its own pathway. This "pseudofeedback inhibition" starves the cell for the end product. When large numbers of cells are inoculated in the presence of the analog, rare mutants resistant to its inhibitory effect are readily selected, and the cause of their resistance is found to be the production of an altered, nonresponsive regulatory enzyme. Such altered enzymes are also less responsive to the signal provided by the normal end product. The important consequence is that these strains build up an excessive level of that end product in the cell, "wastefully" excreting it into the culture medium. (Indeed, the use of microbes for the commercial production of many desired metabolic products, such as amino acids, citric acid, or antibiotics, depends on a distortion of the regulatory mechanisms of these domesticated organisms, resulting in less efficient conversion of fuel into their own growth, and increased conversion into the products demanded by their masters.)

The molecular mechanism underlying this allosteric regulation of enzyme action by a specific small molecule is discussed in the chapter by Atkinson. Here I would like to stress that positive as well as negative allosteric effects have been observed; this would markedly increase the opportunities for regulatory interactions within the complex metabolic network. Moreover, in the several systems whose kinetics have been closely examined, cooperative effects between identical regulatory sites on a multimeric protein have been observed. These result in a second-order or higher-order response, rather than a linear response, to increasing concentrations of inhibitor. Such higher-order response clearly narrows down the range within which the cell maintains the concentration of its metabolities, just as the sigmoid oxygen dissociation curve of the multimeric protein hemoglobin permits the animal to transfer a large fraction of the oxygen from arterial

blood to the tissues with only a modest drop in pO_2. Indeed, it is of interest to note that this shape of the oxygen dissociation curve, discovered many decades ago and of great interest to physiologists, is not a special mechanism developed late in evolution; rather, it is an application of a principle that evolved in primitive, single-celled organisms. Surely the nervous system, in delicately regulating its thresholds for neuronal discharge, will employ liberally this principle of higher-order response to variations in the concentration of signaling molecules.

ENZYME LOCATION Thus far, regulatory mechanisms in bacteria have been worked out with so-called "soluble enzymes," which appear in the supernatants of disrupted cells after high-speed centrifugation. Many enzymes, however, are now known to be membrane bound or particulate, as Lehninger[8] and Reed[9] have emphasized. Moreover, Dr. Leive and I obtained evidence,[10] from the distribution of exogenous and endogenous diaminopimelate in components of *E. coli*, that even an apparently "soluble" enzyme, concerned with the incorporation of this metabolite into a cell-wall precursor, is actually preferentially located toward the periphery of the cell. Even the bacterial cell is much more than a bag of enzymes, and it is evident that topographical organization must play a dominating role in the highly specialized cells of the nervous system.

Enzymes can thus have three specific kinds of sites, concerned with catalysis, allosteric regulation, and location. I would like to venture the speculation that in highly differentiated cells the responses of allosteric sites may influence not only enzyme action but enzyme location.

REGULATION OF RNA AND PROTEIN SYNTHESIS We have seen that bacteria, through selection for rapid growth and for versatile adaptation to varying conditions, have developed an elaborate system of controls to avoid overproduction of various specific enzymes and small molecules. Maaløe[11] was the first person to look closely into the possibility that the formation of DNA and RNA might be similarly regulated and adjusted to different circumstances. Table 1, taken from the thesis of his student Kjeldgaard,[12] is concerned with the relation between growth rate and cell composition. When the same bacterial strain is grown on different foods (which support growth at different rates), and the cells are harvested while in steady-state exponential growth, their composition varies systematically with growth rate.

In particular, at a fixed temperature the rate of protein synthesis in these steady-state cells remains strictly proportional to the concentration of ribosomes (measured as concentration of ribosomal RNA [rRNA] per unit weight), over a tenfold range in growth rate. At the same time, the concentration of transfer RNA (tRNA) remains relatively invariant. These results suggest that in balanced growth every ribosome is working, on the average, at a constant rate, the total rate of protein synthesis thus depending on the concentration of ribosomes. Moreover, in order to work at this fixed rate each ribosome must encounter charged tRNA molecules (aRNA) at a fixed rate; hence, the tRNA concentration surrounding the ribosome remains fixed, regardless of growth rate.

As might be expected on grounds of cellular economy, this fixed rate of ribosomal activity is essentially a maximal rate; for when a cell suspension is "shifted up" from a poor carbon source to a better one, providing a better supply of amino acids and of energy for their polymerization, the rate of protein synthesis does not rise at once, but remains proportional to the ribosome concentration. In contrast, this shiftup produces an immediate, marked increase in the rate of net synthesis of RNA (i.e., of rRNA, which constitutes the bulk of the stable RNA whose

TABLE I

RNA distribution and protein synthesis in Salmonella typhimurium *at various rates of balanced growth.*[12]

Carbon source	Growth rate, μ (Generations/hr)	DNA (μg/mg bact. dry wt)	rRNA / DNA	tRNA / DNA	Protein / DNA	Protein synthesis per hr: Per unit RNA	Protein synthesis per hr: Per unit rRNA
Broth	2.4	30	8.3	2.0	22	3.7	4.5
Glucose	1.2	35	3.9	2.4	21	2.8	4.6
Glycerol	0.6	37	2.4	2.4	21	1.8	3.6
Glutamate	0.2	40	0.9	2.1	21	1.0	3.3

synthesis is being measured) as is shown in Figure 3. This "irritability" of rRNA synthesis is similarly shown in a "shiftdown" from a good to a poor carbon source: protein synthesis naturally slows down because of the impaired supply of amino acids and energy, but net RNA synthesis almost completely ceases; and it resumes at the new steady-state rate only when the new steady-state ratio of protein of RNA has been reached (Figure 3).

THE MECHANISM OF REGULATION OF rRNA SYNTHESIS This is still a challenging problem. Any model would have to explain not only the responses just described, but also two other findings: (1) Starving an auxotrophic mutant for a required amino acid causes an immediate cessation of net synthesis of rRNA as well as of protein; whereas (2) blocking of protein synthesis by the addition of chloramphenicol (or tetracycline or streptomycin) results in continued and even stimulated rRNA synthesis for some time. Since these drugs block protein synthesis at a stage *after* the charging of tRNA with amino acids, whereas starvation for a given amino acid *prevents* charging of the corresponding tRNA, Kurland and Maaløe[14] suggested that uncharged tRNA, of any species, is the regulatory substance whose level modulates the over-all rate of RNA synthesis, perhaps by acting on RNA polymerase.

While this hypothesis is ingenious, it presents certain difficulties. First, it requires implausibly high normal levels of charging of tRNA, in order to permit the uncharging of one species (through amino acid starvation), accompanied by increased charging of the 19 other species (which cannot be discharged under these circumstances), to result in a significant increase in the total level of uncharged tRNA. Second, it does not account for the varying ratios in the rates of synthesis of the different classes of RNA under various conditions, shown for tRNA/rRNA in Table 1, and implied for mRNA/rRNA by the divergent shifts in protein and RNA synthesis shown in Figure 3. Accordingly, my colleagues, Mrs. E. Ron and Dr. R. Kohler, and I have investigated an alternative possibility: that the region of the DNA responsible for rRNA synthesis functions as an operon, whose operator responds to the end product of the pathway, free ribosomes, just as amino acid biosynthetic enzymes respond to their end product, aRNA,[15] and purine or pyrimidine pathways respond to the corresponding end products, nucleoside triphosphates.[16] We have found support for this hypothesis in experiments with a strain of *E. coli* that Stent and Brenner[17] have shown to be "relaxed" in the control of RNA synthesis—i.e., to have lost its normal response, so that it continues to synthesize RNA even during amino acid deprival. When a normal, "stringent" strain is starved for an amino acid, its polysomes are largely broken down and converted to free

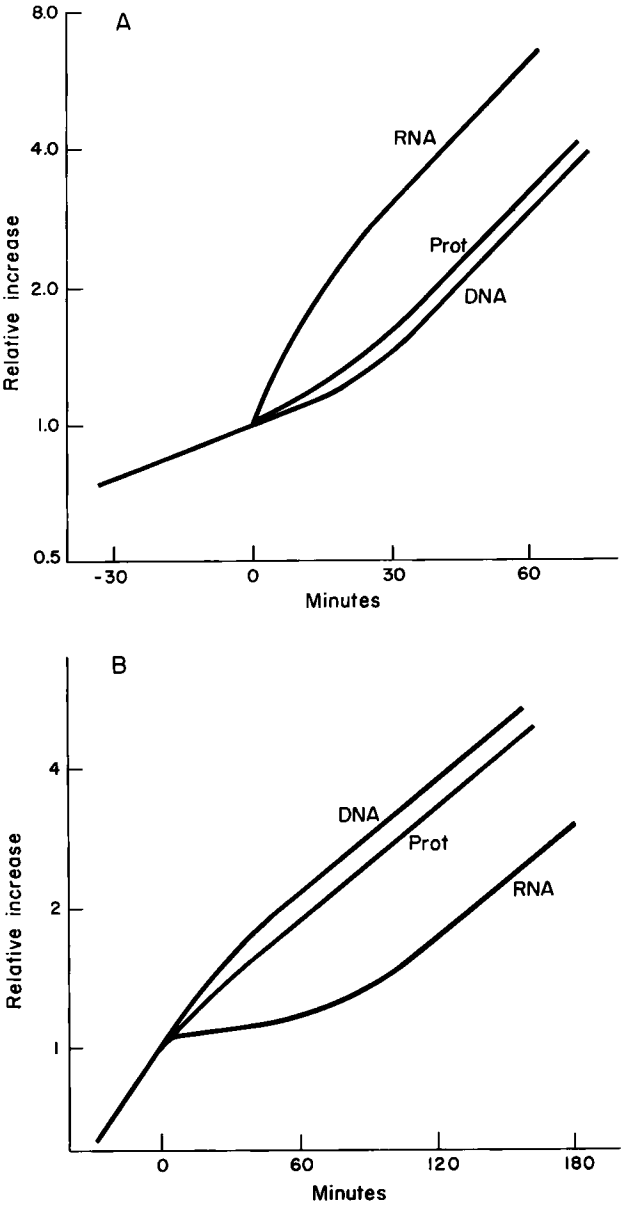

FIGURE 3 Net synthesis (exponential scale) of protein, RNA and DNA. (A) "Shift-up" to a richer medium at 0 time; (B) "shift-down" to a poorer medium. Idealized curves, with the value of each component per ml. of culture normalized to 1.0 at 0 time. (From Neidhardt, Note 13)

ribosomes, while net RNA synthesis ceases[18,19]; but when the "relaxed" strain is treated in the same way it maintains its polysome level[19] and continues to synthesize RNA. Morris and DeMoss[20] have independently made the same observation and come to the same conclusion. Dr. Stent (personal communication) has suggested another interpretation: that the loss of polysomes on amino acid starvation in one strain, and their maintenance in the other, sim-

ply reflect a difference in the levels of mRNA available for polysome formation. The problem is clearly not yet settled.

REGULATION OF DNA SYNTHESIS Although DNA synthesis obviously must be regulated during the morphogenesis of the nervous system, as in all morphogenesis, this regulatory process has no evident special role in the function of the nervous system. I shall therefore briefly summarize the picture in bacteria.[11] The system to be regulated is the following: each bacterial chromosome, which is a closed circle of DNA, begins replication at a fixed initiation point, and the "growing point" then moves continuously around the circle at approximately 10^8 nucleotides per second. When the round of replication is completed, the two new circles separate.

The regulation of this process has some special features. Completion of an incompletely replicated chromosome evidently has a high priority in the cell's economy. If protein synthesis or RNA synthesis is blocked, or if the energy supply is impaired, any currently active round of replication still proceeds to its completion as long as any supply of precursors and energy is available. The initiation of a new round of replication, however, requires at least a brief pulse of synthesis of a new protein. The nature of the required protein is unknown.

In the synthesis of DNA and of protein, and presumably also that of RNA, we can see a common feature that distinguishes macromolecule from small molecule synthesis: under ordinary conditions, with an adequate supply of precursors, the rate of extension of a growing macromolecule is constant; hence the rate of synthesis of a given class of macromolecules (or of inducible or repressible specific macromolecules) is governed by control of chain initiation, and not by direct control of enzyme activity.

The regulatory mechanisms briefly summarized here are now well known to students of mammalian cell physiology. Let us now turn to some other pertinent aspects of microbial genetics and physiology that may be less familiar.

Information storage in bacteria

Few would doubt that information is stored in the nervous system in molecular form; but this very general proposition is merely a restatement, clothed in modern garb, of the conclusions of monistic philosophers some centuries ago: that mind cannot exist without matter. For a useful discussion of the subject of information storage we must focus upon more sharply defined propositions, and we must be sure that the word "molecular" has the same meaning for all parties to the dialogue. In general, two distinct types of mechanisms have been now recognized as theoretically possible. Are blocs of input information in the nervous system *concentrated* on macromolecular tapes, as in genetic systems, with many bits of information sequentially coded in a single nucleic acid or other macromolecule? Or is the information *dispersed*, as in the circuits of a computer, with different bits of input stored (as molecular changes) in the switches of different neurons, resulting in modification of their future behavior?

The present unsettled state of the neurosciences with respect to this fundamental problem recalls the state of bacterial genetics 25 years ago, when a very similar question had to be answered before the miracle of molecular genetics could emerge. It may therefore be useful to review briefly the history of the study of bacterial variation and to describe two classical experiments that established the crucial distinction between *genotypic* and *phenotypic* information.

PHENOTYPIC AND GENOTYPIC ADAPTATION After microbiologists had developed, during the 1870's, the principles and the techniques required for isolating and maintaining pure cultures, they were surprised to find that their cultures were still not free from variation, even though protected from adventitious contamination. When organisms were isolated from nature and were repeatedly transferred in artificial culture media in the laboratory, the appearance of their colonies and even various biochemical properties were often found to change. The mechanism was a mystery: for many decades, until the 1940's, bacteria were believed to possess some vague, plastic mode of inheritance, unrelated to the processes being discovered in the brilliantly developing science of genetics. Several reasons for this persistent misconception are evident: the cytological techniques available did not permit the recognition of discrete bacterial nuclei until relatively recently; gene transfer and recombination, which is essential for any genetic analysis, did not become accessible in bacteria until the mid-1940's; and it was confusing that precisely the same adaptive change could be brought about in two ways—much as a given component of behavior in higher organisms is instinctive in some species and learned in others.

The problem of understanding adaptive mechanisms in bacteria is illustrated by the behavior of *E. coli* cells grown on different carbon sources. Cells grown on glucose lack the enzyme β-galactosidase, which splits lactose to glucose and galactose, but when a sizable, although not visible, inoculum (e.g., 10^7 cells) is placed in a flask of medium containing lactose as the sole carbon source, overnight incubation yields a turbid culture whose cells do possess the

enzyme. This alteration can be produced by two mechanisms, phenotypic and genotypic, which can be distinguished by a simple analysis of the kinetics of change in the cell population.

In one case we have cells that are genetically Lac$^+$, i.e., they possess the *capacity* to make the enzyme, but they do not make it except under appropriate inducing conditions (e.g., when given lactose as the sole carbon source). Appropriate tests with such a population show that within a few minutes of transfer to inducing conditions *all* the cells begin to make the enzyme. In the second case, in contrast, we start with cells that are genetically Lac$^-$, i.e., mutants that lack the genetic capacity to make β-galactosidase. However, this strain can rarely backmutate to Lac$^+$; hence, an inoculum of 10^7 cells may contain *a few* Lac$^+$ cells. In the lactose-containing medium these cells will adapt phenotypically at once and will then multiply exponentially, while the bulk of the population will remain stationary for lack of a suitable carbon source. By morning, the progeny of the Lac$^+$ backmutants will constitute essentially the entire population of the culture.

The end result is the same in the two cases, but the kinetics of appearance of cells containing β-galactosidase will be very different. It should be stressed that in both cases the cells have received *information* from the lactose of the medium, and this information has been used to bring about a phenotypic change in enzyme composition, within the range made possible by the genotype. In the second case the medium has further *selected* the progeny of a rare cell with an altered genotype. In both processes the environment has selected for the formation of certain gene products: the selection takes place both at the level of genes within a cell (phenotypic adaptation) and at the level of cells within a population (genotypic adaptation). However, the environment has not *directed* any change in the information coded in the macromolecules in the cell.

ABSENCE OF DIRECTED MUTATION How can we be sure that the lactose has played only a selective role in the above experiment, and has not directed a specifically adaptive genotypic change? Many workers have found it hard to accept the overwhelming role of chance mutations in genotypic adaptation, because the changes in the population seem so swift, on an anthropomorphic time scale, and are so admirably adaptive to the organism's new needs. Moreover, since lactose can get into the cell whose Lac$^+$ mutations we have just described, how do we know that it does not somehow direct a change in the structure of a gene rather than a change simply in its activity? A similar problem is seen with drug resistance: when an inoculum of 10^{10} cells from a streptomycin-sensitive stock gives rise to ten colonies on a streptomycin-containing plate, the inoculum could already have contained ten spontaneous mutants to resistance—or the drug could have induced the change from sensitivity to resistance, but with such a low efficiency that only one in 10^9 cells survived to undergo the change.

An unequivocal solution to this problem was provided in 1943 by Luria and Delbrück, who developed the method of *fluctuation analysis* to show that mutations to bacteriophage resistance, or to drug resistance, arise spontaneously and are then simply selected by the antimicrobial agent.[21] Figure 4 diagrams a modification of this approach by Lederberg,[22] which demonstrates in a simple, direct way that drug-resistant cells appear in clusters in a medium that lacks the drug. This clustering clearly indicates descent from a common progenitor, which must accordingly have mutated to streptomycin resistance several generations before transfer—i.e., before exposure to streptomycin. This simple but elegant experiment has separated (in time and space) the appearance of mutant cells, in the absence of streptomycin, from their subsequent identification in its presence.

It required years for the implications of this experiment to be generally accepted: as Luria remarked in an early review[23] (too early to foresee the possible impact of molecular genetics on other aspects of biology), bacteriology was the last stronghold of Lamarckism. Indeed, acceptance of the now conventional distinction between genotypic and phenotypic information is still not quite universal. It may

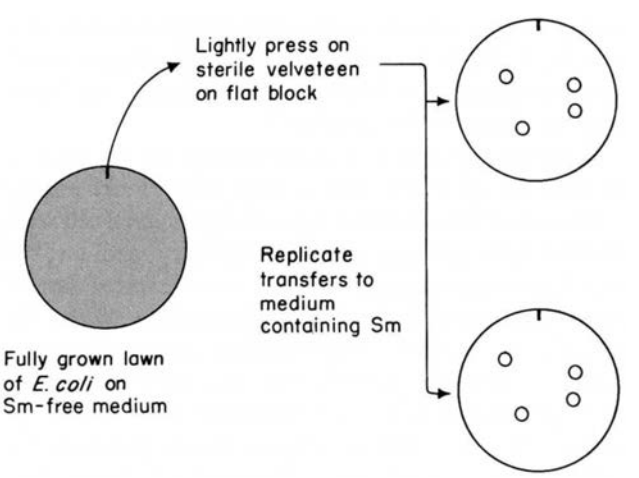

FIGURE 4 Demonstration of spontaneous mutation to resistance to streptomycin (Sm.) A population of cells derived from a single sensitive cell was grown on a drug-free solid medium. Replica plating[22] of the resulting lawn demonstrated the presence of clusters of resistant cells. This result indicates that resistant mutants, each giving rise to such a cluster, appeared in the absence of the drug.

be instructive, for example, to note that Sir Cyril Hinshelwood, Nobel Laureate for his work in physical chemistry, became interested in biology some years ago and produced a book,[24] as well as numerous papers over many years in the *Proceedings of the Royal Society*, which provided logical and experimental support for a Lamarckian view of enzyme adaptation in bacteria. To the best of my knowledge, however, this work, despite its ingenuity and its brilliant literary quality, has not been assimilated into the increasingly coherent structure of microbial and molecular genetics.

APPARENT EXCEPTIONS Since the infinitely complex material of the nervous system can provide apparent exceptions to almost any general mechanisms, it seems useful to show how even in bacteria several phenomena suggesting molecular Lamarckism were observed but were eventually reconciled with the general formulation summarized above.

First, *transfer of genes* may mimic directed mutation. For example, in Japan a few years ago it was found that certain strains of *Shigella dysenteriae* and other enteric bacteria carried genes for multiple drug resistance and transmitted this property to other strains in mixed cultures.[25] The change, however, involves an *addition* to the recipient's DNA, rather than a directed mutation; the addition depends on attachment of the genes for resistance to a "resistance transfer factor" (RTF), which is an episome (bloc of accessory DNA) closely related to Lederberg's fertility (F) factor. Such an episome apparently causes the formation of a long, narrow conjugation bridge between two cells (F-pilus), like the tail of certain bacteriophages, which serves to transfer the DNA of the replicating sex factor from the donor to the recipient.[26]

A second apparent exception is based on *the action of penicillin on cell wall formation*. This drug blocks normal formation of the rigid basal layer of the bacterial cell wall, and thus lyses growing cells in an ordinary medium; but in a hypertonic medium the cells are converted instead into viable, osmotically fragile spheroplasts. When the penicillin is removed, most organisms resume their normal form on further growth, but some grow indefinitely as stable, pleomorphic, wall-defective cells called "L-forms." Since the normal cells are almost quantitatively converted to stable L-forms in these species,[27] we are clearly dealing with a mass alteration in a heritable property, rather than with a rare mutation. The obvious explanation is that the morphogenesis of cell wall in certain strains requires the presence of some normal wall as a "primer" for the synthesis of more of the same. This phenomenon is indeed an example of a directed heritable change; but, as this mechanism can only eliminate a preformed potentiality of the cell, its range of expression is very restricted, and it does not alter the information encoded in a macromolecular sequence in the cell.

The final example seems quite close to the problems of the neurosciences, for it involves a persistent effect of *an induced change in cell permeability*. As will be discussed further by Stent[3] and Kennedy,[28] β-galactosides induce the formation not only of the intracellular enzyme β-galactosidase, but also of a specific, active transport system for β-galactosides. Moreover, the same induction is brought about by β-thiogalactosides; and these compounds, which (unlike β-galactosides) are not attacked by the enzyme, can accumulate in the cells. Growth in the presence of 10^{-3} M methyl-β-thiogalactoside (MTG) causes *E. coli* cells to become fully induced (for both the enzyme and the transport system),[29] whereas 10^{-5} M is too dilute to cause any induction; but once cells have been induced by 10^{-3} M MTG, further growth in the presence of 10^{-5} M results in indefinite *maintenance* of the induced state. Thus cells of the same genotype, growing in 10^{-5} M MTG, may remain either induced or not induced, depending on their recent history.

The explanation is based on the autocatalytic effect of the transport system on its own induction: once cells possess this system they can take up an ineffective external concentration of inducer and raise it to an effective internal concentration. It has been suggested that this persistent phenotypic adaptation may be a useful model for the persistence, or positive feedback, required in the usual processes of differentiation.

MAJOR PRINCIPLES OF MOLECULAR GENETICS These pioneering studies provided a firm conceptual foundation for the future edifice of molecular genetics. As the rapidly growing upper stories of this edifice, richly supplied with intricate and ingenious detail, are highly visible to all biologists today, I shall merely note what seem to be the most fundamental, novel principles revealed in this development.

(1) The *replication* of the genetic material (generally DNA, but RNA in some viruses) is based on the complementarity of the Watson–Crick double helix.

(2) All *genetic* information is encoded in a one-dimensional nucleic acid tape, using a three-letter code whose details are now almost completely known[30]; and the information is decoded by transcription into RNA, followed by translation into polypeptide.

(3) This one-dimensional information also accounts for specific *three-dimensional structures*. Thus, the secondary and the tertiary structures of proteins result from spontaneous folding of their polypeptide chains, in a specific manner determined by the sequences of those chains; and

all other biological structures, however complex, are formed by similar spontaneous aggregation of proteins and of the varied products of their enzymatic activities. For some complex structures, however, such as cell wall or membrane, primer may be necessary for reproducible aggregation in the proper configuration, and in this restricted way the environment may heritably influence the specificity of three-dimensional structures.

(4) All other *phenotypic* information (i.e., environmental influence on cell composition or structure) is incorporated in a cell through selective influences on the activity of various genes (operon regulation) and on the activity of their products (allosteric enzyme regulation). This mechanism furnishes a plausible model for the elementary events of differentiation (which must be further supplemented by some mechanism, absent from bacteria, for stabilizing the altered phenotypic pattern).

(5) The individual molecular events underlying genetic phenomena are all extraordinarily *simple*; complexity arises from the variety of these steps and from their patterns of organization.

IMPLICATIONS FOR THE NERVOUS SYSTEM Against the above background of the history of molecular genetics, it seems to me exceedingly implausible that in neural learning newly received information is encoded in new macromolecular sequences that are not already coded in the genomes of the neurons. Indeed, I can only view learning, in general terms, as an extraordinarily subtle extension of differentiation, closely analogous to the input of information in a computer. According to this view, each experience alters certain switches (i.e., presynaptic, postsynaptic, or other elements affecting neuronal function) in a particular set of interconnected cells. The alteration may be produced by an enzymatic change in a component of the switch; by adding a new component or subtracting an old one; or by changing the environment provided by the cell. But whatever the mechanism, the threshold for future closing of the switches is altered in such a manner that when suitably triggered, the set of altered cells can now recall a pattern of neural activity remarkably similar to that of the original input. Moreover, the molecular changes responsible for these altered thresholds must be capable of persistence, but with variable duration. Finally, while this model does not prescribe that all neurons must use precisely the same chemical changes in encoding a bit of information, it does seem likely that a limited variety would be sufficient to meet the demands of the system.

This is a very general model. It does not specify the nature of the persistent changes in cell structure brought about by experience, but it does insist that these changes can be brought about only by familiar kinds of environmental influences on a cell: on the amount of various gene products formed; on their stability or retention; on their conformation; and on their catalytic or transporting activity. I would further suggest, to be a little more concrete, that a provocative molecular model for a stable, triggered alteration in function is the activation of a proenzyme by the enzymatic removal of a short peptide, resulting in a striking change in the conformation (and the enzymatic activity) of the remainder of the molecule. Examples include pepsinogen, trypsinogen, and components of the blood-clotting system and the complement system of plasma.

The reasons for preferring a distributed rather than a concentrated pattern of memory may be summarized as follows:

(1) It seems reasonable to extrapolate to the cells of the nervous system the distinction between phenotype and genotype, so essential for the progress of genetics.

(2) What we know about the biosynthesis of informational macromolecules, their alterations by mutation, and the one-way flow of genetic information, makes it difficult to conceive a plausible mechanism for transducing electrical messages to a cell, or pulses of a synaptic transmitter, into successive bits of specific sequence in a macromolecule.

(3) If such coding could somehow be accomplished, it is even harder to see how the linear decoding of such a taped engram could be fast enough to meet the needs of the central nervous system—in contrast to the branching spread of impulses through a network of connected cells, each containing part of the stored engram.

(4) Decoding of the known genetic tape requires a large capital investment in specialized machinery (Table I). Such an investment would not be economical unless a large amount of information had to be coded in a single cell: the conventional method of storing phenotypic information is already available for distributing small bits among many cells, and it is much less expensive.

(5) The reported association of learning with new RNA and protein synthesis has been suggested as possible evidence for a macromolecular tape; but such new syntheses could equally be required for the alteration of a cellular switch.

(6) Since the molecular-tape hypothesis has been under discussion for several years, its further heuristic value would seem to require its development into a considerably more detailed and specific model than has yet been proposed.

(7) In addition to the above arguments *against* a molecular tape, an even more impressive argument, already pointed out by Palay,[31] is the evidence from the structure of the nervous system *for* a dispersal of information among

innumerable cells. This argument is based on the presence of a fantastic number of neurons, each of which integrates many impulses received through hundreds or even thousands of dendritic synapses, but each of which can then respond to this information only by firing or not firing its single axon in a given time period. If such a cell had encoded a large amount of neural information, in a genelike manner, how could it efficiently distribute that information through monotonous spike discharges in its one axon?

The argument may become clearer if we consider a hypothetical nervous system with 1/1000 as many cells, but each with 1000 axons. In such a cell the encoding of a large amount of information might be useful, as it could govern a wide range of possible expressions of activity of a cell through the firing of its axons in different patterns. In the real world, however, the anatomy of the neuron cries out for the distribution of any block of information among many cells, with only a small bit in each; the secret evidently lies in the intercellular connections and in their thresholds of activity.

Coda

In conclusion, although the arguments brought up in the latter half of this paper are surely not novel, the issue is still apparently a live one, and it seems much too crucial to leave to informal discussions. I hope my experience as a bacterial geneticst justifies my presuming to comment on a model that clearly has been inspired by the successes of molecular genetics. Indeed, I may even feel a bit embarrassed by the thought that the dazzling light from this field may be blinding people in another.

It is surely desirable to study the nervous system at a molecular level, as well as at more conventional levels. But the nature of the experiments will depend on the models that are taken seriously; and I am convinced that the hope of a rapid shortcut, bypassing a step-by-step development to get directly at the postulated informational macromolecules, is based on taking home the wrong message from the microbial and genetic experience. My reading of the message would not be that all biological information is encoded in macromolecular sequences: rather, genotypic information is encoded in this way, while phenotypic information is encoded in selection and arrangement of gene products. It would follow that neuroscientists have yet to work out a good deal of detailed biochemistry, if I may use an old-fashioned word.

Biochemical studies of the nervous system will surely advance on many fronts. But for the problem of memory the key question would appear to be the nature of the substances, either structurally incorporated in synapses or floating around them, that modify their future behavior. To be sure, the mammalian genome must consecrate an enormous number of genes to the nervous system, and the search for the corresponding variety of specific macromolecules in different neurons also holds out promise—but for another problem: the molecular basis for directing the morphogenesis of the nervous system.

Summary

Regulation of specific enzyme synthesis, and allosteric inhibition of enzyme action, are discussed with particular reference to their physiological significance. In addition, the mechanisms that regulate the over-all rates of synthesis of protein, RNA, and DNA are described.

It is pointed out that the earlier difficulties in discriminating phenotypic and genotypic adaptations in bacteria closely parallel the current difficulty in deciding whether memory is encoded in the central nervous system in a macromolecular tape or in a network of cells with altered synapses. The history of the solution of the bacteriological problem is briefly reviewed, and reasons are presented for considering the macromolecular tape an implausible mechanism.

Conformational Change and Modulation of Enzyme Activity

DANIEL E. ATKINSON

THIS CHAPTER DEALS with some mechanisms by which metabolism appears to be regulated. Although it is clear that such mechanisms have relevance for the study of nervous system function, it is not obvious at present how direct are the analogies that can be drawn, and on how many levels they will hold. Cells of the nervous system are metabolically functioning cells, which have arisen from less specialized cell types. Clearly they share with other cells the problem of maintaining metabolic integrity and coordination, and there is no reason to doubt that the mechanisms by which regulation is obtained are similar to those found in cells generally. It seems very likely that a thorough knowledge of cellular metabolism, including metabolic regulatory mechanisms, will be a necessary prerequisite to understanding the specialized biochemistry underlying the specialized functions of cells in the nervous system. As Davis points out in his chapter (pages 113 to 122) in this book, it is unlikely that neuron function can be understood in any quick and easy way that bypasses the fundamental biochemistry. Further, the possibility should be kept in mind that the specialized control functions of neurons and their associated cell types may have evolved from molecular control mechanisms of the type to be discussed here. In any case, these mechanisms illustrate types of behavioral responses at the molecular level that the living cell has at its disposal.

Just as each type of cell must have evolved from preexisting and, in many cases, less specialized types, specialized proteins such as regulatory enzymes must have arisen from less specialized proteins:

$$\text{protein} \longrightarrow \text{catalytic protein} \longrightarrow \text{regulated catalytic protein}$$

Probably in many cases an evolving enzyme becomes increasingly effective until it reaches a state in which any available mutation leads to a decrease in catalytic activity, but this will not always be true. The immediate effects of mutation are exhibited at the molecular level, but selection obviously occurs at the level of the whole organism. In some cases the over-all needs of the organism will dictate the evolution of an enzyme that is much less than maximally effective, at least under some conditions. Regulatory enzymes illustrate such a case. If an enzyme is to be regulatory, it must function most of the time at less than maximal rate. It now appears that one major type of metabolic regulation involves modulation by low molecular weight metabolites of the activities of enzymes that compete for branch-point metabolites—that is, for compounds that have two or more metabolic fates open to them. Nearly all of the interactions that have been observed—which metabolites modulate which enzymes, and in which direction—are readily rationalized as appropriate for maintaining metabolic stability and integrity. These interactions would, however, appear completely random and arbitrary if the metabolic sequences that they regulate had not been previously explained. Similarily, one may predict that chemical and other types of observations on the specialized cells found in the nervous system will be rationalizable only in terms of extensive knowledge of the fundamental biochemical machinery of the cell.

Many years ago, Sir Frederick Gowland Hopkins expressed the belief that the extremely complex processes of metabolism, then very poorly charted, would be found to consist of simple compounds undergoing discrete, understandable chemical reactions. This statement of faith was a very important one, since only if it were true would the biochemical approach lead to meaningful information. Hopkins' faith was justified in the 1930's by the discovery of several of the sequences of discrete reactions by which carbohydrates are metabolized, and even more strikingly in the decade beginning about 1946, when the biosynthetic pathways leading to most major metabolites of low molecular weight were clarified. Although some biochemists then felt that metabolic biochemistry had become essentially a mature science with no fundamental problems outstanding, almost nothing was known ten years ago as to how these reactions might be integrated and controlled. A chemist would never attempt to synthesize a number of different products simultaneously in the same vessel, but within the limited volume of any living cell several thousand reactions must be occurring simultaneously in an ex-

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quisitely regulated manner. How this potentially chaotic chemical situation is regulated is clearly one of the central problems of life. Without such integrated metabolism there could be no genetic code, no macromolecules with their fascinating physical properties, and assuredly no metazoans to develop nervous systems. The interrelations among metabolic sequences must obviously be far more complex than the sequences themselves. Perhaps for this reason the very existence of the problem was largely ignored by authors of textbooks and reviews.

A little over ten years ago, some workers began to interpret experimental results, and to plan experiments, on the assumption that Hopkins' generalization might apply also to regulation. Among others, Gots,[1] Novick and Szilard,[2] Magasanik,[3] and the Carnegie group[4] supplied the background for the study of regulation at the molecular level. Umbarger deduced from his own[5] and other evidence that the first enzyme involved in the conversion of threonine to isoleucine should be controlled by the level of isoleucine itself. His demonstration in vitro that this relationship in fact existed[6] marked the beginning of study of metabolic regulation at the molecular level. Yates and Pardee[7] simultaneously observed a similar effect in pyrimidine biosynthesis.

Several observations made on these first two examples are usually, although not universally, characteristic of regulatory enzymes. The regulated enzyme usually catalyzes the first committed step in a sequence—that is, the first reaction leading specifically to a product (or a group of products) but having no other metabolic function. The regulated reaction is usually physiologically irreversible; its equilibrium constant is sufficiently large so that the concentration relationships necessary for reversal will never occur in the intact cell.

A very interesting property of many regulatory enzymes, observed by Umbarger in the first case to be discovered, is that the kinetic order of the reaction with regard to substrate is generally higher than one. This can be explained by a brief discussion of types of kinetic responses illustrated in Figure 1. In the simplest case (shown in Figure 1A), the rate of a chemical reaction is directly proportional to reactant concentration; this is called a first order reaction. If more than one molecule of substrate affects the rate-determining step or steps, the rate of reaction will depend on some exponential function of substrate concentration; such a reaction will be of the second order or higher. In this case (Figure 1B) the slope of the rate-vs.-substrate-concentration curve will increase with substrate concentration. When the reaction is catalyzed by an enzyme (or by other surface catalysts with a limited number of reactive sites),a curve of the type shown in Figure 1C is obtained. Such kinetics was explained by several workers

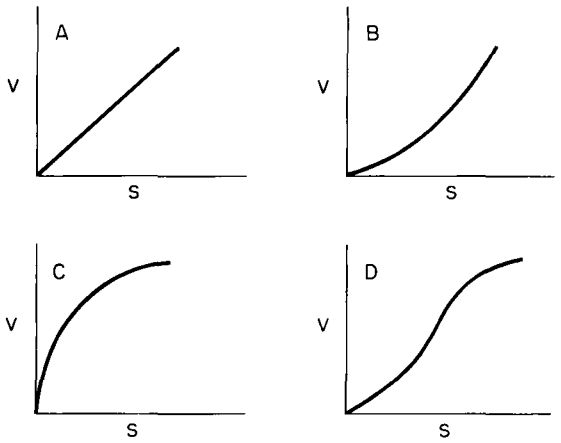

FIGURE 1 Reaction rate as a function of reactant concentration for various types of reactions. A, simple first order reaction; B, reaction of order higher than one; C, simple enzyme-catalyzed reaction; D, enzyme-catalyzed reaction of order higher than one.

as resulting from saturation of catalytic sites, and has come to be known as Michaelis kinetics. It is obvious that if two or more molecules of substrate interact in a rate-determining way with an enzyme, the resulting kinetic curve must share the features of the curves in Figures 1B and 1C. The resulting curve (Figure 1D) will be concave upward at low substrate concentrations but, as with any enzyme-catalyzed reaction, must saturate at high substrate concentrations. This so-called sigmoid curve is characteristic of most regulatory enzymes that have been studied.

Mechanisms

The actual reactions catalyzed by all regulatory enzymes yet studied involve only one molecule of each substrate. In few, if any, cases does it seem reasonable to believe that the modulating metabolite (modifier or effector) interacts directly with either the reactant or substrate or an enzyme-bound intermediate. The problem then arises, how do second molecules of substrate or molecules of modifier affect the kinetics of the reaction? It is usually assumed that these interactions are the result of conformational changes in the enzyme itself and that the relative concentrations of various conformers is a function of the concentrations of substrates and modifiers. Some models that have been proposed to explain the kinetics of regulatory enzymes are classified in Table I.

It is a fundamental assumption of the Michaelis treatment that reaction 1 in the formulation

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} EP \overset{3}{\rightleftharpoons} E + P$$

TABLE I

Some types of models that have been proposed to account for the kinetic behavior of regulatory enzymes (S, substrate; M, modifier)

FUNDAMENTALLY MICHAELIS KINETICS
(Formation of complex not rate-limiting)

1. *Conformational change induced by binding S or M* (Pardee–Koshland)
   a) Subunits explicitly involved (Koshland)
   b) Subunits not explicitly involved (Atkinson)
2. *Conformational change independent of S and M* (Monod, Wyman, Changeux)

NON-MICHAELIS KINETICS
(Formation of complex rate-limiting)

3. *Alternative pathways* (Ferdinand–Dalziel)
4. *Relaxation effects*

is very fast compared to reaction 2: thus free enzyme, substrate, and the enzyme-substrate complex will be very nearly at equilibrium during the course of the reaction. Enzymes for which this relation holds may be said to exhibit Michaelis kinetics.

Most treatments of the kinetics of regulatory enzymes stem from the suggestion by Gerhart and Pardee in 1962[8] that the modifier binds at a specific regulatory site and thereby induces a conformational alteration in the protein. This alteration is assumed to cause a change in the affinity of the enzyme at its catalytic site for the substrate. Koshland[9,10] has proposed for enzymes generally that binding of substrate at the catalytic site is accompanied by at least local conformational changes in the protein. The Pardee model suggests that the binding of some ligands (small molecules or ions that bind at specific sites on the surface of the enzyme) may in the case of regulatory enzymes be sufficiently extensive to cause marked modifications at other sites. This model has served as a basis for nearly all discussions of regulatory enzyme kinetics until very recently. Two specific examples are given only as illustrations. In a theoretical paper, Koshland, Némethy, and Filmer[11] analyzed various patterns of interaction between four subunits, when the conformation of each subunit depends upon whether ligand (substrate in the case of an enzyme; oxygen in the case of hemoglobin) is present or absent. The model deals with only one type of ligand, and thus excludes modifier effects.

An extension of this treatment to cover enzyme-substrate-modifier interactions is in preparation (personal communication). Atkinson, Hathaway, and Smith[12] rationalized experimental results obtained with the diphosphopyridine nucleotide-specific isocitrate dehydrogenase of yeast in terms of a model in which there are at least two catalytic sites, two isocitrate regulatory sites, and two sites that bind the positive modifier, 5′-adenylic acid (AMP). Binding of any component of the reaction (the substrates, isocitrate and diphosphopyridine nucleotide; the modifier, AMP; or Mg^{++} ion) was assumed to increase the affinity of all isocitrate-binding sites for this substrate. An increase in affinity for other ligands was not ruled out, but was not necessary to fit the results. While subunits were not explicitly implicated in this model, one obvious means by which a ligand at one site might affect the conformation around another site is by subunit interaction; thus this model and that proposed by Koshland are not mutually exclusive.

The model proposed in 1965 by Monod, Wyman, and Changeux[13] abandoned the concept of ligand-induced conformational change. Two (or more) forms of the enzyme, differing in affinity for substrate, are assumed to exist in equilibrium in the absence of substrate and modifier. These forms of the enzyme reflect corresponding forms of the identical subunits of which it is comprised, and it is postulated that molecular symmetry is conserved; that is, the subunits of a given molecule will always all be in the same conformational state: if one changes, all must change essentially simultaneously. It is a fundamental postulate of this model that there is no interaction between ligand-binding sites—a postulate that is a chief distinction between this formulation and the more general Pardee model.

Both models satisfactorily rationalize sigmoid reaction kinetics of the type illustrated in Figure 1D; thus simple studies of reaction kinetics in the absence of modifier cannot distinguish between them.[14] The postulation of no interaction between binding sites can, however, be tested by the elegant relaxation techniques developed by Eigen and associates. In this book, Eigen reports relaxation studies on triose phosphate dehydrogenase that appear to establish for this enzyme the situation postulated by Monod, Wyman, and Changeux—two interconverting forms of the protein, with little or no interaction between DPN^+ binding sites.

Relaxation methods yield much more detailed information on enzyme-catalyzed reactions than can be obtained from kinetic studies of the over-all reaction, and the application of such methods to the enzyme-substrate-modifier interactions of some typical regulatory enzymes should give valuable information. Such interactions cannot be studied with triose phosphate dehydrogenase, because this enzyme as yet has no known modifier (although its strategic location in carbohydrate metabolism makes it seem a likely candidate for regulation).

If the Michaelis assumption of rapid equilibration be-

tween enzyme and substrate is abandoned, sigmoid rate curves may result even if there is only one substrate-binding site per enzyme molecule. Ferdinand,[15] starting from an earlier treatment by Dalziel,[16] showed that, at certain combinations of kinetic parameters and concentrations, sigmoid kinetics might be exhibited by an enzymic reaction between two substrates if the order of their addition to the enzyme is random, so that two different reaction pathways are available. It also seems possible that an enzyme with a single site might give sigmoid kinetics if its relaxation from a Koshland type of conformational change were relatively slow in terms of the time between enzyme-substrate collisions.[14] Both of these non-Michaelis models require rather special conditions. Further, they suggest an explanation only for sigmoid kinetics with regard to substrate and do not bear on enzyme-modifier interactions; hence they are probably not relevant to the fundamental problems of regulatory enzyme kinetics.

Because there are very few enzymic reactions for which anything is known about the catalytic mechanism, no present explanation of the regulation of such reactions need be taken very seriously; nor should my classification of such explanations, which is presented merely as an indication of the kinds of concepts that have been proposed. In any case, it seems highly unlikely that a single explanation will be found that is valid for all of the very large number of regulatory enzymes. Although we know little about enzymic catalysis, we do know that different enzymes function in widely different ways. In a few cases, covalent compounds are known to be formed between the enzyme and its substrate. Any attempt to generalize this finding to all enzymic reactions would, however, lead to mainly wrong conclusions. Enzymes have evolved diverse catalytic mechanisms. Each regulatory system must have evolved independently after the catalytic properties of the enzyme had been at least partly established. It seems unlikely that a diverse group of proteins, catalyzing a wide variety of chemical reactions by very different mechanisms, would all evolve identical or closely similar modes of regulation.

Regulatory enzymes as control elements

Regulatory enzymes function in nature as individual elements in highly complex control circuits. The properties of each have evolved in the context of the total cellular economy. Fortunately, we may attempt to deduce some in vivo regulatory interactions without understanding the molecular basis for the behavior of the individual enzymes. The performance characteristics of the enzyme are what matter here, just as vacuum tubes and transistors, although working by quite different physical principles, may serve very similar functions in electronic circuits.

A typical performance curve for a regulatory enzyme is seen in Figure 2A. Beginning at point *a* on the control curve, addition of the concentration of positive effector indicated by a single + on the figure would be expected to increase the rate of the reaction to the point indicated by *b*. This would, in fact, occur under usual assay conditions in vitro. However, in the intact cell, the rate of production of substrate by preceding reactions may quite possibly be limiting, in which case substrate could, of course, be used no more rapidly than it was produced. Because the rate could not increase, the system would change to the point indicated by *c*, where the concentration of substrate is markedly less than at point *a*. Probably in most cases in vivo the end result will be intermediate between *b* and *c*; that is, an increase in the concentration of a positive modifier may be expected to lead both to an increase in the rate of the reaction and to a decrease in the concentration of its substrate. The effects on rates have been emphasized in the past, but in the intact cell many modifiers may well exert their most significant effects on the concentrations of other metabolites; these in turn may serve as modifiers for one or more other enzymes, thus participating in regulatory circuits.

Figure 2A is very similar to a graph of characteristic curves for a vacuum tube. A family of generalized triode characteristic curves is seen in Figure 2B. When the tube is tested in isolation, the plate voltage, E_p, may be held constant. In this case an increase in control grid voltage, E_c, will of course cause an increase in tube current, I_p. However, if the tube is used in a typical voltage amplifier circuit, E_p is not constant. Because of voltage drop in the plate circuit, E_p varies as a function of I_p. Indeed E_p is the

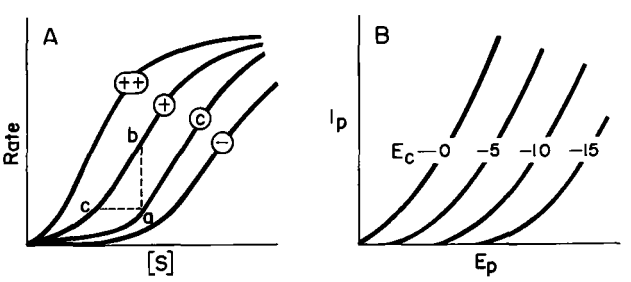

FIGURE 2 Comparison of generalized performance curves for a regulatory enzyme and for a triode. A: rate of an enzymic reaction as a function of substrate concentration. Curve identifications: −, negative modifier added; c, control; +, positive modifier added; ++, higher concentration of positive modifier. See text for significance of lettered points. B: tube current (I_p) as a function of plate voltage (E_p) at several values of control grid voltage (E_c).

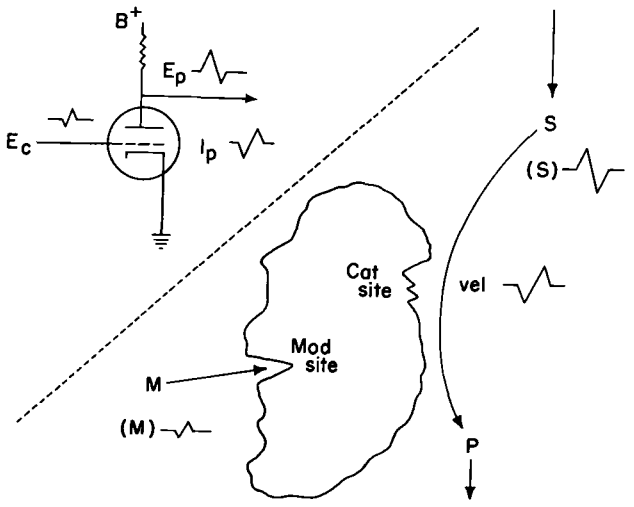

FIGURE 3 Schematic representation of the formal functional analogy between triode and regulatory enzyme. Upper left, triode voltage amplifier circuit. Waveforms indicate relative polarities of changes in: control grid voltage, E_c; tube current, I_p; plate voltage, E_p. Lower right, suggested concentration amplifier circuit utilizing a regulatory enzyme: Mod site, modifier site; cat site, catalytic site; P, product. Waveforms indicate relative polarities of changes in: concentration of positive modifier M; concentration of substrate, S; and reaction velocity, vel.

output of such an amplifier stage. I propose that a similar situation may obtain in the case of many regulatory enzymes in vivo. This formal analogy between the vacuum tube and a regulatory enzyme is illustrated in Figure 3. The squiggles in this figure illustrate the direction of change in voltage, current, concentration, or reaction flux. In the case of the voltage amplifier, a change in control grid voltage causes tube current to change in the same direction. The current, flowing through the plate resistor, leads to oppositely directed changes in plate voltage. For a regulatory enzyme with a positive modifier, a change in modifier concentrations, by causing a similarly directed change in reaction velocity, may be expected to lead to changes of opposite polarity in the concentration of substrate. Some electronic circuits exploit the possibility of voltage amplification, while in others the flow of current through the tube is the regulated variable. Figure 4 illustrates in schematic form a simple voltage amplifier whose output, E_{p_1}, is applied to the control grid of a current amplifier or output stage. This tube is connected in series with a transformer, whose output may drive, for example, a speaker. In the tube–enzyme analogy, concentrations correspond to voltages and reaction rates or fluxes to currents. It is probable that both concentration amplifiers and flux amplifiers, or output stages, exist in the functioning

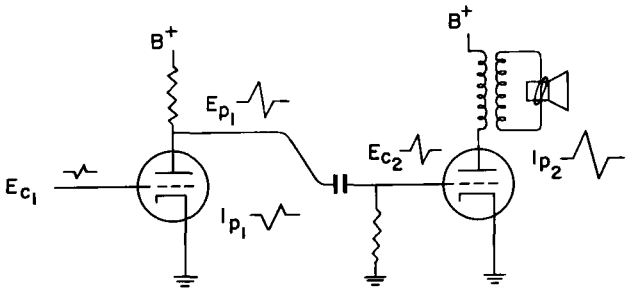

FIGURE 4 Schematic representation of a generalized voltage amplifier with its output (E_{p_1}) coupled to the input (E_{c_2}) of a current-regulating amplifier stage.

cell. This possibility is illustrated in Figure 5. The reactions from A to G represent a main metabolic sequence; conversion of B to C is the first step in an alternative pathway. Variations in concentration of M, the positive modifier for the F ⟶ G conversion, lead to oppositely directed variations in the concentrations of the substrate, F, and its precursor, D. D, in turn, serves as positive modifier for the enzyme catalyzing the conversion of B to C. The analogy with a voltage amplifier driving a power output stage seems obvious: the concentration of M, acting through an intermediate stage, controls the portion of B diverted into an alternate pathway. There would be no reason in general to show this particular relationship between the two stages; the output, or flux-regulated, step need not involve a metabolite in the sequence leading to the other stage. This arrangement was chosen for illustra-

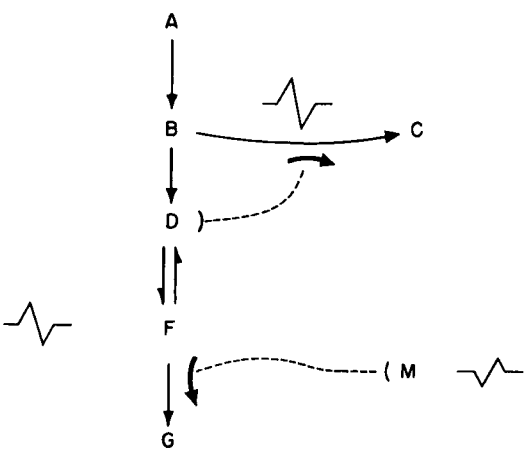

FIGURE 5 Schematic representation of proposed concentration amplifier with its output (concentration of D) coupled to the input (modifier site) of a reaction velocity-regulating amplifier stage.

tion because it appears that just this relationship is found at two important points in the central energy metabolism of typical aerobic cells.

Figure 6 shows a number of regulatory interactions that have been found to affect enzymes of carbohydrate and fat metabolism, and it illustrates a suggestion as to how these effects may interact in partitioning intermediates between oxidation and storage as fat or polysaccharides.[17] The scheme represents the glycolytic and citric acid sequences, with many intermediates omitted for the sake of clarity. The oval near the lower left corner represents the citric acid cycle, a primary supplier of electrons for oxidative phosphorylation, which in typical aerobic cells is responsible for most of the regeneration of adenosine triphosphate (ATP) from the mono- and diphosphates. Since ATP, as the primary energy fuel of the cell, is converted to ADP or AMP in nearly every energy-requiring metabolic reaction, the ATP/ADP/AMP balance is appropriate as a primary control parameter in the cell's energy metabolism.

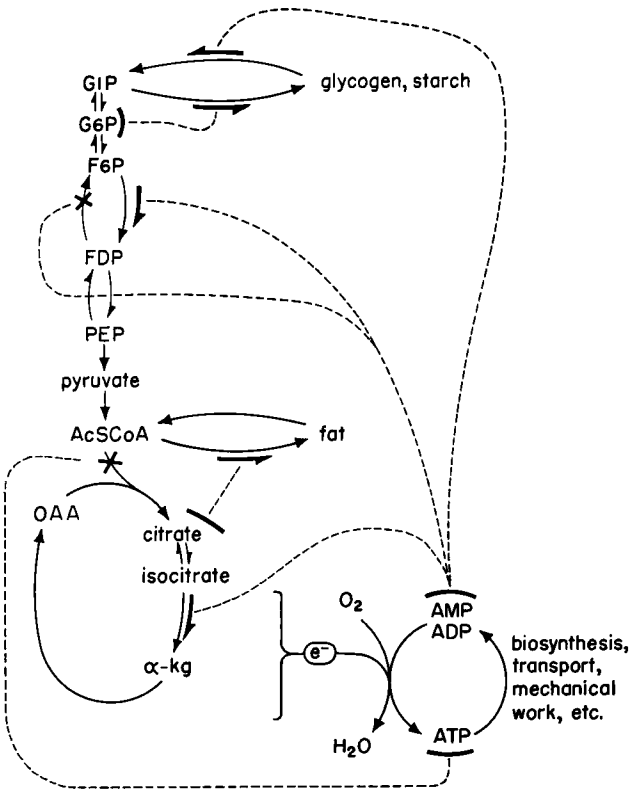

FIGURE 6 Schematic illustration of the role proposed for AMP, ADP, and ATP in regulation of energy metabolism. Broken lines connect effector compounds (indicated by heavy arcs) to the enzymes they modulate. Positive effector action is denoted by a heavy arrow, and negative effector action by a heavy cross. Abbreviations: G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; PEP, phosphoenolpyruvate; AcSCoA, acetyl coenzyme A; α-kg, α-ketoglutarate; OAA, oxaloacetate. Supply of electrons (from oxidative reactions in the glycolytic and Krebs cycle pathways) to the electron transport phosphorylation system is indicated by the symbol e−.

Our interest in this chapter is not in metabolic regulation as such, but rather in the types of regulatory interactions that have evolved. These might be useful in consideration of other types of biological control functions. We will assume without further metabolic discussion that when the concentration of ATP is low the appropriate response is for acetyl coenzyme A (AcSCoA) to enter the citric acid cycle, thus leading to an increased rate of regeneration of ATP. Conversely, when the level of ATP is high, it may be advantageous for the cell to convert much of its AcSCoA into storage fat, which may be mobilized at a later time of need. It appears that a combination of concentration amplifier and output stage has evolved to insure these desirable consequences. When the concentration of ATP is high, that of AMP must necessarily be low. Since AMP is a positive effector for the conversion of isocitrate to α-ketoglutarate, the rate of this reaction will be low and, perhaps more important, the concentrations of citrate and isocitrate should tend to rise. Citrate, in turn, is a positive modifier for the first reaction in the conversion of AcSCoA to fat,[18–20] which will therefore be facilitated under these circumstances. The resulting tendency for AcSCoA to be converted to fat rather than to enter the citric acid cycle when ATP is high is strongly enhanced by the powerful negative effector action of ATP on the enzyme catalyzing the entry of AcSCoA into the cycle.[21] If these deductions are correct, the changing ratio of ATP to AMP concentration continuously modulates the relative percentages of AcSCoA entering the citric acid cycle for immediate utilization in the regeneration of ATP and the portion stored for deferred use. It has been suggested[17] that a similar regulatory relationship plays a role in the control of polysaccharide synthesis. AMP is a positive modifier for the conversion of fructose-6-phosphate to fructose diphosphate. The precursor of the substrate of this reaction, glucose-6-phosphate, serves as positive modifier for the production of polysaccharide from glucose-1-phosphate.

Other regulatory interactions indicated in the figure need not concern us here, except that they suggest the degree of complexity that is coming to be recognized in metabolic control relationships. Work in this field has only begun; it is very likely that we have only scratched the surface and that far more complex interrelationships remain to be discovered. Compounds in this sequence serve as starting materials for the biosynthesis of amino

acids, purines, pyrimidines, and other necessary building blocks of the cell. Each of these biosynthetic sidepaths has its own controls; often several are necessary because a single branch may lead to two or more essential metabolites.

Phosphofructokinase, the enzyme catalyzing the conversion of fructose-6-phosphate to fructose diphosphate, illustrates the degree of complexity that may be found in the regulation of a single enzyme. This enzyme is a simple kinase, catalyzing merely the transfer of a phosphate group from ATP to the oxygen attached to carbon 1 of fructose-6-phosphate. This is a simple displacement reaction, and there would be no reason to expect complexities. However, this enzyme is known to catalyze a reaction that is second order or higher with regard to fructose-6-phosphate, rather than first order, as would be expected; Mg^{++} ion, in addition to participating in the reaction as it does for all kinases, affects the affinity of the enzyme for its substrates; ATP, although a substrate, at higher concentrations is an inhibitor, or negative modifier; AMP, as noted previously, is a positive modifier; citrate is a negative modifier; and NH_4^+ has profound effects on the reaction. There is no obvious metabolic explanation for the effect of NH_4^+, but all of the others are readily rationalized as metabolic controls. The appropriateness of the AMP and ATP effects follows from our previous discussion. The effect of citrate probably represents feedback from the citric acid cycle to the primary control point in glycolosis, which is a major supplier of carbon for the cycle. In addition, fructose diphosphate, although the product of the reaction, is also a positive modifier. This effect may help to insure that the phosphofructokinase kinase reaction and the hydrolysis of fructose diphosphate to fructose-6-phosphate will not occur simultaneously.[14,22]

These effects all seem appropriate for regulation of phosphofructokinase in response to the local conditions and immediate needs of the individual cell, and they are characteristic of phosphofructokinases from most or all types of cells that have been investigated. In addition, the phosphofructokinases of mammalian cells respond to cyclic 3′, 5′-AMP, which is without effect on the enzymes from single-celled organisms. Because the production of cyclic AMP in muscle and heart cells is known to be modulated by epinephrine, this control presumably allows the central nervous system, as one consequence of alarm, fright, or anger, to override local controls and accelerate glycolysis in advance of energy need.

These complex properties of a fundamentally simple reaction illustrate two important generalizations. First, life is highly complex. This is hardly a new or startling assertion, but we are beginning to see levels of complexity previously unsuspected. Second, the controls, although exerted by chemical compounds, are completely unpredictable from any knowledge, no matter how complete, of the chemistry of reactants and products. We deal here with a form of coding. AMP modulates the phosphorylation of fructose-6-phosphate not because of any chemical relationships, but because such a modulation, being functionally advantageous, has been perfected through the evolutionary processes of mutation and selection. Nonbiological reactions are promoted or retarded on the basis of chemical properties of the reactants, catalysts, or poisons and are in principle predictable; the interactions we deal with in the study of regulatory enzymes are purposeful and directive, and in principle are not predictable from the properties of reactants and modifiers. They depend on properties of the enzyme, and these, like any other constituent or property of a living organism, depend on selection. It was Monod, I believe, who first emphasized the significance of this distinction, and pointed out that such coding probably underlies biological phenomena of all types, with notable examples being the expression of genetic information, intracellular metabolic regulation, and hormone action.[23,24]

It is difficult to believe that the complex properties of a molecule like phosphofructokinase (as well as any additional properties yet to be discovered) are all in fact attributable to mutation and selection. The difficulty increases when we consider that thousands of other enzymes and intracellular structural features and processes were evolving simultaneously. As Reed has pointed out in this book, enzymes have evolved not only their catalytic abilities, and not only regulation of these catalytic abilities, but also in at least some cases specific sites for interaction with other functionally related enzymes. Nevertheless, I think that we must believe that these and other intracellular complexities, as well as the superimposed and greater complexities of higher organisms, have indeed been designed by the slow and undramatic processes of mutation and selection. It is hardly surprising that few of those who predict the laboratory synthesis of life during the next decade are either biologists or biochemists.

Phosphofructokinase, one enzyme among many involved in the central energy metabolism of the cell, responds in a functionally appropriate way to variations in the concentrations of at least half a dozen small molecules. It has, of course, a single output: catalysis of phosphorylation of fructose-6-phosphate. We could call this "information processing," if we wished, and facile formal analogies with neuron function might even be suggested. I doubt that either of these courses would be productive. But, in any case, phosphofructokinase, like other regulatory enzymes, is a sensing, responding, and controling system. Cells of the nervous system must have evolved

from cells whose ancestors had long contained elements of this type. It would not be surprising if similar mechanisms underlie some of the specialized activities of neurons and their associated cell types.

Summary

A number of enzymes interact specifically with small molecules (effectors or modifiers) with consequent changes in the kinetic (catalytic) properties of the enzymes. These enzyme-substrate-modifier interactions are not yet understood at the molecular level, but conformational changes are almost certainly involved. The resulting modulations of the reactions catalyzed by such enzymes seem to be major factors in metabolic control. Some of the dynamic characteristics of regulatory enzymes resemble those of vacuum tubes, and this electronic analogy may aid in the analysis of the metabolic control circuits in which regulatory enzymes function.

Dynamic Aspects of Information Transfer and Reaction Control in Biomolecular Systems

M. EIGEN

IN A PREVIOUS paper,[1] presented at one of the NRP work sessions, we defined a scheme of successive terms ranging from simple molecular interaction to the more complex forms of responses found in psychic behavior (Figure 1). At present, any attempt to analyze psychic processes in terms of molecular mechanisms may be premature. However, we can start from some biological processes of which we know the molecular programs and analyze the level at which we must introduce new concepts in order to present a certain degree of "intelligent" behavior. The molecular mechanisms of programming, code reading, and reaction control of cellular biosynthesis offer a promising start.

According to the definitions in the above scheme, we know quite a bit about biomolecular processes up to the level of "identification" and "adaptation." They involve sophisticated molecular mechanisms for code reading and checking, as well as for program transfer and control. We usually do not know how the programs developed during evolution—in other words, we know little about molecular "learning" and "retrieval." But if we understand in detail how the present mechanisms *work,* we might also find out how they *came about.*

Let us start with a molecular analysis of the code-reading and transfer mechanisms at the gene level. Watson, Crick, Nirenberg, Khorana, and many others have established that the program for protein synthesis is laid down in the sequence of the purine and pyrimidine bases of the nucleic acids—adenine (A), guanine (G), thymine (T) or uracil (U), and cytosine (C). The basis of code reading is the complementarity between the nucleobases A and T(U), or G and C, respectively. This complementarity rests on a specific interaction, a chelate-like H-bonding between the bases A and T or G and C, as shown in Figure 2. Why has only this type of interaction been used, although we know that many other combinations are possible (e.g., as depicted in Figure 3)? Apparently in an early stage, when "chemical evolution" had led to the synthesis of corresponding nucleotides, selection for this type of interaction allowed the adaptation of an enzymic recognition site, which had the geometry (and complementarily interacting side-chain arrangement) of a purine–pyrimidine pair. At this level the "reading" is a simple molecular interaction of the purine with the pyrimidine, so there must be some selective interaction that long ago established this type of "complementarity."

Indeed, if one measures the free energies of pair interaction (or the thermodynamic stability constants for the pair formation), one finds the mentioned preferences. Such measurements have been carried out independently by several groups: Rich and coworkers[2] and Küchler and Derkosch[3] measured the infrared absorption of different

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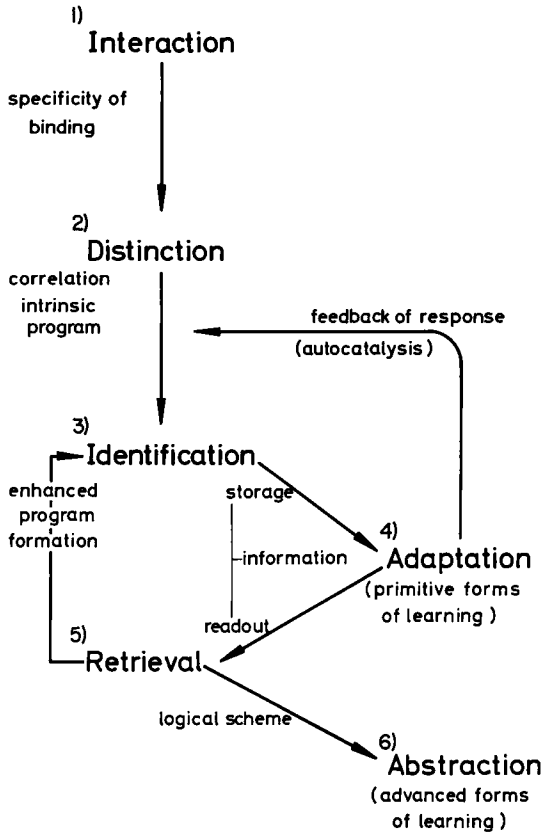

FIGURE 1 Scheme of molecular information storage.

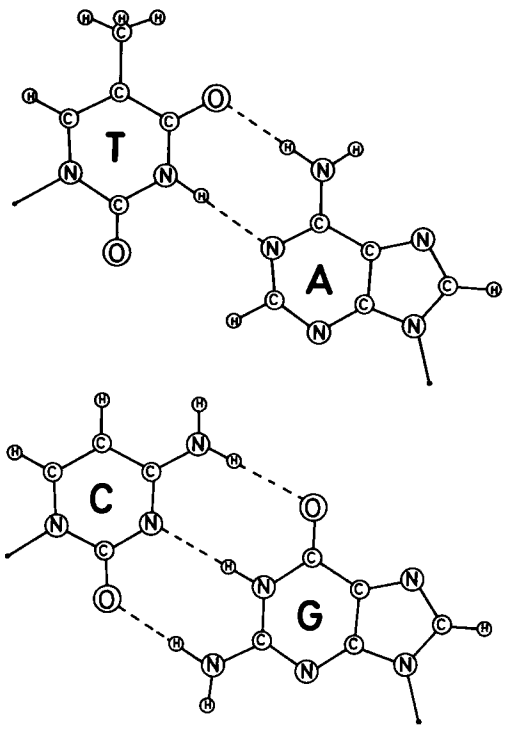

FIGURE 2 The complementary base pairs: thymine-adenine and cytosine-guanine. These two pairs are distinguished by an equivalent geometry of sites, which can be checked by the polymerizing enzyme. This checking process represents the major principle of correct readout in the polymers.

combinations of nucleobases and nucleosides in nonpolar solvents.

In our laboratory, we are using a "dynamic" method,[4,5] and in the presence and absence of a strong electric field we study the dielectric loss in a variety of nonpolar solutions of model compounds, as well as of the nucleosides of A, U, G, and C. Beside the stability constants, these studies yield the rates of pair formation, a quantity we shall need in our further considerations. In all this work,[2,3,5] we had to synthesize derivatives of the nucleobases or nucleosides that are sufficiently soluble in nonpolar solvents.

Let me say a few words about the method.

We know that hydrogen bonds are formed between polar groups and that therefore any H-bond formation will influence the effective electric moment of the system. The process of base pairing, as depicted in Figure 2, actually will lead to a compensation of certain moments, i.e., to a change of the total electrical moment. The process, therefore, can be studied by measurement of the dielectric constant as a function of concentration.

Furthermore, it should also be possible to learn about the rates by studying the dynamic dielectric behavior—the frequency dependence of the dielectric constant or loss factor. But there is a difficulty. Such studies usually must be made with small amplitudes of the alternating electric field in order to allow a linearization of the corresponding relations defining the dielectric constant or loss factor. Those linear relations may reflect the dynamic behavior resulting from orientation of the dipoles; they will not, however, give information about the rate of chemical equilibration. The chemical equilibrium involves a quadratic dependence on electric field strength. If K is the constant of the law of mass action (e.g., for the base pairing), E the electric field strength, R the gas constant, and T the absolute temperature, one obtains:

$$\partial \log K/\partial E = \Delta M/RT$$

where ΔM is the difference of the partial molar moments of reactants and reaction products, i.e., for a reaction $A + B \rightleftharpoons AB$

$$\Delta M = M_{AB} - M_A - M_B$$

At low concentrations and fields not exceeding some 10^5 volts per centimeter, M_i is proportional to the square of

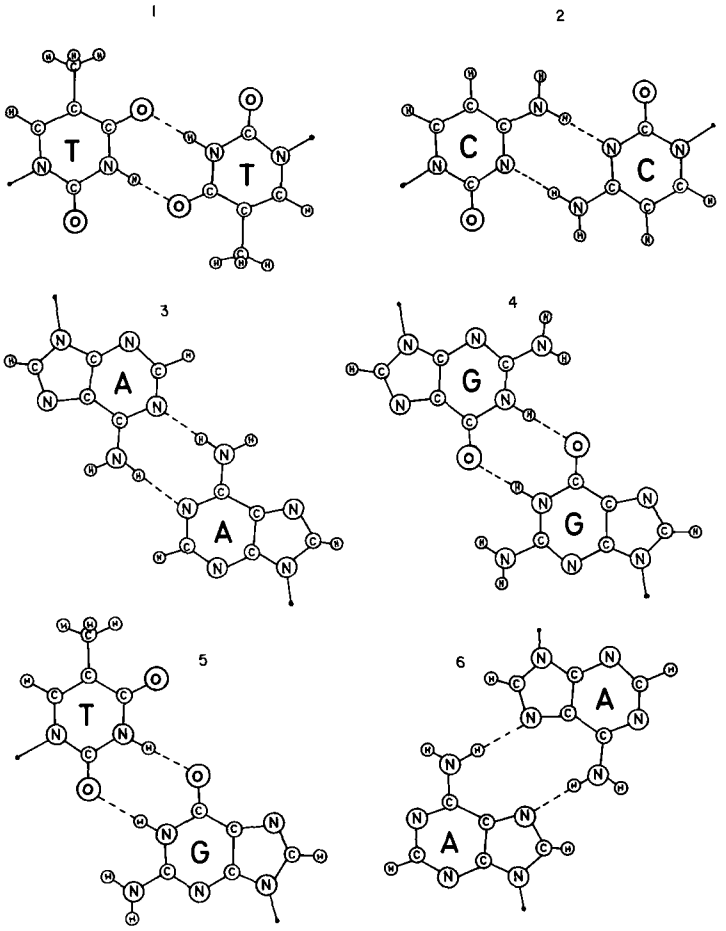

FIGURE 3 Other pair combinations of nucleobases. The binding constants of these pairs are lower than for the "complementary" pair AT; the geometry of sites is different for all of them. The combination AA, as shown in example 6, is used in the double-stranded form of poly-A (which is stable around *p*H 4).

the dipole moment of the component i times the electric field strength; in other words ΔM is a function of E. Upon integration, one obtains a quadratic dependence of log K on E, as shown in Figure 4.

At low fields a variation of E will not lead to a finite change of log K (i.e., the extent of base pairing). In order to fulfill the requirements of small amplitudes of the alternating field, one therefore must superimpose a strong static field, E_0. In our experiments, this field has a strength of about 200,000 volts per centimeter, whereas the alternating field has the usual small amplitudes. If now the period of the alternating field approaches the time constant of chemical equilibration, a phase shift between the alternating electric field and the alternating chemical composition will occur, resulting in a loss of energy. This relaxation process occurs in the frequency range of 1 to 100 megacycles per second (producing a maximum of the chemical increment of the loss factor in this range). Any increment due to relaxation of orientation processes would occur in the microwave region of the spectrum. The chemical increment is small (depending on the dipole moments of the reactants) but can just be observed by very sensitive line-width measurements.[4,6]

We obtained the following results.

1) Among the combinations presented in Figures 2 and 3, the complementary bases A and U, as well as G and C, show the most stable pairs. In accordance with the infrared results of Rich, et al. (who also studied a large variety of substituted bases) the most stable pair found was GC, followed by AU, which is substantially more stable than AA, UU, or CC. Only GG showed a slightly higher stability than the three other iso-pairs.

2) The formation rate of any model pair is diffusion controlled (with some possible steric hindrance in the larger substituted nucleosides). This is equivalent to a reaction time of $< 10^{-9}$ seconds for pair formation in an encounter complex.

3) The lifetime of the pair depends on the stability constants, and varies from about 10^{-6} seconds (for the more stable complexes) to about 10^{-8} seconds (for the less stable complexes).

We may therefore conclude that there is a definite selec-

tivity for the two isometric complementary base pairs, distinguishing them clearly from any other combination. This difference in stability, however, does not amount to much more than one order of magnitude. If this were the only selective principle of code reading in replication, the error rate would be quite high. The formation rate of all pairs is maximal (diffusion controlled) and the lifetime is short (usually below a microsecond). This is important, as wrong pairs must dissociate quickly in order to allow the rapid "trial and error" checking process necessary for a rapid "recognition" in reading.

The question may be raised: Why is complementarity established in just this form (apart from GC having three instead of two H-bonds)? Two factors may account for the selectivity. (1) The H-bonds in the stable pairs involve the least strain; the lone pair of proton-acceptor groups lies in the direction of the bridge proton groups for both H-bonds. (2) The stable pairs involve one H-bond, which is particularly polarized, expressed by a relatively small pK difference of that particular proton donor acceptance pair. (This difference might even be smaller than the pK difference in the isolated compounds due to changes in resonance structure.[7]) We have found pronounced stability maxima in internal H-bonds (e.g., o-hydroxy-azo-compounds, acetylacetone, etc.) when this condition is fulfilled.

In a very early stage of chemical evolution, when polymerizing enzymes were not yet adapted to this specific purine–pyrimidine complementarity, there was already some "force" directing the way in which code reading finally became established with the adaptation of enzymes. To reduce transcription errors to a tolerable limit, the enzymes must match the right fit of the complementary pair. Errors could also be reduced by strengthening the interaction between the complementary units, but this would always slow down the rate of response and, thus, the dynamic performance. Instead, new levels are introduced; these increase the specificity without losing dynamic flexibility. In a similar way, further code-checking devices—repair assemblies, for example—may be introduced at higher stages of evolution to reduce further errors where necessary.

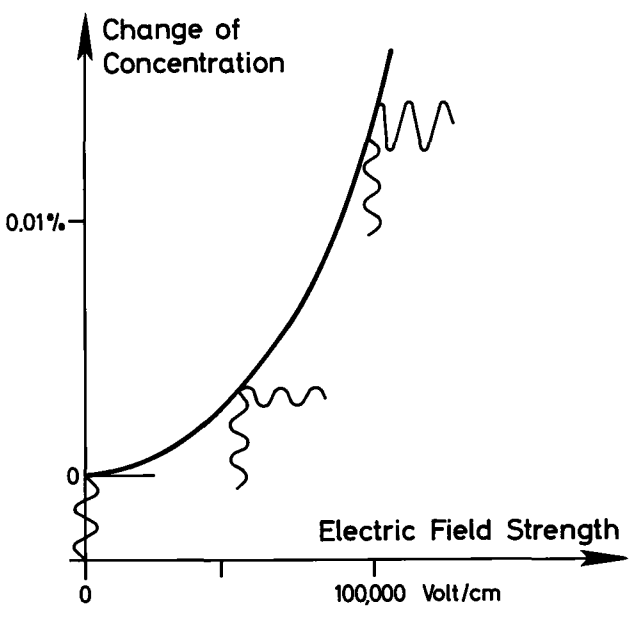

FIGURE 4 The dependence of the mass action constant (log K) for base pairing on electric field strength. A periodical perturbation of the equilibrium can only be enforced at high density of the superimposed D.C. field E_0.

Readout in polymeric systems

We know that the interaction of two polymeric strands of nucleotides cannot be described solely by the H-bonds between the complementary bases. Actually, in aqueous media—as a result of competition with solvent H-bonds—the H-bond interaction is so weak that even at the highest concentrations of monomeric nucleosides no pairing can be observed. In his chapter in this book, Dr. Crothers[8] discusses the additional forces that occur between the planar base pairs in the direction of the long axis of the polymeric double strand. These so-called "stacking" interactions are partly hydrophobic in nature, apart from their entropic stabilization, which is caused by a reduction of degrees of motion. These interactions are even stronger than the H-bonds, and thus are mainly responsible for the stability of the double-stranded oligomeric or polymeric pair. Interactions of polymeric strands can no longer be described by one single mass action constant. The cooperation requires more than one interaction parameter, as is shown by Crothers.[8]

To understand the elementary process of readout in polymeric strands, as well as the nature of complementary interaction in smaller code units, we studied the dynamic behavior of oligonucleotides of different chain length in a series ranging from the tri- to the nonameric nucleotide. As a model system we chose oligo-A, which is known to form a double-stranded helix at *p*H values below 4.5.[9] These studies are described in more detail elsewhere,[10] so here we shall deal only with the results.

If we measure the degree of base pairing by means of the light absorption at 260 millimicrons (hypochromicity), we encounter "melting" curves that become steeper as the degree of polymerization becomes higher. This behavior is seen in Figure 5, in which the absorption change in the helix → random coil transition range is plotted against

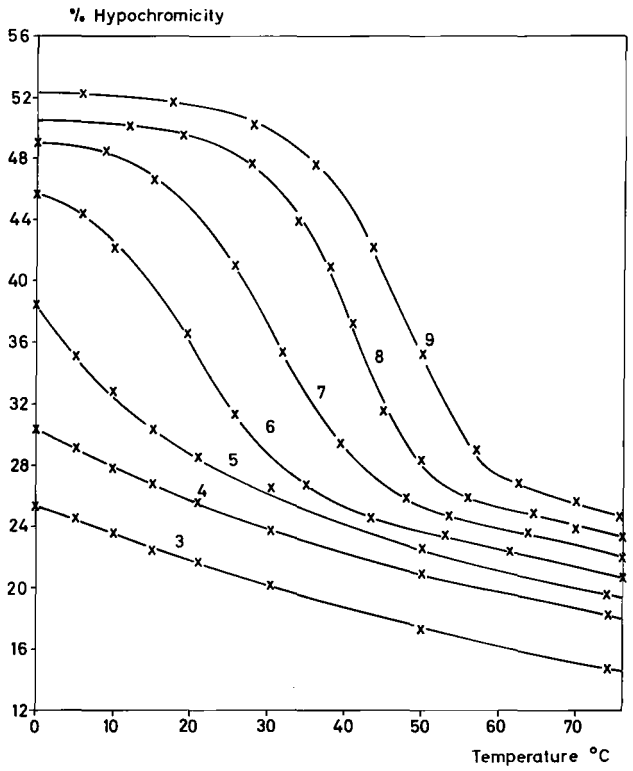

FIGURE 5 Helix-coil transformation as a function of temperature for oligonucleotides (oligo-A, n = degree of polymerisation). The optical density at 265 mμ is characteristic of the helicity. These "melting curves" describe the transition from a double-stranded helix (low temperature) to randomly coiled single strands (high temperature).

temperature for different oligomeric chains. We know from these melting curves that a strong cooperation builds up with the increasing degree of polymerization. If the binding strength per base pair were independent of n (the number of bases per oligomer), we should expect that all oligomers would have one general curve, which could be described by the temperature dependence of the "intrinsic" binding constant (related to one base pair). Apparently this is not so; the first base pair is appreciably less stable than any of the subsequent ones, leading to a difference in free energy for nucleation and propagation of base pairing.

The question is: How many constants are needed for a realistic description of the binding? We expect at least two, one for the formation of the first base pair and one for any subsequent pair, if they are identical. It might well be that the nucleation requires more than two base pairs (before additional binding becomes independent of the number of bonded pairs); in that case we would need correspondingly more parameters. We know, for instance, that the formation of droplets or crystals requires nuclei consisting of quite a large number of molecules. The above question can be answered by rate studies.

In these studies (performed by D. Pörschke[11]), we used the temperature-jump (T-jump) relaxation method.[12] The principle is simple. We start from a point in the melting region and raise the temperature several degrees centigrade (as required) within one microsecond. The subsequent shift in equilibrium is followed by observing the time dependence of the absorption at 260 mμ. A picture of the T-jump apparatus is shown in Figure 6 (heating by high-voltage pulses, observations made spectrophotometrically, registration at Tektronix oscilloscope type 545 with differential input). If only one rate process is present, one observes a simple exponential time dependence, yielding the relaxation time (which is a function of the rate constants and—for reaction orders higher than one—of equilibrial concentrations). If several coupled rate processes are present, one may observe a whole spectrum of relaxation times that are related to the normal modes of reaction.[13] An example of such a relaxation spectrum is presented below with the discussion of allosteric enzymes.

With the oligonucleotides the following results were obtained:

1) Each oligomeric compound in aqueous solution showed only one relaxation time. This means that only one reaction is observable, and it must be an "all-or-none" process. That is, either the two strands are separated completely or are combined to a helix with the maximum number of base pairs. This all-or-none behavior is expected from statistical mechanical considerations[14] to a high degree of approximation for oligomeric compounds with $n < 10$ at the relatively low temperatures of the melting region. It does not hold for higher degrees of polymerization.

2) Accordingly, the inverse of the relaxation time shows a linear dependence on concentration of oligomers (Figure 7). This is a clear indication for a second-order rate process.[13] The two single strands combine to a double strand in a quasi one-step reaction (all-or-none). This reaction really consists of many steps of subsequent base pair formation, and can be treated as one step by steady-state assumptions, because the intermediate concentrations of partly paired double strands are negligibly small. On the other hand, poly-A (with $n > 100$) shows a distribution of concentration-independent relaxation times. This is because of the first-order rearrangement of base pairing among the many intermediates in partly paired double strands, which (in poly-A) are present at detectable concentrations.

3) A linear dependence of the reciprocal relaxation time on concentration of the reaction partners yields the rate

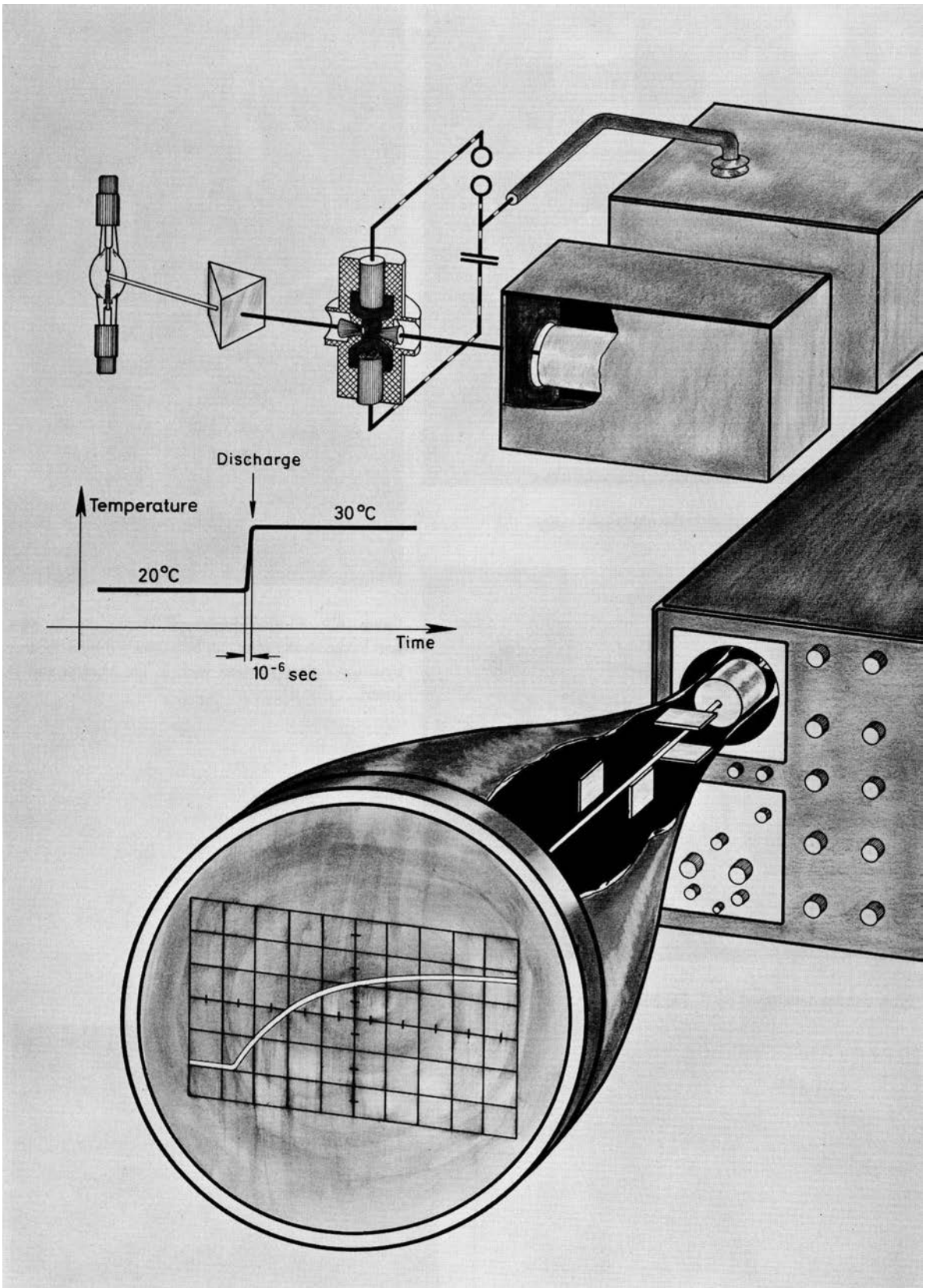

FIGURE 6A Scheme of temperature-jump equipment.

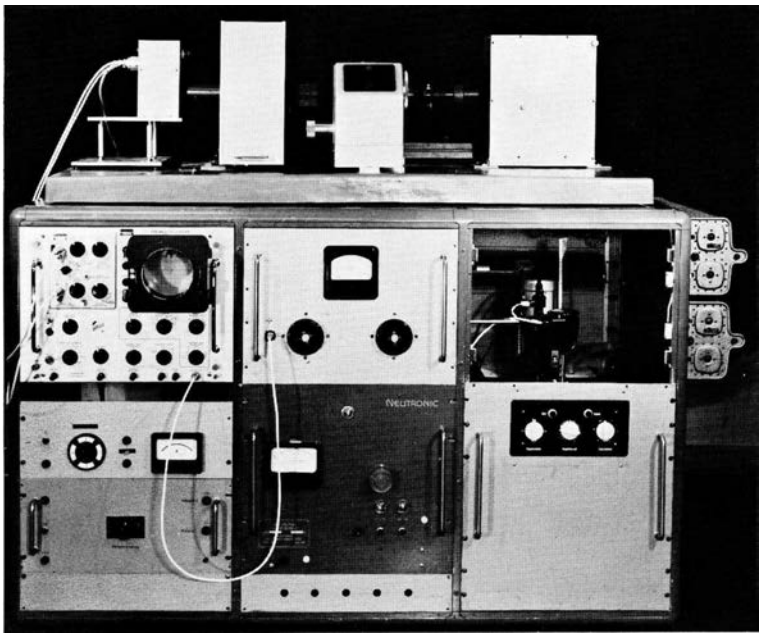

FIGURE 6B Older model for observation in the ultraviolet range.

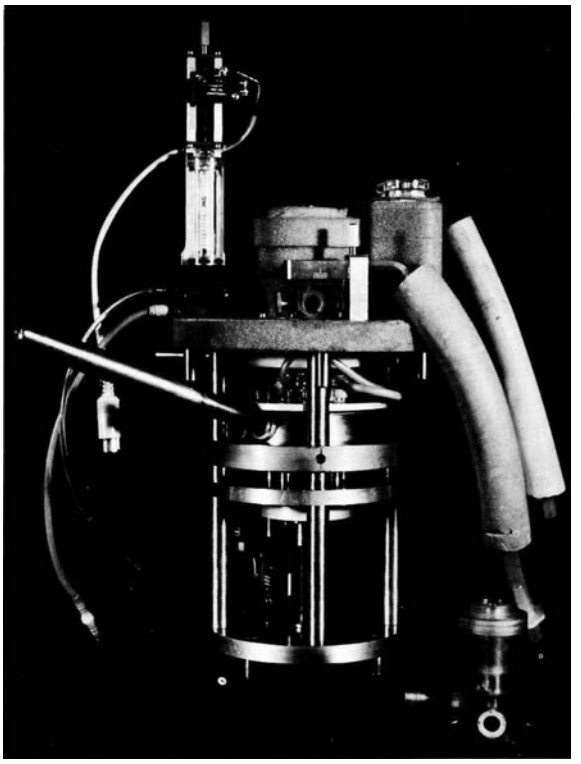

FIGURE 6D Flow-T-jump cell (Here a rapidly mixed system in the steady state can be perturbed by a T-jump; construction in cooperation with L. De Maeyer and B. Havsteen).

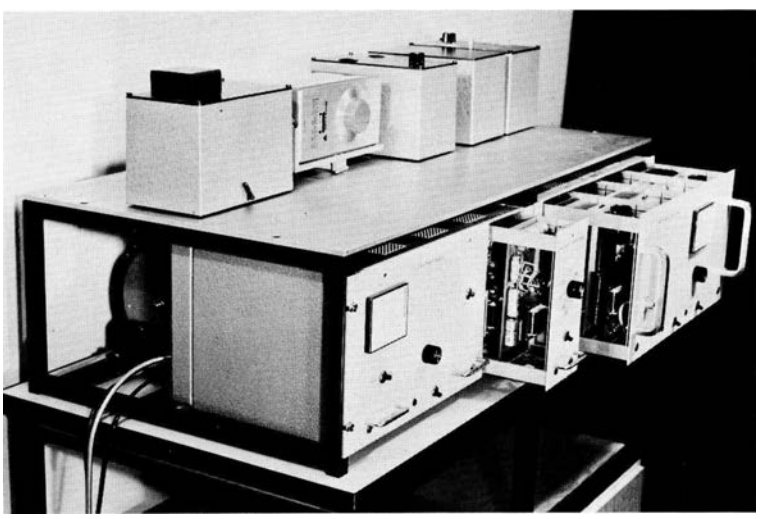

FIGURE 6C New model developed by L. De Maeyer.

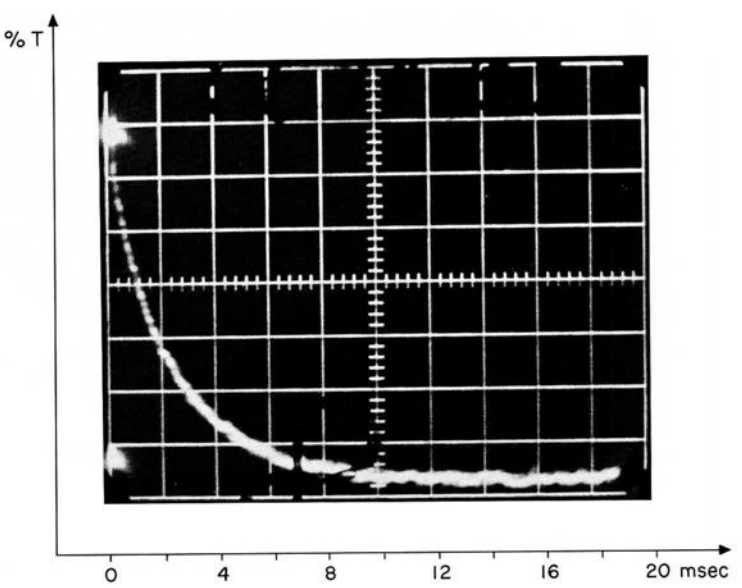

FIGURE 6E Oscillogram of relaxation of helix-coil transformation of oligo-A as obtained by a T-jump experiment.[10,11]

constant of the recombination process k_R (for the double-stranded helix formed from separated single strands), whereas the intercept is a measure of the dissociation rate constant k_D. (k_R has the dimension M^{-1} sec^{-1}. Therefore it occurs in the term that depends linearly on concentration, so that the product yields a reciprocal time. k_D refers to a quasi first-order dissociation process and has the dimension $second^{-1}$). The rate constant k_R has the order of magnitude of $10^6 M^{-1} sec^{-1}$, only very slightly dependent on n (the degree of polymerization) for $n \geqslant 4$. Apparently the formation of a nucleus of only four base pairs is sufficient and is rate limiting for the subsequent "zippering through" to the completely paired helix. The rate constant k_D (between 1 and 10 sec^{-1}) also showed little variation when measured at the same degree of helicity (which, however, occurs at different temperatures for the different oligomers, as shown in Figure 5). This result is trivial. Because k_R does not vary too much and k_R/k_D is the stability constant for the (all-or-none) complex formation, k_D must vary as little as k_R when measured under equivalent equilibrium conditions.

4) However, both rate constants show quite different temperature dependencies. k_R *decreases* with increasing temperature, yielding an apparent activation energy of about 6 kilocalories per mole (independent of n). On the other hand, k_D *increases* with temperature (as rate constants normally do), but shows very large activation energies (about 30 kilocalories per mole for n = 6) increasing with n (by about 6 to 7 kilocalories per mole of base pair). If all measurements were made at the same temperature, k_R would have shown no dependence on n, but k_D would have shown a strong dependence. The conclusions from these results are as follows:

The recombination reaction—although it shows similar rate constants for all oligomers with $n \geqslant 4$ — is not diffusion controlled, as was the single base-pair formation. The relatively low value of $10^6 M^{-1} sec^{-1}$ as well as the negative (apparent) heat of activation suggest that a pre-equilibrium is involved. The rate-limiting step should be the same for all $n \geqslant 4$, which means that the "nucleation length" does not involve more than four base pairs. At first, it would seem reasonable to assume only two base pairs as the nucleus for the propagation of helix formation. Actually, all statistical theories were worked out for two parameters:[14,15] that is, s and σ, where s represents an equilibrium stability constant for base pair formation next to a nucleated pair region, and $\sigma \cdot s$ is the corresponding equilibrium constant for the formation of the *first* base pair. The equilibrium constant for the whole all-or-none pairing reaction would then be $\sigma \cdot s^n$. If we denote the rate constant for the rate-limiting propagation step by $k_{prop.}$, the rate constant of recombination would be $\sigma \cdot s \cdot k_{prop.}$, and the rate constant of dissociation would be $k_{prop.}/s^{n-1}$. If, as was assumed in the statistical treatment, σ is temperature independent and s decreases with temperature (as stability constants usually do), an assumed heat of reaction of about –6 kilocalories per mole would lead to formal agreement with the experimental data. This interpretation, however, has some weak points:

1) $k_{prop.}$ must involve some positive activation energy, even if it amounts to only a few kilocalories per mole.

2) The stability constant for the first base pair ($\sigma \cdot s$) can not involve a negative heat of reaction, which would correspond to the observed negative activation energy of 6 kilocalories per mole in k_R. The only interaction involved in the first pair is H-bonding. Because of interference with solvent H-bonding, the enthalpies of reaction in aqueous media are known to be positive, i.e., disfavoring association. Even if the enthalpies of reaction were negative in the present case, one would have to assume quite unrealistic entropy terms to compensate for the enthalpy terms, as no association of the nucleotides in aqueous media can be observed.

3) The melting temperatures, as taken from Figure 5, can easily be related to s and σ, assuming an all-or-none equilibrium. The values determined from the experimental equilibrium data suggests that not σ alone but the product σs^k (where k is between 1 and 2) is temperature independent. We conclude, therefore, that σ is associated with a positive ΔH, which overcompensates the negative ΔH value associated with s.

Thus, the experiments suggest that the nucleation length does not involve two, but rather three (to four) base

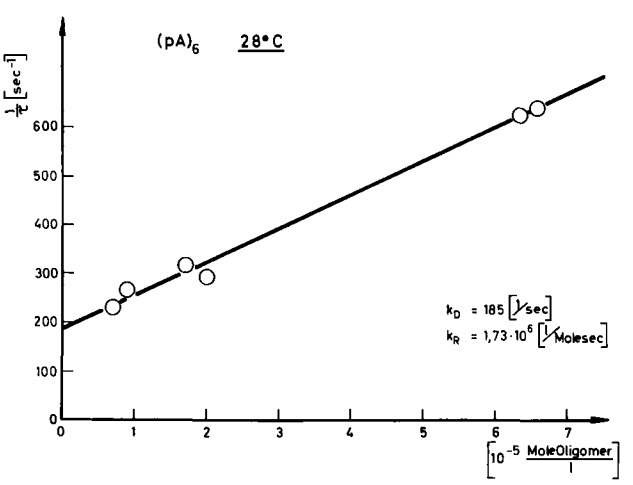

FIGURE 7 Inverse relaxation time ($1/\tau$) for helix-coil transformation of oligo-A as a function of the concentration of single-stranded oligo-A. The linear dependence suggests a second-order recombination process for two single strands to form a double-stranded helix (cf. Notes 10 and 13).

pairs; in other words, the model requires at least two (to three) σ parameters. This leads to a consistent explanation of all experimental results. The σ-value of statistical theories then would correspond to our products $\sigma_1\,\sigma_2$ or $\sigma_1\,\sigma_2\,\sigma_3$.

In the recombination process, the complete double-strand formation cannot start before a stable nucleus of at least three base pairs has formed. The propagation then is characterized by a rate constant of about 10^7 to 10^8 base pairs per second. The reverse step, or unzippering, is somewhat slower in the transition range, given by $k_{prop.}/s$.

The most surprising result is that not more than three to four base pairs are required to form a stable nucleus. This figure also corresponds to the optimum for a code unit. Any number of base pairs fewer than three would correspond to a large loss of specificity and stability; any number larger than three would mean too little dynamic flexibility. Mispaired (but partly complementary) codon-anticodon complexes would stay together too long and block the readout. We see from the experimental data that in aqueous solution a quintet codon would stay with its anticodon for about a tenth of a second; this figure becomes even larger for a nonpolar medium or in the interior of a ribosome complex. Of course, so far these studies have been performed only for the oligo-A model system. The accurate numbers still must come from more extensive studies with all possible nucleotide combinations.

Another important result is the high "reading" rate. As Crothers has shown,[16] reading in long-chain polynucleotides may occur not only at open ends, but also by means of untwisted loops at any position in the double-stranded helix. The unpaired region in the loop can easily migrate along the molecule by untwisting one loop end at the expense of retwisting the other end. Each base pair can open within less than 10^{-6} seconds and for AT in less than 10^{-7} seconds. If the loop is more than three base pairs away from the ends, it will move with equal probability in either direction. In equilibrium, the number of loops formed equals the number that disappear. The small extension of the "nucleation length" then means that there is a constant distribution of loops over the whole strand, except for a few base pairs at the ends or around double-strand imperfections (single-strand breaks, noncomplementary pairs, odd bases). The whole dynamics of readout is similar to the molecular dynamics in crystals. Lattice imperfections, dislocations, and misfits (i.e., deviations from the ideal crystal structure) determine the dynamic behavior. The crystal does not have to melt to undergo transformations. Similarly, the "linear crystal" of a double-stranded helix can perform all its dynamics far below the melting point under "physiological conditions."

Although most of the details are still missing, a picture of the dynamics of programmed information transfer in nucleic acids is now emerging. However, as much "intelligence" may be involved in these processes, everything must be predetermined entirely by programs. If we are asked how these programs can change, how they can collect experience and adapt to it, we can give no conclusive answer.

Two main problems remain to be solved: one is that of *evolutionary adaptation;* the other is the *control function* at the level of molecular information transfer and its potentiality for delivering instructions. The second problem is of special interest in connection with the chapters in this book, and its solution requires more detailed considerations about enzyme control. Again, at this early stage, we prefer to discuss some concrete mechanisms rather than to speculate about general concepts.

Information transfer and enzyme control

Nucleic acids are ideal for storage and transfer of information because of the linear arrangement of the code units. Relatively simple molecular machinery can carry out all the processing. On the other hand, the simple geometrical shape of double-stranded helices keeps them from any fancy kind of control function. (It is possible that the spatially folded sRNA molecules represent some exception.) On the other hand, proteins, with their enormous multiplicity of spatial structures and shapes, provide the ideal molecular machinery for any type of processing and control function, although they are not at all suited for coding. One may well say that life could have evolved only after a feedback correlation between the "legislative" function of nucleic acids and the "executive" function of proteins had developed.

Proteins recognize substrate with high precision. Their large binding capacity and catalytic power is the result of spatial folding, whereby information from distant positions in the primary sequence (some hundred amino-acid residues) can be brought into close contact with the substrate to be identified. The rate of binding is almost as high as for base pairing (10^6 — $10^8 M^{-1}sec^{-1}$); the lifetime of the complex is usually short enough not to interfere with the rate limitation by the turnover mechanism (usually $<10^{-3}$ seconds). Furthermore, the flexible secondary and tertiary structures allow a rapid adjustment to more or less favorable positioning of certain groups, and thus involve the potentiality of adaptation and control. Actually, most enzymes whose sequence of reaction steps has been studied by relaxation spectrometry show such structural rearrangements, with half times from fractions of seconds to fractions of milliseconds. The most pronounced ex-

amples of molecular feedback and control are represented by "allosteric enzymes," on which attention has been focused in this book. Let us consider such a mechanism in more detail.

A particularly simple example would be an enzyme consisting of several (e.g., four) identical subunits. Binding of a substrate usually would be governed by the simple law of mass action and characterized by a hyperbolic binding curve, as depicted in Figure 8A. Similarly, steady-state kinetics of catalytic turnover according to Michaelis would lead to a correspondingly shaped rate curve.

On the other hand, binding of substrate by cooperative interaction of the subunits would change the character of these curves. As is known from binding of oxygen to hemoglobin (Figure 8B), those binding curves can become sigmoidal, indicating "cooperation" in binding. This means low affinity at low oxygen concentration and high affinity in concentrations at which hemoglobin becomes partly saturated. (Cf. also the cooperative binding as depicted in Figure 5.)

Several theories have been proposed to describe this behavior. Adair[17] assumed a set of four different and successively increasing binding constants. This certainly can describe empirically any sigmoidal curve. However, it might be possible that the number of intrinsic parameters can be reduced, similar to the treatment of helix coil transformations where the n-subunit system (n being a large number) could be represented by only two or three intrinsic parameters.

Such a model was proposed by Monod, Wyman, and Changeux,[18] and is represented in a generalized form in Figure 9. This model requires only three parameters. R and T denote two isomeric states of the enzyme that can

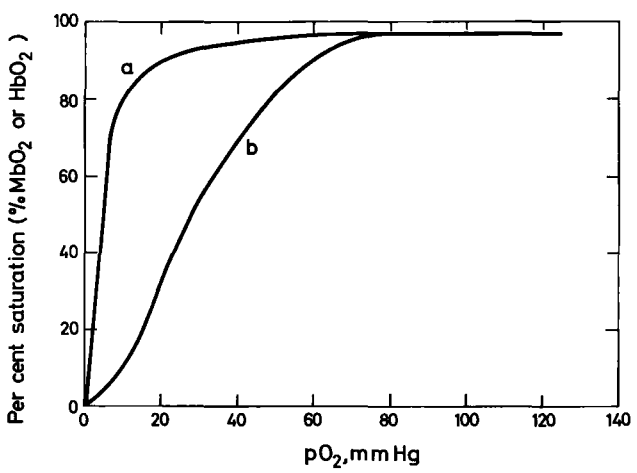

FIGURE 8 Hyperbolic and sigmoidal binding curves for oxygen binding to myoglobin (a) and hemoglobin (b).

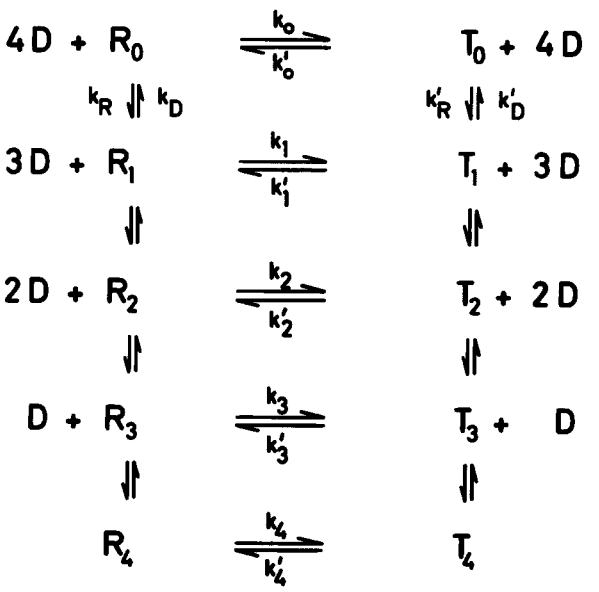

FIGURE 9 Generalized reaction scheme for allosteric model of Monod, et al (Note 18).

bind the substrate. The affinity for the substrate (D) in the R-state is high, whereas in the T-state it is low (or even negligible), but T is the preferred state in the absence of binding. The subunits in either the R- or the T-state are "degenerate," so that only two intrinsic binding constants, one for any subunit in the R-state and one for any subunit in the T-state, must be assumed. In addition to these two constants, we need one parameter to describe the transformation from R- to T-state—possibly a conformation change—which is assumed to be extremely cooperative, i.e., all-or-none (all subunits are either in the R or the T conformation). The two simplifying assumptions—that is, the complete degeneracy of R-states or T-states for binding and the all-or-none transformation from R to T derived by Monod from symmetry considerations—have been subject to criticism.[19] It is obvious that a decision about the mechanism cannot be derived from equilibrium studies. However, relaxation spectrometry can provide the tool for elucidation of such complex reaction mechanisms. Instead of looking at the system after all steps have equilibrated and characterizing it by one integrant number, we expand the rapidly perturbed equilibrium function on the time axis, and thus we may see each step equilibrating with its own characteristic rate.

What do we expect for a system such as that depicted in Figure 9? If the system is characterized by three intrinsic parameters it should yield three characteristic time constants. They would have a particularly simple form, if the all-or-none conformation change (R $\leftrightarrow$ T) is the

slowest step. Let us assume this condition and see how the system would "relax" after perturbation. At the first instant, the two conformations would be frozen in; each would start to bind substrate with a characteristic rate. Usually one of them—often the more affine form—will show a shorter relaxation time. There will be only one *detectable* time constant for the binding at all R-subunits. This can be represented by a second-order reaction between "free" substrate molecules (D) and unoccupied R-binding sites ($4R_0+3R_1+2R_2+R_3$). The reverse process is a first-order dissociation from all occupied R-sites ($R_1+2R_2+3R_3+4R_4$). According to considerations in the preceding chapters, the inverse relaxation time ($1/\tau_I$) should be a linear function of the sum of the concentrations of free substrate and unoccupied binding sites. The slope will yield the "intrinsic" rate constant of recombination to R-states, and the intercept will yield the intrinsic rate constant of dissociation from R-states.

Similarly, the binding by the less affine form (T) should yield an analogous dependence for the inverse relaxation time ($1/\tau_{II}$), i.e., an increase with the sum of free substrate plus unoccupied T-binding site concentrations. The latter term, however, must be corrected by a factor that results from coupling to the more rapidly established R-state equilibrium. Both equlibria are coupled via the substrate concentration; in other words, while T-states react with the substrate, the change of substrate concentration modifies the more rapidly equilibrating R-state binding. These factors (which would also modify $1/\tau_I$ if both relaxation times were of the same order of magnitude) can easily be calculated.[20] Again, for the T-state binding there is only one detectable relaxation time for all four subunits, and it yields the intrinsic rate constants of recombination and dissociation (the ratio of which also yields the intrinsic binding constant).

The third relaxation time now must be characteristic of the first-order conformation change. Nevertheless, it may be substrate-concentration dependent, as the substrate binding equilibria will rapidly rearrange during the transformation: $R \leftrightarrow T$. At very low concentrations of substrate we expect no binding, so the system will be in the T_0- and R_0-state only. Similarly, at very high concentrations of D, where R and T are saturated, the system will be in the R_4-state and T_4-state only. In both cases we expect first-order transformations and therefore concentration-independent $1/\tau_{III}$ values. In general, however, these are different for both cases. Sigmoidal characteristics will result only if (at low concentration) the T_0-state is more stable than the R_0-state, whereas at high concentrations of D the system favors the R_4-state more than the T_4-state. This is equivalent to the fact that the rate of $R_0 \rightarrow T_0$ is larger than that of $T_0 \rightarrow R_0$, but the rate of $R_4 \rightarrow T_4$ is smaller than that of $T_4 \rightarrow R_4$. If we raise the concentration of D to a level at which R starts to saturate, $1/\tau_{III}$ will decrease from the constant value (given by $k_{RT_0} + k_{TR_0}$) to another constant value $k_{TR_0} + k_{RT_4}$ (but usually $k_{RT_4} \ll k_{TR_0}$). If we then reach the level at which T saturates, $1/\tau_{III}$ will again change to $k_{TR_4} + k_{RT_4} \approx k_{TR_4}$. It might well be that saturation of the less affine form does not change the rate constants k_{TR} detectably. If so, this latter step in $1/\tau_{III}$ should not be detectable. In any case, $1/\tau_{III}$ should remain constant at higher concentrations. Furthermore, $1/\tau_{III}$ should be independent of the enzyme concentration throughout the whole concentration range (at constant "free"-substrate concentration).

With such a relaxation spectrum we should have an excellent tool to test the nature of this simple type of allosteric control mechanism. The existence of the first two relaxation effects, for which $1/\tau$ increases linearly with concentration, should give detailed information on binding in both states. From a comparison at low and high concentrations (low and high saturation), we should learn whether the simplifying assumptions of degeneracy, or intrinsic binding constants, are fulfilled. The third relaxation time should inform us about the existence of a conformation change. If $1/\tau_{III}$ decreased from one constant level to another, we should expect two conformations with first-order transformations. The sharpness of the decrease of $1/\tau_{III}$ decides about the cooperativity. An all-or-none transformation of a four-subunit system requires fourth-power terms in substrate concentration. On the other hand, any model that assumes only one binding form would never yield this type of $1/\tau_{III}$ relation. For such a model, all $1/\tau$ values should more or less increase with the "free" substrate concentration. Moreover, the nonexistence of any of the simplifying assumptions in the RT-model (degeneracy, all-or-none transformation) would result in a higher complexity of the relaxation spectrum, e.g., more than three relaxation times, or curvature changes, in $1/\tau$ relations. If, on the other hand, a relaxation spectrum like the one described above could be detected, one could well conclude that Monod's model is the simplest to account for the experimental facts and that—even if a more complex mechanism is involved—it is still a good approximation.

Obviously, quite some luck is required for one to encounter a system agreeable enough to show three relaxation times with concentration dependencies that can be analyzed explicitly in such a simple manner. Dr. Kirschner in our group had such luck with the enzyme glyceraldehyde-phosphate-dehydrogenase, a key enzyme in the glycolysis pathway. He also was fortunate enough to isolate this enzyme from yeast instead of from muscle—fortunate because only the former allows the complete

analysis in a concentration range that is easily accessible to relaxation spectrometry. Details about these investigations are published elsewhere.[21,22] Figures 10 to 15, however, show how exactly the behavior described in this chapter is reflected by the enzyme.

From these studies Monod's assumptions about symmetry seem to be met completely, although according to Koshland, Némethy, and Filmer,[19] nonsymmetric models in which the simplifying assumptions of Monod are not fulfilled should not be excluded a priori. It may well be that both models are possible in principle, but that symmetric models had a better chance to survive during evolution. Multisubunit enzymes seem to be products of "adaptation" or "learning." The binding and cooperation of subunits is a product of successive mutation and selection. Any symmetric model would have advantages, for improvement of subunit interaction becomes effective at more than one side. Although it would be premature to draw general conclusions from only one example, two

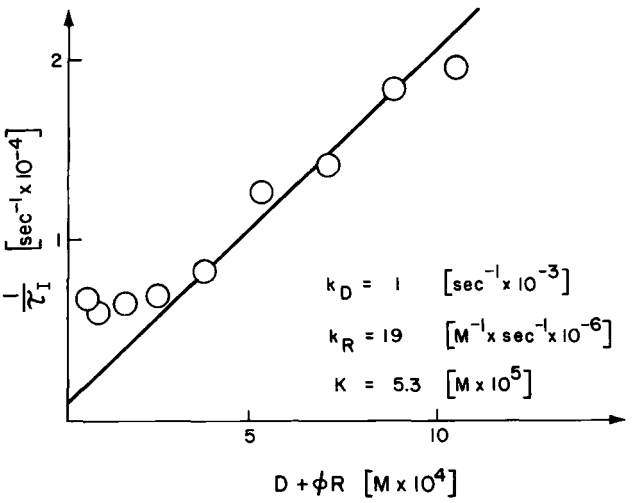

FIGURE 11 Inverse relaxation time τ_I as a function of free substrate (D) and free R-binding site (ϕ_R) concentration. The approximately linear plot suggests a second-order binding reaction. Deviations at low concentrations due to *p*H-dependence.

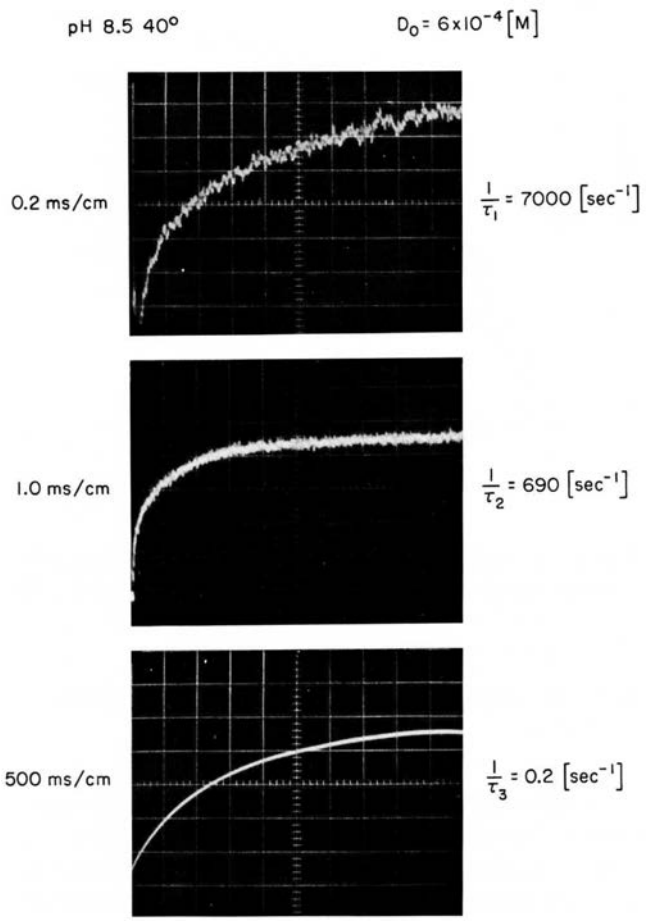

FIGURE 10 Oscillogram of the relaxation spectrum of DPN-binding to glyceraldehyde-phosphate-dehydrogenase.

conclusions are possible: (1) Monod's model represents a useful working hypothesis; (2) techniques now available (such as relaxation spectrometry) allow a complete analysis of such molecular models of control function.

Summary and epilogue

Certainly, much further work must be done before it will be possible to draw more general conclusions about the mechanism of control of information transfer in biological systems. The success of the idea of molecular information transfer in genetics has provoked much speculation about the possibility of "instruction" or "learning" at the molecular level. So far we know well the *scheme* or code of genetic information and some of its use in ontogenesis and phylogenesis, but we know very little about the underlying mechanisms. The situation is comparable with that of chemistry at the beginning of this century, when the schemes—for instance, the periodic table and the relations of constant and multiple proportions in chemical reactions —were known, but the "how" remained obscure.

We should realize that the only truly striking example of molecular instruction in biology—the adaptation of a molecular code producing improved responses—is found in the fact of evolution. It may well be that any other type of learning is a selective process at the molecular level, initiated by external stimuli but guided by programs formed in evolution and turned on and off by molecular and supramolecular control mechanisms that govern quantities rather than qualities. All the known facts about the significance of RNA and protein synthesis, as well as

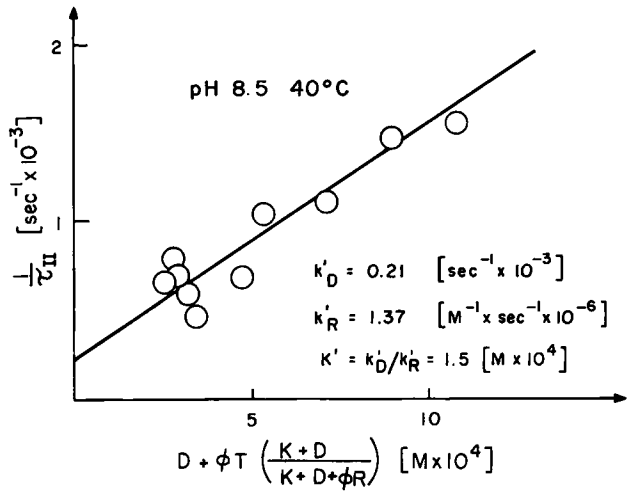

FIGURE 12 Inverse relaxation time τ_{II} as a function of free substrate (D) and free T-binding site (ϕ_T) concentration, corrected for equilibrium of R-binding. The linear plot, again, suggests a second-order binding process.

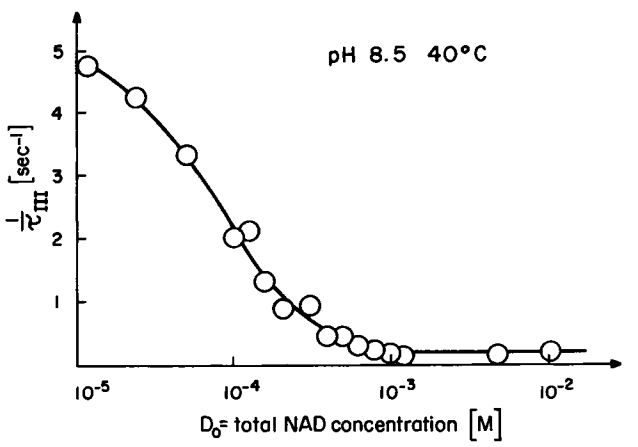

FIGURE 13 Inverse relaxation time τ_{III} as a function of substrate concentration (log [D]). Only one step occurs suggesting a decrease of k_{RT} for increasing saturation. This decrease is governed by fourth-power terms of substrate concentration suggesting "all or none" conformation changes. No change of k_{TR} is observed. This result is confirmed by flow experiments. τ_{III} remains constant at high concentration.

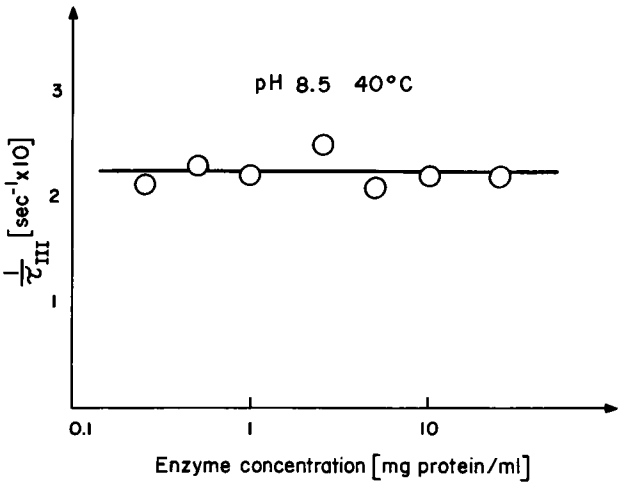

FIGURE 14 Inverse relaxation time τ_{III} as a function of enzyme concentration. The constant value suggests a first-order process.

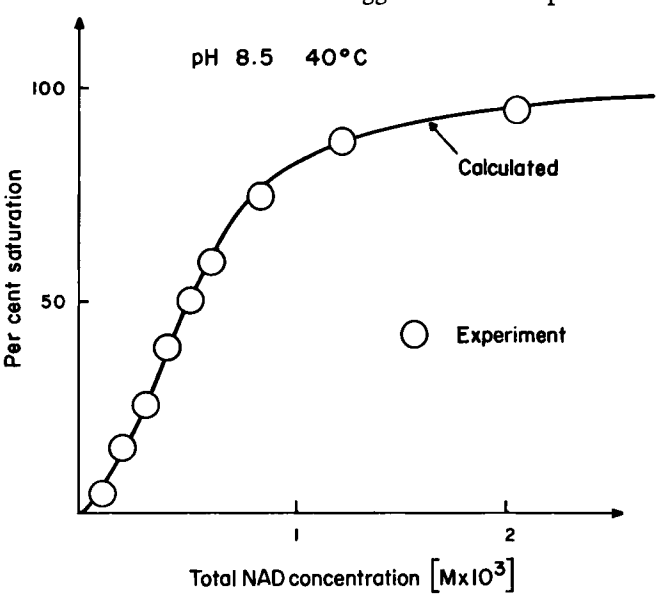

FIGURE 15 Saturation curve for DPN binding to glyceraldehyde-phosphate-dehydrogenase. The curve—constructed with the parameters obtained from relaxation experiments—agrees well with the experimental points.

Note: For details of experimental conditions of Figs. 10 to 15, cf. Note 21.

those about molecular specificity in the neuronal network, do not necessarily suggest that instruction reaches the molecular level except by such control of pre-existing programs. However, we cannot exclude other possibilities at the present time.

In this connection, I should like to emphasize that the several seconds to several minutes it takes to consolidate labile information in the central nervous system is equivalent to that of biosynthesis of whole nucleic acid or protein molecules, not *de novo*, but under the control of genetic programs. In order to learn which possibilities are at the disposal of nature, we must study the mechanisms of biosynthesis and their control, their self-organization, and their guidance by external (such as electrical) stimuli.

The Genetic Code

MARSHALL NIRENBERG

THE ORDER OF THE FOUR BASES IN DNA corresponds to the order of the twenty amino acids in protein. Perhaps the simplest way to describe the nature of the code is to describe the process of protein synthesis.

Two chains of DNA, running in opposite directions, interact with one another and form a double helix; bases on one chain hydrogen bond with bases on the other chain. (The specificity of base pairing, postulated by Watson and Crick, is shown in the chapter by Dr. Davidson, this volume; adenine interacts with thymine, and guanine with cytosine.)

The genetic information corresponding to the amino acid sequence of protein is transcribed from DNA into single-stranded molecules of messenger RNA (mRNA). An enzyme, RNA polymerase, attaches to an initiator site on the DNA template and catalyzes mRNA synthesis until a terminator site is reached. A molecule of mRNA may correspond to two or more proteins. The structures of initiator and terminator sites on DNA, and mechanisms for selecting the DNA strand to be read remain to be clarified. The newly synthesized strand of mRNA corresponds to only one of the two DNA strands.

The over-all process of protein synthesis is illustrated diagramatically in Figure 1. The steps required for the release of nascent chains of mRNA from DNA templates, the attachment of mRNA to ribosomes, and the initiation of protein synthesis are being studied intensively in many laboratories. Such studies are of great interest, for it seems probable that the synthesis of some species of mRNA and protein may be regulated at an early stage of protein synthesis.

Messenger RNA is translated into protein on ribosomes. Amino acids become attached to adaptors, termed transfer RNA (AA-tRNA). Each AA-tRNA specifically recognizes mRNA codewords, probably by hydrogen bonding among three bases that comprise the tRNA anticodon and three bases in the mRNA codon. One or more species of tRNA serve as adaptors for each of the twenty amino acids. An amino acid is linked to an appropriate species of tRNA by specific AA-tRNA synthetase enzymes. Each cell contains at least twenty species of AA-tRNA synthetases—one for each amino acid.

An *E. coli* 70S ribosome consists of two subunits, 30S and 50S particles. A ribosome may attach to the 5′-terminus of mRNA, and then the first and second mRNA codons are recognized by appropriate AA-tRNA molecules, which bind to ribosomes. The first amino acid is transferred to the amino group of the second AA-tRNA. The first tRNA then is released from the ribosome and the ribosome "moves" along the mRNA to the next codon, possibly shifting the second codon and the peptidyl-tRNA to the positions formerly occupied by the first codon and first tRNA. The peptide chain increases in length by one amino acid at a time, and the RNA message is read sequentially, three bases at a time, from the 5′-terminus toward the 3′-terminus.

Ribosomes contain at least two kinds of sites for AA-tRNA; one site for tRNA bearing the first amino acid or the nascent polypeptide chain; the second site for AA-tRNA bearing the next amino acid to be incorporated. Two enzymes apparently are required for the initiation of protein synthesis; i.e., the formation of the first dipeptide. Three additional enzymes and GTP are required for further elongation of the peptide chain. Each step is under intensive investigation, for the mechanisms have not been fully defined.

Studies on the genetic code can be divided into two phases. Initially, base compositions of RNA codons were determined by directing cell-free protein synthesis with randomly ordered synthetic polynucleotides; during the second phase, base sequences of RNA codons were determined by directing binding of AA-tRNA to ribosomes with trinucleotide templates of known sequence.

In the first phase, protein synthesis was shown to be dependent upon mRNA. Randomly ordered polynucleotides containing different combinations of bases were synthesized with the aid of polynucleotide phosphorylase, and then were used to direct cell-free protein synthesis. By determining the relative amount of each amino acid directed into protein by the polynucleotide and the base content of the polynucleotide, base compositions of fifty-three RNA codons were determined and the general nature of the code was defined. The code was shown to be highly degenerate, i.e., alternate codons correspond to one amino acid. Three bases in mRNA constitute one codon. Codons are read sequentially in a non-overlapping fashion. Messenger RNA functions catalytically. One mole-

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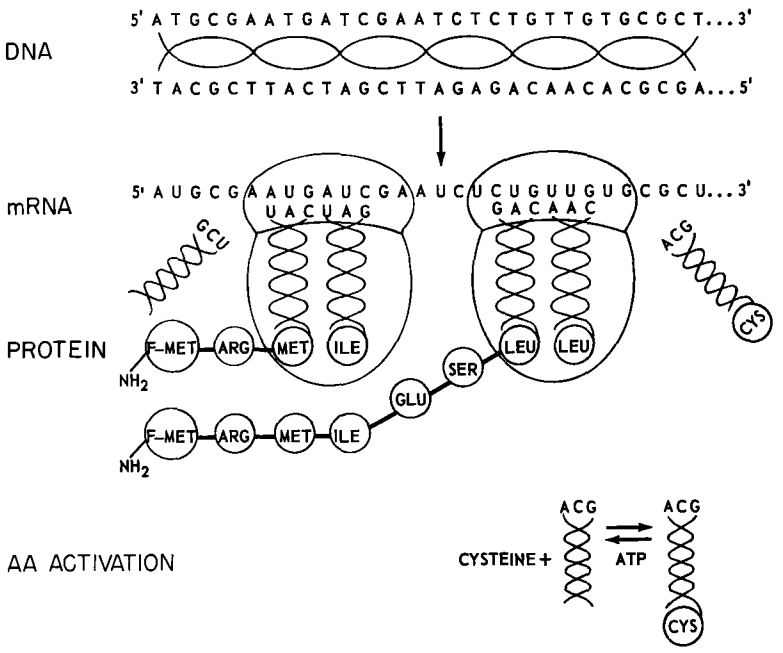


FIGURE 1 Diagramatic illustration of steps in protein synthesis.

cule of mRNA may direct the synthesis of many proteins. However, some messages apparently are destroyed more rapidly than others, and it seems probable that some messages are read more often than others.

The specificity of protein synthesis usually is high, but errors soon were detected in the cell-free protein synthesizing system. The frequency of error is a function of Mg^{++} concentration, *p*H, temperature, tRNA concentration, amino acid concentration, and so forth. These findings were unexpected and at first were thought to be in vitro artifacts. However, an in vivo error in protein synthesis had been demonstrated by Yanofsky, and it soon became clear that some codons are translated with higher specificity than others, and that the specificity of codon recognition can be altered by modifying part of the apparatus required for codon translation. This concept is important and is discussed more fully later in this chapter.

Alternate codons for the same amino acid usually contain two bases in common and thus are structurally related to one another. Common bases were assumed to occupy the same base position within synonym triplets. Although base compositions of codons had been determined, base sequences of codons still were unknown. A different experimental approach was devised to decipher the order of bases within triplets. Trinucleotides of known sequence were used as templates to direct the binding of AA-tRNA to ribosomes prior to peptide bond formation. Hence the process of codon recognition rather than protein synthesis was studied. Earlier studies in the laboratories of Schweet, Lipmann, and Kaji and Kaji had shown that polynucleotides stimulate binding of AA-tRNA to ribosomes, and these findings set the stage for codon sequence studies.

A rapid, convenient method for studying the codon recognition process was devised; it depends upon the formation of AA-tRNA-ribosome-codon complexes. Ribosomes are incubated with C^{14}-AA-tRNA and trinucleotide templates for a short time, the reaction then is diluted and poured through a cellulose nitrate disk. The disk retains ribosomes and AA-tRNA bound to ribosomes. Unbound AA-tRNA is removed readily by washing.

At the time the technique was devised, fewer than half of the trinucleotides had previously been isolated or synthesized. Two enzymatic methods for oligonucleotide synthesis were developed: the polynucleotide phosphorylase method by Leder, Singer, and Brimacombe, and by Thatch and Doty; and the pancreatic RNase method described by Heppel, Whitfield, and Markham and refined by Bernfield. Elegant chemical methods were devised by Khorana and his associates.

A different approach to the sequence problem was devised by Khorana and his associates. In a remarkable series of studies that required years of effort, polydeoxynucleotides and polyribonucleotides with alternating doublets or triplets of known sequence were synthesized and then were used to direct cell-free protein synthesis. Polyribonucleotides with alternating triplet sequences directed the synthesis, in vitro, of three kinds of homopolypeptides, each corresponding to one of the three possible phases of reading. Polyribonucleotides with repeating doublet se-

quences stimulated the synthesis of polypeptides with two amino acids in alternating sequence.

Nucleotide sequences of RNA codons

The 64 trinucleotides now have been synthesized and their template activities for *E. coli* AA-tRNA have been determined. Nucleotide sequences of codons are shown in Table I, and degeneracy patterns in Table II. Almost all trinucleotides correspond to amino acids. In most cases, synonym codons differ only in the base occupying the third position of the triplet. Hence, degeneracy is logical. Patterns of alternate third bases of degenerate codons are: U = C; sometimes A = G; on other occasions G is the only acceptable base; U = C = A = G. In several cases, alternate bases may occupy the first position of synonym codons.

One consequence of logical degeneracy is that many mutations leading to single base replacements in DNA at sites corresponding to third bases of mRNA codons do not result in amino acid replacements in protein. Hence, many mutations are silent. Also, the code is arranged so that the effect of error is minimized, for often only one base per triplet is recognized incorrectly. Amino acid replacements in protein then are restricted, sometimes to amino acids with similar properties, for amino acids that are structurally related or arise from common biosynthetic precursors often correspond to similar RNA codons (for example, the aspartic acid [Asp] codons GAU and GAC are similar to the glutamic acid [Glu] codons GAA and GAG). In Table I, potential amino acid replacement as a result of single base changes can be read by moving horizontally or vertically from the amino acid in question, but not diagonally. Because the mutation frequency is usually low and most mutations result in single base replacements, codon ancestry often can be traced by comparing amino acid sequences of related proteins isolated from different organisms. Predictions also can be made about other replacements that may have occurred in the past or may occur in the future.

Mechanism of codon recognition

Cells often contain multiple species of tRNA for the same amino acid. Soon after the code was found to be degenerate, the specificity of multiple species of tRNA for alternate codons was examined. Two species of leucine (Leu) tRNA were examined and were found to recognize different Leu-codons. Recently this question was reinvestigated using the tRNA-ribosome binding assay. The results show that many species of tRNA are capable of recognizing two or more synonym codons.

TABLE I

Nucleotide sequences of RNA codons

1st Base	2nd Base U	2nd Base C	2nd Base A	2nd Base G	3rd Base
U	PHE*	SER*	TYR*	CYS*	U
	PHE*	SER*	TYR*	CYS	C
	leu*?	SER	TERM?	cys?	A
	leu*, f-Met	SER*	TERM?	TRP*	G
C	leu*	pro*	HIS*	ARG*	U
	leu*	pro*	HIS*	ARG*	C
	leu	PRO*	GLN*	ARG*	A
	LEU	PRO	gln*	arg	G
A	ILE*	THR*	ASN*	SER	U
	ILE*	THR*	ASN*	SER*	C
	ile*	THR*	LYS*	arg*	A
	MET*, F-MET	THR	lys	arg	G
G	VAL*	ALA*	ASP*	GLY*	U
	VAL	ALA*	ASP*	GLY*	C
	VAL*	ALA*	GLU*	GLY*	A
	VAL	ALA	glu	GLY	G

Nucleotide sequences of RNA codons were determined by stimulating binding of *E. coli* AA–tRNA to *E. coli* ribosomes with trinucleotide templates. Amino acids shown in capitals represent trinucleotides with relatively high template activities compared to other synonyms shown in lower case. Asterisks (*) represent base compositions of codons which were determined previously by directing protein synthesis in *E. coli* extracts with randomly ordered synthetic polynucleotides. F-Met, represents N-formyl-Met-tRNA which serves as an initiator of protein synthesis. TERM represents possible terminator codons. Question marks (?) indicate uncertain codon function.

Table III contains a summary of synonym codons recognized by purified tRNA fractions obtained either by countercurrent distribution or by column chromatography. Purified tRNA fractions recognize sets of synonym codons. Codons comprising a set differ only in the third base. The following patterns of alternate third bases were found: C = U; A = G; G; U = A = C; A = G > U.

After a molecule of tRNA is released from its DNA template, many bases are modified by enzymes. Conversion of embryonic to mature tRNA may require 20 or more enzymatic reactions, hence many intermediate forms of tRNA corresponding to the same gene may be present. In addition, cells often contain several genes for tRNA corresponding to the same amino acid.

Holley and coworkers recently reported the base sequence of yeast alanine (Ala) tRNA. A sample of yeast

TABLE II

Patterns of degenerate codons for amino acids

U C ••A G ••U C	U C ••A G ••G (A?)	U C ••A G	U C ••(A)	U ••C	A ••G	••G	U C•• A (G)
SER	ARG LEU	GLY ALA VAL THR PRO	CYS ILE	ASP ASN HIS TYR PHE	GLU GLN LYS TERM?	MET TRP	F-MET

Solid dots represent the first and second bases of trinucleotides: U, C, A, and G indicate bases which may occupy the remaining position of degenerate codons. In the case of F-Met (N-formyl-methionine), dots represent the second and third bases. Parentheses indicate codons with relatively low template activities.

TABLE III

Codon patterns recognized by purified sRNA fractions

	Alternate acceptable bases in 3rd or 1st positions of triplet				
C U	A G	G	U C A	A G (U)	Possibly only 2 bases recognized
$TYR_{1,2}$ UA(C/U)	LYS AA(A/G)	LEU_2 CUG	ALA^{yeast} GCU/GCC/GCA	ALA_1 GCG (U)	LEU CU(U/C)
VAL_3 GU(C/U)		LEU_3 UUG	$SER_{2,3}^{yeast}$ UCU/UCC/UCA	$VAL_{1,2}$ GUG (U)	$LEU_{1,2,3}$ UU(U/C)
		MET_2 AUG	$F\text{-}MET_1$ AUU/AUG (C) (G)		LEU_1 (U)UG
			TRP_2 UGG (A)		

At the top of the table are shown alternate bases which may occupy the third or first positions of degenerate sets. Purified tRNA fractions from *E. coli B* and corresponding codons are shown below. Parentheses ( ) indicate codons with relatively low template activity.

Ala-sRNA of high purity (> per cent) was obtained from Holley, and its response to trinucleotide codons was determined. The relation between concentration of yeast or *E. coli* C^{14}-Ala-tRNA and response to synonym Ala-codons is shown in Figure 2A and B. At *limiting* concentrations of purified yeast Ala-tRNA, at least 59, 45, 45, and 3 per cent of the available C^{14}-Ala-tRNA molecules bind to ribosomes in response to GCU, GCC, GCA, and GCG, respectively. Responses of unfractionated *E. coli* C^{14}-Ala-tRNA were 18, 2, 38, and 64 per cent, respectively. Since the purity of the yeast Ala-tRNA was greater than 95 per cent, the extent of binding indicates that one molecule is capable of recognizing three, possibly four, synonym codons. The data also demonstrate marked differences in the relative response of yeast and *E. coli* Ala-tRNA to synonym codons, particularly in the case of GCG.

By correlating the base sequence of yeast Ala-tRNA with the sequence of mRNA codons, estimates can be made concerning the structure of the Ala-tRNA anticodon and the mechanism of codon recognition. All of the evidence is consistent with antiparallel hydrogen bonding between the Ala-tRNA anticodon, inosine-guanine-cytosine (IGC), and Ala-codons in mRNA. Watson-Crick hydrogen bonding would be expected between GC in the first and second positions of Ala-codons in mRNA and corresponding bases in the tRNA anticodon, and alternate pairing of inosine in the anticodon with U, C, or A, but not with G in the third position of Ala-codons. Crick has proposed a mechanism that would enable a base in a tRNA anticodon to pair with alternate bases occupying the third position of synonym mRNA codons by a "wobble mechanism." As shown in Table IV, U in the tRNA anticodon may pair with either A or G occupying the third position of mRNA codons; C pairs with G; A with U; G with C or U; and inosine with U, C, or A. In

TABLE IV

Alternate base pairing

tRNA Anticodon	mRNA Codon
U	A G
C	G
A	U
G	C U
I	U C A
rT	A G
Ψ	A G (U)
DiHU	No Base Pairing

Alternate base pairing. The base in a tRNA anticodon shown in the left-hand column forms antiparallel hydrogen bonds with the base(s) shown in the right-hand column, which usually occupy the third position of alternate mRNA codons. Relationships for U, C, A, G, and I of anticodons are "wobble" hydrogen bonds suggested by Crick.

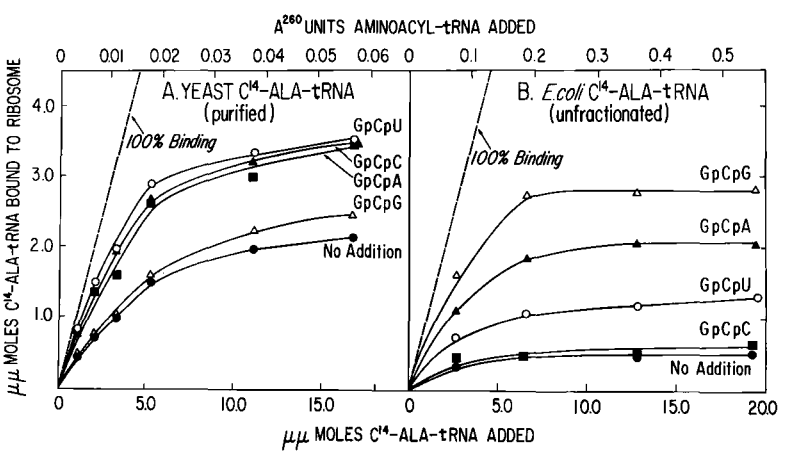


FIGURE 2 Relation between the template activities of trinucleotides in stimulating Ala-tRNA binding to *E. coli* ribosomes and the concentrations of purified yeast C^{14}-Ala-tRNA (Part A) and unfractionated *E. coli* C^{14}-Ala-tRNA (Part B).

addition, it is possible that pseudouridine in a tRNA anticodon may hydrogen bond alternately with A, G, and, less well, with U. Dihydrouridine in an anticodon may be unable to hydrogen bond with bases in mRNA but may be less repelled by pyrimidines than by purines. Possibly hydrogen bonds then form between the two remaining bases of the codon and corresponding bases in the anticodon.

Zachau and colleagues have shown that yeast serine (Ser) tRNA 1 and 2 contain, in the position appropriate for the anticodon, the sequence IGA. Our studies agree well, for Ser-tRNA fractions that resemble those of Zachau recognize UCU, UCC, and UCA, but not UCG. Ingram and coworkers have shown that purified valine (Val) tRNA fraction from yeast contains the sequence IAC, which corresponds to three Val-codons, GUU, GUC, and GUA. In addition, Madison and coworkers have determined the base sequence of yeast tyrosine (Tyr) tRNA, and have suggested that the sequence, GψA, serves as the anticodon, which would correspond to UAU and UAC mRNA codons.

In summary, patterns for amino acids often represent the sum of two or more codon patterns recognized by different tRNA species. Specific tRNA patterns, in turn, often result from alternate pairing between a base in the anticodon and corresponding bases in the appropriate position in messenger RNA codon.

Universality

The results of many studies indicate that the genetic code is largely universal. However, the fidelity of translation can be altered in vivo by extragenic suppressors, and in vitro by altering components or conditions required for protein synthesis. Thus, cells sometimes differ in specificity of codon translation. To investigate the fine structure of the code recognized by AA-tRNA from different organisms, nucleotide sequences and relative template activities of RNA codons recognized by bacterial, amphibian, and mammalian AA-tRNA (*E. coli*, the South African clawed toad [*Xenopus laevis*], and guinea pig liver, respectively) were determined. Acylation of tRNA was catalyzed in all cases by aminoacyl-tRNA synthetases from corresponding organisms and tissues; *E. coli* ribosomes were used for binding studies.

A summary of results is shown in Table V. Almost identical translations of nucleotide sequences to amino acids were found with bacterial, amphibian, and mammalian AA-tRNA. However, *E. coli* AA-tRNA sometimes differs markedly from *Xenopus* and guinea pig AA-tRNA in relative response to synonym codons. Differences in codon recognition are shown in Table VI. The

TABLE V

Nucleotide sequences of RNA codons recognized by AA-tRNA from bacteria, amphibian liver, and mammalian liver

1st Base	2nd Base U	2nd Base C	2nd Base A	2nd Base G	3rd Base
U	PHE	SER	TYR	Cys	U
	PHE	SER	TYR	Cys	C
	(leu, phe?)	SER	term ?	[Cys]	A
	leu,[F-MET]	[SER]	term ?	TRP	G
C	leu	PRO	HIS	ARG	U
	leu	PRO	HIS	ARG	C
	leu	PRO	gln	ARG	A
	leu	PRO	gln	[ARG]	G
A	ILE	THR	asn	[SER]	U
	ILE	THR	asn	[SER]	C
	[ILE]	THR	LYS	[ARG]	A
	MET,[F-MET?]	THR	[LYS]	[ARG]	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	gly	A
	VAL	[ALA]	GLU	gly	G

Universality of the genetic code. Nucleotide sequences of RNA codons were determined by directing binding of AA-tRNA from *E. coli*, *Xenopus laevis* liver (South African clawed toad), and guinea pig liver. Codon assignments in capitals indicate that the trinucleotide sequence is recognized by bacterial, amphibian, and mammalian AA-tRNA. Codon assignments in lower case indicate that the trinucleotide sequence was assayed only with *E. coli* AA-tRNA and was recognized correctly. Cys-codons, however, were assayed with and were recognized by both *E. coli* and guinea pig liver Cys-tRNA. Data are from R. Marshall, C. T. Caskey, and M. Nirenberg.

following trinucleotides had little or no detectable template activity for unfractionated *E. coli* AA-tRNA, but served as active templates with *Xenopus* and guinea pig AA-tRNA: AGG, CGG, arginine; AUA, isoleucine; AAG, lysine; AGU, AGC, serine; and UGA, cysteine. Those trinucleotides with high template activity for *E. coli* AA-tRNA but low activity for *Xenopus* or guinea pig liver AA-tRNA were: UUG, N-formyl-methionine (*E. coli*); GCG, alanine; and UCG, serine. Possible differences also were observed with ACG, threonine; AUC, isoleucine; CAC, histidine; GCC, alanine; and GUC, valine.

TABLE VI

Species dependent differences in response of AA-tRNA to trinucleotide codons

Codon		tRNA: Bacteria (*E. coli*)	tRNA: Amphibian (*Xenopus laevis* liver)	tRNA: Mammalian (Guinea pig liver)
ARG	AGG	±	++++	++
	CGG	±	++++	++++
MET	UUG	++	±	±
ALA	GCG	++++	±	++
ILE	AUA	±	++	++
LYS	AAG	±	++++	++++
SER	UCG	++++	±	++
	AGU	±	+++	+++
	AGC	±	+++	+++
CYS	UGA	±		+++

Possible differences: ACG, THR; AUC, ILE; CAC, HIS; GUC, VAL; and GCC, ALA.

No differences found: Other degeneracies for the amino acids cited above and codons for ASP, GLY, GLU, PHE, PRO, and TYR.

The following scale indicates the approximate response of AA-tRNA to a trinucleotide relative to the responses of the same AA-tRNA preparation to every other trinucleotide for that amino acid (with the exception of Gly-tRNA which was assayed only with GGU and GGC).

++++	70–100%
+++	50–70%
++	20–50%
±	0–20%

No species-dependent differences were found with aspartic acid, glycine, glutamic acid, phenylalanine, proline, and tyrosine codons.

Thus, some degenerate trinucleotides were active templates with tRNA from each species studied, whereas others were active with tRNA from one species but not from another.

Nucleotide sequences recognized by *Xenopus* skeletal muscle argenine-, lysine-, methionine-, and serine-tRNA were determined and compared with sequences recognized by corresponding *Xenopus* liver AA-tRNA preparations. No differences between liver and muscle AA-tRNA were detected, either in nucleotide sequences recognized or in relative responses to synonym codons.

It should be emphasized that most of the major differences observed were between *E. coli* and higher organisms rather than between amphibian muscle and amphibian liver, or between amphibian liver and mammalian liver tRNA.

Fossil records of bacteria 3.1 billion years old have been reported. The first vertebrates appeared approximately 510 million years ago, and amphibian and mammals 355 and 181 million years ago, respectively. The genetic code thus may have been functional 3 billion years ago; almost surely the code is more than 600 million years old. Several investigators have suggested that the code may have changed relatively little since the time organisms as complex as bacteria had evolved. However, factors that modify components required for codon translation may influence the specificity of codon translation to some extent.

The remarkable similarity in codon base sequences recognized by bacterial, amphibian, and mammalian AA-tRNA suggests that most, if not all, forms of life on this planet use almost the same genetic language, and that the language has been used for at least 500 million years, possibly with few major changes. The species-dependent differences observed may reflect changes in the codon recognition apparatus acquired after cells had evolved, which may enable cells to store additional genetic information and become more highly differentiated. Certain aspects of embryonic differentiation may even be dependent upon changes in codon recognition. The studies described are recent ones, so additional information is needed to assess this hypothesis. At the present time, the biological consequences of a modifiable translation apparatus are largely unknown.

Regulation of protein synthesis and codon function

Most codons correspond to amino acids; however, some codons serve in other capacities, such as initiation, termination, or regulation of protein synthesis. Only a few codons have been assigned special functions thus far. This area of investigation is of great interest, for it seems probable that protein synthesis sometimes is regulated on ribosomes at the translation level and that additional codon functions will be found.

CODON FREQUENCY AND DISTRIBUTION IN MRNA

Some synonyms probably occur more frequently in mRNA than in others, or are distributed nonrandomly within a chain of mRNA and among different species of mRNA. Because multiple species of tRNA corresponding to the same amino acid often recognize different codons, different sets of tRNA could be used for the synthesis of two proteins with the same amino acid composition. The generally accepted view that the code is an amino acid

code is somewhat misleading. It is a tRNA code, and it is not known whether multiple tRNAs responding to different synonyms for one amino acid serve the same function. One possibility currently being investigated is that alternate codons for the same amino acid may differ in template activity, specificity, stability of codon-ribosome-tRNA complexes, etc., and that such differences may regulate the rate of synthesis of certain proteins.

CODON POSITION Each codon may occur in three chemically distinct forms in RNA and DNA, depending upon position, as a 5′-terminal-, 3′-terminal-, or internal codon. Substituents attached to terminal or internal ribose hydroxyl groups may have profound effects upon template activity of codons. As shown in Table VII, relative template activities of oligo U preparations, at limiting oligonucleotide concentrations, are as follows: p-5′-UpUpU > UpUpU > CH_3O-p-5′-UpUpU > UpUpU-3′-p > UpUpU-3′-p-OCH_3 > UpUpU-2′,-3′-cyclic phosphate. Trimers with (2′-5′) phosphodiester linkages, (2′-5′)-UpUpU and (2′-5′)-ApApA, do not serve as templates for phenylalanine- or lysine-tRNA, respectively. The relative template efficiencies of oligo A preparations are as follows: p-5′-ApApA > ApApA > ApApA-3′-p > ApApA-2′-p.

These studies led to the proposal that RNA and DNA contain three classes of codons, differing in structure: 5′-terminal, 3′-terminal, and internal codons. 5′-terminal and perhaps also 3′-terminal codons may serve, together with neighboring codons, as regulatory regions, possibly operator regions.

Many enzymes have been described that catalyze the transfer of nucleotides, amino acids, and other molecules to or from terminal sugars of nucleic acids, so modification of terminal hydroxyl groups was suggested as a possible mechanism for regulating the reading of RNA or DNA.

INITIATOR AND TERMINATOR CODONS The initiation of protein synthesis is under intensive study currently, for it seems probable that initiation may sometimes limit the rate of protein and mRNA synthesis. Synthetic rates may be regulated at the level of initiation.

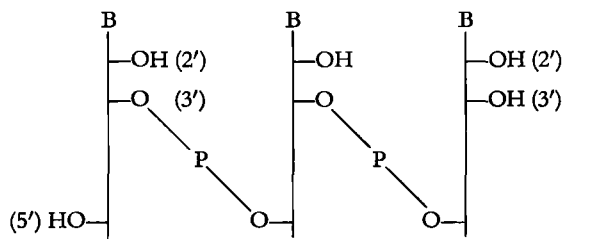
As discussed earlier, ribosomes apparently attach to the 5′-terminus of mRNA (or close to the terminus) and initiator codons specify the first word to be read and, thereby, the phase with which subsequent words are read. Transfer RNA recognizes codons at two ribosomal sites; site 1, occupied either by initiator AA-tRNA or peptidyl-tRNA, and site 2, occupied by AA-tRNA. Recent studies have shown that a codon at site 1 may have a different meaning from that at site 2.

E. coli contains two species of methionine-tRNA; Met-tRNA 1 can be converted enzymatically to N-formyl-Met-tRNA 1 and responds to AUG, UUG, and to a lesser extent, to GUG. Met-tRNA 2 does not accept formyl moieties and responds primarily to AUG. Most *E. coli* proteins contain N-terminal methionine. The N-terminal amino acid is the first to be incorporated; the C-terminal amino acid, the last.

N-formyl-Met-tRNA 1 serves as an initiator of protein synthesis in *E. coli*. The degeneracy pattern is unusual, for alternate bases occupy the first position of synonyms. This probably is a consequence of interactions between ribosome and codon. The affinity of N-formyl-Met-tRNA 1 for ribosomal site 1 appears to be higher than that of Met-tRNA 2.

TABLE VII

Relative template activity of substituted oligonucleotides



Oligonucleotide	Relative template activity
p-5′-UpUpU	510
UpUpU	100
CH_3O-pUpUpU	74
UpUpU-3′-p	48
UpUpUp-OCH_3	18
UpUpU-2′,3′-cyclic p	17
(2′-5′)-UpUpU	0
OLIGODEOXY T	0
p-5′-ApApA	181
ApApA	100
ApApA-3′-p	57
ApApA-2′-p	15
(2′-5′)-ApApA	0
OLIGODEOXY A	0

Relative template activities of substituted oligonucleotides are approximations obtained by comparing the amount of AA-tRNA bound to ribosomes in the presence of limiting concentrations of oligonucleotides compared to either UpUpU for C^{14}-Phe-tRNA, or ApApA, for C^{14}-Lys-tRNA (each designated as 100%).

The codons AUG and UUG correspond to N-formyl-Met-1 at ribosomal site 1; however, at ribosomal site 2, these codons correspond to Met-tRNA 2 and Leu-tRNA, respectively.

Two non-dializable factors are necessary for initiation. They cannot be replaced by transformylase preparations or by other enzymes required for protein synthesis. Their function is unknown.

UAA and UAG may serve as terminator codons ( ). To date, terminator tRNA corresponding to UAA or UAG has not been found. The mechanism of termination is unknown.

Mechanisms for regulating transcription during translation on ribosomes have been proposed. Recent studies by Newton, Beckwith, Zipser, and Brenner and by Lodish and Zinder indicate that the synthesis of mRNA and protein may be regulated by the positions of an initiator codon relative to a terminator codon in mRNA. When the distance between initiator and terminator codon is relatively small, protein and mRNA synthesis corresponding to the message is severely repressed. Ribosomes may attach to partially synthesized mRNA; in turn, they are attached to DNA or RNA templates. Thus protein synthesis may be initiated before the nascent chain of mRNA detaches from its DNA template. Transient intermediates of factors which DNA-nascent mRNA and ribosomes have been isolated, and the in vitro formation of such complexes has been studied. Factors influencing the rate of initiation of protein synthesis may affect the release of mRNA from its template and hence may *selectively* alter the rate with which that template is transcribed. The process has been termed *coupled regulation.*

CODON ACTIVITY AND SPECIFICITY Trinucleotides with little activity for AA-tRNA, in studies thus far, are: UAA, UAG, and UUA (perhaps CUA also). Additional codons, discussed in the section "Universality," serve as active templates with tRNA from one organism, but not from another. However, some trinucleotides that appear to have little template activity may be more active at internal positions of mRNA. Among the many possible explanations for low template activity of trinucleotides in binding assays are: special codon function; relative position of codon in mRNA; appropriate species of tRNA absent or in low concentration; the presence of additional species of tRNA that compete for codons or for ribosomal sites; high ratio of deacylated tRNA to aminoacyl-tRNA; aberrant concentration of Mg^{++}, etc., or aberrant time or temperature of incubation.

In summary, terminal codons may differ structurally and functionally from internal codons of identical sequence; a codon may have alternate meanings, depending upon the ribosomal site at which it is read; a codon may serve several functions simultaneously, such as specifying the beginning of a protein, an amino acid, and the phase with which subsequent codons are to be read; the meaning of a codon may change; alternate codons for the same amino acid often differ markedly in template activity, specificity, and stability of codon-ribosome-AA-tRNA complexes.

Variable translation

The meaning of a codon often depends upon the state of the codon translation apparatus. By altering one of the components required for codon translation, such as ribosomes, tRNA, or enzymes catalyzing AA-tRNA formation, it is possible to change the accuracy, efficiency, rate of translation, or the meaning of a codon. Extensive modification of the codon translation apparatus may often be lethal, whereas subtle modifications may sometimes permit cell growth, yet profoundly influence the rate or course of cellular differentiation. We now know that the code is largely universal but that certain codons are translated with higher specificity in some cells than in others. The translation apparatus of one cell may differ in many ways from that of another. The reading mechanism is plastic and can be modified relatively easily: for example, one or two molecules of the antibiotic streptomycin may bind to a ribosome and distort the orientation of a site required for codon recognition. The frequency of error then is high. Also, under certain circumstances, the rate of protein synthesis may be greatly inhibited. Translation errors are non-random, for in many cases, two out of the three bases per codon are translated correctly. Interestingly, different analogs of streptomycin induce different kinds of errors.

A mutation in a gene for a ribosomal protein may result in the production of altered ribosomal protein, which in turn may affect the specificity or rate of protein synthesis. Similarly, a mutation that alters a gene corresponding to a species of tRNA may result in the production of tRNA differing in specificity for codons or in other properties. The term "suppression" is a general one used to denote such a phenomenon (and other phenomena as well).

MODIFICATION OF CODON RECOGNITION DUE TO VIRAL INFECTION Sueoka and Kano-Sueoka have shown that infection of *E. coli* by T2 bacteriophage results, within one to three minutes, in the modification of one or more species of Leu-tRNA present in the *E. coli* host. Concomitantly, *E. coli,* but not protein synthesis, is inhibited. Pro-

tein synthesis is required, however, for modification of Leu-tRNA.

In collaboration with the Sueokas, we have studied the specificity of Leu-tRNA for codons before and after infection. The results show that a species of *E. coli* Leu-tRNA that responds to CUG is modified within one to three minutes after infection by T2 phage, and then responds to poly UG, but not to CUG or to any trinucleotide containing U and G. Further studies are needed to determine whether this modification turns off host, but not viral, protein synthesis.

Many compelling questions must still be answered. For example, it is possible that certain aspects of embryonic differentiation may be dependent upon changes in codon recognition. The biological consequences of a plastic codon translation apparatus are still largely unknown. Codon function, particularly as it relates to the regulation of protein synthesis and the selective retrieval of genetic information, is also a relatively unexplored field.

Summary

In conclusion, one may ask whether basic principles relevant to the problem of information processing by neurons have been derived from the study of genetic information processing. Elsewhere in this volume, Dr. Davis stated eloquently one of the most important genetic principles. Only a few *kinds* of units are required for the storage of genetic information. A large amount of complex information is stored by arranging these base units in different sequences. The second principle is that relatively few recognition mechanisms are employed. Third is that virtually all forms of life on this planet use essentially the same mechanisms for storage and retrieval of genetic information, for the mechanisms have been retained as higher forms of life evolved from lower forms. Fourth, genetic messages are relatively stable; during evolution, messages are slowly modified and many new messages are added to the old.

Induction and Repression of Enzyme Synthesis

GUNTHER S. STENT

IN THIS ARTICLE we shall consider how the expression of genetic information is regulated so that each gene produces the optimal amount of its product for harmonious and economic function of the cellular enzymatic ensemble. The discussion will be confined entirely to gene regulation in bacteria and will concern the functional expression of only two small groups of the many bacterial genes, namely those that are concerned with the fermentation of the sugar, lactose, and with the synthesis of the amino acid, histidine. This highly parochial view of the altogether ecumenical subject of gene regulation is not without didactic merit, however, because the regulatory aspects of bacterial lactose and histidine genes are now paradigms of the general control of gene function.

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Enzymatic induction

It was known by the turn of the century that the enzymatic properties of microbes depend on the medium on which they have grown, in that microbes can be "trained" for, or adapted to, growth in a variety of different environments. Thus in 1900, F. Dienert[1] found that yeast contains the enzyme galactozymase when depending on the carbohydrates lactose or galactose for its carbon and energy sources, and that it loses this enzyme when the yeast is transferred to a glucose substrate, where galactozymase is not required for growth. In the 1930's, H. Karström[2] studied the formation of several carbohydrate-splitting enzymes in bacteria and divided them into two classes: *adaptive* enzymes, whose formation proceeds only in the presence of their substrates in the medium, and *constitutive* enzymes, whose formation proceeds regardless of the nature of the medium. In 1938, Yudkin[3] published a review of enzyme adaptation among microbes in which he proposed a simple, mass-action model of adaptation. He sug-

gested, in line with some then-current notions of enzyme synthesis, that enzymes are formed from enzyme precursors, with which they exist in equilibrium in the cell. Yudkin supposed, furthermore, that in the case of constitutive enzymes, this equilibrium favors the enzyme and that in the case of adaptive enzymes, the equilibrium favors the precursor. Combination of the adaptive enzyme with its substrate, however, displaces the equilibrium to lead to enzyme accumulation.

Yudkin's review opened the modern era of the study of adaptive enzymes, even though later the theory he proposed turned out to be wrong. In 1946, Jacques Monod began work on the adaptive formation of the enzyme β-galactosidase of *E. coli*, the lactase by means of which bacteria split lactose into galactose and glucose (Figure 1). This enzyme hydrolyzes lactose, its presumed "natural" substrate, as well as a rather wide range of other β-galactoside compounds. From 1946 to 1956, the studies of Monod and his collaborators on the formation of β-galactosidase brought into focus and defined the nature of enzyme adaptation. In a 1953 manifesto signed by its then-leading students,[4] enzyme adaptation was rechristened "enzyme induction."

Study of the nature of the inducers to whose presence *E. coli* responds with the synthesis of β-galactosidase showed that some β-galactosides are inducers without being substrates of the enzyme, whereas others are substrates without being inducers. This finding led to the conclusion that action of the enzyme on the inducer is neither necessary nor sufficient to induce enzyme synthesis. In-

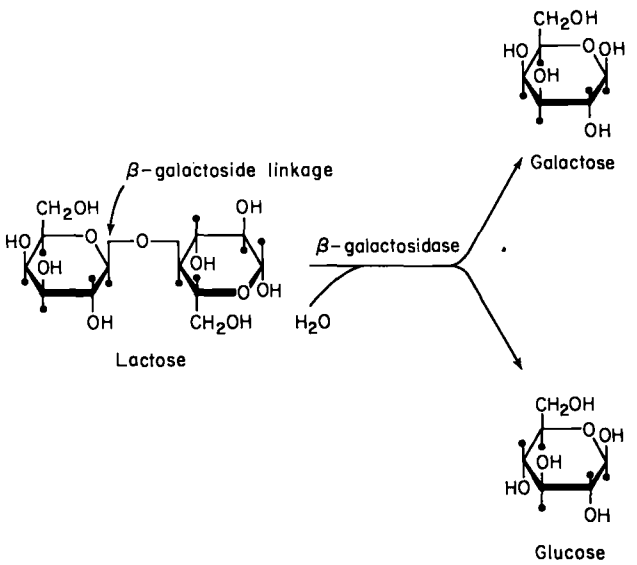


FIGURE 1 The hydrolysis of the sugar, lactose, catalyzed by the enzyme β-galactosidase.

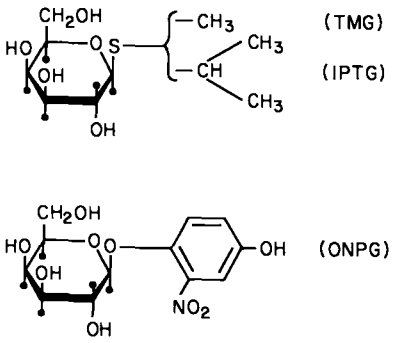


FIGURE 2 Structure of two non-metabolizable inducers of β-galactosidase, thiomethyl galactoside (TMG) and isopropyl thiogalactoside (IPTG), and of the chromogenic enzyme substrate o-nitrophenyl galactoside (ONPG) used in the enzyme assay.

deed, this first suggested (although it did not rigorously prove) that, contrary to the proposal of Yudkin, the enzyme is not the "target" of its inducer in the induction process. The most important practical consequence of these studies, however, was the discovery that nonmetabolizable sulfur analogs of ordinary β-galactosides, including methyl- and isopropylthiogalactoside, are inducers of high potency (Figure 2). Such sulfur analogs made possible a meaningful study of the true kinetics of the induction process under gratuitous conditions in media containing a carbon-energy source (such as glycerol or succinate), in which bacterial growth does not depend on the level of β-galactosidase that they may happen to contain, and inducer concentration remains invariant. It was found that under gratuitous conditions the cells start forming β-galactosidase at a constant and maximal rate within a few minutes after a saturating amount of inducer has been added to the growth medium. That is, $\Delta Z/\Delta B = p$, where Z is the amount of enzyme and B the bacterial protein in the culture. As soon as the inducer is removed from the medium, p falls to a value less than a thousandth of that when a maximal inducer concentration is present. Thus it became apparent that during bacterial growth, a constant proportion p of new protoplasm is β-galactosidase, the value p depending on the inducer concentration in the growth medium[5] (Figure 3). Once purification and isolation of β-galactosidase had shown it to be a rather large protein with an approximate molecular weight of 800,000 and its specific enzymatic activity per milligram protein was established, Z and B could be expressed in the same units, and p became the fraction of total cellular protein synthesis devoted to the manufacture of β-galactosidase. As seen in Figure 3, p has the value of 0.066 in the presence of maximal inducer concentration; that is, in maximally

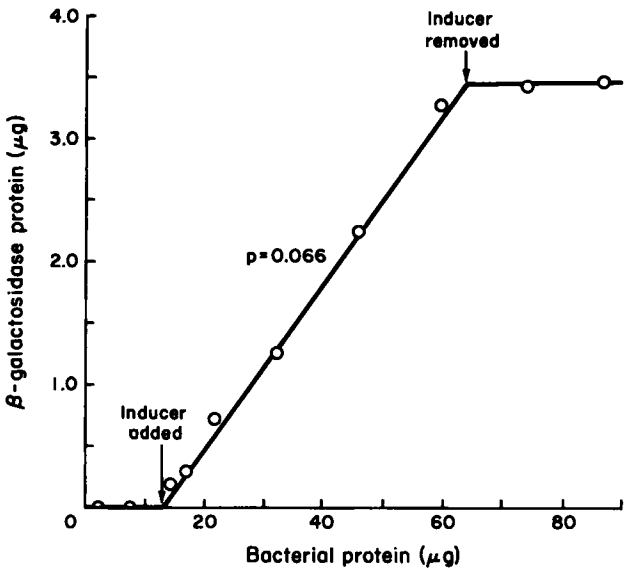


FIGURE 3 Induction kinetics of β-galactosidase under gratuitous conditions. The parameter *p* expresses the ratio of the increments of enzyme and total protein in the culture. (From Monod, et al., Note 5)

induced cells, 6.6 per cent of all protein manufactured is the one enzyme, β-galactosidase.

The induction kinetics seen in Figure 3 strongly suggested that the induced appearance of β-galactosidase represents its *de novo* synthesis, rather than a substrate-induced conversion of any pre-existing intracellular enzyme precursor. To prove this point, an earlier isotope tracer experiment[7] that had finally ended precursor-conversion notions in the domain of bacterial virus multiplication, was adapted to the problem of enzyme induction[6] (Figure 4). For this purpose, *E. coli* cells were grown in a medium supplemented with radiosulfur ^{35}S and devoid of any galactoside inducer. The radioactive bacteria were then switched to a nonradioactive medium lacking ^{35}S but containing an inducer, in order to initiate β-galactosidase formation. Isolation and purification of the β-galactosidase from these bacteria revealed that the enzyme formed under this labeling program contained no ^{35}S. Hence, the enzyme cannot be derived from a protein precursor that existed in the cell prior to the addition of inducer, because the amino acids cysteine and methionine in the precursor protein would have contained radiosulfur. Thus the inducer appears to instruct the cell to start assembling the β-galactosidase polypeptide directly from amino acid building blocks, and it seemed clear that the explanation of this mechanism should provide important clues for understanding protein synthesis.

Not long after it was found that induced formation of β-galactosidase represents a *de novo* protein synthesis, it was discovered that the activity of this enzyme, although necessary, is not sufficient for utilization of lactose by intact *E. coli* cells. A second enzymatic activity, quite distinct from hydrolysis of the galactoside, was found to exist. This activity allows the lactose to enter the cell.[8] Formation of the enzyme responsible for this activity (*galactoside permease*) is also inducible, because the presence of galactoside inducers is required for its synthesis. Thus *E. coli* grown in the absence of lactose are not only unable to ferment it, but until synthesis of an adequate permease level has been induced, they do not take up lactose from the medium. Some years after the discovery of the permease, a third distinct enzymatic activity pertaining to lactose metabolism in *E. coli* was identified. The responsible enzyme, galactoside transacetylase, catalyzes the transfer of an acetyl group from acetyl coenzyme A to the galactose moiety of β-galactoside.[9] Like synthesis of galactosidase and permease, synthesis of the transacetylase enzyme proceeds only in the presence of β-galactoside inducers. What physiological role, if any, is actually played by the transacetylase is not known, except that its presence is not required for apparently normal lactose fermentation.[10]

Genetic control of enzyme formation

Not long after Monod began his studies on the physiology of lactose fermentation in *E. coli*, J. Lederberg started to work out its genetic basis.[11] By 1948, Lederberg had iso-

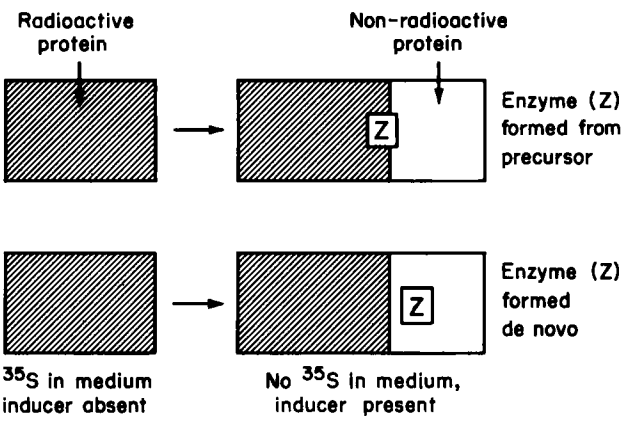


FIGURE 4 Schematic representation of the radiosulfur ^{35}S transfer experiment. The rectangles represent the mass of bacterial protein, which has increased by two-thirds after removal of ^{35}S from and addition of inducer to the medium. The enzyme (z), represented by the small squares, appearing upon induction should be mainly radioactive if it were derived from pre-existing precursors. It should be entirely non-radioactive if it were formed *de novo*.

lated a collection of lactose-negative (Lac^-) mutants of *E. coli*, which are unable to ferment lactose. Further work on these mutants showed that some of them owe their Lac^- character to an inability to produce β-galactosidase. (In order to test for the presence of the enzyme in these experiments, Lederberg invented an ingenious assay method that uses the colorless lactose analog o-nitrophenyl-β-galactoside [Figure 2] as substrate for β-galactosidase. Hydrolysis of this galactoside yields the intensely yellow o-nitrophenol, whose color attests to presence of the enzyme.) In succeeding years, more and more Lac^- mutants were isolated, characterized, and located on the bacterial genetic map by crossing experiments. The physiological basis of some of these mutants appeared to be complicated, whereas that of others was rather simple. The simplest were of two types: $z^-y^+a^+$ mutants that lack galactosidase (z) but possess permease (y) and transacetylase (a); and $z^+y^-a^+$ mutants that lack permease but possess galactosidase and transacetylase. (A z^+y^- mutant lacking permease is called *cryptic*, because its galactosidase activity is not shown by the intact cell and becomes manifest only in a cell extract.) On the basis of these mutant types and other genetic findings, it was possible to identify three closely linked and probably contiguous Lac genes on the *E. coli* chromosome (Figure 5): the galactosidase gene z, the permease gene y, and the transacetylase gene a. There is now little doubt that these three genes specify the primary structure of three enzyme proteins.

Among Lederberg's mutants there also was one in which formation of galactosidase did not depend on the presence of inducer: this constitutive mutant synthesized the enzyme in the absence of lactose or other galactosides.[12] Such constitutive mutants are now called i^-, in contradistinction to the inducible i^+ wild type. Later studies showed that i^- mutants are constitutive for synthesis of their permease and transacetylase as well as for their galactosidase, i.e., the mutation from i^+ to i^- has altered the functional regulation of z, y, and a genes.[13] Genetic mapping of various i^- mutations showed that they pertain to a separate gene, the i regulator gene, closely linked to, but distinct from, the other three Lac genes (Figure 5).

The repressor

How is it that constitutive i^- mutants synthesize their Lac enzymes in the absence of any exogenous inducer? One obvious possibility seemed to be that the mutation from i^+ to i^- leads to the formation of an internal inducer that allows the cells to synthesize their Lac enzymes without externally added galactosides.[14] Search for such internal inducers in constitutive mutants was, however, without success. When, in the late 1950's, the work of Wollman,

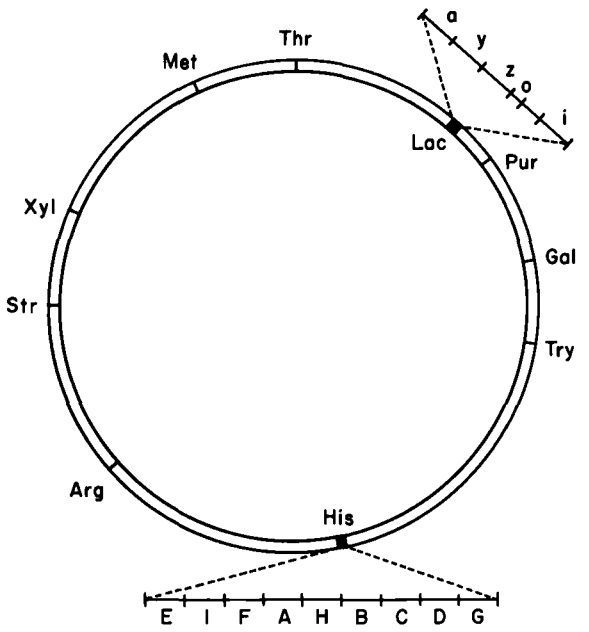


FIGURE 5 The circular chromosome (or genetic map) of the bacterial species *Escherichia coli* and *Salmonella typhimurium*. The symbols refer to some genes concerned with synthesis of amino acids and purines, fermentation of sugars, and resistance to an antibiotic. The Lac region is expanded to show the relative situation of the closely linked genes a, y, z, and i controlling respectively the formation of galactosidase, permease, acetylase and repressor, and of the operator gene o. The His region is expanded to show the relative situation of the genes lettered A–I, which control the formation of the enzymes that catalyze the ten successive reaction steps in the biosynthesis of the amino acid, histidine.

Jacob, and Hayes[15] revealed that in bacterial conjugation the DNA of a male donor bacterium migrates into the female recipient cell to form a transient merozygote endowed with the female cytoplasm, an experiment became possible whose result, rightly or wrongly, led to the abandonment of the internal inducer idea. In this experiment, Pardee, Jacob, and Monod[16] mated normal i^+z^+ male bacteria grown in the absence of any inducer to i^-z^- female bacteria in an inducer-free medium and followed the appearance of galactosidase activity in the culture. The result of this experiment is shown in Figure 6. Prior to mating, there is no galactosidase in the culture, because the inducible i^+z^+ male bacteria have not been exposed to inducer and the constitutive i^-z^- female bacteria cannot form the enzyme at all. After about one hour of conjugation, however, by which time the DNA bearing the i^+z^+ Lac genes of the male donor bacterium has entered the female cytoplasm, galactosidase synthesis begins and proceeds for about an hour, after which it stops. This experiment showed that the merozygote formed upon entrance

of the i^+z^+ donor genes into the i^- recipient cytoplasm acts at first as a constitutive cell, since its z^+ gene forms galactosidase in the absence of inducer. But as soon as the i^+ gene has had an opportunity to express itself, constitutive function of the z^+ gene ceases, and the zygote is converted to the inducible state, which requires the presence of inducer for further galactosidase synthesis. Hence, the i gene of the inducible i^+ wild type appears to give rise to a cytoplasmic product, the repressor, which exerts a negative effect on Lac enzyme synthesis, i.e., "inhibits information transfer from structural gene (or genes) to protein."[16] Galactoside inducers then exert their effect by neutralization, or inactivation, of this repressor, and i^- constitutive mutants can dispense with external inducers—not because they possess internal inducers, but because they lack an active repressor.

Relatively little progress was made in identifying the i gene product, or repressor, for many years following the first postulation of its existence, although some further information concerning its nature did emerge from the ensemble of known i gene mutant phenotypes. Both temperature-sensitive[18] and amber-suppressor sensitive[19] mutations in the i gene were found—i.e., mutants whose character is constitutive (i^-) at temperatures higher than 37° or in the absence of an amber, or nonsense, suppressor gene in their genetic background and whose phenotype is inducible (i^+) at low temperatures or in the presence of a suppressor gene. Therefore it seemed most plausible that the i gene product was a protein. In addition, other i gene mutants, called i^s, were found that need much higher inducer concentrations for enzyme induction than does the i^+ wild type (or cannot be induced at all and hence appear as Lac^- phenotypes). Because of this and because the thermal stability of the repressor in yet other i mutants is raised by the presence of inducer,[21] it could reasonably be inferred that the i gene product, as originally postulated, does interact with the inducer. Other inferences, such as that the i gene product is metabolically unstable or that it combines with a nucleic acid component to constitute the active repressor, still seem less well established. In any case, Gilbert and Müller-Hill[22] have recently reported the first successful in vitro identification of the i gene product by assaying for its binding of radioactive inducers. Further progress in clarifying the nature of the repressor can now be expected.

Operator and operon

Granted that the i gene repressor inhibits information transfer unless neutralized by a galactoside inducer, what, then, is the target in the cell toward which the repressor directs its negative role? In reply to this question, Jacob and Monod made a most important doctrinal advance in their now classical 1961 review[17]: they proposed that the three closely linked Lac genes z, y, and a, which are subject to coordinate control by the i gene product, form an operon, in that they share a second gene of regulation, their operator, or o, as shown in Figure 7. The operator, whose genetic site forms one extremity of the operon linkage group, is the target of the repressor. The operator can exist in two states, open and closed. It is open as long as it is free of repressor and it is closed as soon as it has combined with the repressor (the repressor, for its part, can combine with the operator only as long as it has not interacted with inducer). Closing of the operator prevents transcription of messenger RNA of all genes of the operon, and, *a fortiori,* synthesis of any of the corresponding enzyme proteins.

Taking the protein nature of the repressor for granted, Jacob and Monod[23] then showed how the effect of its interaction with the inducer and its interaction with the operator can be understood. They proposed that the repressor is an allosteric protein that possesses two specific sites. One of these sites is affined to all, or part, of the exact nucleotide sequence of the operator gene, cognate to the repressor, and the other site is affined to the cognate inducer molecule. Combination of repressor and inducer at the second allosteric site reduces the operator affinity of the first site, and thus frees the operator from its embrace by the repressor.

Jacob and Monod were able to adduce convincing genetic proof of their topological notions of the operon by isolating constitutive mutants that evidently carry mutations in the operator locus. For this purpose, they had constructed a variety of partial diploid bacteria that carried two sets of Lac genes in their genome, one set on the normal chromosome and another set on the sex factor F. Table I presents a comparison of induced and noninduced levels of galactosidase and transacetylase in a variety of diploids with the corresponding levels in relevant haploid genotypes. Rows 1 and 2 show induced and noninduced levels of the two enzymes in haploid bacteria of $i^+z^+a^+$ inducible and $i^-z^+a^+$ constitutive genotypes. It can be seen that in the inducible strain the presence of inducer raises the level of galactosidase a thousandfold and the level of transacetylase a hundredfold, whereas in the constitutive i^- strain both enzymes achieve high levels in the absence of any inducer. Row 3 confirms the inference made from the experiment with transient zygotes (Figure 6) that i^+ produces an active repressor, since the $i^+/F\ i^-$ heterozygous diploid is of inducible, and not constitutive, phenotype. (The higher induced levels of both enzymes in the diploid bacterium reflect a gene dosage effect, because, compared to haploid, the partial diploid carries twice as many z and a genes relative to the rest of its genome.)

TABLE I

Relative levels of galactosidase and galactoside transacetylase in various E. coli *genotypes (Modified from Jacob and Monod[16])*

Genotype	Galactosidase(z)		Galactoside-transacetylase(a)	
	noninduced	induced	noninduced	induced
1) $i^+z^+a^+$	0.1	100	1	100
2) $i^-z^+a^+$	100	100	90	90
3) $i^+z^-a^+/F\ i^+z^+a^+$	1	240	1	270
4) $i^sz^+a^+$	0.1	1	1	1
5) $i^sz^+a^+/F\ i^+z^+a^+$	0.1	2	1	3
6) $o^cz^+a^+$	25	95	15	100
7) $o^+z^-a^+/F\ o^cz^+a^-$	180	440	1	220
8) $i^so^+z^+a^+/F\ i^+o^cz^+a^+$	190	210	150	200

Rows 4 and 5 show that the i^s genotype carries a mutant repressor of greatly reduced affinity for the inducer galactoside; the $i^s/F\ i^+$ diploid attains only very low levels of both enzymes upon induction. Rows 6 and 7 present the enzyme levels in a new kind of mutant, o^c, or *operator-constitutive*. In a haploid, the o^c mutant is only partly constitutive, in that only part of the maximal enzyme levels are achieved in the absence of inducer. In a heterozygous diploid, however, the effect of the o^c mutation is evidently confined to the expression of the genes coresident with it on the F sex factor. Synthesis of galactosidase by the z^+ gene carried by the sex factor is partially constitutive, whereas synthesis of transacetylase by the a^+ gene carried by the o^+ bacterial chromosome remains inducible. (The a^- mutant gene of the o^c sex factor bears a mutation in the transacetylase structure, and hence cannot contribute to synthesis of that enzyme under any conditions.) Finally, Row 8 shows that o^c appears to correspond to a mutation of the operator gene that has reduced its sensitivity to closure by the repressor, because synthesis of the enzymes in the i^so^+/i^+o^c diploid is not impeded by the presence of the mutant i^c repressor. The behavior of the ensemble of genotypes of Table I is thus in full accord with that to be expected from the model in Figure 7.

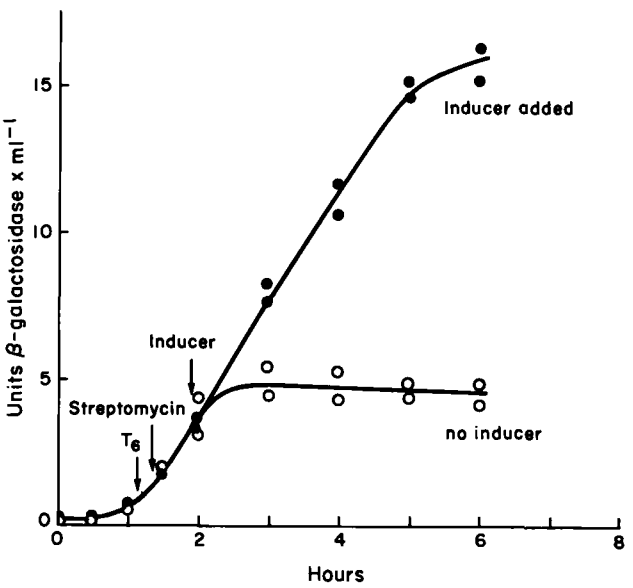


FIGURE 6 Appearance of β-galactosidase in zygotes formed by conjugation of i^+z^+ male bacteria with i^-z^- female bacteria, in the absence of inducer (open circles). The curve labeled "inducer added" (closed circles), and the arrows (indicating the times at which T6 bacteriophage, streptomycin, and inducer are added to the culture), refer to a control part of this experiment. This demonstrates that in the presence of inducer these zygotes can continue the synthesis of β-galactosidase after enzyme synthesis in the absence of inducer has come to a halt. (From Pardee, et al., Note 16)

Further studies on the nature of o^c mutants showed that they correspond to deletions of genetic material lying between the i and z genes. This finding agrees with the idea that the repressor carries an affinity for the nucleotide sequence of the o gene,[24,25] because genetic deletion of that sequence would evidently remove the repressor target from the operon and render function of its genes constitutive. Another dramatic demonstration of the justice of Jacob and Monod's general topological considerations of operon structure was provided by the more recent finding that very long deletions of o^c type, which remove all genetic material between the z gene and the Pur gene concerned with purine synthesis (Figure 5), not only free the Lac operon genes from control by galactoside inducers but also place their expression under the control of metabolites in the pathway of purine biosynthesis.[26]

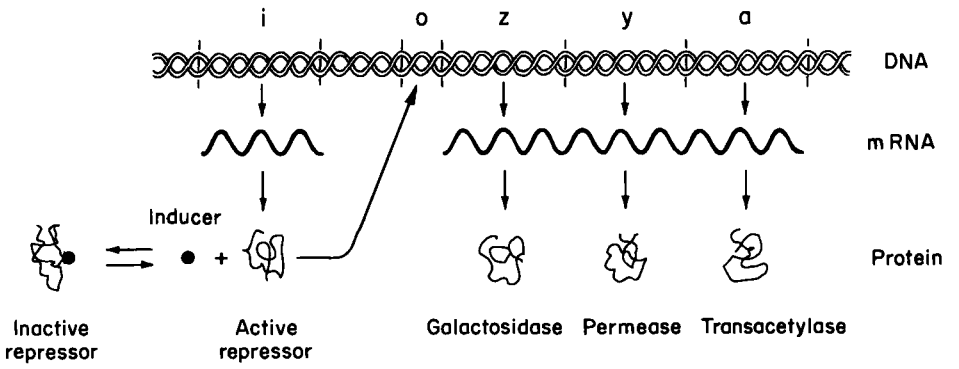


FIGURE 7 The original operon model of Jacob and Monod,[17] as applied to the Lac genes of *E. coli*.

Control of messenger RNA synthesis

In addition to explaining an ensemble of facts known at the time of its formulation, the operon model shown in Figure 7 made an important prediction: the presence of galactoside inducers should raise not only the level of Lac enzymes in the cell but also the level of specific messenger RNA molecules homologous to the DNA of the Lac operon. Experimental tests of this prediction became possible when it was found that messenger RNA may form specific molecular hybrids with its homologous DNA when mixtures of the two polymers are heated and then slowly cooled.[27] Such hybrids provide a means by which the amount of messenger RNA corresponding to a particular gene can be specifically assayed in the presence of the myriad other messenger RNA species that may exist in the same cell. It was shown by means of this assay that in the presence of galactosides, i+ bacteria contain a much greater quantity of messenger RNA capable of forming hybrids with the DNA sector representing the Lac operon than in their absence.[28,29] This result appeared to prove that the primary action of the repressor really does concern the inhibition of messenger RNA formation, i.e., *regulation is achieved at the level of transcription.* In their 1961 review, Jacob and Monod had also considered the possibility that the operator might be transcribed onto messenger RNA and recognized as a repressor target in its ribopolynucleotide rather than its deoxyribopolynucleotide guise. In this case, combination of repressor and operator would have prevented the *translation* in protein synthesis of the already synthesized messenger, rather than its transcription. This alternative, which would have predicted the same levels of Lac messenger RNA whether inducer is present or not, now no longer seemed worthy of serious consideration.

However, these measurements of messenger RNA level contained a sleeper that eventually demanded a reappraisal of the meaning of the results. In 1961, Jacob and Monod had reported the discovery of *operator negative,* or o^0, mutations, which abolish expression of all three genes of the Lac operon. These mutations, like o^c operator-constitutives, cluster at the i proximal extremity of the Lac operon and, in heterozygous diploids, confine their effect to genes coresident with them on the same genetic structure. Therefore it seemed reasonable to suppose that these o^0 mutations exert their effect by permanently closing the operator gene. And just as predicted by this notion, the messenger assay experiments showed that o^0 mutant bacteria do not contain detectable quantities of Lac messenger RNA, even in the presence of inducing levels of galactosides. More detailed studies of these o^0 mutants later showed, however, that they do not carry any operator mutations at all. Instead, the o^0 mutations map within the z gene and are highly polar amber, or nonsense, mutations at whose site synthesis of the galactosidase polypeptide is precociously terminated. The Lac^- phenotype of these o^0 mutants can be suppressed, and full or partial function restored to the Lac operon by amber suppressor genes whose effect is certainly at the level of messenger translation.[30] Thus it must be concluded that, appearances to the contrary, the o^0 mutation does not present a permanent block to transcription of the Lac genes.

Translational control of messenger RNA levels

How, then, is one to reconcile the inference that o^0 mutants can synthesize Lac messenger RNA with the failure of the direct chemical tests to show its presence? One by no means unreasonable resolution of this quandary would be to suppose that untranslatable messenger RNA molecules carrying a nonsense mutation are so rapidly destroyed in the cell that they escape detection in the DNA–RNA

hybrid test.[28,30] Another possible explanation would be that there exists a feedback connection between messenger synthesis and function: messenger might be synthesized on the DNA template only as long as it can work in protein synthesis.[31] This latter hypothesis is supported by the finding that RNA is formed by the in vitro action of RNA polymerase at a chain growth rate about two orders of magnitude slower than its in vivo rate and, furthermore, does not spontaneously dissociate from its DNA template.[32,33] Hence it would appear that, in vivo, an active process, like the movement of ribosomes over the nascent messenger, is required for high-speed function of the polymerase enzyme and for liberation of the messenger RNA from its parent operon, as shown in Figure 8. The model shown in this figure, in which translation of, and polyribosome formation on, the nascent messenger begins before its synthesis is complete, requires that the chemical direction of messenger synthesis be the same as the direction of its translation. In fact, this requirement is met, since both processes are now known to proceed from the 5′ terminus of the RNA molecule to its 3′ end.[33] Thus, by precociously terminating translation of the z gene protein, o^0 amber mutations in the z gene would also bring to an untimely end transcription of the remainder of the Lac operon messenger and hence prevent expression of the distal y and a genes.

The possibility of a translational control of the intracellular level of messenger RNA prompted a re-examination of the question whether the primary action of the repressor is, after all, to be explained by an inhibition of transcription by operator closure. It now became once more permissible to think that the primary action of the repressor could be the inhibition of operon translation and that the increase in Lac operon messenger observed after addition of galactoside inducers is merely an epiphenomenon of an increase in translatability of the messenger engendered by neutralization of the repressor.

Repression of enzyme synthesis

Positive enzymatic adaptation, i.e., the induction of synthesis of an enzyme by the presence of its substrate, had been known for more than half a century when, in 1953, *negative* enzymatic adaption was discovered, i.e., the inhibition of synthesis of an enzyme by the presence of the product of the reaction it catalyzes. The first such case was the finding by Monod and Cohen-Bazire[34] that in *E. coli* tryptophan and some of its structural analogs inhibit the synthesis of tryptophan synthetase, the last enzyme of the biosynthetic pathway of that amino acid. Within the next few years other examples of negative adaptation were uncovered, many of them concerning anabolic enzymes of pathways of amino acid, purine, and pyrimidine biosynthesis. This phenomenon was given the name enzyme repression,[35] as distinctive from enzyme induction.

Today, one of the most intensively studied examples

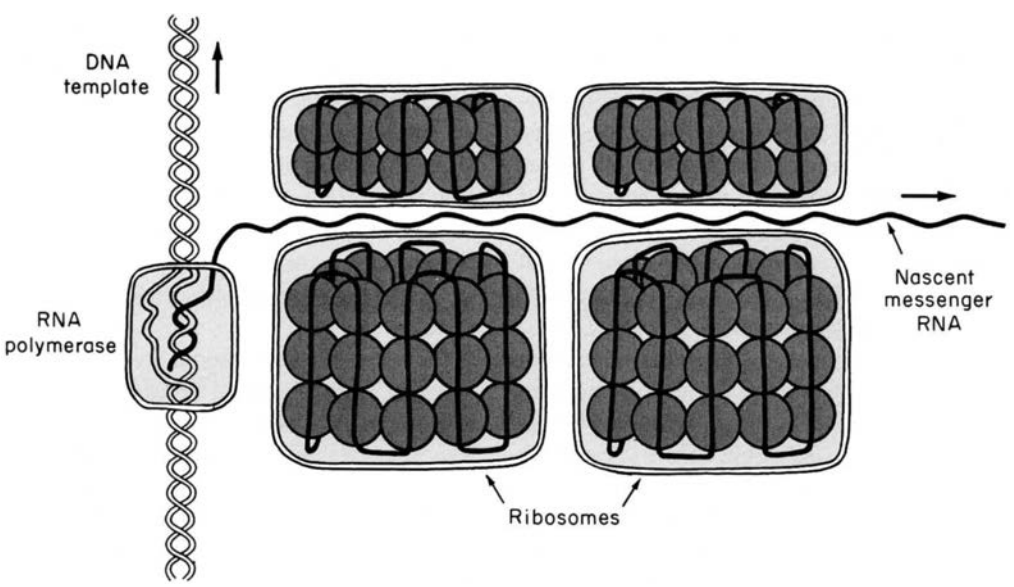
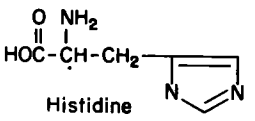


FIGURE 8 Schematic representation of the formation of polyribosomes on nascent messenger RNA and of the active removal of messenger RNA from its DNA template by protein synthesis. (From Stent, Note 33)

of enzyme repression is that of the ensemble of enzymes of *Salmonella typhimurium*, which, in ten successive biosynthetic steps, converts phosphoribosyl pyrophosphate and adenosine triphosphate into the amino acid, histidine.



The enzymes and biochemical intermediates of this pathway were characterized by Ames, Hartman, and their collaborators. These workers also isolated more than a thousand mutants of *S. typhimurium* that require histidine in order to determine the nature of their enzymatic deficiencies and to map the chromosomal location of their mutant sites.[36,37] The result of these genetic studies is shown in Figure 5. It can be seen there that the His genes (designated by the letters A to I) coding for the enzymes catalyzing the individual reaction steps of histidine synthesis cluster in one small region of the chromosome. Ames and Garry[38] discovered that the synthesis of the enzymes of this pathway is repressed by histidine. That is, bacteria growing in conditions under which their intracellular histidine concentration is very low contain levels of histidine enzymes that can be 25-fold greater than the corresponding levels present at very high histidine concentrations. The control of production of these enzymes is strictly coordinate, in that the ratio of the activity of one enzyme to that of another always remains constant, even though, depending on the growth conditions, the amount of each enzyme can vary over a wide range.

Since the coordinate repression of the histidine enzymes by histidine appeared to be formally analogous to the joint induction of the three lactose enzymes by galactosides, Jacob and Monod[17] proposed that an ensemble of closely linked genes for amino acid biosynthesis also constitutes an operon. The only modification of their model of Figure 7 necessary to explain enzyme repression was to suppose that in the case of a repressible operon the spontaneous operator-affinity of the repressor is low and becomes high only after combination of the amino acid with the second allosteric site. In other words, the repressor of repressible enzymes requires activation before it can close its cognate operator gene. This concept received further support from the isolation of nonrepressible *S. typhimurium* mutants that produce maximal levels of the histidine enzymes even in the presence of high histidine concentrations, i.e., mutants that are the formal analogs of constitutive mutants of the Lac operon. These nonrepressible mutants, furthermore, fall into at least two classes. The mutant sites of one class are found in a sector of the chromosome far from the histidine region and can be presumed to be mutations in the regulator gene coding for the repressor (i.e., to be analogs of i^- mutants). The mutant sites of another class map within the histidine region, at one of its extremities just outside the G gene controlling the first reaction step (Figure 5), can thus be presumed to be mutations of the operator gene (i.e., to be analogs of o^c mutants).

Transfer RNA and regulation

It was thus concluded that enzyme induction and enzyme repression are but opposite sides of the same coin, the main difference between the two phenomena apparently residing in the nature of the repressor. However, further study of amino acid repression led to the surprising discovery that for amino acids to repress formation of their biosynthetic enzymes, they must first be attached to their cognate transfer-RNA molecules. For, in the case of the histidine operon, one of the classes of nonrepressible (i.e., constitutive) bacterial mutants previously thought to contain defective repressor (i.e., to be analogs of i^-) turned out to contain a defective histidine-activating enzyme.[39] Furthermore, mutant bacteria known to carry a heat-sensitive activating enzyme for the amino acid valine turned out to become derepressed for valine biosynthesis enzymes at high temperatures.[40] Of course, this might mean merely that the allosteric repressor is activated, not by the free amino acid, but by the corresponding aminoacyl-transfer RNA. However, this would seem a strange way for the cell to do business, as one might have thought that the biosynthetic control system would be designed to allow complete aminoacylation of every transfer-RNA species while holding the pool of endogenously synthesized amino acids to a minimum. Thus the idea insinuates itself that perhaps here a control system somewhat different from that envisaged by Jacob and Monod is operative—one in which, as first proposed by Ames and Hartman in 1963,[37] special species of transfer-RNA molecules regulate the rate of enzyme synthesis at the level of messenger RNA translation.

One conceivable scheme by which repression of the histidine enzymes could be envisaged is that the nucleotide sequence in the peptide-chain-initial portion of the first gene of the histidine operon contains a special codon (base-pair triplet) that speaks to a special species of transfer RNA. This transfer RNA would be "special" in that at low intracellular histidine concentrations it is aminoacylated with some amino acid other than histidine through action of the normal activating enzyme of that amino acid. Under these conditions, the first gene is translatable and the entire histidine operon is derepressed. At high intracellular histidine concentrations, however, the special

class of transfer RNA is aminoacylated preferentially with histidine through action of the normal histidine-activating enzyme. Once attached to that special class of transfer RNA, the histidine molecule would be subject to modification by a "repressor" enzyme that converts histidine into a peptide chain terminator, e.g., by esterification of its α-amino group. Under these conditions, the first gene is not translatable beyond the special codon and, by virtue of the polarity effect of nonsense, or chain-terminating, codons, expression of the entire operon is repressed.

How can the special nature of this facultative chain-terminating codon be recognized by its cognate special transfer RNA? It is, of course, possible, although perhaps not too plausible, that of the 64 possible triplet codons one triplet is wholly consecrated to the regulation of each independently repressible amino acid operon. A second, somewhat more plausible, possibility is that the codon is recognized as being special by its singular location within the nucleotide sequence of the first gene of the operon. For instance, according to a suggestion once made by Bruce Ames, the special codons might be so situated in the message that because of steric restrictions imposed by the transfer RNA molecule called for by the *preceding* "normal" codon, only the special, but not the normal, transfer-RNA species could fit into the ribosomal site of the next "special" codon. That some kinds of neighbor interactions between successive transfer-RNA molecules do seem to exist in the messenger decoding process is indicated by the apparent inability of the "special" formylmethionyl peptide-chain-initiator transfer-RNA molecules to insert methionine into the body of polypeptide chains, as if that transfer-RNA species could not tolerate being the successor of any other transfer-RNA molecule in the ribosomal amino acid assembly line.[41]

What becomes of repressor and operator under this view of enzyme regulation? Evidently, there would be no operator at all in the strict sense of the model of Jacob and Monod, since the target of the repressor is not any segment of the DNA, or even any part of its RNA transcript, but a species of transfer RNA. Instead, the genetic region identified with the operator on the basis of constitutive, or nonrepressible, o^c mutations would correspond to the domain of the special codon (or codons) whose mutational deletion from the genetic message would remove the facultative obstacle to translation and consequently to expression of the operon. It should be noted that this particular model of control of enzyme synthesis at the level of messenger RNA translation predicts the modification of some amino acids bound to transfer RNA and the synthesis of many abortive short peptide chains by operons that happen to be repressed. In accord with this prediction it has been found recently[42] that a significant fraction of *E. coli* transfer-RNA molecules is esterified in vivo to substances other than their homologous, free amino acids. Among these substances are not only the peptide chain initiating compound formylmethionine, but also other amino acid derivatives and short oligopeptides.

Summary

Whether or not Jacob and Monod's original notions of repressor and operator, and the direct control of messenger formation ultimately turn out to be correct, their operon concept is likely to remain one of the landmarks in the landscape of molecular biology. But what is the possible relevance of such control mechanisms of protein synthesis to the neurobiological problems to which these articles are consecrated? As it does to many other people, so it also does seem to me most unlikely that such macromolecules as nucleic acids and proteins, or even their supramolecular ensembles, could serve as memory tapes of readily retrievable information acquired by the nervous system during the life of the individual. Instead, it seems rather more likely that this information is reflected in the state of the synapses of the neural network. Hence, learning would entail selective modification of particular synapses, so that signals arriving at the presynaptic ending of the modified synapse contribute in either greater or lesser degree to the firing of the recipient neurons. These putative modifications, facilitative or inhibitory, could very well involve synthesis of specific proteins whose formation is induced, either directly or indirectly, by arrival of the transmitter substance discharged by the presynaptic axon. Thus each use of a synapse might leave a proteinaceous trace in the neighborhood of its postsynaptic side that would alter the weight the recipient neuron would place on future signals it might receive through that channel. In that case, enzyme induction would account not only for the harmonious and economic function of bacterial cells and, as is often thought nowadays, for the basic mechanisms of cellular differentiation, but also, for good measure, for the molecular basis of memory.

The Recombination of DNA Molecules

C. A. THOMAS, JR.

FOR A NUMBER of years we have been working on the anatomy of DNA molecules liberated from various bacteriophage. These studies have involved some physical chemistry, a little genetics, and a fair amount of electron microscopy. At the first level, our conclusions and those of others are very simple: all of the larger bacteriophage (excepting only the very small ϕx and M13 phage) contain a single linear duplex DNA molecule. This same conclusion applies to the mammalian viruses—vaccinia, pseudorabies, and adeno—but excepts the small viruses, polyoma and papilloma, which contain circular duplex molecules. Among the bacteriophage DNA molecules, all of the species so far studied contain (largely) two uninterrupted polynucleotide chains. The exception to this rule is T5 DNA and certain related phage DNA molecules.[1] These molecules contain four nicks (or interruptions) at genetically determined positions along the molecule. This situation may reappear with certain of the *subtilus* bacteriophages.

While these exceptions interest us very much, they will only indirectly concern us here, as our attention will be centered on those species of the viral DNA molecules that contain continuous chains.

Now, these viral DNA molecules are more than discrete physicochemical entities; they are also discrete informational entities. Many of the bacteriophage show a very high specific infectivity, and because of this one may be sure that nearly every virus particle contains a DNA molecule that encodes the information necessary to make a new virus particle. This being the case, one would suppose that if we were endowed with atomic spectacles, the sequence of nucleotides (reading from left to right) would be exactly the same (neglecting copy errors) in each and every molecule. This seems to be the case with a number of species of phage DNA molecules; on the other hand, some species are circular permutations of a common sequence. Finally, all of the species of phage DNA molecules that we have studied begin and end with the same sequence of nucleotides: the genetic text is terminally repetitious. This simple idea is conveyed by the sentences at the top of the facing page.

C. A. THOMAS, JR. The Johns Hopkins University, Baltimore, Maryland

The genetic texts of some viral DNA molecules are nonpermuted (all the same) and others are permuted cyclically. Both types can be terminally repetitious.

I would like to describe these experiments for you—mainly because the kinds of interactions that are taking place are undoubtedly related to the interactions that are responsible for the recombination of DNA molecules, which is the physical basis of genetic recombination. Our interest in genetic recombination has come about through these molecular-recombination experiments. As we shall see, these experiments themselves are of some interest, but of equal importance are their *controls*.

The annealing of complementary polynucleotide chains to reform a duplex DNA molecule

The Watson–Crick duplex structure of DNA molecules needs no introduction. It has been properly said that the major part of modern molecular biology is merely working out the consequences of the model. When this duplex structure is exposed to elevated temperature or unfavorable solvents or *p*H values, the highly ordered duplex is disorganized and the two-component single chains completely separate from each other (except in those easily recognized cases in which the two chains are held together by an artificial crosslink). Once they have separated, they can maintain their separate kinetic individuality and can be examined by a variety of means. However, if the solvent conditions (temperature, *p*H, or the presence of anions like ClO_4) are adjusted so that the single chains expand and can interpenetrate each other, a surprising thing happens: duplex molecules are efficiently reformed, provided the sequence of the rejoining strands is complementary. This phenomenon, called "renaturation" (being the converse of "denaturation"), was first observed with bacterial DNAs by Marmur and Doty, who demonstrated the reformation of duplex structure and the recovery of transforming activity. The efficiency and specificity of this *annealing* process are truly astonishing. The single chains derived by denaturing a phage DNA will rapidly anneal even when incubated at 10^{-2} μg/ml for a brief period. Most aspects of the kinetics of annealing have not yet been thoroughly studied, and this high efficiency remains a puzzling phenomenon. In Figure 1, some of the possible salient features of the reaction are outlined.[2]

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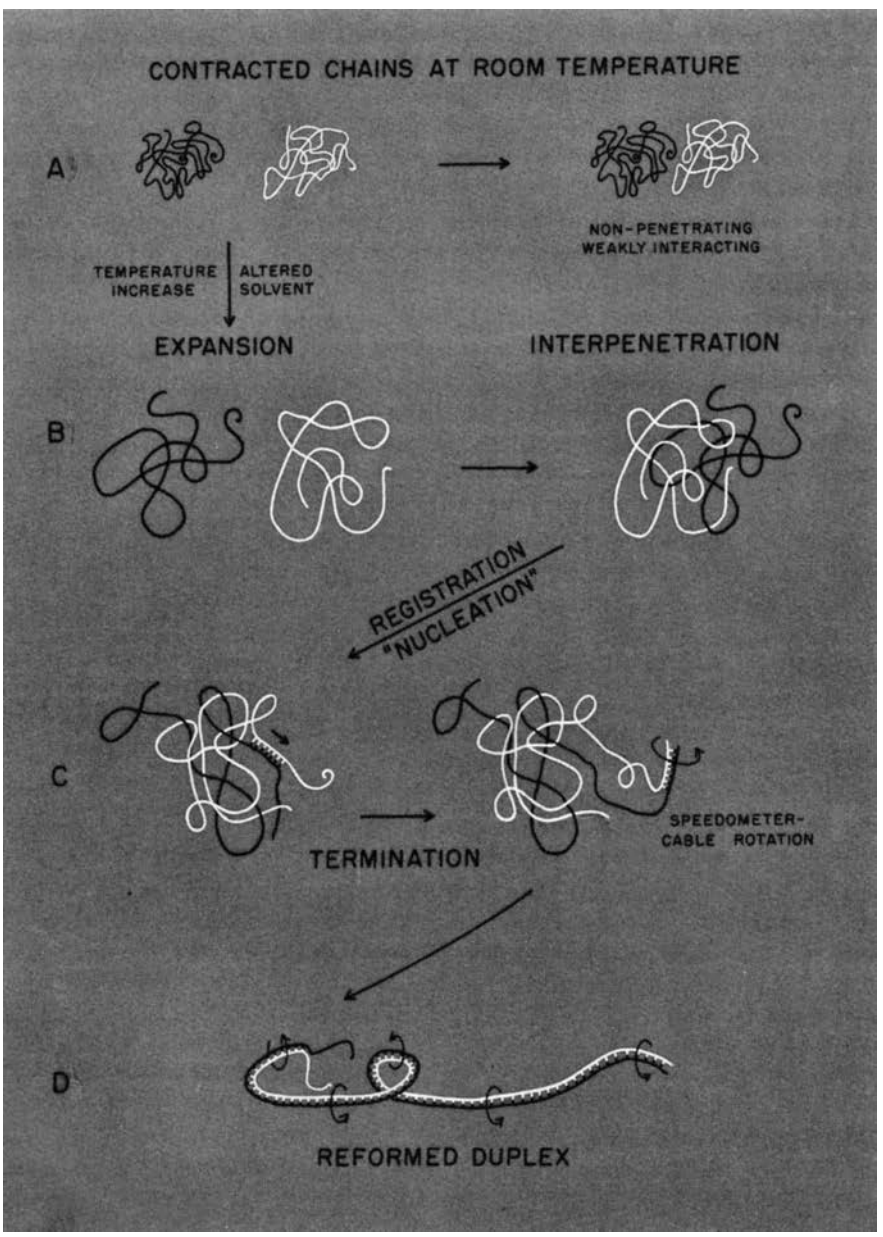


FIGURE 1 How complementary polynucleotide chains find each other. A. Contracted single chains are nonpenetrating and weakly interacting. B. Upon altering the solvent or temperature they expand and interpenetrate. C. Nucleation is accomplished by union of one pair of complementary regions. It could terminate as shown. D. The molecule is then free to "zip up" by speedometer-cable rotation.

NUCLEATION BY COOPERATION DNA molecules containing interchain crosslinks will rapidly reform the duplex molecule, so the rate-limiting step appears to be the establishment of such a "nucleus" by the reunion of two short complementary segments. This need only be 10 to 20 nucleotide pairs long, as this number is sufficient to form a duplex, the stability of which is nearly equal to the stability of a duplex of infinite length. Suppose that p is the probability that a given nucleotide, together with its context of 10 to 20 neighboring nucleotides, will collide with *any* other nucleotide on the complementary chain. If there are M nucleotides in both chains, the probability that this nucleotide will collide with its exact textuary complement will be $p(1/M)$. If this applies to one nucleotide, it must apply to all. Therefore, the probability that at least *one* of the M nucleotides will collide with its textuary complement is $p(1/M).M$ or p. The probability that one of the M nucleotides will collide with its complement is just equal to the probability that a given nucleotide will collide with any other on the complementary chain.

This is analogous to the well-known "birthday problem," in which one calculates the chance that any two people in a group of N individuals have a common birthday. The result is always astonishing: there is a better-than-even chance that two people will have the same birthday if there are only 23 individuals in the group. Perhaps we are surprised at the efficiency of annealing and the results of the "birthday problem" for essentially the same reason.

THE ANNEALING OF NON-PERMUTED SINGLE CHAINS FROM T3 AND T7 T3 and T7 DNA molecules are about 12.5 μ long and have a molecular weight of about 25×10^6. An example of a linear T7 DNA molecule (visualized by the method of Kleinschmidt[3]) is shown in Figure 9A. Their component single chains can be separated in strong alkali. Upon reneutralization and annealing, simple linear duplex DNA molecules are reformed. The contour length of these molecules is identical with that of untreated molecules.[4] The contour lengths of these molecules can be measured provided the magnification is known. The lengths of many molecules have been assembled into a histogram shown in Figure 2. The reformed molecules have a length distribution that is identical with untreated T7 DNA molecules. We interpret this according to the scheme shown in Figure 3, which shows simply the separation and reconstitution of linear duplex T7 DNA.

Circularly permuted phage DNAs

One might expect this simple situation to prevail for all phage DNA molecules. Such is not the case! For example, when native, linear P22 DNA is denatured and annealed, more than half the molecules have the structure shown in Figure 4.[5] Also, with native, linear T2 or T4 DNA one finds circular structures comprising 20 per cent of all molecules (Figure 5).[6] Our interpretation of these results is outlined in Figure 6. These molecules are shown as being permutations of each other; also each is terminally repetitious, as I will show later. These terminal repetitions are left out of the duplex and are visualized as two bushlike structures connected to the circular molecule. The spacing between these bushes gives us some information regarding the variety of permutations present.[7]

Now for the controls. We have fragmented T2 to various sizes and treated the fragments with alkali in parallel with those tubes containing unbroken molecules. No significant number of circles of any contour length were ever found among the fragments. Wherever circular molecules are found they always have a contour length nearly equal to that of the untreated linear molecules.

Before leaving this subject, let me add that physical permutation in T2 and T4 has now been established in two different ways.[6,8] These results are in exact accord with a model proposed by Streisinger and his collaborators to account for certain genetic results with this phage.[9] This agreement has been a gratifying example of mutual confirmation by genetic and physical experimentation.

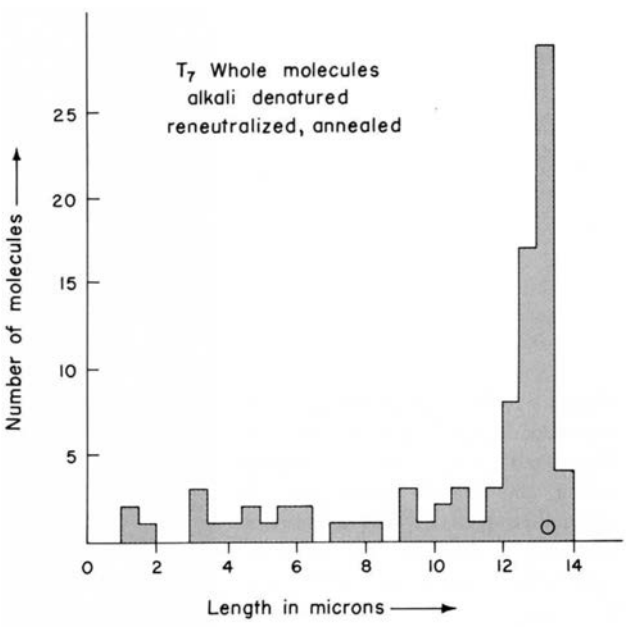


FIGURE 2 Histogram of molecular lengths of denatured and annealed T7 DNA molecules.

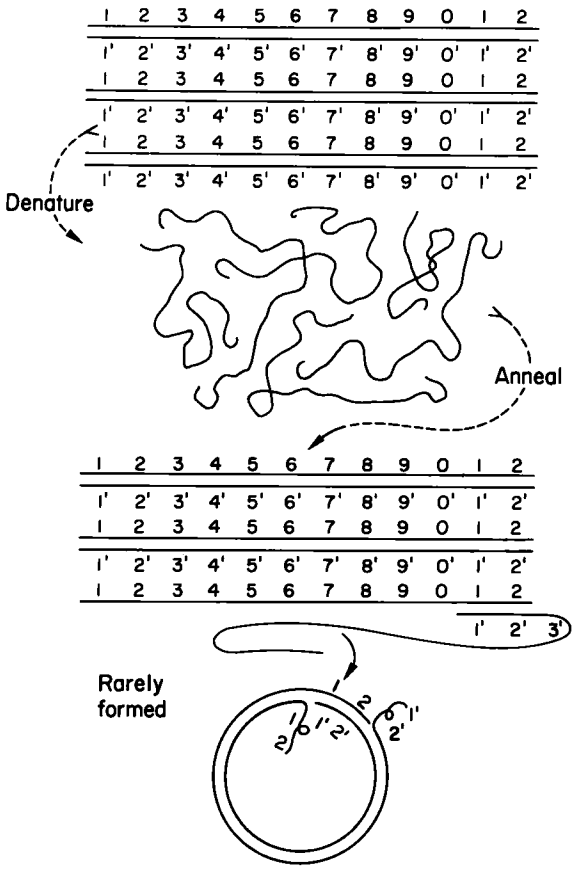


FIGURE 3 Denaturation and reformation of nonpermuted linear duplexes. Such a collection should yield nonpermuted single chains that would be expected to reunite to form linear molecules of the original length and sequence. A short terminal repetition could in theory lead to the production of a circular molecule. One was found in this experiment, but whether this arose by this mechanism or by the coincidental overlap of the simultaneously unwound ends of a linear molecule is not certain.

Terminal repetition

CIRCLES AND CONCATENATES As indicated in Figure 6, T2 DNA molecules are terminally repetitious, that is, the genetic text found at the beginning of the DNA duplex starts over again and runs for not more than 2 μ, where it is terminated by the physical end of the molecule. The evidence for this comes from experiments done by MacHattie et al. at The Johns Hopkins.[7] Charles Richardson, the discoverer of exonuclease III, gave us this enzyme and showed us how to use it.

The experimental scheme is shown in Figure 7. Exonuclease III has unusual specificity in that it removes nucleotides one at a time from the 3'-ended polynucleotide chain, provided it is in a duplex DNA molecule. Its action, therefore, is just the reverse of the normal DNA polymerase.[10] As can be seen in Figure 8, the action of this enzyme leaves 5'-ended single chains exposed at the terminals of the DNA molecule. If the beginning sequences are repeated at the end of the molecule, this process exposes single chains that are *self-complementary;* if they anneal intramolecularly, circular structures result. If they anneal intermolecularly (and in the case of T3 or T7 they can, as these are a nonpermuted collection of molecules and all molecules have the same complementary ends), long repeating genomes formed by the head-to-tail joining of various DNA molecules can result. We have called these structures "concatenates" (Figure 8). Obviously, the relative abundance of these two structures depends upon the concentration of the DNA molecules at the time of annealing. In Figures 9A and 9B are two T7 DNA molecules after an annealing treatment: the first was not treated with exonuclease III, the second had about 6 per cent of its nucleotides removed by the enzyme. Circle formation is very efficient (more than 50 per cent circles) with T3 or T7 DNA molecules when about 1 per cent of the nucleotides have been removed and when annealing is conducted at 1 μg/ml. If annealing is conducted at 7.5 μg/ml, some circles and also many concatenates are formed. Figure 10 shows a fourfold concatenate of T7 DNA and a circular dimer of T7. Figure 11 displays the length distribution of concatemers found before and after degradation by exonuclease III.

THE OVERLAP You will recall from Figure 7 that if exonuclease III degradation proceeds beyond the length of the terminal repetition, two single-chain regions are exposed on either side of the duplex segment formed by

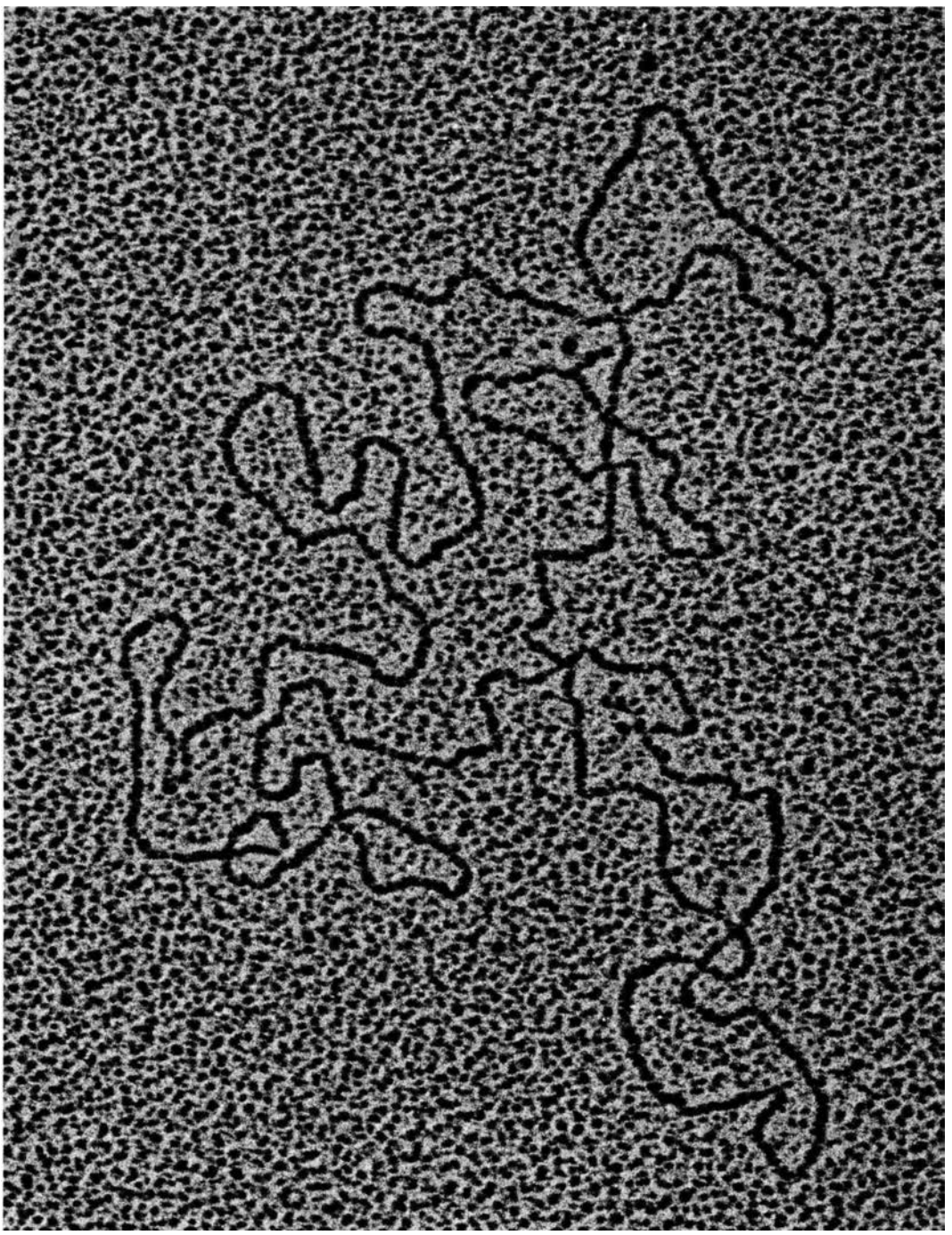


FIGURE 4 Artificial circle of P22 DNA formed by denaturation and annealing of the original linear P22 molecule.

the complementary ends. The protein film technique does not allow these single-chained regions to be seen very clearly. The result is what appears to be two "gaps" on either side of the duplex segment. We have 50 examples of such molecules; somewhat less than half of these are convincing. An example is shown in Figure 12. The preparation from which this molecule was selected had about 6 per cent of its nucleotides released by exonuclease III. The length of the duplex segment between these gaps is 0.10 $\pm$ 0.03 μ long, corresponding to 0.7 per cent of the genome or 260 nucleotide pairs. This is our best estimate of the length of the terminal repetition in T7. Our information on T3 is less extensive, but the terminal repetition appears to be of equivalent length in this phage.

CONTROLS In order to conclude that the unbroken molecule is terminally repetitious on the basis of circle formation, we must demonstrate that molecules that are *not* terminally repetitious do not form circles, even though terminal single chains are exposed by exonuclease III. Now, a priori, we could not be certain that any molecule was not terminally repetitious, or sufficiently so to allow cyclization. We finally selected fragments of the same molecule assuming (and hoping) that sequences found at the ends were not found in the middles. A mixture of unbroken and fragmented molecules was prepared and the mixture then partially degraded with exonuclease III. The results for T3 and T7 DNA are shown in Figure 13. Here it can be seen that neither fragments or whole molecules will form circles when no degradation has occurred. On the other hand, after 4 per cent of the nucleotides have been released from the mixture, the whole molecules will form circles efficiently, yet the fragments will not. This is true even though all shear-broken ends are highly susceptible to the action of the enzyme. Thus, the ends of the intact molecule are special; any arbitrary terminal single chains do not interact to form circles. We are tempted to attach special importance to these controls, because they indicate that sequences found at the ends of the molecule are not found in the middles. This is not a proof, but it struck a responsive chord because we were entertaining the notion that phage DNA consisted of nonrepetitive nucleotide sequences for quite a different reason.

GENERALITY These experiments have been extended to P22 DNA, and it develops that this molecule is easily cyclized after about 3 per cent of its nucleotides have been removed. Experiments with adeno virus DNA show a low frequency of circles after (but not before) exonuclease III degradation.[10a] We are attempting to extend these studies to different types of viral DNA molecules. At present all viral DNAs that can be tested properly show terminal repetition. Both permuted (T2, P22) and nonpermuted (T3, T7, λ) viral DNA molecules are terminally repetitious, and it would not surprise us to learn that this is a general rule for all DNA-containing viruses.

Intracellular cyclization and concatenation

Soon after the injection of a phage DNA molecule into the host cell, the molecule can assume a circular form. This is the case with λ, P22, and perhaps other phages as well. It is reasonable to suppose that the first step occurring in vivo is the same as that occurring in vitro after partial exonuclease III degradation: cyclization after injection probably results from the annealing of complementary terminals. There seems to be one difference, however. The gaps are rapidly sealed, resulting in two continuous (circular) polynucleotide chains that are topologically linked. This leads to the formation of a twisted form, called the superhelix, observable when the molecules are examined in ordinary solvents. This superhelical form was first discovered by Vinograd and his collaborators in polyoma virus DNA,[11] and has been found in ϕx replicative form[12] and intracellular P22[11a] and λ.[13]

Intracellular concatenates are least well identified. Such a long, rapidly sedimenting, shear-fragile structure does appear to be formed during the replication of T2,[14] λ, and T5 DNA.[15] These structures could be formed in the same way as they are formed in vitro, or they could be a direct product of replication.

Thus, circular and concatenated DNA molecules can be produced artificially or naturally within infected cells. When circles or concatenates are formed, the initial genes find themselves physically adjacent to the terminal ones. According to the operon theory one would expect that the control of gene expression might reflect these new connections.

The recombination of DNA molecules

From any point of view, the joining of complementary terminals to produce a circle or a concatenate does represent a kind of recombination event. Whether these experiments are directly related to genetic recombination is not yet known, but we believe they are.

The mechanism of genetic recombination is still one of the outstanding unsolved problems in molecular biology. Meselson's crosses with density-labeled λ DNA molecules indicate clearly that genetic recombination takes place by the "breakage and rejoining" of fragments derived from alternate parents.[16] This rejoining process is so precise that

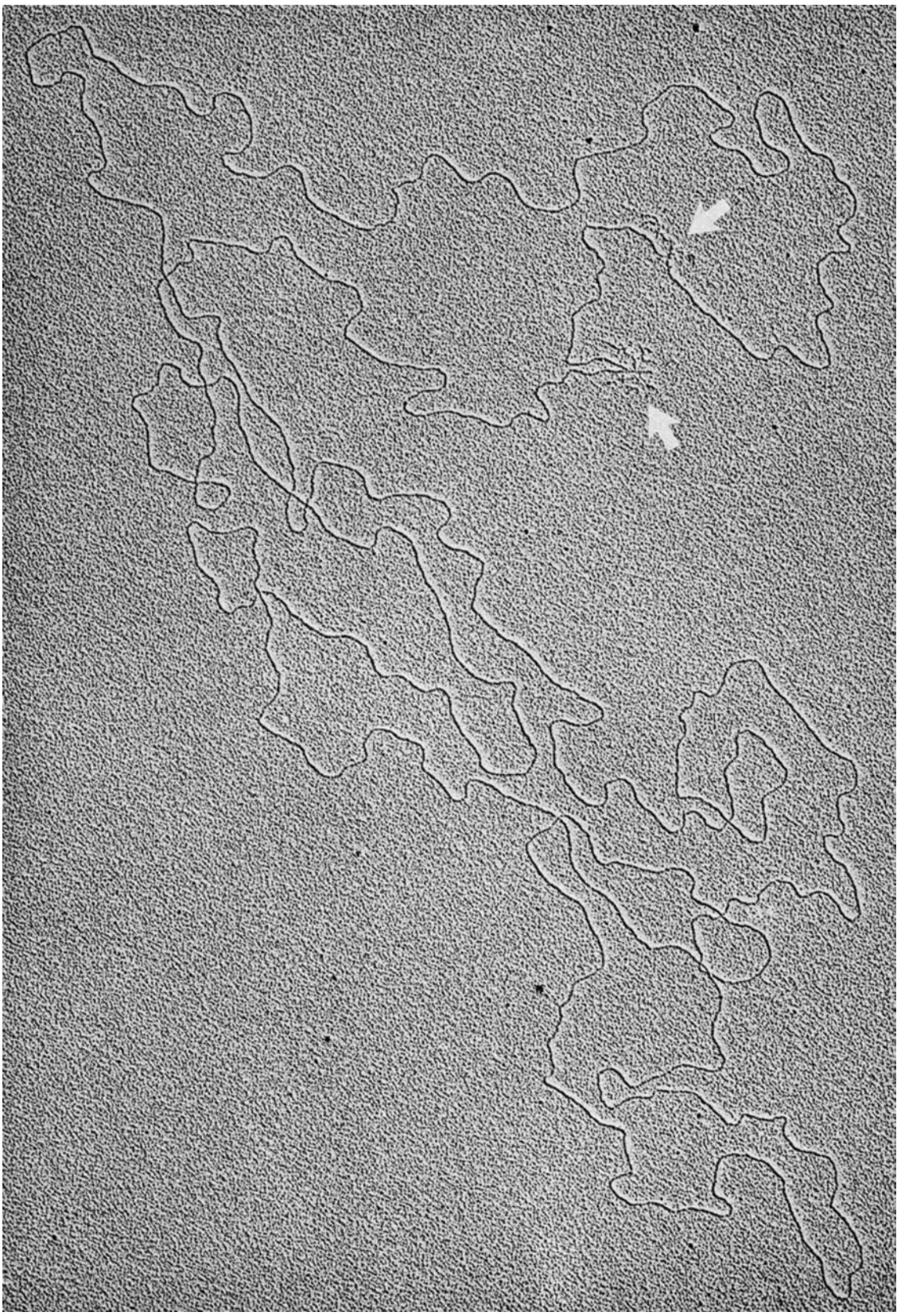
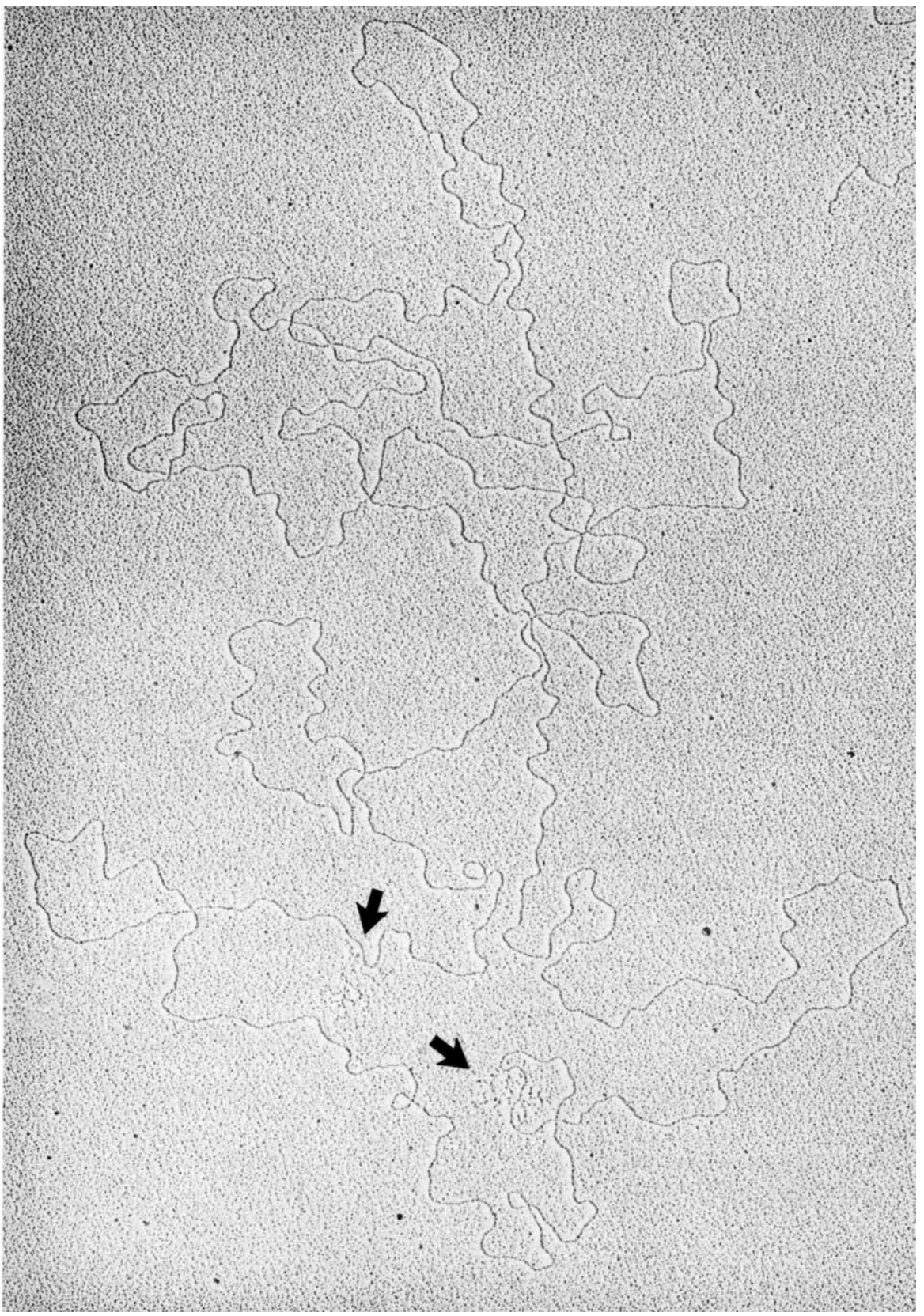


FIGURE 5 Artificial circles of T2 DNA. The contour length of these molecules is close to 55 μ. Notice the two bush-like structures on each molecule. These are thought to



be the single-chained terminal repetitions that can find no partner. "Interbush" distance in molecule A is substantially different from that in molecule B, above. × 36,000.

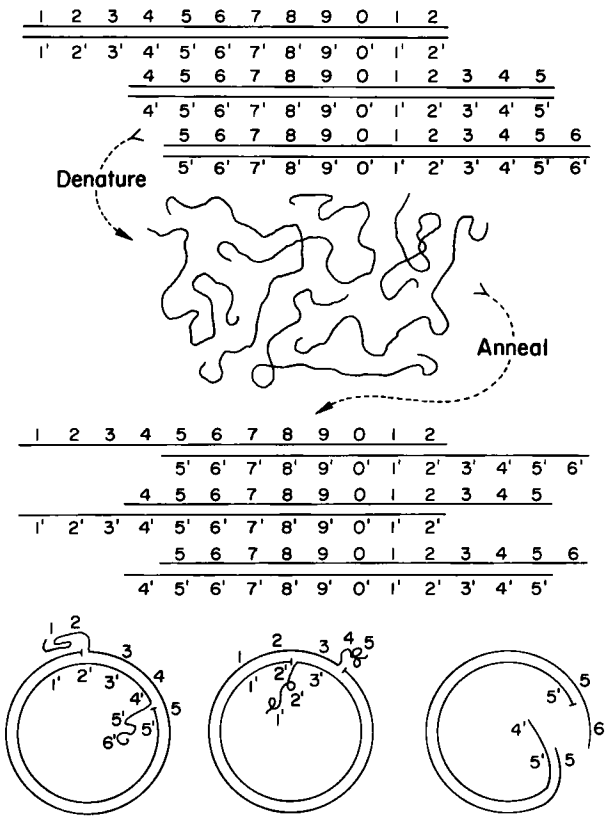


FIGURE 6 Circle formation by a permuted, terminally repetitious collection of duplex genomes. The contour distance between the single chains depends on the *relative* permutation of the component single chains.

not a single nucleotide is gained or lost in the process. To use the older language: the crossing event is almost always "equal"—that is, between strictly homologous regions of the chromosome. If this were not true, recombination would be highly mutagenic, which it is not. Although there are a few ritualistic dissenters, there is only one known process and one available idea to explain this unusual specificity: the annealing of complementary polynucleotide chains.

Before embracing this idea too warmly, remember that this assumption relegates any homologous pairing of duplex DNA molecules or the synapsis of more organized chromosomes to a secondary role that is not intimately related to the recombination event. It is unfortunate that the ideas of synapsis, so well established in the chromo-

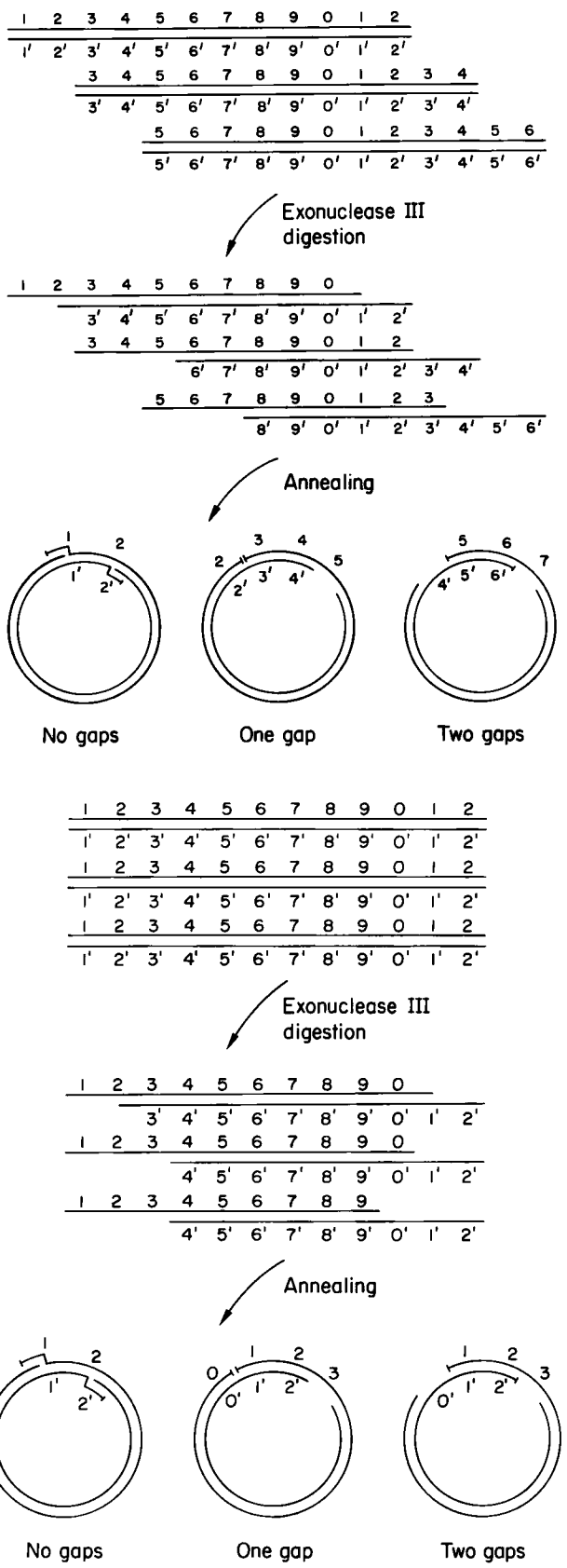


FIGURE 7 Experimental scheme of the test for terminal repetition. A. Permuted collection like T2, 4. B. Nonpermuted collection like T3, 7.

somes of higher organisms, have been grafted to the recombination of DNA molecules. There is no evidence at all that two duplex DNA molecules can pair in such a way that their nucleotide texts are in exact register. Since the outside of the duplex is so regular in chemical structure, it would appear that there is no specific force available by which the homologous pairing of duplex molecules can take place. Therefore, at some stage in the recombination process, single polynucleotide chains must be exposed and made available for duplex formation with another single polynucleotide chain derived from another parent.

Once complementary single chains are exposed, the chance that they will find each other, reunite, and form a duplex is very much greater than generally supposed. For example, λ DNA molecules close into circles (and the gaps are sealed) almost immediately after injection.[13] P22 DNA molecules take a little longer (about 6 minutes), but this is still rapid, because protein synthesis appears to be necessary to accomplish intracellular cyclization.[11a] Therefore, the assumption that seems nearest the truth is the extreme one: if complementary chains are exposed (and confined to a small volume such as a bacterial cell) they will anneal very swiftly. Hence, the first crucial step is the exposure of single polynucleotide chains.

THE EXPOSURE OF COMPLEMENTARY POLYNUCLEOTIDE CHAINS From a chemical point of view exposure can take place in two ways. The chains can unwind, or one chain can be degraded by exonuclease, leaving its complement exposed. Preceding these events, the continuity of one or both of the polynucleotide chains can be broken by an endonuclease. Partial unwinding might conceivably occur without chain breakage at all. These possibilities are summarized in Table I. All of the published (and unpublished) speculations of which I am aware can be fitted into one of the five categories shown here. These five ways of exposing single polynucleotide chains are depicted in Figure 14.

Some general features begin to appear. If exposure is accomplished by an exonuclease, then the elementary recombination event is obliged to be nonreciprocal; both recombinant types cannot be made in a single act. Reciprocal recombination can only come about by some unwinding of the two polynucleotide chains. A mechanism that is basically reciprocal (O—U, or 1—U) could generate an apparently nonreciprocal recombinant by the secondary loss of one or the other recombinant type. A mechanism that is basically nonreciprocal (1—E, 2—E, or 2—U) could generate apparently reciprocal recombinants by a secondary nonreciprocal event that is in some degree correlated with the first, involving a third parental DNA duplex (two of one parental type and one of the second parental type). Thus, in principle, an experiment that can clearly determine whether the recombination of DNA molecules is reciprocal could eliminate a large group of possible mechanisms. On the other hand, it may be difficult to devise an experiment that eliminates possible secondary events with certainty.

Unwinding presents a special difficulty. It is not clear why a DNA molecule should unwind, or locally denature, only to anneal again under less favorable circumstances. If the chains do unwind, there must be some process forcing them apart. This process could be the DNA polymerase. Such an exposure scheme is depicted in Figure 15 which is a member of group 1—U (Table I).

RE-ESTABLISHMENT OF CHAIN CONTINUITY Once two duplex fragments have rejoined, there are still imperfections in the resulting structure. There are gaps or regions of exposed single chains; there are overlaps and

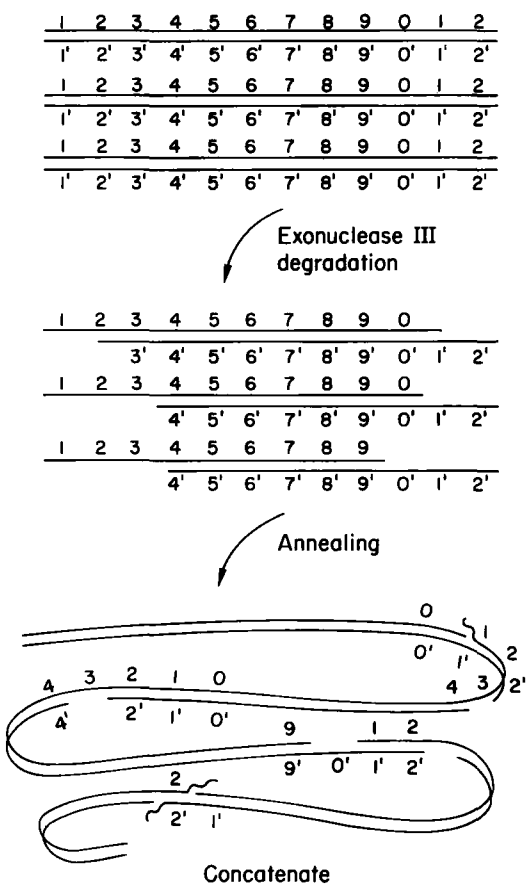


FIGURE 8 Concatenate formation. In a non-permuted collection, all ends are mutually self-complementary and can join to produce repeating structures called concatenates.

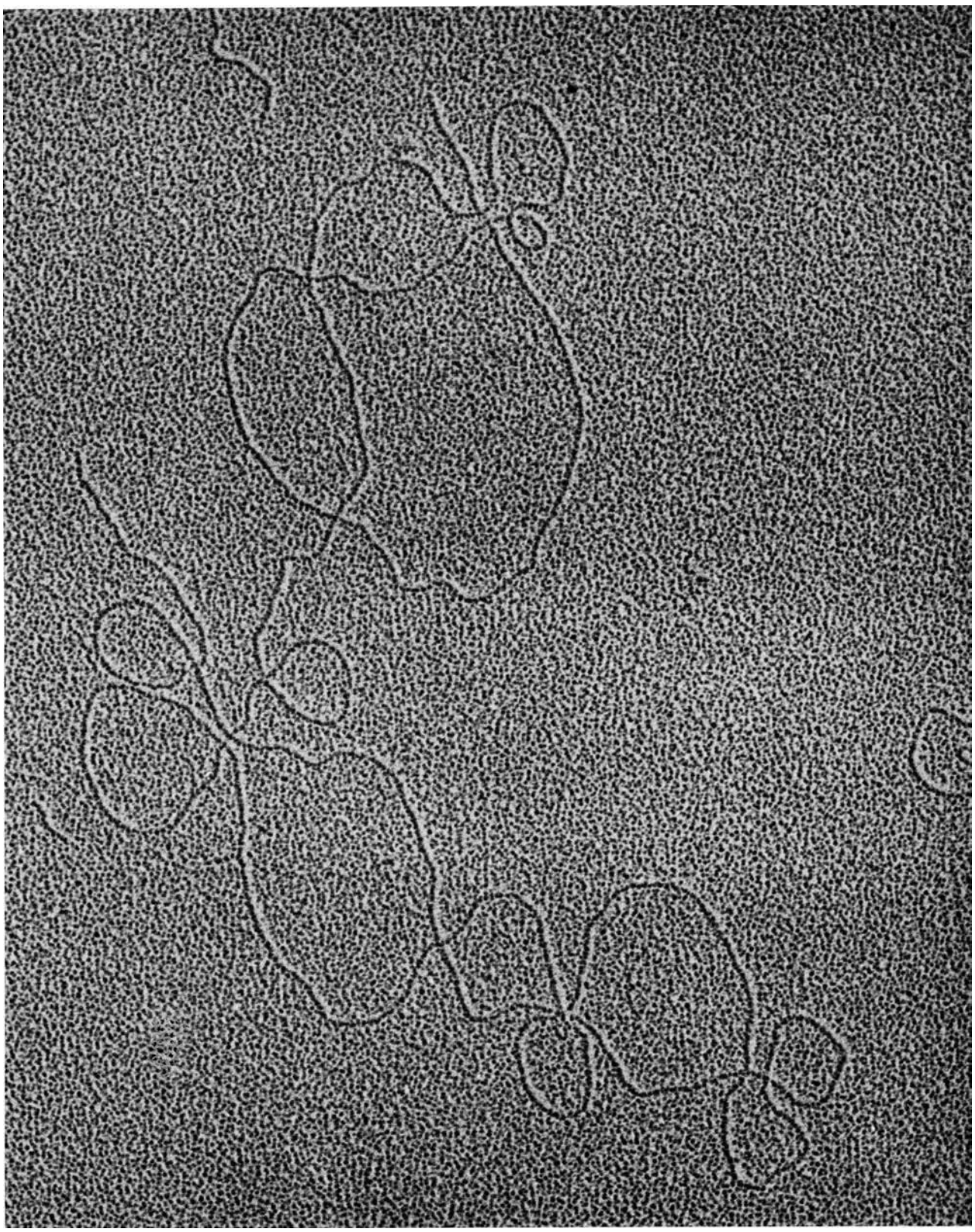


FIGURE 9 T7 DNA molecules, annealed. A. Before partial exo III degradation.

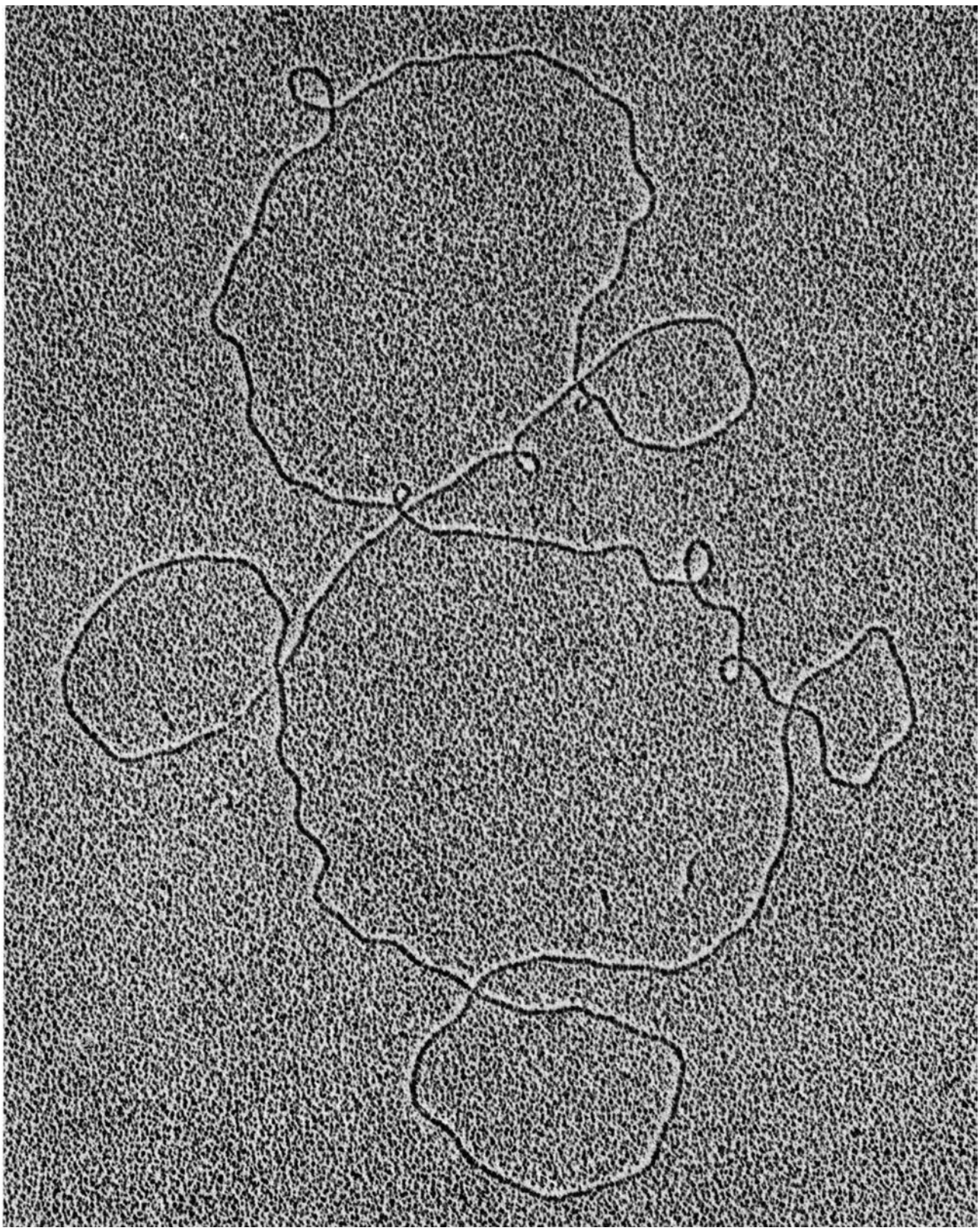
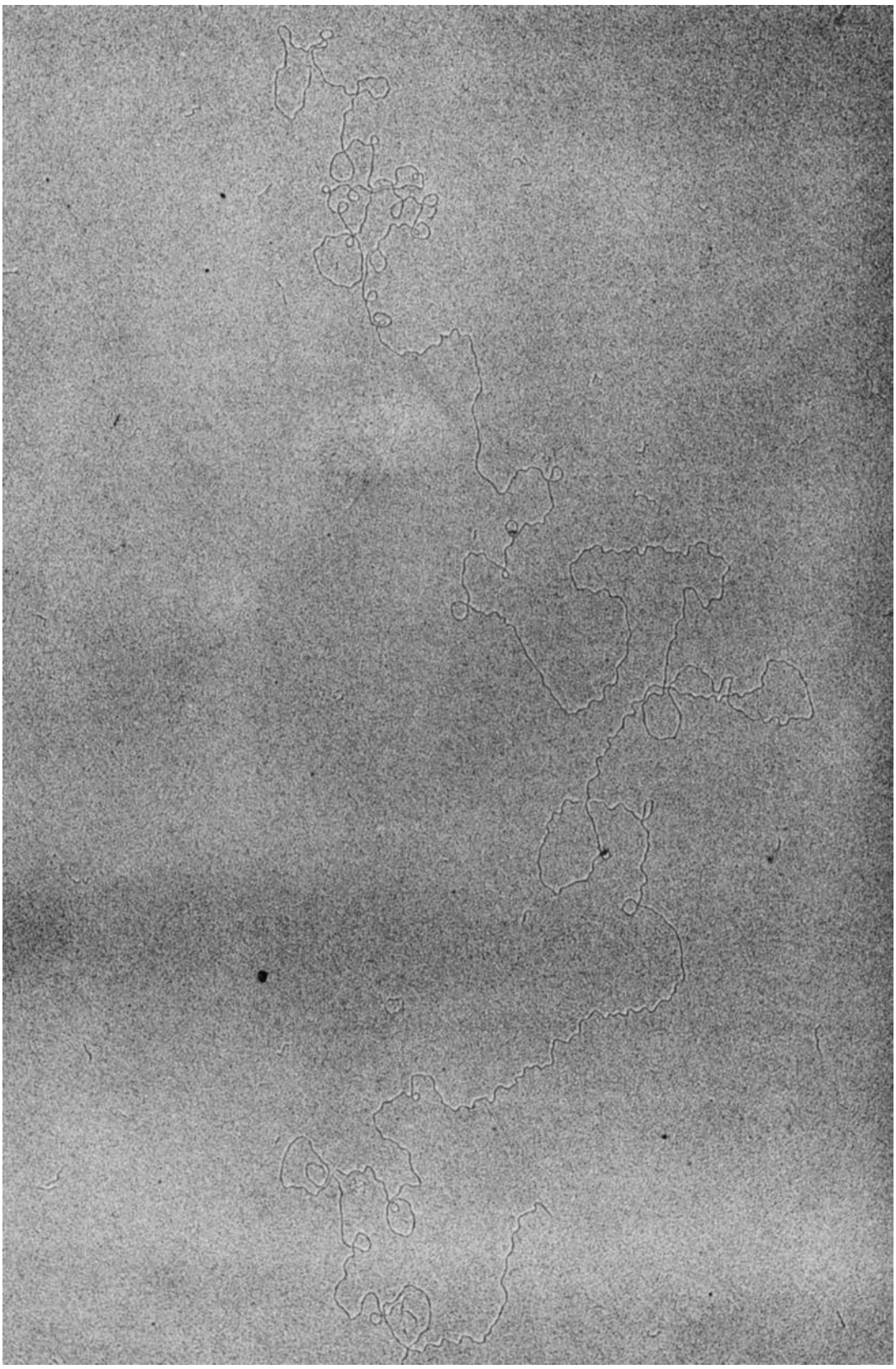


FIGURE 9B After partial exo III degradation.



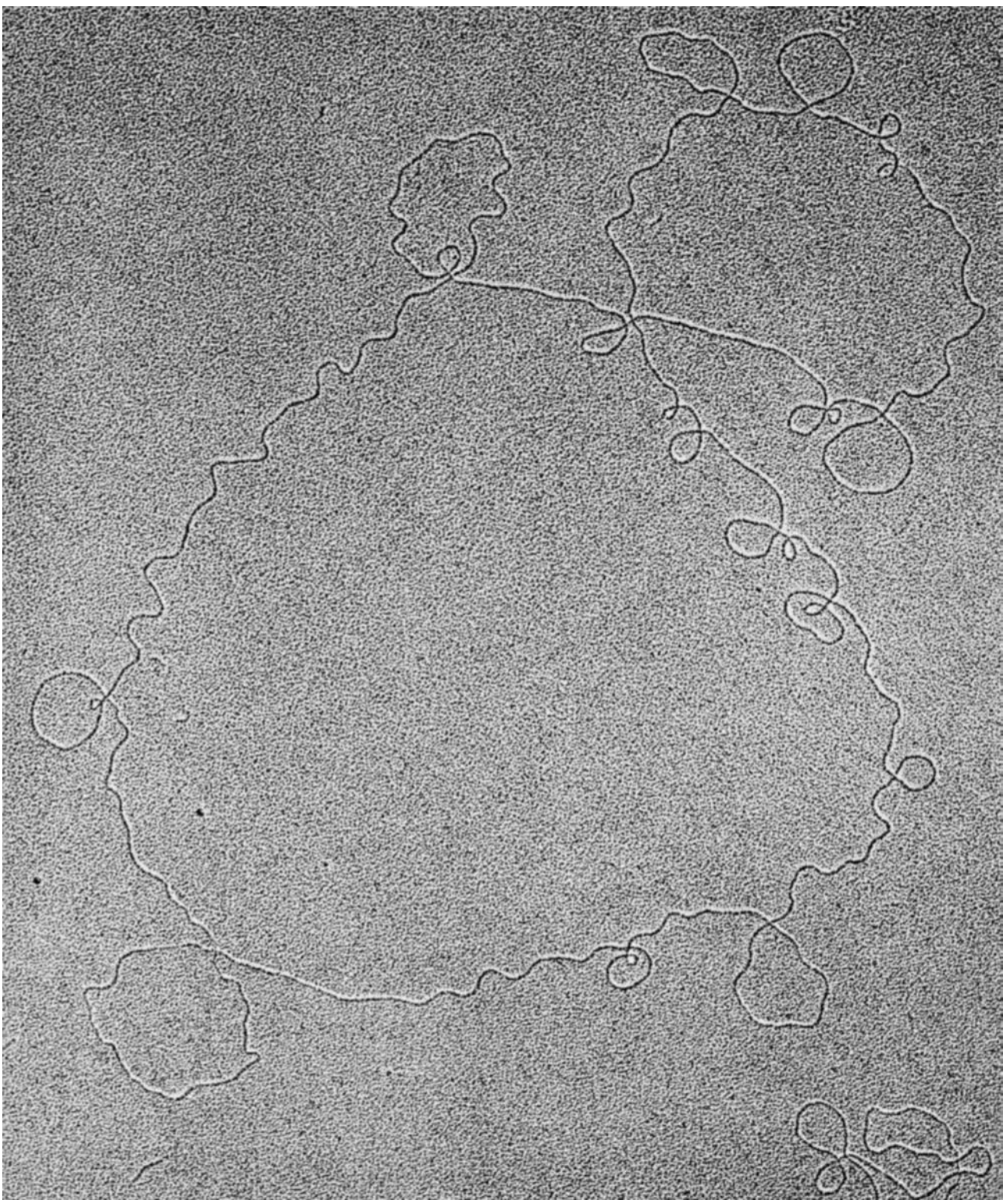


FIGURE 10 T7 Concatenates. A: (*at left*) A four-fold linear concatenate.
B: (*above*) A circular dimer.

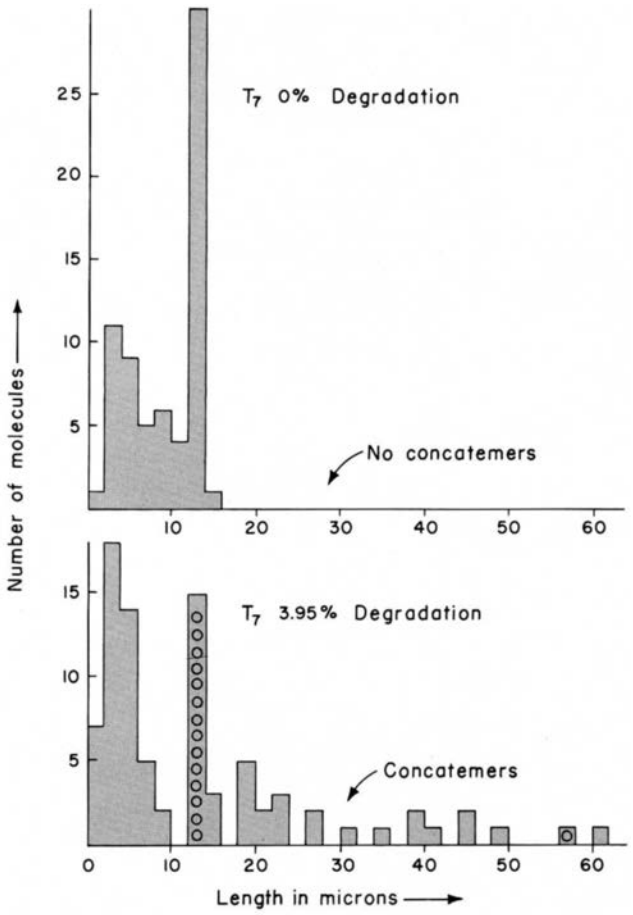


FIGURE 11 Length distribution of T7 concatenates. A mixture of fragments and whole molecules was annealed at 7.5 γ/ml; (upper) before partial exo III degradation, (lower) after partial exo III degradation.

nicks. These present a chemical, not a conceptual problem. The gaps are uniquely suited templates for DNA polymerase, and the overlapping polynucleotide chains are sensitive to known exonucleases. There is one step that has not yet been identified in vitro—the sealing reaction by which the continuity of a nicked polynucleotide chain is re-established. On the other hand, there is now much evidence that such a sealing action does exist in normal and virus-infected cells.[11a]

MINIMUM RECOGNITION LENGTH Having chosen to believe that annealing is the only basis for the specificity of recombination, we have reiected the notion of homologous pairing, and fixed our ideas on homologous joining. Since this takes place by the union of complementary polynucleotide chain segments, one is led naturally to the question: how many nucleotides are involved in this process? As the interaction of these single-chain regions or segments provides the only means by which two duplex fragments can recognize each other, the number of nucleotides involved can be termed the *recognition length*. Now, it is not necessary to think that the entire text of the DNA molecule is involved in this recognition process; only a short region (of variable length) is necessary and this is already implied in Figure 14. While there is no definitive way to set an upper limit to the recognition length, the lower limit can be set by energetic and informational considerations. As indicated previously, a duplex segment 10 to 20 nucleotide pairs long has considerable stability, yet this stability decreases sharply below 10. The intracellular environment may tend to stabilize or destabilize the DNA duplex, but it is unlikely to change this estimate very much. For our purposes it is sufficient to conclude that the energetically stable minimum length is small, and is small in vivo.

NONREPEATING SEQUENCES Whatever the minimum value of the recognition length may be, no two identical recognition lengths can exist in the same molecule without serious consequences. These take the form of deletions, insertions, episome formation and inversions, as is discussed shortly. These serious consequences may be balanced by attendant temporary advantages to the cell or virus, but under selective pressure (which cannot be properly defined in this case) I would imagine that repetitious recognition lengths have been eliminated during evolution. Therefore, the *Principle of Nonrepetition** reads: "A DNA molecule that is stable under recombination cannot contain any ordinary or inverted repetitions."

Clearly, a repetition must be defined in terms of some number of nucleotides. This number cannot be as small as two or three, because there must be abundant repetition of doublets and triplets throughout the DNA molecule. It must be some larger number, say f nucleotides.

It is easy to calculate the number of different kinds of sequences f nucleotides long: there are 4^f. If one is to avoid both ordinary and inverted repetitions, then there are about $1/2 \cdot 4^f$ different f-mers.

The next question is whether these $1/2 \cdot 4^f$ f-mers can be joined together to produce long nonrepetitive sequences. It is not obvious that they could be so joined, since all overlapping sequences must also be nonrepetitive. This can be proved by graph theory.[17] The longest sequence that can be constructed in such a way that no sequence f units long repeats is:

*This is given a dignified name to compensate for the fact that there is not a *shred* of evidence to support it!

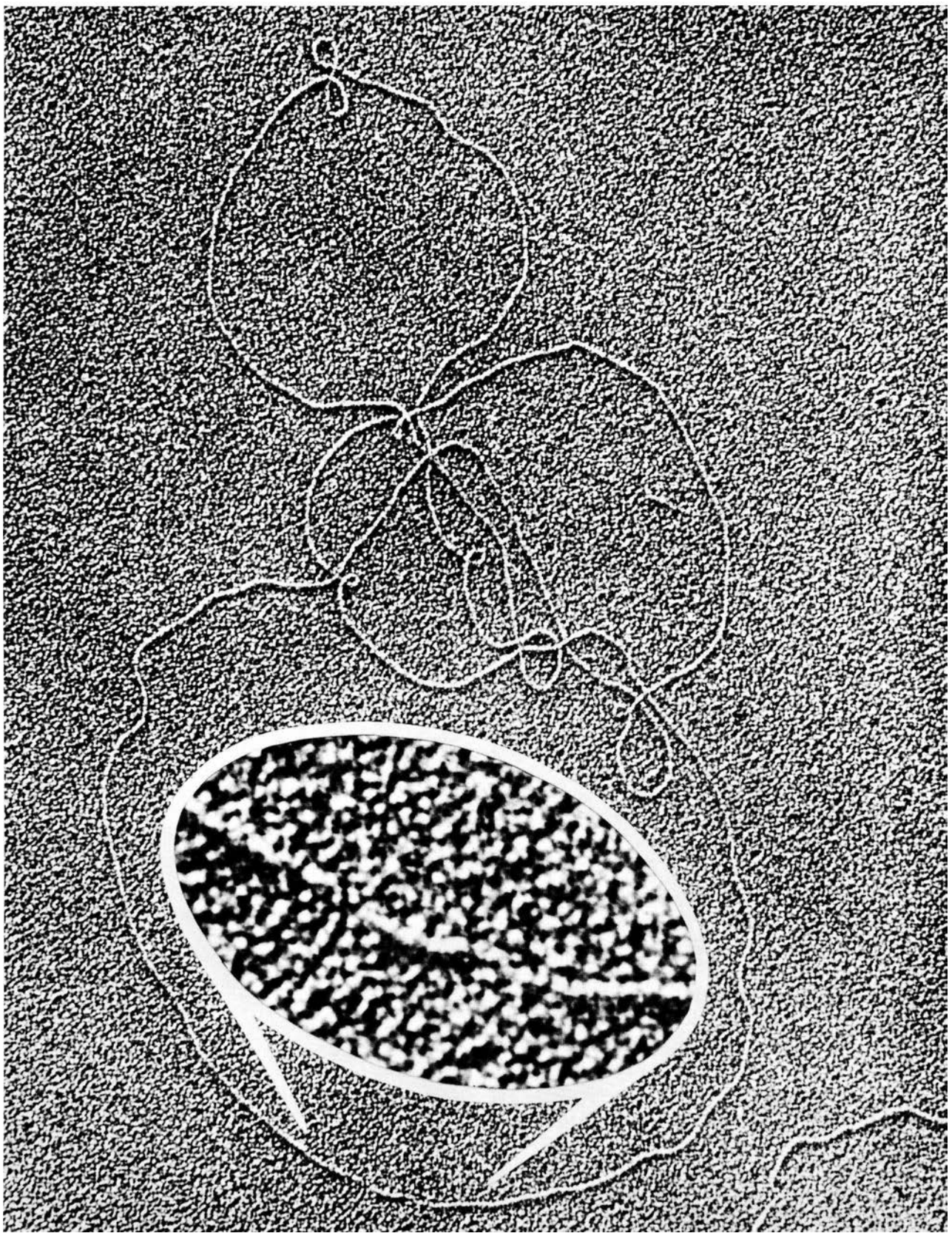


FIGURE 12 Duplex terminal repetition bracketed by single chains.

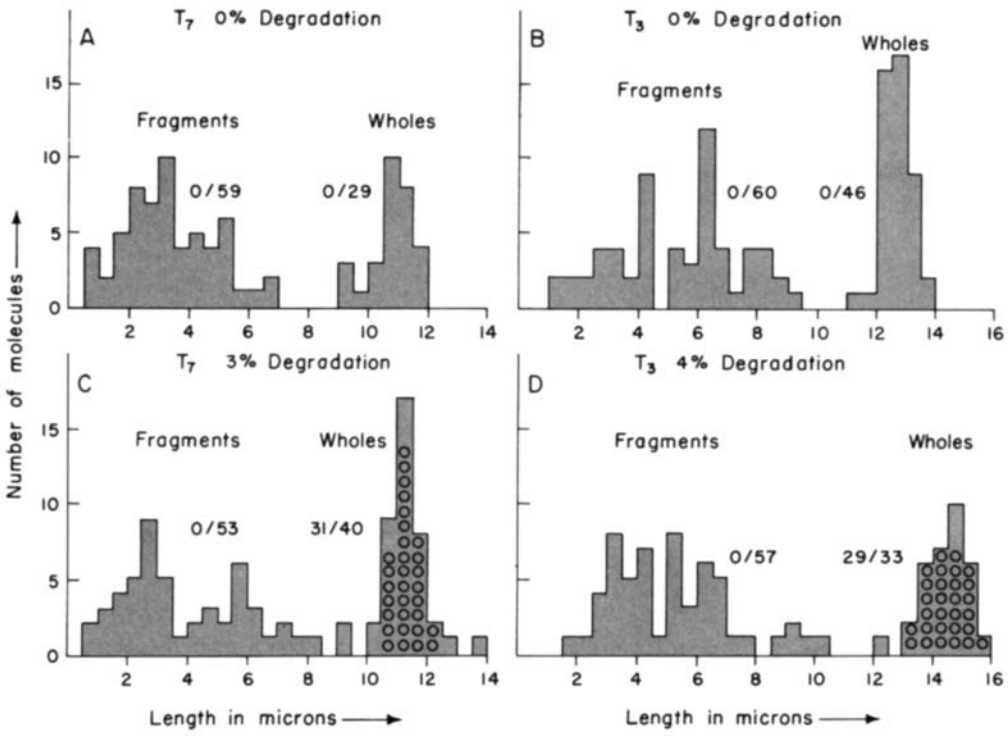


FIGURE 13 Cyclization of T3 and T7 DNA by exonuclease III. Length distributions measured by electron microscopy on a mixture of intact molecules with shear-produced fragments. A and B: controls, annealed only. C and D: the same mixtures, exonuclease III degraded and annealed. The circles (O) in the histogram represent circular molecules. Note that only full-length molecules form circles, and then only after partial degradation.

$$1/2 \cdot 4^f + 2^{f-1} + f - 1, \text{ if f is even}$$
$$1/2 \cdot 4^f + f - 1, \text{ if f is odd}$$

or approximately $1/2\ 4^f$.

Longer DNA duplexes cannot be constructed without having repetitious f-mers. These molecules are said to be at the nonrepetition limit defined by f.

Salmonella and *E. coli* contain DNA molecules of at least 4×10^6 nucleotide pairs. If it is assumed that any part of this molecule is free to recombine with any other part, then

$$1/2 \cdot 4^f \geqslant 4 \times 10^6$$

and

$$f \geqslant 12.$$

Thus, both the energetics and informational requirements indicate that the minimum recognition length must be at least 12 nucleotides long.

If the nonrepetitive length, f, is specified to be very large, then the Principle of Nonrepetition will invariably hold. However, its usefulness as a theory disappears. It will be easier to prove the theory wrong if the assertion is made that f is small. Because f can be as small as 12 nucleotides, we suppose that it *is* as small as 12 to 15

TABLE I

Mechanism of PNC exposure	Number of PNC breaks at any given point in the text		
	0	1 (nick)	2 (chop)
EXONUCLEASE	impossible	model 1-E nonreciprocal	model 2-E nonreciprocal
UNWINDING	model 0-U reciprocal	model 1-U reciprocal	model 2-U nonreciprocal

Note: PNC = (single) polynucleotide chain.

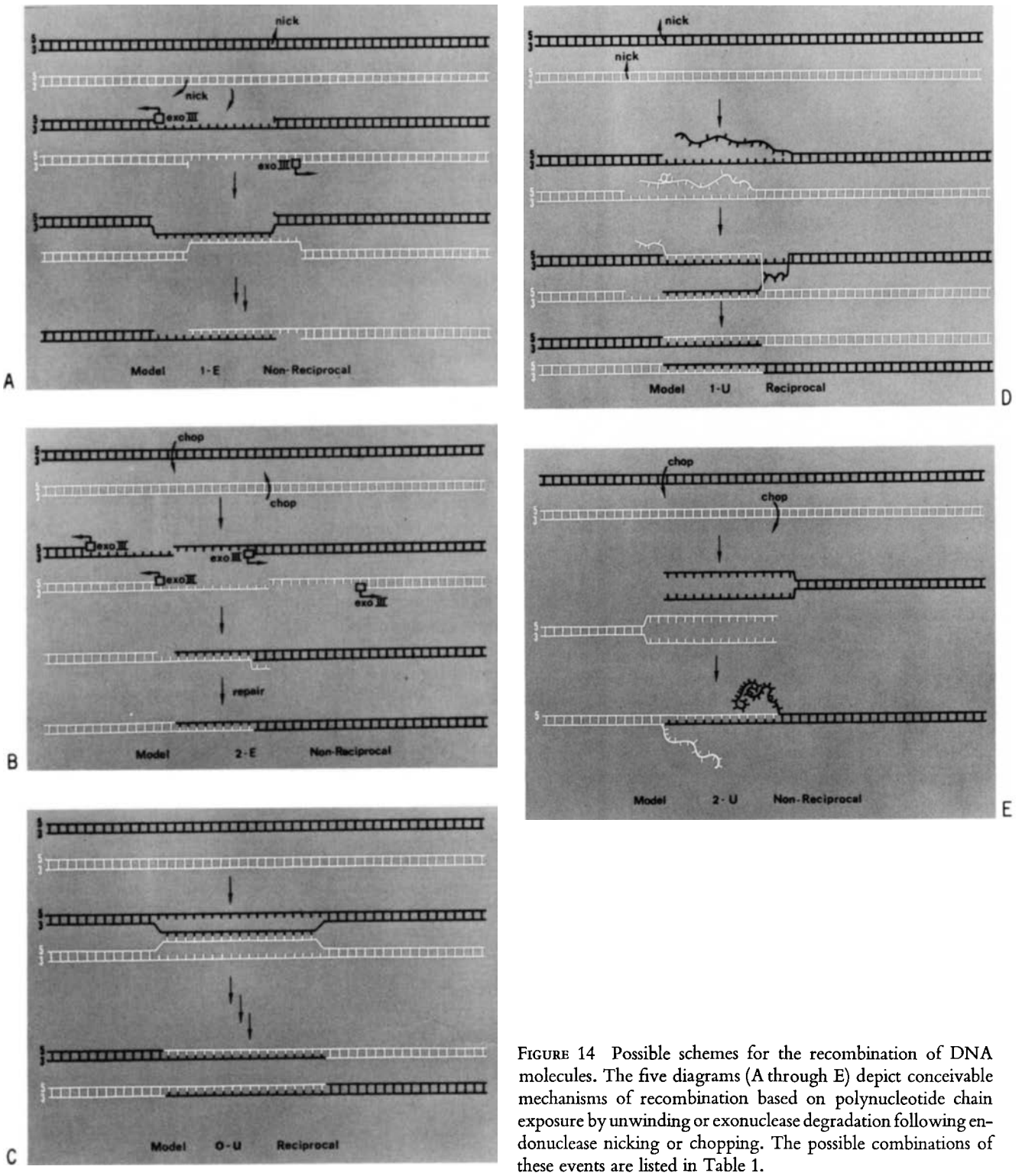


FIGURE 14 Possible schemes for the recombination of DNA molecules. The five diagrams (A through E) depict conceivable mechanisms of recombination based on polynucleotide chain exposure by unwinding or exonuclease degradation following endonuclease nicking or chopping. The possible combinations of these events are listed in Table 1.

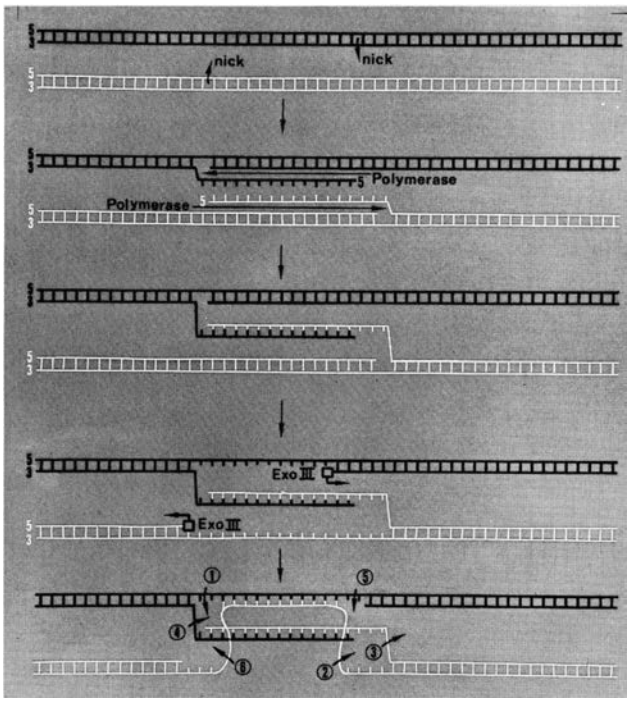


FIGURE 15 A special model of Class 1-U in which unwinding is driven by polymerase.

nucleotides, and that all freely recombining genomes are nonrepetitive at this level. As you can see, such a hypothesis has direct consequences for RNA and peptide sequences. For example, if two *coli* sRNA molecules can be shown to have the same or nearly the same sequences, yet are templated from different genes, we must reject the Principle of Nonrepetition. This rejection will require a re-evaluation of the recognition length and could change the course of our current thinking about recombination.

If nonrepetition is correct, this does not imply that different genes could not template proteins containing the same tetra or quinta peptide sequences. There might be very useful peptide sequences that are employed for similar tasks in unrelated proteins. However, these need not be encoded by the same sequences of 12 to 15 nucleotides in DNA. Degeneracy in the genetic code provides an ideal way to reconcile the requirement of nonrepetition with the structural requirements for protein function.

THE CONSEQUENCES OF REPETITION In order to demonstrate the effects of repetitions, I have used the "chop and nibble" model 2 — E (Table I) depicted in Figure 14. Here the chains are chopped at random by an enzyme like endonuclease I and then nibbled by an enzyme like exonuclease III. This chopping-and-nibbling model applied to a collection of identical DNA molecules is shown in Figure 16. This model results in the relatively efficient recovery of reassembled full-length DNA molecules.[2] However, it can only work if there are no repeating recognition lengths. If there were two identical recognition lengths, then ambiguity would result. This is shown more clearly in Figure 17.

Appropriately located "chops" can lead to deletions, or to the formation of circular DNA molecules. The segments bearing the same complementary terminals could be inserted any number of times, resulting in duplications. The effect of inverted repetitions is shown in Figure 18. These can lead to inversions, an event that could be very

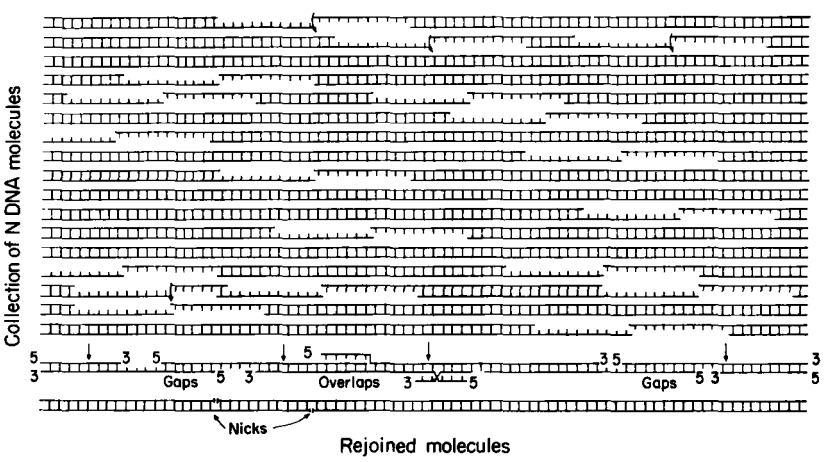


FIGURE 16 The Chop and Nibble Model for molecular recombination. Here one supposes that an enzyme like endo I breaks both chains thereby exposing sites for exo III reaction.

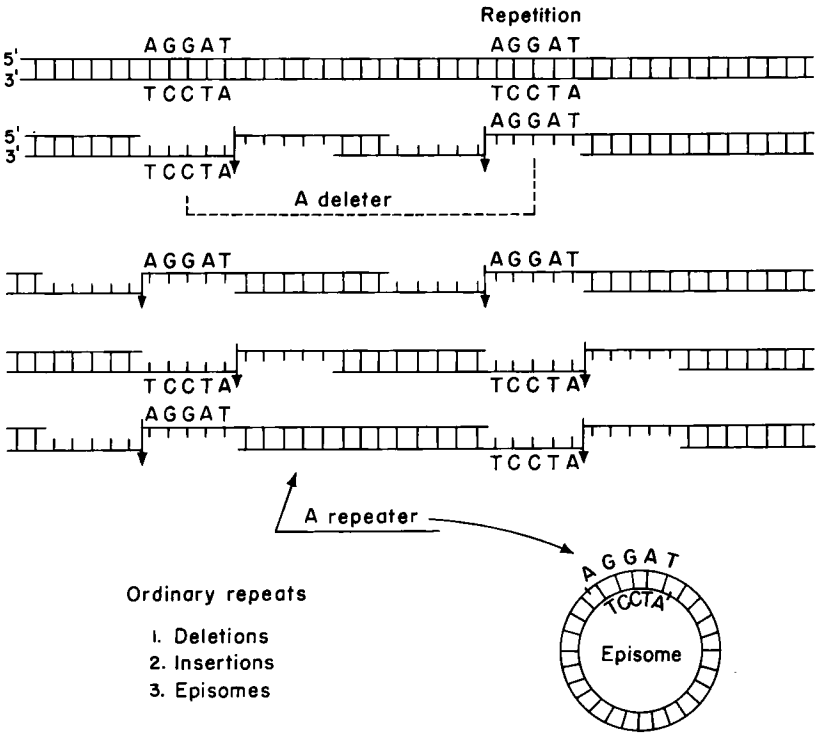


FIGURE 17 The consequences of ordinary repetitions. The *minimum recognition length* is at least 12 nucleotides, but for this illustration of shorter, more pronounceable, sequence of 5 letters is shown to occur at two places in the DNA duplex. The molecule is "chopped" and "nibbled" by enzymes as specified in the text. The exposed complementary chains anneal. In this way the sequences TCCTA...5′ and 5′... AGGAT can be exposed by chops in the insides of the repetitions. Upon annealing the region *between* the repetitions is deleted along with one of the repetitious sequences. If chops occur outside the repetitions, then a "repeater" segment is formed. This segment bears a 5′ . . . AGGAT on the left and a TCCTA. . .5′ on the right. It may insert itself any number of times or even unite with its own end to form a circular DNA molecule. In the event that this "episome" is able to control its own replication, it could maintain its independence from the chromosomal DNA. However, since it contains a sequence that is identical with that found in the chromosome (namely, AGGAT) it will be susceptible to reinsertion by recombination at a single unique site.

dangerous to the cell if they were not properly transcribed.

These consequences do not follow from the particular model used to demonstrate them; they follow from the assumption that recombination is based on the act of recognition between two complementary polynucleotides.

There are some pleasing things about believing that the nonrepetitive length is short and that the genome is near its nonrepetition limit. Point mutations will occasionally convert a nonrepetitive sequence into one that is repetitive with another elsewhere in the molecule. Under these conditions, recombination will lead to deletion mutants. By the same token, a point mutation could lead to the production of an episome, which, if properly endowed, could be considered to be a primitive virus DNA molecule.

ORGANIZED CHROMOSOMES There are good reasons for a cell to have as much DNA as possible. More DNA allows for greater biochemical potentiality and regulatory flexibility. However, a freely recombining genome is restricted by the repetition limit ($1/2 \cdot 4^f$). To possess more DNA, a cell must be able to increase f, which may not be chemically possible, or it must resort to compartmentalization. This can be accomplished by taking on symbiotic "boarders" like the κ or λ particles in paramecium or perhaps the plastids like mitochondria; or compartmentalization may be realized by more highly organized chromosomes. The role of higher chromosomes may be to isolate structurally (during synapsis) different regions of the DNA molecule during the time that recombination takes place. This would allow for distant repetitions between sequences that are locally nonrepetitive.

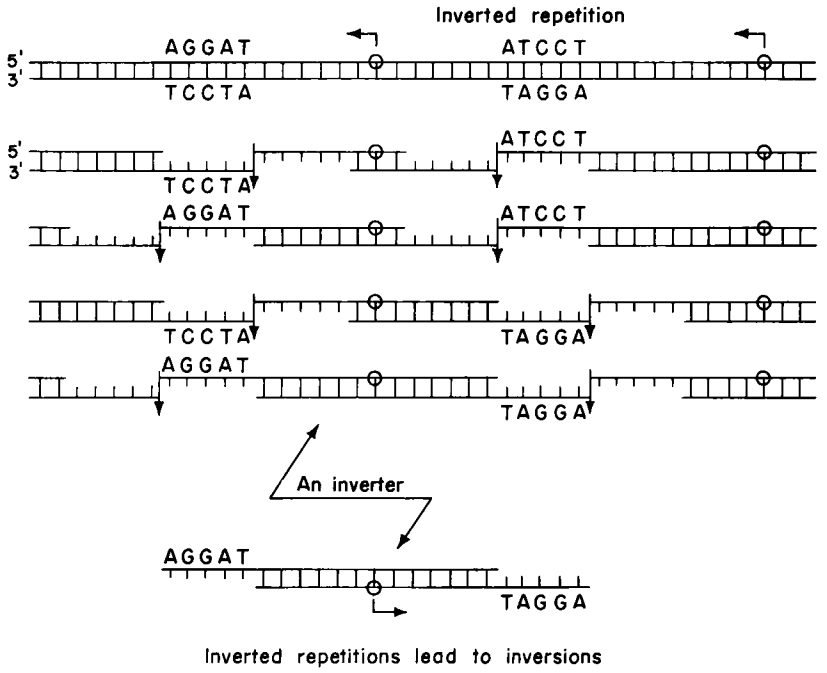


FIGURE 18 The consequences of inverted repetitions. If the inverted repetition of AGGAT, namely, ATCCT, is present in the same molecule, then the "chopping" and "nibbling" action will expose a segment which is 5′ . . AGGAT on the left and . .TAGGA. . . 5′ on the right. When this segment is inverted, it again reads as before. Therefore it could be inverted and reincorporated into the DNA molecule. The circle with an arrow (↰) outside the interval between the repetitions signifies the hypothetical initiation site for transcription. Inverted message is undoubtedly nonfunctional. If the initiation site for transcription is within the inverted region, normal message would be expected.

Summary

Some species of virus DNA molecules have sequences that are circular permutations of a common sequence; other species have nonpermuted DNA molecules. Examples of both types can be shown to begin and end with the same sequence of nucleotides (terminal repetition). These conclusions are based upon the conversion of linear molecules into circular ones by annealing self-complementary, terminal, single chains. Nonpermuted molecules also form concatenates.

These events are a kind of molecular recombination that may be directly related to the mechanics of genetic recombination. Making the likely assumption that they are, some general properties of models for recombination based on annealing of complementary chains have been examined. This inquiry has led to the requirement that freely recombining DNA molecules are so composed that no stretch of 12 to 15 nucleotides repeats anywhere in the molecule. If correct, nonrepetition is an important syntactical rule for the organization of genetic information in DNA molecules. When this rule is violated, unusual structures can result. Viral DNA molecules violate this rule because they are terminally repetitious.

The Biology of the Immune Response

G. J. V. NOSSAL

LIKE THE NERVOUS SYSTEM, the immune defense system has reached a sophistication in the mammals that is not paralleled by lower orders. Our first step, in this brief review, will be to examine the evolution of the system. Next, we will identify and characterize the cells and organs that make up the system, and devote special attention to a consideration of immunological memory. Finally, we will attempt to draw some parallels between problems at the moving edge of immunology and neuroscience.

Evolution of the immune system

Even unicellular organisms have a defense mechanism against invasion by microbes. It takes the form of a primitive phenomenon called phagocytosis, whereby certain cells ingest and destroy foreign bodies and wastes. Electron-microscopic analysis has now allowed the sequential steps in the phagocytic event to be defined clearly. First, the organism to be phagocytosed is absorbed fairly firmly to the surface of the cell. Next, the plasma membrane of the cell is invaginated and a pouch develops, buds off from the surface, and forms a little vesicle or bag in the cytoplasm, lined with the original outer aspect of the membrane. Finally, pre-formed vesicles containing strong lytic enzymes fuse with the phagocytic vesicle and digest the particle. In this way, the particle can be eliminated without the necessary digestive enzymes ever spilling into the cell's cytoplasm proper.

This effective mechanism appears to have been the standby defense mechanism throughout many millennia of evolution. It is still present in mammals, in which two major classes of phagocytic cells—the macrophages and the polymorphonuclear leucocytes—exist. The interrelationships, both functional and phylogenetic, between phagocytosis and antibody formation, are not yet clear. A progressively more complex array of nonspecific defense mechanisms developed, including anatomical barriers, a variety of serum factors, special enzymes and secretions, and so forth. However, none of these exhibited the *specificity* that characterizes a mechanism like antibody formation.

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Then, during the Devonian period, some 400 million years ago, at the level of the primitive cartilaginous fishes (cyclostomes), something new appeared to happen. The only two remaining examples available for study are the hagfish and the lamprey. Through the work of Good and his collaborators,[1] we now know that the hagfish contains in its circulation a new type of cell—a lymphocyte—which we will describe more closely below. However, extensive testing revealed that these fish could *not* form antibodies, nor could a specific immune mechanism for graft rejection be demonstrated.

Then, in the lamprey, three associated developments were noted. Focal collections of organized lymphoid tissue appeared in the spleen and bone marrow; a thymus could be identified; and, although feeble, a clear-cut adaptive immune response could be demonstrated.

What do we mean by an adaptive immune response? Essentially, it is the new development of a population of cells or molecules that react with a foreign substance in a specific way and fail to react with other, unrelated foreign substances. The two chief examples of immune phenomena for the present discussion are the formation of humoral antibodies and the destruction of tissue grafts. Between the cyclostomes and the elasmobranchs, including the sharks, there is a sharp rise in the intensity of immune responses. Sharks have a well-developed thymus in relation to the gill pouches, and a new group of cells, the plasma cell family, can be readily identified in the spleen. Ultracentrifugal analysis of the serum shows the presence of an antibody molecule of great size—its molecular weight is about 950,000; its sedimentation rate is 19 Svedberg units (19S)—which resembles one of the major classes of antibodies found in mammals. In some sharks and amphibians, there is *partial* immunological memory. In other words, the second reaction to a given antigenic stimulus some weeks after the first may be greater than the first reaction. However, quite clearly this memory faculty is poorly developed in these species and many negative as well as positive experiments have been reported.

In the amphibian, structures termed jugular bodies appear. These are the homologues of mammalian lymph nodes. However, analysis of the serum shows the presence of a new class of antibody molecules of much lower molecular weight (*circa* 160,000) with a sedimentation constant of 7 Svedberg units. These appear later than the 19S anti-

bodies, and resemble the 7S or immunoglobulin G type (IgG) antibodies. Thus, the chief functional difference in the immune response of amphibians and mammals is the absence of convincing secondary responses.

In the monotremes, or egg-laying mammals, clear-cut immunological memory can be demonstrated.[2] This appears to be associated, at least in part, with the emergence in the lymph nodes of special collections of primitive cells that we term germinal centers. In the marsupials and placental mammals, the lymphoid system has undergone further specialization, and we must now discuss its anatomy and physiology in more detail. No evidence indicates clearly that the immune system of humans differs in any vital respect from that of lower mammals such as the mouse or rat, and most of our examples will be chosen from these latter animals.

The anatomy and physiology of the lymphoid system

This must be viewed at three levels (Figure 1). The basic stem cells from which immune reactive cells or "immunocytes" are generated reside in the bone marrow, a fact that has only recently become apparent.[3] In this way, immunocytes resemble other circulating blood elements such as red cells, phagocytic cells such as polymorphonuclear leucocytes and probably monocytes, and blood platelets. The morphology of the immunocyte precursors in bone marrow is not known.

Interestingly, before such stem cells can participate in immune reactions, they must receive "instruction" from the second-level immune organ, the thymus. This organ is still incompletely understood. It consists of a cortex composed mainly of lymphocytes and a medulla composed of epithelial cells. The cells of the medulla are believed to secrete a hormone or hormones responsible for conferring immunocompetence on the bone-marrow-derived cells. The thymic cortex, in which lymphocytes proliferate at a rapid rate, seeds a few progeny cells into the other lymphoid organs, but the number of lymphocytes that leave the thymus is only a small proportion of the total number born there. The function of the thymic cortex remains obscure.

The third level of lymphoid organ is the area of chief interest to us at the moment—the level at which direct confrontation between antigen and reactive immunocyte occurs.[4] Here we are dealing with "peripheral" lymphoid tissue such as lymph nodes and spleen (Figure 2). These tissues have an elaborate and multicomponent architecture. Moreover, they are functionally connected with each other by means of a peculiar circulation. The lymphocyte cells leave the lymph nodes via an efferent lymphatic vessel and, after percolating through a number of other lymph nodes, finally reach a main lymphatic trunk and enter the venous blood stream. Then it can be shown that a substantial proportion "home" to the lymphoid tissues, employing an unusual route. They exit from the circulation through the postcapillary venule, a vein with a high, cuboidal, and obviously rather specialized endothelium. Here they actually enter the cytoplasm of the endothelial cell and leave it again on the other side.

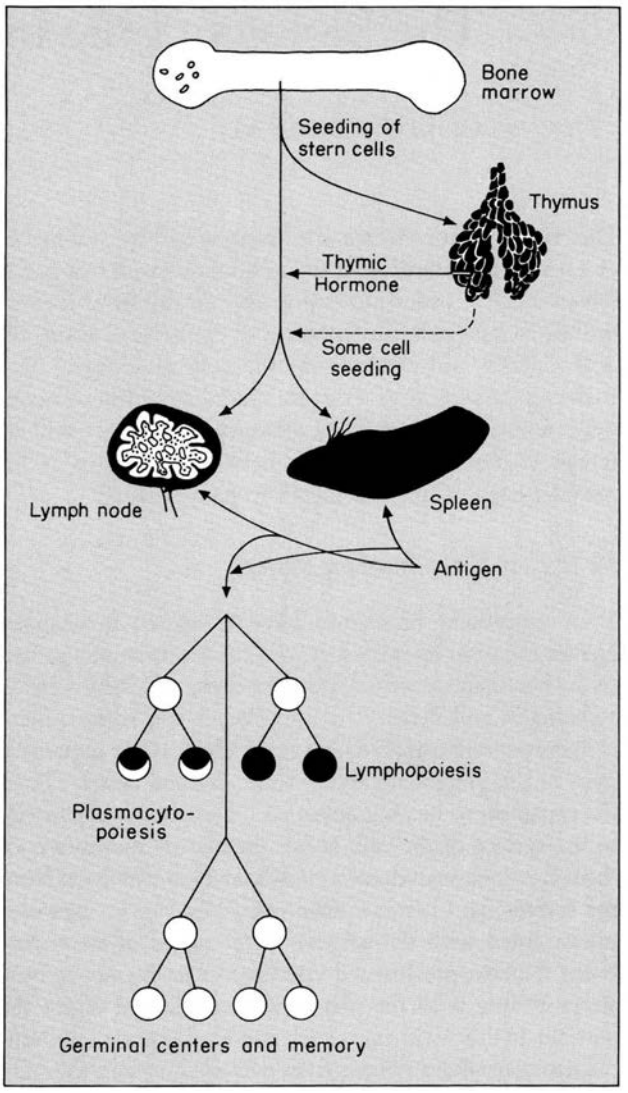

FIGURE 1 Cell cycles in immunity. A schematic representation of the three levels of organs involved in mammalian immune responses.

In the third level, or peripheral, lymphoid tissues we strike two main families of cells—those belonging to the immunocyte group, chiefly lymphocytes and plasma cells, and those belonging to the reticuloendothelial sys-

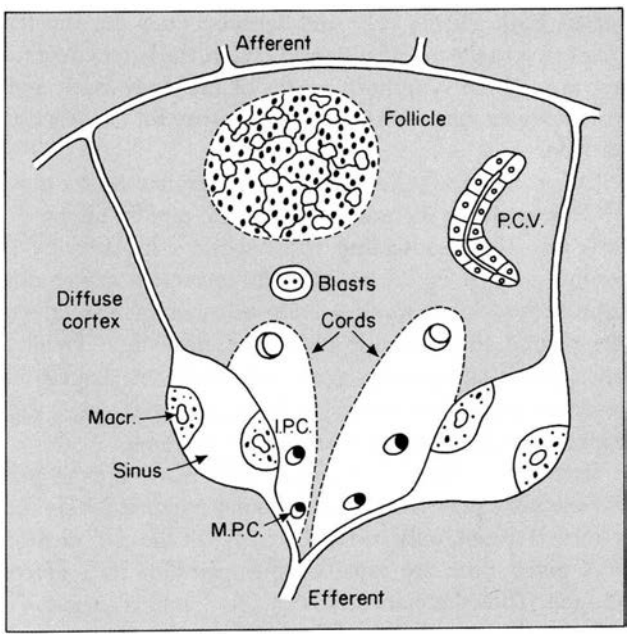

FIGURE 2 Organization of lymph node. Some microanatomical features of mammalian lymph nodes. PBL, IPC, MPC = plasma blast, immature and mature plasma cells. PCV = postcapillary venule. Macr. = macrophage.

tem, chiefly the scavenger macrophages and reticular cells. The lymphocytes vary in maturity. The most rapidly dividing—the large lymphocytes, or blasts—have a pale nucleus, prominent nucleoli, and a cytoplasm containing numerous mitochondria and free ribosomes, but little else. They have progeny known as medium lymphocytes, which are smaller and divide more slowly. These, in turn, have progeny known as small lymphocytes, which are still smaller and have a very scanty cytoplasm with few inclusions of any sort. Small lymphocytes were thought to be end cells, incapable of further division, until Gowans[5] showed that under appropriate circumstances of antigenic stimulation they could reverse their life history and turn back into large, primitive, dividing cells. Many workers believe that this sequence of events is a necessary part of every immune response. The small lymphocytes themselves are the effector cells in the destruction of such foreign tissue as skin grafts.

Similarly, the other primary immunocyte histological type, the plasma cells, vary in their maturity, creating a sequence—plasmablast, immature plasma cell, and mature plasma cell[6] (Table I). This family of cells is responsible for the formation of circulating immunoglobulins or antibodies. Unlike the lymphocytes, these cells cannot reverse their life history. They are highly specialized, born to form only one specific type of antibody. If one immunizes an animal with antigens A, B, and C, the whole animal turns out three populations of antibody molecules—anti-A, anti-B, and anti-C. However, a simple cell from such an animal will form either anti-A, anti-B, or anti-C, but not two (with rare exceptions) or all three antibodies. Furthermore, while each of the three populations of antibody molecules is itself a heterogeneous mixture, the antibody made by a simple plasma cell is probably much more homogeneous, possibly representing a single molecular species.

The scavenger-type cells can only be understood in terms of their location in the lymph node microenvironment. These are the cells that actually capture the antigen. In Figure 2, we can see that the lymph, which carries antigens from the tissues to the nodes, first reaches a circular sinus or channel running right round the outside of the node. The antigen can then take one of two pathways. It can stream into the sieve spaces known as medullary sinuses, or it can penetrate through the inner wall of the circular sinus into the substance of the cortex, or outer shell, of the node.

In both of these areas, specialized antigen-capturing cells can be found. In the medulla, there are macrophages that take up the antigen and rapidly sequester it into various types of vacuoles, or inclusions. In the cortex, there are rounded areas, known as lymphoid follicles, that contain reticular cells with long branching processes, which actively capture antigens. However, these cells retain a large proportion of the captured antigen on the cell surface, without jetting it into digestive vacuoles. A convenient depot is set up, allowing plenty of opportunity for contact between antigen retained on cells and surrounding lymphocytes.

It is not yet possible to state with certainty which of these two classes of antigen-capturing cells plays the more important role in the induction of antibody formation. I

TABLE I

Characteristics of plasma cells

1. One cell makes one antibody.
2. All plasma cells are the result of antigen-induced mtotic division.
3. The mature plasma cell is a highly differentiated end cell, with low RNA and without DNA synthesis.
4. Most plasma cells live a few days only; a small proportion live for months or years.
5. Each plasma cell contains thousands of polysomes that make the component chains.
6. The antigen content of such cells is very low (210 molecules) and may be zero.

favor the view that the inductive event may be the result of surface contact of antigen, retained on the follicle reticulum, and pre-formed antibody molecules located on the surface of certain lymphoid cells. This surface reaction could lead to a series of derepressive events resulting in proliferation and differentiation toward the plasma cell morphology. Antigenic induction need not involve information transfer—an idea difficult to grasp at first, and which will be dealt with extensively in the next two chapters of this volume. Stimulation of cell proliferation by antigen–antibody union at the surface of a lymphocyte is a plausible mechanism, which has been experimentally verified in a different context. When lymphocytes are treated in vitro by an antilymphocyte serum (in the absence of complement), transformation to a blast cell morphology and the beginning of DNA synthesis occur.

Antigen-induced proliferation of lymphoid cells

Whatever the detailed mechanism of antigenic stimulation may be, it is clear that a series of mitotic divisions in lymphoid cells must follow to create populations of immunological effector cells. Some aspects of this mitotic burst are described on the lower part of Figure 1 and in Figure 2. Three related phenomena occur. New large, medium, and small lymphocytes are formed, probably largely in the diffuse cortex, and these mediate cellular immune reactions. New families of plasma cells are formed; although it is probable that these gain their original stimulus in the cortex, perhaps in the follicles, the majority of the cells soon move to the medulla into areas known as medullary cords. There extensive further proliferation takes place. This final proliferative phase does not seem to require either antigen in the plasma cells or antigen-laden macrophages near them. Although we cannot be certain of the biochemical events that occur when antigen meets a single reactive cell, we can state that a clonal expansion results. This creates perhaps 500 antibody-forming plasma cells.

Immunological memory and germinal centers

The third effect of antigen in lymph nodes is to create the germinal center, a new morphological entity that was not present before antigenic stimulation. This develops in relation to the anlage, the primary follicle, with its contained antigen.[7] Primitive lymphoid cells invade the follicle, proliferate, and compress the antigen-capturing web into a crescentic "cap." The nest of primitive cells grows until it may contain many hundreds of blast cells, all dividing rapidly. Where these progeny go, and what they do, is not known for certain. We believe that they consist of both plasma cells and lymphocytes, the former migrating to the medulla after injection, the latter circulating around the lymphoid organs of the third level, and possessing an enhanced or special reactivity for the original antigen.

One reason for believing that the germinal center may be important for memory is that it can rapidly discharge cells into the surrounding tissue when stimulated by a second antigen injection. Also, the germinal center can capture them much more actively when antigens are given the second time than it could the first time. Finally, ontogenetic and phylogenetic considerations suggest an association between capacity to form germinal centers and capacity to give a classical secondary response.

Recently developed techniques have allowed us to put the memory phenomenon on a sound numerical basis. In a normal spleen, only one in 10^4 to 10^5 of the cells present at a given time are capable of responding to a given antigen. Thus there are relatively few "antigen-reactive" cells in the population. During the primary immune response, not only antibody-forming cells are generated. The number of antigen-reactive cells rises by a factor approaching 100. Moreover, this is a specific effect. The new, antigen-reactive cells respond to a reinjection of the original antigen, but not to an injection of an unrelated antigen. We may thus regard these memory cells as irreversibly committed to reactivity with one particular antigen.

The molecular basis of memory

It seems clear that "memory" is a statistical concept depending on the presence of a greater pool of reactive cells. It further seems likely that these reactive memory cells represent some of the progeny of the original smaller numbers of reactors—presumably those that did not differentiate into plasma cells. It remains for us to discuss the molecular basis on which this special reactivity to the original antigen might rest.

F. M. Burnet,[8] in his clonal selection theory, put forward the idea that lymphocytes might be genetically preadapted to react to only one, or to a restricted number, of antigens. When the "right" sort of antigen for a particular cell came along, the cell would be stimulated to multiply and the size of the reactive clone would be enlarged. The basis of heterogeneity among cells might be somatic mutation. Alternative and more sophisticated genetic models can be constructed.[9] These postulate that the original cell is multipotent, but its progeny, by some genetic process, are able to respond solely to a further administration of the same antigen. A third possibility, in my view not so likely, is that informational nucleic acids

stream from the macrophage that captured the antigen, conveying to a lymphoid cell instructions for the manufacture of the corresponding antibody. In some way, the lymphoid cell incorporates the instructions into its machinery in heritable form, and its progeny display enhanced reactivity. While it is clear that memory cells, with special reactivity for a given antigen, do exist, we must confess that the molecular mechanisms that confer memory on them are entirely speculative.

Problems shared between neurosciences and immunology

From the foregoing, it is obvious that there are many problems shared between the neurosciences and immunology. First, in both contexts the clear role of nucleic acids in memory has been postulated but not firmly established. Second, the nature of the inductive mechanisms that set in motion the synthesis of specific nucleic acids and proteins is not understood. In particular, the question of whether "new" nucleic acid molecules are made in some manner, contrary to the accepted Crick dogma, must be faced. Third, another facet that has received much attention in neuroscience but little in immunology concerns specific pharmacological mediators of induced responses. This is one area of potential interest in which the immunologist could learn much from the neurosciences. We are beginning to pinpoint the microanatomical sites for immune induction and to realize that at least two cell systems participate. We know little or nothing about the pharmacological events occurring between them, and the methods and conceptual approach of the neuroscientist could be most helpful.

SUMMARY The evolutionary aspects of specific immune responses show that the function first developed in the cyclostomes. It gained progressively greater sophistication in the elasmobranchs and amphibians and reached its full flowering in the mammal.

The organs important for immune responses are at three levels. Stem cells arise in the bone marrow. The thymus "instructs" them to become reactive to antigens. The peripheral lymphoid organs—lymph nodes and spleen—are the sites of direct interaction between antigen and immunocyte, and of the proliferative events that lead to antibody production. Thus, the microanatomical organization of lymph nodes is obviously important in correct functioning.

Immunological memory is that property of the immune system that allows a heightened reactivity to the *second* administration of a given antigen. It appears to depend on the creation of a population of memory cells. These are probably the progeny of cells stimulated by the first antigen injection. Such heightened reactivity is probably a genetic trait of the cells concerned, but the mechanism by which this is acquired is not known.

Immunological memory may depend in part on special structures, the germinal centers.

Antibody Structure and Diversity: Implications for Theories of Antibody Synthesis

G. M. EDELMAN

THE ADMINISTRATION OF an antigen to vertebrate organisms may elicit a number of different manifestations of the immune response, including delayed hypersensitivity, immune tolerance, and humoral antibody production.[1-4] All of these share the property of specificity. Specificity implies that a given manifestation of the immune response is elicited by the particular chemical structures present on the antigen (the antigenic determinants) originally employed and not by chemically unrelated antigenic determinants.

One of the fundamental problems of immunology is to determine the basis of this specificity. The solution would describe how information on the three-dimensional structure of the antigen is processed by the immunologically competent cell to result finally in production of the specific molecules that mediate the immune response. The molecular species that mediate delayed hypersensitivity and tolerance are not known. Only in the case of humoral antibody production are we in a position to examine the nature of specificity at the molecular level.

This article is concerned with a description of antibody structure and with some hypothetical mechanisms of specificity in the immune response. Although at present there is no direct connection between immunological specificity and specificity in the nervous system, some fruitful ideas may be generated by comparing the two biological systems. For example, in both systems there is specific recognition of a wide range of structures, and also storage of the information acquired.

Two main types of theory of antibody formation have been proposed to explain the origin of specificity.[5-8] Instructive theories postulate that the three-dimensional structure of the antigenic determinant contributes new information not previously encoded in the protein synthetic machinery of antibody-producing cells. Selective theories propose that the requisite information is already present in these cells; the three-dimensional structure of the antigen provokes the selective synthesis of appropriate complementary antibodies. There is another way of stating the distinction between the two types of theory: instruction by the antigen would introduce new types of diversity in antibody structure; selection would not. Thus, a central issue is to determine the mechanisms by which diversity of structure is achieved at the specific antigen-combining sites of the antibody molecules. This issue cannot be joined, however, until some important general structural properties of immunoglobulins are described.

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Immunoglobulin structure

Antibody activity is found in a complex group of serum proteins,[9] which are collectively designated as immunoglobulins.[10] There are several known classes of immunoglobulins; some of their properties are listed in Table I. These classes are distinguishable in terms of their molecular characteristics, such as the kinds of polypeptide chains of which they are composed. If immunoglobulins from one animal species are used as antigens in another species, antibodies directed against them may be formed. These antibodies may be used to show that immunoglobulins in different classes have distinctive antigenic determinants. The immunoglobulin molecules in all classes are multichain structures.[11-13] This provides a basis for recently proposed nomenclature[10] that attempts to reflect fundamental similarities and differences of the classes at the level of their polypeptide chains (Table II).

The molecules within each class consist of two types of chains that are under the control of different genetic loci.[14,15] Light chains have molecular weights in the neighborhood of 23,000[16-18] and are found in at least two antigenic types. Both types of light chains are present in all immunoglobulin classes. On the other hand, the heavy chains appear to be distinctive for each class. The known molecular weights are 55,000 for γ chains (those in γG immunoglobulins)[16] and 70,000 for μ chains (those in γM immunoglobulins) as seen in Table II.[19,20] The carbohydrate portion of immunoglobulins is attached covalently to the heavy chains[21]; as yet no function has been found for it.

Perhaps the most striking chemical feature of antibodies

TABLE I

*Properties of the Major Immunoglobulin Classes**

Class	Sedimentation coefficient	Moleulcar weight	Carbohydrate content (%)	Polypeptide chains	Molecular formula
γG	6.6S	1.5×10^5	2.5	Light: κ or λ Heavy: γ	$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
γA	6.6S	1.5×10^5‡	5.7	Light: κ or λ Heavy: α	$\alpha_2\kappa_2$ or $\alpha_2\lambda_2$
γM	19S	9×10^5	9.8	Light: κ or λ Heavy: μ	$(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$

* An additional class, γD immunoglobulin, has recently been described (Rowe, D. S. and Fahey, J. L., *J. Exp. Med.*, *121*, 185, 1965). It has a sedimentation coefficient of 7S. It is not included in the table because it has not been extensively studied in several animal species.

‡ Aggregates of higher molecular weight are also found in the γA immunoglobulins.

is their chemical heterogeneity.[9] This is reflected in their electrophoretic mobilities, amino acid composition, content of amino terminal and carboxyl terminal amino acids, and binding constants for antigen.[4] Evidence is accumulating that the fundamental basis of the heterogeneity of antibodies rests in differences in amino acid sequences in their polypeptide chains.

What is the reason for heterogeneity? It is probably a reflection of two major functions of antibodies: their capacity to bind to a variety of structurally unrelated antigens, and their biological role in different reactions such as complement fixation, fixation to the skin, placental transfer, and opsonization.[22,23] I will not describe further these biological functions of antibodies. It should be noted,

TABLE II

Nomenclature and Properties of the Polypeptide Chains and Fragments of Immunoglobulins (see Figure 2)

Chain or Fragment	Molecular weight	Present in or derived from	Special properties
Light chains: (κ chains or λ chains)	23,000	All immunoglobulin classes	Identified with Bence-Jones proteins
Heavy chains:			Contain carbohydrate moieties
γ chains	55,000	γG immunoglobulins	—
α chains	?	γA immunoglobulins	—
μ chains	70,000	γM immunoglobulins	—
Fab fragment	50,000	γG immunoglobulins	Consists of a light chain and amino terminal portion of heavy (γ) chains. Contains one combining site.
Fc fragment	40,000	γG immunoglobulins	Crystallizable. Consists of carboxyl terminal portion of γ chain.
Fd fragment	25–30,000	γG immunoglobulins	Contains amino terminal half of γ chain.

however, that there are structures responsible for these functions on portions of the antibody molecule other than the antigen-combining site.[24]

The origin of the chemical heterogeneity of the combining site is fundamental to a solution of the central problem of specificity. Before considering the nature of the site, it will be useful to describe in some detail the structure of a single class of antibodies, the γG immunoglobulins.

The structure of antibodies of the γG class

On the basis of detailed chemical studies, a number of similar models have been proposed for the γG antibody molecule.[13,25,26] One model is depicted in Figure 1. The molecular envelope within which the individual chains are folded is shown in an extended form, although it is possible that the molecule possesses a certain amount of flexibility.[27] Little information is available on the details of folding of the polypeptide chains; it is fairly certain, however, that there are few or no α-helical structures present.[28]

A main feature of the model is that it is a multichain structure in which both heavy and light polypeptide chains play a role in forming the active sites.[26] The antibody consists of two identical light chains and two identical heavy chains arranged as two light–heavy pairs, or half-molecules, linked through the heavy chains. Each chain is linked to its neighbor by a single disulfide bond and by noncovalent interactions. The half-molecules are assumed to have a two-fold rotation axis between them. The antigen-combining sites are placed at the ends of the molecule[29] and may be thought of as crevices between the light chains and heavy chains.

The chemical degradation steps upon which the above picture of the antibody molecule is based are summarized in Figure 2. Reduction of the interchain disulfide bonds followed by exposure to solvents that disrupt noncovalent interactions (urea, weak acids) results in dissociation of the chains.[11,12,21] Neither step alone is sufficient for dissociation. Mild reduction followed by treatment with dilute hydrochloric acid leads to separation of half-molecules.[30] Hydrolysis with the proteolytic enzyme papain[31] cleaves the heavy chains, producing two Fab fragments (containing the combining sites) and one Fc fragment.[10] Treatment with pepsin yields a product that resembles two Fab fragments linked by a disulfide bond.[32]

Separated light and heavy chains will reassociate to form molecules that are grossly similar to γG immunoglobulin.[33-36] Although the chains will reassociate without forming interchain disulfide bonds, these bonds can also be re-formed.[33] It is possible to produce hybrid molecules using light chains from antibodies of one specificity and

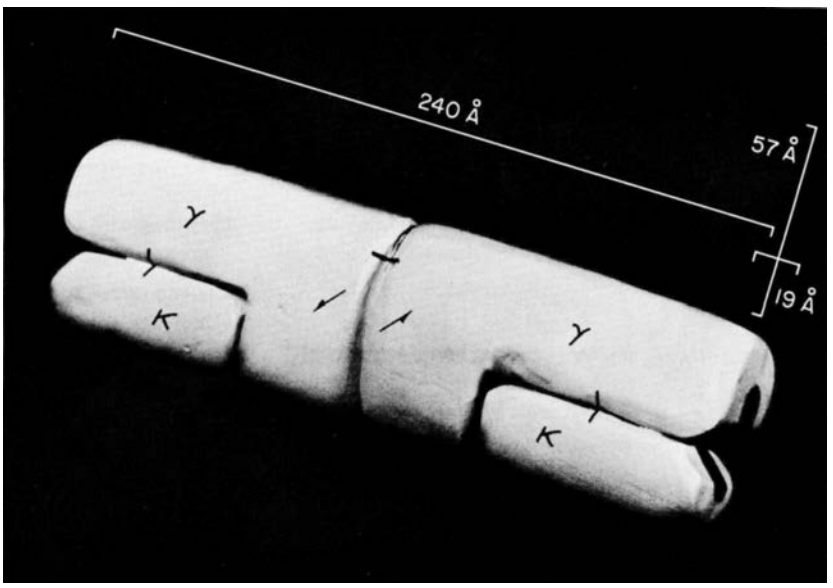

FIGURE 1 Model of an antibody molecule belonging to the γG immunoglobulin class. γ—heavy polypeptide chain. κ—light polypeptide chain. The single lines drawn on the model represent interchain disulfide bonds. The darkened area at the right represents one of the two combining sites. Arrows on the heavy chains indicate the presence of a two-fold rotation axis.

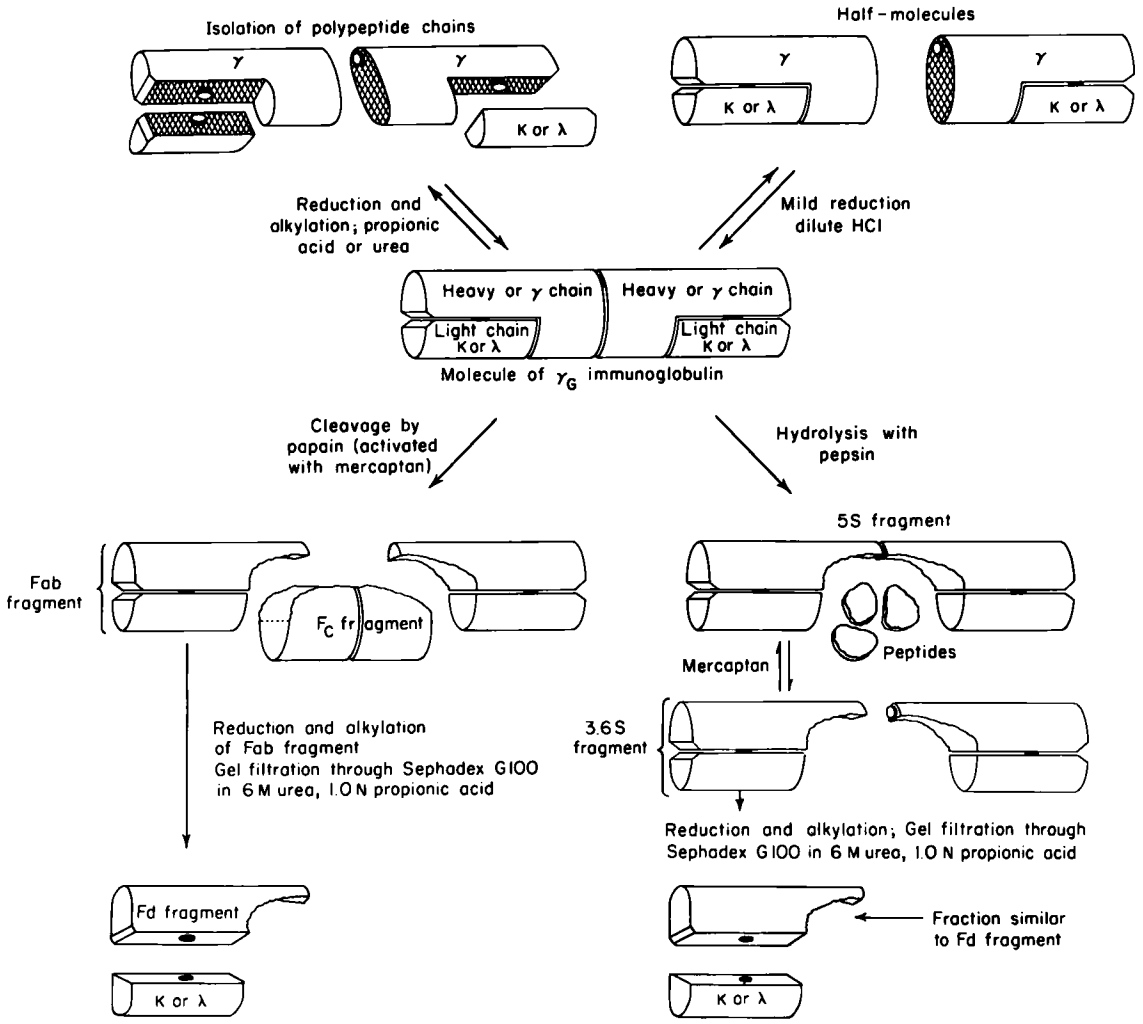

FIGURE 2 Degradation of the γG immunoglobulin molecule to chains and fragments (see Table II).

heavy chains from antibodies of a different specificity.[33,34] Such experiments have a bearing on the nature of the antibody-combining site.

The antibody-combining site

In Figure 1 the antibody-combining site was assigned dimensions based on the measurements of Kabat in his studies of human antibodies directed against dextran.[37] Compared to the over-all dimensions of the molecule, the site is relatively small, although it may differ in size in different antibodies. Studies on the thermodynamics of binding of haptens (antigens of low molecular weight) have suggested that the site is complementary in shape to the antigenic determinant group.[4]

Antibodies consist of different kinds of polypeptide chains; this raises the question of whether light chains or heavy chains or both together control the specificity of the combining site. The question has not been completely answered, but the preponderance of evidence indicates that chain interaction[13,26] is required for formation of the site. The evidence comes from two different lines of investigation—reconstitution of active molecules from light and heavy chains and site-labeling experiments.

If purified antibodies of two different specificities (1 and 2) are dissociated into their constituent chains, four different kinds of molecules may be constructed from the chains. These include the reconstituted molecules consisting of the original chains, H^1L^1 and H^2L^2 (homologous mixtures), and hybrid molecules of types H^1L^2 and H^2L^1. These products and the chains themselves may be tested for activity and compared with the original antibodies. A

typical experiment is illustrated in Table III. Light chains show no activity, whereas heavy chains retain some of the original activity toward the homologous antigen. The important result is that homologous mixtures show considerable reconstitution of activity and this activity is always greater than that of any hybrid mixture.[38-40] These experiments suggest that structures on both the homologous light and heavy chains are necessary for a complete and specific combining region.

It is not, however, apparent from such studies whether both light and heavy chains contribute amino acid residues directly to the combining site (shared site) or whether one chain influences the folding of the site on the other chain (modulated site), as shown in Figure 3A. Evidence that the site is shared comes from affinity-labeling experiments.[41-43] In these experiments, a hapten containing a functional group capable of reacting with amino acid residues in the site is mixed with purified antibody to that hapten. After reaction, the chains are separated and the amount of hapten covalently bound to each is assessed. It has been found that both the light and the heavy chains of the antibodies directed against a particular hapten are labeled specifically. Moreover, peptides containing the haptenic label have been obtained from both light and heavy chains.[42] These peptides have two outstanding characteristics. They are hydrophobic and they show evidence of extensive chemical heterogeneity. Both of these findings are consistent with previous evidence on the nature of the combining site.[4]

TABLE III

*Reconstitution of Antibody Activity by Admixture of Light and Heavy Chains of Guinea Pig Antibodies to the Dinitrophenyl (DNP) Hapten**

Sample	Binding of C^{14} dinitrophenol cpm†
Ab	900
Ab-P	790
Ab-RA	710
Ab-RAP	480
H(DNP)	380
L(DNP)	negligible
H(DNP) + L(DNP)	580§
H(DNP) + L(γ)	240

Abbreviations: Ab—antibody; H(DNP)—heavy chain from anti-DNP antibody; L(DNP)—light chains from anti-DNP antibody; L(γ)—light chains from immunoglobulin of animal obtained prior to immunization; P—exposed to 0.5 N propionic acid; RA—reduced alkylated, RAP—reduced and alkylated and exposed to propionic acid for the same length of time as the separated chains.

* See ref 39.

† cpm—counts per minute calculated to be bound per unit absorbency of protein at 280 mμ after extrapolation of data from equilibrium dialysis experiments to infinite hapten concentration.

§ Maximal value expected if contributions of heavy and light chains were only additive is 340 cpm per unit absorbency at 280 mμ.

With this evidence in mind, one might consider that the polypeptide chains of specific antibodies have three regions of importance.[26] They are: a site region, a hypothetical modulating region, and an interchain binding region (Figure 3B). The site region would consist of those amino acids in the polypeptide chains that actually contribute residues to form the combining site. The modulating region would consist of amino acid residues that affect the shape of the site indirectly by altering the folding of the chains. The interchain binding region would consist of amino acid residues that are responsible for interaction between the chains to make half and whole molecules. It has been suggested that the interchain binding region does not vary greatly from antibody to antibody in the same class.[33,34,39] This stems from the observation that hybrid molecules may be formed readily from chains of different antibodies. On the other hand, the site and modulating regions might be expected to show considerable differences in amino acid sequence among different antibodies. Studies on the amino acid sequences of immunoglobulin chains have proceeded to the point at which some decisions may be made about these expectations.

Amino acid sequence and the diversity problem

A decade ago it was a tenable hypothesis that all antibodies had the same amino acid sequence and that differences in specificity were the results of differences in folding of the polypeptide chains.[44] This hypothesis is no longer tenable. Several experimental findings have made it necessary to assume that the specificity of antibodies is related to differences in amino acid sequence within their polypeptide chains. Starch gel electrophoresis of the chains of different antibodies showed striking differences in pattern.[45] Careful analyses revealed that different antibodies have significant differences in their amino acid composition.[46] Site-labeling experiments of the type described above showed extensive heterogeneity of labeled peptides even within a group of antibodies directed against the same hapten.[41,42] Active Fab fragments of antibodies that were completely denatured after cleavage of all disulfide bonds were found to regain activity by refolding in the absence of antigen.[47] This latter experiment would suggest that, as in the case of ribonuclease,[48] the major determinant of active native three-dimensional structure is the amino acid sequence of the protein. This is inconsistent with certain instructive theories[44] in which it is postulated

that the active site of antibodies is formed by folding around the antigen, which serves as a template.

All of these findings have provoked efforts to determine the amino acid sequence of antibodies or their polypeptide chains. In view of the enormous heterogeneity of these proteins, however, the task cannot be approached directly. Fortunately, homogeneous immunoglobulins do exist as a result of an accident of nature. In multiple myeloma, a disease that occurs spontaneously in man[49] and that may be experimentally induced in mice,[50] tumors of the plasma cells produce large amounts of either γG or γA immunoglobulins. Although these proteins are relatively homogeneous and have not been shown to possess activity as antibodies, they are in every other respect similar to normal immunoglobulins.

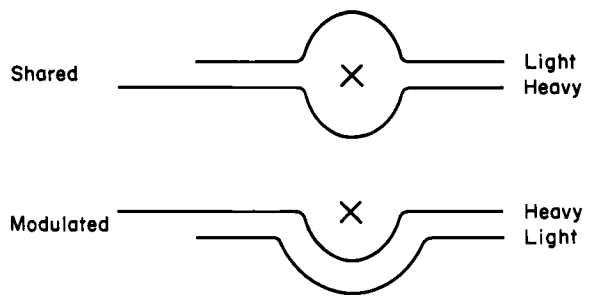

FIGURE 3A Ways in which the conformation of an antibody combining site may be influenced by interaction between light and heavy chains. X—designates the location of the site.

Amino acid sequence analysis must proceed at the level of the individual polypeptide chains of a multichain protein. A fortunate circumstance has made this task easier in studies of the immunoglobulins. In multiple myeloma, Bence-Jones proteins are excreted in the urine, sometimes in copious amounts. It has been shown that Bence-Jones proteins are free light chains of the type found in the myeloma proteins of a given individual.[18,51] Most studies of the amino acid sequence have been carried out on Bence-Jones proteins.[52-54] A number of striking facts have emerged from these studies, which tend to confirm and sharpen previous ideas about the origin of specificity and diversity in antibody molecules.

A comparison of the partial amino acid sequences of seven different human Bence-Jones proteins (light chains) is given in Figure 4. The polypeptide chains consist of 212 to 214 amino acid residues. The most important feature of this comparison is that there are many differences in the stretch of amino acids from the amino terminus (residue 1) to residue 105, whereas in the stretch from residue 106 to residue 212 (the carboxyl terminus) the sequence is al-

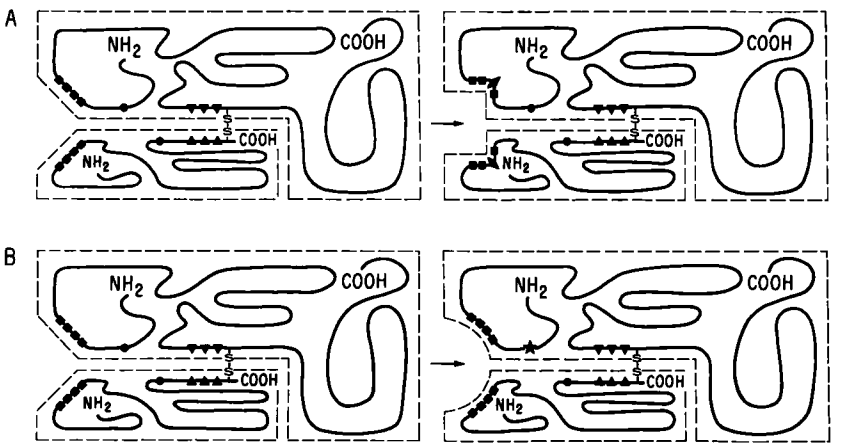

FIGURE 3B Schematic illustration of regions of importance for specificity of the γG antibody molecule. (Only half-molecules are shown; the site is to the left.)

■ or ♦—amino acid residues in the site region.

● or ★—amino acid residues in the modulating region.

▼—amino acid residues in the interchain bonding region.

A) Differences in conformation of the site resulting from a change in the amino acid sequence of the site region. B) Differences in conformation of the site resulting from a change in the amino acid sequence of the modulating region. The disulfide bond (S–S) linking the carboxyl terminus of the light chain to the heavy chain is also illustrated.

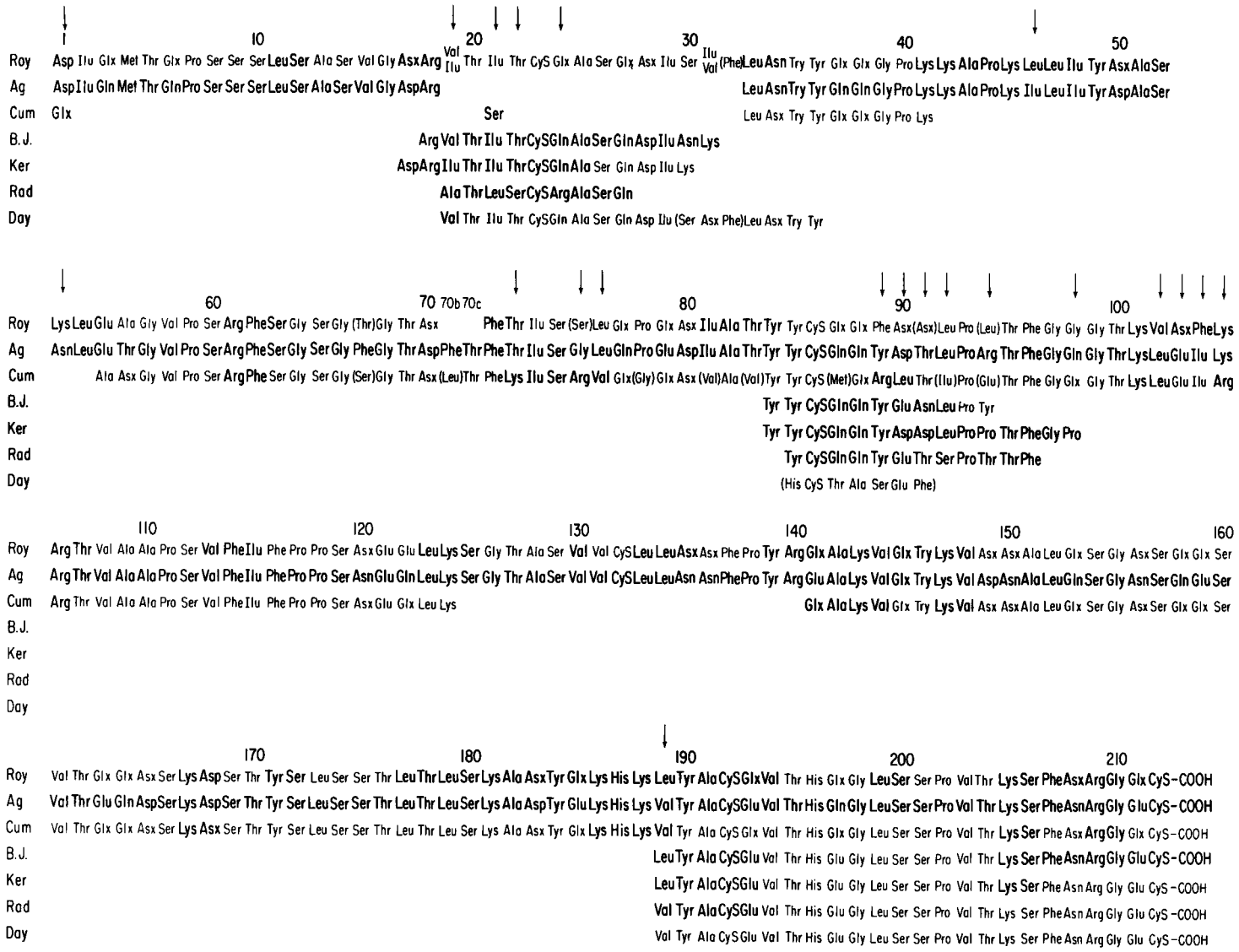

FIGURE 4 Comparison of partial amino acid sequences of seven different human Bence-Jones proteins (light chains). All of the samples are κ chains, i.e., are of antigenic type K. The residues printed in boldface have been assigned definite sequences; those in lighter face are assigned by homology on the basis of peptide compositions. The symbols Glx and Asx are used where it is not certain whether glutamic acid or aspartic acid occur as amino acids or their amides. The numbering system is based on that used for protein Roy (Note 52). Vertical arrows (↓) are placed at positions where it is *definitely* known that an interchange of amino acids has occurred. Sources of data: proteins Roy and Cum (Note 52); protein Ag (Notes 53, 71); proteins B.J., Ker, Rad, and Day (Notes 54, 56).

most identical. Only at position 189 is a valine residue in protein Ag replaced by a leucine residue in protein Roy (Figure 4). This has led to the idea that there is a variable portion and a constant or invariant portion in all light chains. Other structural studies have led to the conclusion that the amino terminal region of the light chain is involved in the combining site of antibodies,[55] whereas the carboxyl terminal region is probably concerned with binding the light to the heavy chain. The carboxyl terminal half-cystine forms part of the disulfide bond that links one type of light chain to the heavy chain.[56] As far as is known, heterogeneous light chains from normal immunoglobulins have the same sequence in their carboxyl terminal portions.[54]

The position of the disulfide bonds linking portions of the light chain to other portions has been established.[54,56] A curious structure involving two intrachain loops emerges (Figure 5). This observation and the delineation of variable and constant portions prompts one to ask if the halves are homologous, i.e., related by evolution from a single gene. Fitch[57,58] has described a procedure for testing the evolutionary homology of two proteins. In this method a computation is made of the minimal number of nucleotides in the genetic code that must be altered to

convert one sequence to the other. This value is then compared with that expected by chance alone. F. A. Dodge has examined the variable and constant halves of protein Ag using Fitch's method. Two regions appeared to have sequences that were possibly related. They are indicated in Figure 5. The relationship is marginal, however, and the over-all impression is that there is no strong homology between the variable and constant regions.

Comparable studies on heavy chains have not proceeded as far, both because of their size and because, in contrast to Bence-Jones proteins, there are difficulties in obtaining large amounts of homogeneous material. The few experiments that have been performed suggest that the heavy chain of γG immunoglobulin (γ chain) will also have a common portion in the carboxyl terminal region and variable portions in the amino terminal region.[59] Singer and Doolittle[43] have noted that heavy chains and light chains have some homologous sequences. On these grounds, they postulate that genes coding for heavy chains arose in evolution from genes coding for light chains.

The sequence variations found in the chains of immunoglobulins have no parallel in other proteins so far studied. Although other proteins show differences in sequence (e.g., the normal and abnormal hemoglobins), multiple substitutions usually are not seen, and the presence of a variable region sharply demarcated from an invariant region is not as obvious as it is in Bence-Jones proteins.

The evidence so far accumulated indicates that immunoglobulins have an enormous diversity of amino acid sequences. The reasonable assumption is that this diversity is related fundamentally to the enormous range of specificities of antibodies.

Possible mechanisms for diversity

The classic work of Ingram[60] has demonstrated that a genetic difference between normal men and those with sickle-cell anemia is reflected in a single amino acid substitution in their hemoglobins. Extensive studies on viral systems,[61] on the enzyme tryptophan synthetase,[62] and on the genetic code[63] have shown that mutations in structural genes result in changes in the amino acid sequence of their protein products. The question to be considered here is whether the enormous diversity of immunoglobulins also arises by this means or whether additional mechanisms are required.

There is no doubt that at least some portions of immunoglobulins are under direct genetic control. The immunoglobulins of humans,[14] rabbits,[15] and mice[64] have all been shown to have genetic differences, the so-called allotypes. These differences are detectable by serological methods and appear to be inherited in classical Mendelian fashion. Thus, heavy chains are determined by genetic loci that differ from those determining light chains. (The detailed description of the genetically controlled serological or allotypic differences among immunoglobulins will not be undertaken here [see notes 14 and 15].) The total number of such loci so far discovered is relatively small,[14] and as yet no clear-cut relationship between antibody specificity and genetic control has been established.[12,65] However, a discussion of the diversity of immunoglobulins must be concerned with just these two facts.

Such a discussion is difficult at the outset, because we do not know how many structurally different antibodies are required to account for all known serologic specificities. The number is not likely to be less than 10,000 and may in fact exceed 10^6. To underline the difficulty, it might be pointed out that no two myeloma or Bence-Jones proteins from humans or mice have been found to be alike. The exact number of samples examined is not available, but it exceeds several hundred.

The multiple structural differences in immunoglobulins could be accounted for hypothetically by one or more of three major mechanisms. The first is gene duplication and accumulation of point mutations by classical routes of mutation and selection.[66,67] The second postulates somatic alteration in the structural genes for immunoglobulins, and includes somatic mutation,[68] chromosomal rearrangement, and intragenic crossing over.[69] The third mechanism[70] invokes special coding triplets in the portion of the

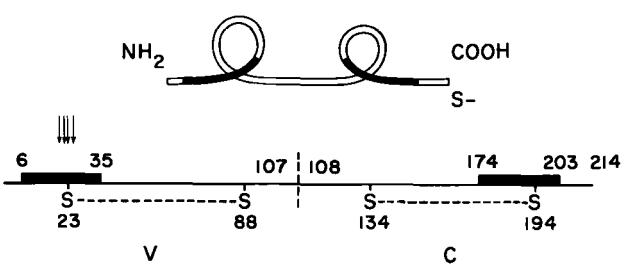

FIGURE 5 Schematic diagram of human light chain indicating positions of intrachain disulfide bonds (refs 54,56). Also indicated (▬) are stretches in the variable and constant regions which may be homologous. Vertical arrows refer to positions of interchanges found in one of these stretches. The top figure shows the loops on the variable (V) and constant (C) halves formed by the intrachain disulfide bonds. S– represents the half-cystine residues involved in linking the light (κ) chain to a heavy chain. The numbering system is based on protein Ag and thus from residue 71 onwards the number of an amino acid residue is two higher than the corresponding number used for protein Roy (see Figure 4). NH_2—amino terminus. COOH—carboxyl terminus.

gene corresponding to the variable region. It is assumed that the special triplets bind to anticodons in those transfer RNAs that lack unique specificity for their corresponding activating enzymes. In this way, different amino acids would be inserted by the same triplet. Features of more than one of the three mechanisms may be required for a complete explanation.

The diversity in immunoglobulin sequence could be accounted for by the existence of separate structural genes for each different chain. Examination[71] of recent data on sequences of light chains has indicated that there are 22 definite positions of interchange for all proteins so far studied. Altogether there are 32 interchanges of amino acids. Twenty-six of these are compatible with single nucleotide changes in the codons for an amino acid pair. The remainder require changes in two nucleotides, as pointed out by Titani, et al.[71] The genes must contain corresponding regions in which mutations have occurred, giving rise to the variations in the final amino acid sequence (Figure 6).

If one assumed that each of these genes also contains the information for the invariant portion of the sequence, then *all* of the genes must have identical regions. This is difficult to reconcile with present data on allotypes. Allotypic differences appear to be related to single amino acid substitutions in the invariant portion of the chains. For example, the so-called Inv factor is a+ when the amino acid at Position 189 (see Figure 4) is leucine. The factor is b+ when leucine, at this position, is replaced by valine.[52,56,71] Genetic analysis[14,15] suggests that there is little or no recombination among the different allotypic genes. If there were many copies of each allotypic gene, as would be required by the hypotheses above, one would expect a high recombination frequency. Such recombination would make it unlikely that a homozygous allotype could be maintained. But homozygosity of allotypes is often seen. For these reasons, the requirement that a definite portion of the light chain remain invariant except for the allotypic difference is more easily met if two kinds of genes are postulated. One kind would code for the variable region and the other for the constant region. This has been proposed by Dreyer and Bennett.[67] We will call these the V (variable) genes and C (constant) genes.

Dreyer and Bennett did not consider in detail how a large number of V genes might have arisen, and how the C gene might have remained unchanged. At the outset, there is a mechanism whereby the number of different V genes required might be considerably reduced.[26] This mechanism is suggested by the observation that both light and heavy chains are needed to form a complete combining site. If there were p different genes for light chains and q different genes for heavy chains, and if the folding of any particular combination is unique, then pq different combining sites are possible. If, on the average, m different conformations were possible for any combination of chains, then the number of structurally different sites would be mpq. In the absence of evidence to the contrary, it is assumed here that $m = 1$.

It is possible that more than one combination of different light and heavy chains may be able to form sites with more or less the same specificity. This *degeneracy* would reduce the number of different specificities that could be formed by interaction of a given number of light and heavy chains. If there were 10^3 genes for light chains and 10^3 genes for heavy chains, the maximum number of different sites would be 10^6. If, on the average, any given specificity could be generated by ten different combina-

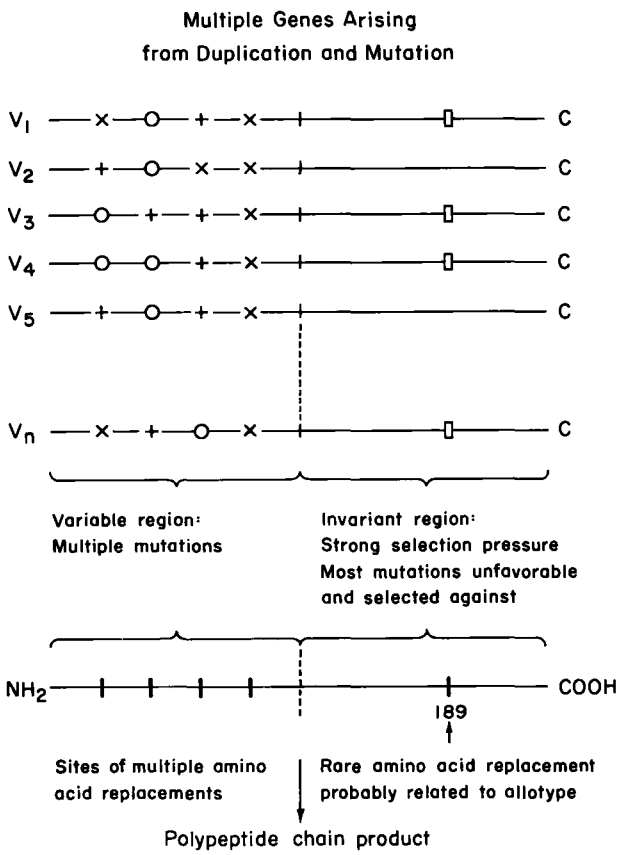

FIGURE 6 Illustration of the hypothesis of multiple genes (V) for variable regions of light chains combined with a single gene (C) for common region. Multiple genes are assumed to have arisen by duplication. X, O, +, sites where mutations have occurred in codons in V genes. □—site of mutation in C gene influencing allotype of light chain. NH_2—amino terminus of light chain. COOH—carboxyl terminus of light chain. A similar picture is envisioned for the heavy chain.

tions, 10^5 completely different kinds of sites could be formed by the products of 2000 genes.

Although the assumption of the chain-interaction hypothesis reduces the number of genes required, the origin and maintenance of possibly as many as 2000 or more different V genes must still be accounted for (Figure 6). It is assumed that a series of V-gene duplications occurred (similar to those postulated for hemoglobins).[60] The borderline homology discussed in the previous section raises the possibility that the V gene arose in evolution by partial duplication of the C gene. An additional assumption must be made to account for the stability of the C genes and the variability of the V genes. Individual mutations in V genes that code for site regions or modulating regions are postulated to have *in themselves* neither selective advantage nor selective disadvantage. There might, however, be a selective advantage for accumulating a large *number* of different mutations. On the other hand, mutations in C genes would be subject to stringent selection pressure, inasmuch as C genes code for the interchain binding regions and for physiological functions of the antibody molecule other than antigen binding. These functions include the capacity to fix complement, to bind to cells, to opsonize, etc.

According to the above explanation there would be selective advantage for those animals that produce the largest numbers of different polypeptide chains capable of forming *whole* immunoglobulin molecules, each of which is also capable of complete physiologic function. The diversity of antibodies is generated without any direct intervention of the various antigenic determinants to which they may ultimately be directed. There is only one requirement: mutations in V genes affecting the site regions or modulating regions must not affect the conformation of other functional regions of the molecule determined by C genes. A large number of possible sites could be generated by accumulated point mutations in V genes, even if the site were relatively small and fixed in its location. Thus, if 15 amino acid residues contributed directly to the site, genetic variations could account for 20^{15} different sites, as there are 20 different amino acids. The presence of amino acid substitutions in the modulating region would increase this number greatly.

A second possible mechanism of diversification could be the result of alteration of the genetic information by some form of somatic mutation. A detailed hypothesis based on somatic crossing over has been proposed by Smithies.[69] He pointed out that sequence variation could be accounted for by short-range, inverted duplications of genes in the cells of the immune system. The accumulating evidence on the sequence of light chains does not appear to support this ingenious mechanism.[53,56,71] Although it has the advantage of generating large numbers of different amino acid sequences from a small number of genes, it also has disadvantages. The information for the large number of antibodies required would have to be generated within the lifetime of the organism. It is difficult to see how selection against unfavorable structures could take place in so short a time.

Brenner and Milstein[68] have suggested another means by which somatic variation might occur in a small number of genes, each of which codes for the entire light chain. It is assumed that an exonuclease attaches to the constant portion of the gene and hydrolyzes one of the complementary strands of DNA for a variable length. Repair by a DNA polymerase then takes place in such a manner that errors of replication occur in the region corresponding to the variable part of the light chain. These errors are transmitted after cell division; one daughter cell receives the unchanged light-chain gene and the other daughter receives the changed gene. Repetition of this process leads to the observed diversity of sequence. This hypothesis has the advantage of explaining the constant and variable regions in terms of the specificity requirements of an enzyme. It does not explain why the errors appear in fixed positions as single-base substitutions in most of the interchanges so far examined.

A third possible mechanism for diversity has been proposed by Potter and his co-workers.[70] The number of genes coding for antibody chains may be relatively small, but they may contain unusual codons. In this case, only one gene would be required to code for both V and C regions. There may be a set of transfer RNAs corresponding to the special codons and specific only for them. If each of the special transfer RNA molecules could insert more than one amino acid at positions corresponding to these codons (perhaps as a result of differences in the corresponding activating enzymes), a large number of different sequences could arise. For example, if there were special codons at 20 different places in the portions of the gene corresponding to the variable region, and if at each of these locations two different amino acids could be inserted in translation, there would be 2^{20} different possible sequences that could be synthesized. This hypothesis assumes that the transfer RNA molecules or activating enzymes are not those involved in the synthesis of other cellular proteins. Perhaps the strongest criticism of this hypothesis is that it requires the postulation of an unconventional mechanism for control of protein synthesis. Moreover, it has been pointed out[71] that in the light-chain sequence, arginine is replaced by six different amino acids, and that leucine and isoleucine are each replaced by five different amino acids. It is difficult to see how the transfer RNA could be so nonspecific. Nevertheless, the hypothe-

sis can be tested by direct experiment, and a demonstration that special transfer RNAs are present in lymphoid cells would be of great significance.

The hypothetical mechanisms described above can be divided into those in which multiple genes arise by mutation and selection in evolution, and those in which there is some means of introducing variability in a single gene or a few genes within the lifetime of the organism. A mechanism that invokes both multiple genes and somatic variations has been formulated by J. A. Gally and me.[72] We assume that multiple tandem duplications of genes corresponding to the variable regions occurred in evolution. The number of genes need not be very great; perhaps one hundred would suffice. After duplication, point mutations would have accumulated in a number of the genes. Aside from these mutations, however, the genes are homologous. It is postulated that somatic crossing over occurs in the homologous regions. Crossing over is made more probable by the homology and by the fact that the duplications are tandem. As shown in Figure 7, new sequences may be generated by such crossing over. At the same time, each product would appear to have been generated strictly by point mutation. This hypothesis would be strengthened if it were found that certain Bence-Jones proteins contain combinations of amino acid interchanges that are found singly in other Bence-Jones proteins. Somatic crossing over has been observed in human leukocytes,[73] particularly when they are treated with mitomycin.[74] These observations have not as yet been related to variations in immunoglobulin structure. Nevertheless, they suggest the possibility that somatic recombination may occur in these cells.

If two different kinds of genes, V and C, are necessary for the synthesis of a single polypeptide chain, the information for the variable and constant portions must be combined in the correct order. The halves of the chains might be synthesized separately and joined by an enzyme with peptidase activity. The messenger RNAs corresponding to each half might be joined before the chain is synthesized. Finally, the two genes might be joined in a fashion similar to phage insertion.[67] So far, there is no conclusive evidence to support or exclude any of these possibilities. None of them is required if variation occurs by break and repair (Brenner and Milstein) or by modulating transfer RNA's (Potter). There is an additional observation not accounted for by any theory: in the human proteins so far examined, the lengths of the variable and constant regions appear to be the same (Figure 4). This would imply that the V and C genes, or the V and C regions of a single gene, have the same length. If a C gene is inserted as an episome in place of one of the tandem duplicated V genes shown in Figure 7, the need for specificity may require

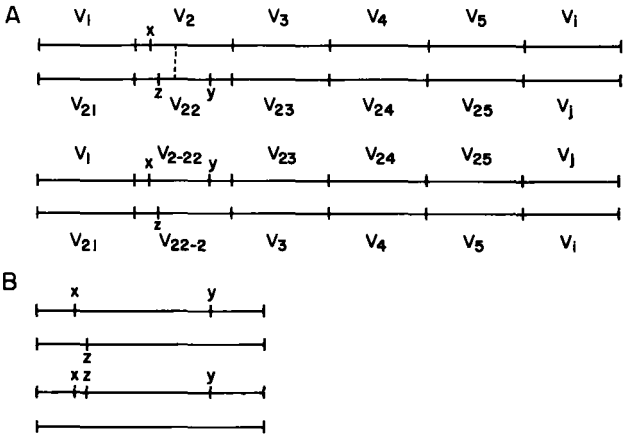

FIGURE 7 Hypothetical mechanism of homologous crossing over between V genes which have undergone tandem duplication. a) Result of a single crossing over between gene V_2 and V_{22}. x,y,z-sites of point mutations. Crossing over occurs in the homologous region between x and y. b) Results of single crossing over in region between x and y (top two genes) and of single crossing over in region between x and z (bottom two genes).

that the two genes be the same length. Another possibility is that a gene corresponding in size to a C gene doubled during evolution. The doubled gene would then have undergone mutation in the V region, as shown in Figure 6. At present, however, it must be admitted that there is no compelling explanation for the apparent equality.

The foregoing discussion has ignored the problem of control of synthesis of specific antibodies [see articles by Nossal and Jerne in this volume]. Nevertheless, it may be useful if a hypothetical scheme[66] is put forth here to show how a mechanism of diversification might be correlated with other stages of specific antibody synthesis.

This scheme is divided into four steps (Figure 8): (1) diversification of sequence; (2) chain interaction; (3) initial recognition of antigen; and (4) maturation and replication of cells containing "proper" subsets of chains to produce specific antibodies. I favor the view that diversification results from mutation and selection in evolution, invoking a combination of tandem duplication, accumulated point mutations, and somatic crossing over to achieve a large number of different sequences. After the chains are synthesized they interact to form a large number of different antibodies. Some molecules of this large set may be directed toward the same antigen, but the set of antibodies is orders of magnitude larger than the set of heritable genes required for synthesis of the light and heavy chains. Antigen molecules are probably recognized by appropriate members of this huge collection of antibodies. This requires that low levels of antibodies or their

chains be present before the antigen is introduced. Cells containing the "correct" types of chains are stimulated to mature, divide, and produce additional amounts of antibodies of the specific type. This theory is selective; the antigen is not required at any fundamental step of protein synthesis, and its chief function would be to stimulate cells to produce additional amounts of specific antibodies.

Some speculations on the relation of immunological specificity to specificity in the nervous system

Even with the relatively large amount of information available on antibody structure, our understanding of the mechanism of specificity remains highly speculative. It is therefore extremely hazardous to venture comparisons with mechanisms of specific recognition in the central nervous system, in which both the types and the roles of the macromolecules that might be involved are largely unknown. One possibly fruitful analogy may be drawn, however. If specific protein molecules are required at some stage of information transfer in the central nervous system, it might not be too surprising to find that they show a diversity of sequence and heterogeneity similar to those of antibodies. In addition, it might be expected that the proteins are multichain structures. This latter structural principle (which has become prominent in discussions of control mechanisms and allostery) is also capable of generating a large number of specific conformations from the products of a relatively small number of genes. It might, therefore, be predicted that brain proteins, if they have a role in information processing, will be multichain proteins of high molecular weight. Certain brain proteins might be expected to show a degree of heterogeneity as great or greater than that of antibodies.

Whether these predictions are fulfilled or not, the immunological system provides a well-studied model of molecular recognition in the interaction of the organism with the outside world. At the very least, the kinds of problems generated in examining the immune response must ultimately be faced in analyzing the nervous system at the molecular level.

Summary

One of the outstanding properties of antibodies is their capacity to interact specifically with a wide variety of chemically different antigens. The molecular basis of this specificity remains unknown, although it appears to have its origin in the great diversity of three-dimensional structures among antibody molecules. Recent work on the structure of antibodies has sharpened our view of this problem.

Hypothetical Scheme of Stages in Specific Antibody Synthesis

Diversification of sequence by mutation, crossing over, or special translation mechanisms		Complementation and chain interaction to form molecules and sites	Recognition of antigen	Maturation of cells containing proper subsets of chains and antibody synthesis
Complementing genes	Polypeptide chains			
	$light_1$	$light_1\ heavy_6$		Antibodies to A:
	$light_2$	$light_3\ heavy_2$	Antigen A	($light_3\ heavy_2$)
cistron(s) for light chains	· · · ·	$light_{12}\ heavy_{26}$		($light_{14}\ heavy_5$)
	·	$light_{14}\ heavy_5$		
	$light_p$			
	$heavy_1$	$light_{32}\ heavy_{17}$		Antibodies to B:
	$heavy_2$	$light_{32}\ heavy_4$	Antigen B	($light_{32}\ heavy_{17}$)
cistron(s) for heavy chains	· · · ·	$light_{18}\ heavy_5$		($light_{32}\ heavy_4$)
	·	$light_{22}\ heavy_{16}$		($light_{18}\ heavy_5$)
	$heavy_q$			

FIGURE 8 Hypothetical scheme of stages in specific antibody synthesis.

Antibodies have been found to be multichain structures consisting of light chains and heavy chains. By means of chemical and enzymatic degradation of antibodies, those portions of the chains responsible for antigen binding have been localized. Both chains appear to be required for complete specificity of antigen binding. Other studies have suggested that the three-dimensional structure of the antigen-combining site depends on the amino acid sequence of the light and heavy chains.

Complete amino acid sequence analysis cannot be performed on antibodies because of their chemical heterogeneity. It is possible, however, to analyze homogeneous immunoglobulins produced by tumors of plasma cells (multiple myeloma). In particular, Bence-Jones proteins (light chains) have been subjected to extensive analysis. The results suggest that the light chain has a variable region that differs from antibody to antibody and a constant region that is similar in antibodies of a given class.

These facts have stimulated several hypotheses on the origin of diversity among antibodies. Genetic and extragenetic mechanisms have been proposed and briefly reviewed. It is suggested that the diversity arose from tandem gene duplication, accumulation of point mutations, and homologous somatic crossing over. Mutations in those genes that code for the variable regions are assumed not to be selected against, and have no effect on the physiologic function or over-all structure of the antibody molecule. Mutations in genes coding for the constant portion are subjected to severe selection pressure, as they are assumed to alter the capacity to form intact molecules as well as the capacity to carry out essential physiological functions such as complement fixation, opsonization, etc.

The hypothetical mechanisms of diversification of antibody structure may be useful in formulating hypotheses to explain macromolecular patterns in the nervous system.

Antibodies and Learning: Selection versus Instruction

NIELS KAJ JERNE

UNTIL LESS THAN ten years ago, there was an almost unanimous consensus among immunologists that antibody formation was equivalent to a learning process in which the antigen played an instructive role. The main basis for this belief was that the number of different antibody specificities, or the number of different antibody molecules that one animal can produce, is so large that it would be impossible for a cell nucleus to accommodate genes for this entire range of potentialities for protein synthesis. The number of different antigens is immense. Every species of animal, for example, must have several species-specific antigens. Against any one of these millions of antigens the immune system of one individual animal can produce a specific antibody. Therefore, the argument went, the number of different antibodies that an animal can produce must be virtually unlimited. Furthermore, the work of Landsteiner and his school[1] had shown that an animal produces antibodies even against artificially synthesized substances (haptens) that were made in a chemical laboratory and had never before existed in the world. The immune system of an animal could not possibly have anticipated the arrival of such antigens and must therefore have been "instructed" by the antigen itself in the formation of antibody. The instructive mechanism proposed was that the antigen, after having entered a competent cell, guides the tertiary folding of polypeptide chains into globulin molecules, thereby imposing upon those molecules a conformation complementary to a surface region on the antigen.[2-5]

Instruction versus selection in antibody formation

In contrast to this view, a selection mechanism was proposed, based on a logical argument concerning "recognition." The precision of recognition by the immune system

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can be illustrated by examining the antigenic properties of the "constant" part of the kappa light chain of human immunoglobulin. This constant portion comprises 107 amino acids (numbered 108 to 214) at the carboxyl end of the light chain, which have been found to be identical in sequence in all individual cases so far examined, except for amino acid number 191. In some individuals, this is valine; in others it is leucine. This difference is known as an allotypic difference. Those individuals that have valine at the 191 position belong to allotype Inv b^+, whereas those that have leucine at this position belong to allotype Inv a^+. This allotypic difference was detected by immunological methods involving the formation of allotype specific antibodies.[6] The immune system is thus capable of recognizing the replacement of one amino acid within a long sequence of amino acids of a protein molecule. It is a characteristic feature of the immune system that an animal does not normally appear to produce antibodies against its own circulating antigens, which *do* elicit antibody formation when injected into a different animal. How does the animal recognize that an antigen arriving in its tissues is, in fact, an antigen against which antibody should be produced and not one of its own antigens to which no response is desired?

The immune system, then, must not let itself be stimulated to produce antibodies before having recognized that the antigen with which it is confronted differs from its own antigens. In order to recognize its own antigens, which differ among themselves in innumerable ways, the animal would have to possess a large set of self-recognizing molecules, and it would not be able to decide that a given antigen is its own before this entire set had been applied—a scrutiny, moreover, that would have to be interminably repeated. It would therefore seem impossible for the animal to recognize its own antigens. The recognizing agent must recognize foreign antigens, and the obvious molecules to accomplish this task are antibody molecules.

It follows that an animal cannot be stimulated to make specific antibodies, unless it has already made antibodies of this specificity before the antigen arrives. It can thus be concluded that antibody formation is a selective process and that instructive theories of antibody formation are wrong.[7-10]

Many immunologists were not convinced by the logical argument presented above, and only because of direct experimental evidence, accumulated during the last three or four years, have instructive theories of antibody formation finally been abandoned. Antibody molecules have been shown to consist of two identical heavy polypeptide chains and two identical light polypeptide chains.[11] It has also been shown that these polypeptide chains are assembled on ribosomes[12] and that the specificity of an antibody molecule is determined by the primary structure of its polypeptide chains.[13,14] This leaves no room for instructive action by the antigen. Furthermore, it has been demonstrated that certain antibody-producing cells, which turn out more than 1000 antibody molecules per second, contain no antigen.[15] The experimental methods would have detected the antigen if more than ten molecules of antigen had been present per cell. At one ribosomic site, a light or heavy polypeptide chain cannot be synthesized in less than 15 seconds. Therefore more than 100,000 ribosomes in an antibody-producing cell must simultaneously be able to turn out specific chains in the absence of antigens.

Although it is thus clear that the antigen plays a selective and amplifying role, we do not yet know by what mechanism the selective stimulus is transmitted. All we know with certainty is that, one day and later after having injected an antigen into an animal, we can find, in its spleen or lymph nodes, cells that are both multiplying and producing specific antibody. The simplest assumption would seem to be that an animal contains, among its population of 10^9 to 10^{12} lymphocytes (depending on species and age), a very large number of subpopulations, each of which is capable of being stimulated by certain antigens to grow, divide, and produce antibody-secreting cells among their offspring (perhaps because on their surface the cells display the antibodies they can synthesize).

Cellular dynamics in immunology

This picture can be illustrated by experiments that make use of an agar plaque method for counting, in a cell suspension obtained from a mouse spleen, the number of cells secreting a certain antibody. A certain amount of such a suspension—say 10^6 mouse spleen cells, as well as 4×10^8 sheep red blood cells (SRC)—is added to 2 milliliters of fluid 0.7 per cent agar at 45° C. The mixture is immediately poured into a petri dish, where it solidifies into a layer less than 1 mm thick. Each spleen cell is now surrounded by many SRC in a fixed position in the agar layer. If a spleen cell, during incubation of the petri dish at 37° C, secretes antibody molecules directed against an antigen of the SRC surface, the molecules diffuse into the agar and become fixed to the SRC in the immediate surroundings. Red blood cells, to the surface of which an antibody has attached, are said to be sensitized. Such sensitized cells will lyse in the presence of a serum factor called complement. By flooding the petri dish with complement after one-hour incubation, the sensitized SRC will lyse and lose their hemoglobin. Thus, around each mouse spleen cell that secretes hemolytic antibodies against sheep red blood cells, a pale plaque, visible to the naked eye, appears. Microscopic observation reveals the antibody-producing

lymphocyte, or plasma cell, in the precise center of each plaque.[16,17]

The spleen of an untreated eight-weeks-old inbred mouse contains about 80 plaque-forming cells (PFC) among a total of about 1.5×10^8 spleen cells. These 80 PFC produce antibody against sheep red blood cells, although the mice have never experienced sheep antigen. As always in immunological observations, there is a great variation among individual animals. The normal level of 80 PFC is an average. Among a group of 50 apparently identical mice, the normal level could range from, say, 10 to 500 PFC.

We now give each of a few hundred mice one injection of 4×10^7 SRC into a tail vein. Every day we sacrifice 20 mice and determine the total number of plaque-forming cells in each spleen. Twenty-four hours after the antigen injection, the number of PFC starts to rise above the normal level, proceeding exponentially to reach an average of 10^5 PFC per spleen at four days, after which there is a rapid decline.

The following two observations appear to support the assumption that the exponential rise in PFC between day one and day four reflects cell multiplication.

If, on day two, an animal is given one microgram of colcimid per gram body weight and is killed three hours later, about 20 per cent of the PFC in its spleen is found to have been arrested in metaphase of mitosis. If, on day three, the spleen cells are suspended for 30 minutes in vitro in a medium containing tritiated thymidine, about 55 per cent of the PFC can be shown by autoradiography to have synthesized DNA, whereas the remainder have not.

The rate of appearance of PFC after an intravenous dose of 4×10^7 SRC corresponds to a cell-doubling time of seven hours. Although smaller doses of SRC evoke smaller responses, it is not possible to obtain a larger response than that elicited by 4×10^7 SRC, even if the dose is increased to 4×10^8 or 4×10^9 SRC. Each dose within this hundredfold range produces the same maximum response, indicating that all cells capable of being stimulated by sheep red blood cell antigen are maximally engaged.

The experiments described above can be repeated with similar results if rabbit red blood cells are used as antigen. The antibodies produced by mice against SRC and rabbit red blood cells do not cross-react. Also, the PFC that arise after a mouse has been injected with SRC do not form plaques in agar with rabbit red blood cells, and vice versa. Furthermore, the following experiment shows that the class of cells in the mouse spleen initially stimulated by an SRC injection is different from the class of cells initially stimulated by a rabbit red blood cell injection. The number of PFC against rabbit red blood cells appearing after a single injection of 10^6 rabbit red blood cells is the same, whether or not 10^8 SRC are injected simultaneously. The two types of antigen clearly do not compete for the same target.[18]

In summing up, we can conclude that the mouse spleen possesses, among its more than 10^8 cells, small classes of cells that can be stimulated to grow and divide by particular antigens, and that antibody-secreting cells arise from these subclasses by cellular proliferation.

We can now try to estimate the number of cells in a mouse spleen that belong to the class that can respond to SRC antigen. We have reasons to believe that these cells are not the PFC that form the normal level in nonstimulated animals. First, the normal level PFC are mostly plasma cells, whereas the cells that respond to a primary antigen stimulus are probably small lymphocytes. Second, the magnitude of the response of individual mice appears unrelated to their normal level of PFC. We therefore do not believe that the normal PFC belong to the class of cells that can respond to a primary stimulus of SRC antigen, nor that they become the ancestors of the PFC arising after a stimulus.

The exponential curve describing the appearance of PFC extrapolates below the normal level of PFC at less than 24 hours after the sheep red blood cell stimulus, suggesting that the average size of the class of responding cells might be less than 80. Other experiments appear to leave little doubt, however, that the size of the class of initially responding cells is of the order of several thousand. These experiments involve (1) the exponential decay of this class of cells after increasing exposure of nonstimulated mice to X-radiation, and (2) the transfer of a small fraction of the cells from a normal mouse spleen to the spleen of a mouse that has been rendered immunologically incompetent by X-irradiation.[19-21] Thus, only a small fraction of the immediate descendants of the initially responding cells are PFC, i.e., secrete an antibody that can cause lysis of sheep red blood cells.

The initially responding cells and most of their immediate descendants might then display or secrete antibodies of a degree of specificity that enables the antigen to stimulate these cells to divide, but which is not serologically recognizable. This might also explain the finding that certain serologically unrelated antigens, such as different *Salmonella flagellar* antigens[22] and different protein subunits of lactodehydrogenase,[23] can induce immunological tolerance with respect to each other. In both of these cases, tolerance may be due to the removal of a class of initially responsive cells that display cross-specific antibodies not detected by serological methods.

The picture that emerges for the initial stages of antibody formation is that the antigen first selects a class of initially responding cells. These are stimulated to dif-

ferentiate and to divide. For each division they may require a new antigenic stimulus. All daughter cells do not necessarily produce the same antibody, and the antigen preferentially stimulates those descendant cells that produce the antibody best fitting the antigen.

This brings me to another relevant immunological phenomenon, the increase in "avidity" of the antibodies produced during the time following an antigenic stimulus. In the early days of immunology, the term avidity was introduced to express the degree of firmness of the bond formed between serum antibody and antigen in vitro. If the antigen dissociated easily from the antigen–antibody complex on dilution, the antibody was said to be of low avidity. Avidity is thus a measure of the goodness of fit of antibody toward the antigen. It was shown that the antibody present in animal serum is a heterogeneous population of molecules varying widely in avidity, and that the average avidity of the antibody present in an animal increases after repeated antigenic stimulation, or even with time after a single primary stimulus.[24,25]

Recent studies have confirmed that antibody molecules produced by one animal shortly after a single primary antigenic stimulus have a lower average association constant with respect to the antigen than later, and that this applies to a single class of antibody (IgG) with respect to a single antigenic determinant.[26]

This indicates that a further selection of cells producing better-fitting antibody takes place among the descendants of the cells first stimulated after the initial antigenic stimulus. Avidity does not increase as quickly after a large dose of antigen as after a small dose. As might be expected, a large dose of antigen is less selective, and in extrapolation we might relate tolerance following excessive doses of antigen to the absence of progressive selection. The immunological tolerance observed after repeated minimal doses of antigen might be caused by stimulation of the entire class of available responding cells, followed by a decay of their descendants because of the absence of antigen needed for further stimulation.

The assumption underlying the above discussion, that the descendants of the initially stimulated cells require further antigenic stimulation in order to continue to multiply, is supported by the finding that the exponential rate at which PFC appear in the spleens of mice after one intravenous injection of SRC decreases with decreasing antigen doses. Thus the doubling times of PFC per spleen after one dose of 4×10^7, 4×10^6, 4×10^5, and 4×10^4 SRC are 7, 9, 21, and 36 hours, respectively. The simplest explanation of this remarkable fact is that the cells require, and must therefore wait for, a new antigenic stimulus for each division.

In the picture developed above, a continuing selective role has thus been assigned to the antigen. Among the general population of antigen-responsive cells, a particular antigen first selects the small class of cells that can respond to the primary presence of that antigen. These cells are stimulated to grow and divide. A secondary selection by the antigen then takes place among the differentiating descendants of these cells, resulting in the production of increasingly better-fitting antibodies.

Instruction versus selection in learning

In accordance with this general picture of the selective role of antigen in antibody formation, the view that antigen acts instructively at the intracellular level has been abandoned. It may be useful, therefore, to examine in a broader biological context whether there exist situations in which instructive mechanisms operate or to which the term "instruction" is applicable. It would seem that an answer to this question requires a specification of the organizational level at which a process is described. Thus, although the mechanism by which an antigen brings about antibody formation in the tissues of a mouse must be purely selective, the antigen does not select a mouse. When viewing the situation at the level of the entire mouse, we may still say that the antigen "instructs" the animal to produce an adequate antibody.

Similar reasoning can be applied to examples from other areas of biology. For instance, in the case of the selection by streptomycin of streptomycin-resistant mutants among a population of bacteria, it is clear that the streptomycin molecules did not cause these mutants to arise. They were already present before the streptomycin arrived; no instructive role can therefore be assigned to streptomycin. On the level of the entire bacterial culture, however, we may still say that streptomycin instructs the transition to streptomycin resistance.

Let us consider a more complicated example of Darwinian selection. A large population of brown moths spend a major part of their time sitting on a factory wall of the same color. These moths are the prey of certain birds. Now the wall is repainted white. One or two years later we observe that the moths sitting on the wall are likewise white. In this case, the signal that entered into the system, i.e., the color change, was not even received by the moths, but by the birds. The mechanism by which the color change in the moths came about was obviously selective, in that moths of lighter color were already present among the original population before the signal arrived. Again we might say, however, on the level of the entire system, that the signal "instructed" the population of moths to mimic the color change.

A clear example of an instructive process would be the role of messenger RNA in protein synthesis. The mes-

senger RNA molecules arriving in their ribosomal habitat do not select already existing protein molecules and may therefore be said, at the organizational level of protein, to play an instructive role. The messenger RNA does recognize and select, however, already available subunits, namely, species of amino acid-charged transfer RNA. At this lower level, therefore, the process is a selective one.

I will finally turn to the question of the analogies between the immune system and the central nervous system.

Both systems have a history that develops during the lifetime of the individual. Each antigen that makes its appearance irreversibly changes the immune system. In the same way, the state of the central nervous system reflects the experience of the individual.

The immune system appears to be learning by responding to antigens entering from the outside world. The central nervous system also appears to learn in response to sensory signals.

Like the central nervous system, the immune system appears to have a memory that enables it to benefit from previous experience. It produces more and better antibodies if the antigen enters a second time, or repeatedly.

The experience gathered by the immune system of an individual cannot be transferred to its progeny. As with the central nervous system, each newborn must start, so to speak, from scratch.

In the remaining, speculative part of this paper, I shall try to make the most of these analogies. Let us consider the kappa light chains of the antibodies of mice and man. Each of these light chains consists of a "constant" sequence of 107 amino acids and a "variable" sequence of 107 amino acids.

The constant part of human light chains is identical in all individuals and in all antibodies they produce. It differs, however, from the constant part of mouse light chains by some 40 amino acid substitutions that have obviously arisen by mutation during phylogeny. The variable part of human light chains, on the contrary, differs between different antibody molecules of one individual, and the differences are similar in nature to the differences between mouse and man in the constant part. This is reminiscent of the old saying that ontogeny mimics phylogeny: phylogenic differences between species in the constant part of the light chain are mimicked by the ontogenic plasticity of the variable part.[27-30]

Similarly, in the central nervous system, instincts are fixed in one species, but each individual (particularly man) has also a plasticity in learning capacity, which mimics the total of all phylogenically developed instincts of different species. In the immune system, the constant part of the light chain is obviously laid down in the DNA of the zygote, and it is equally clear that there is DNA in the zygote that represents the variable part of the light chain, although, ontogenically, this DNA may exhibit an immense plasticity.

In the central nervous system, instincts are also obviously encoded in the zygote, most probably in the DNA. But if DNA acts only through transcription into RNA and translation into protein, and if the phenotypic expression of instincts is based on particular arrangements of neuronal synapses, then DNA through RNA and protein must govern the synaptic network in the central nervous system.

Analogous to the utilization of the diversity of the variable part of the antibody light chain in the immune system, it would seem probable to me that, in the central nervous system, learning from experience is based on a diversity in certain parts of the DNA, or to plasticity of its translation into protein, which then controls the effective synaptic network underlying the learning process. I would, therefore, find it surprising if DNA were not involved in learning, and envisage that the production by a neuronal cell of certain proteins, which I might call "synaptobodies," would permit that cell to enhance or depress certain of its synapses, or to develop others.

Pursuing these analogies even further, we might now ask whether one can distinguish between instructive and selective theories of learning in the central nervous system. Looking back into the history of biology, it appears that wherever a phenomenon resembles learning, an instructive theory was first proposed to account for the underlying mechanisms. In every case, this was later replaced by a selective theory. Thus the species were thought to have developed by learning or by adaptation of individuals to the environment, until Darwin showed this to have been a selective process. Resistance of bacteria to antibacterial agents was thought to be acquired by adaptation, until Luria and Delbrück showed the mechanism to be a selective one.[31] Adaptive enzymes were shown by Monod and his school to be inducible enzymes arising through the selection of pre-existing genes.[32] Finally, antibody formation that was thought to be based on instruction by the antigen is now found to result from the selection of already existing patterns.

It thus remains to be asked if learning by the central nervous system might not also be a selective process; i.e., perhaps learning is not learning either.

Several philosophers, of course, have already addressed themselves this point. John Locke held that the brain was to be likened to white paper, void of all characters, on which experience paints with almost endless variety.[33] This represents an instructive theory of learning, equivalent to considering the cells of the immune system void of all characters, upon which antigens paint with almost endless variety.

Contrary to this, the Greek Sophists, including Socrates, held a selective theory of learning. Learning, they said, is clearly impossible. For either a certain idea is already present in the brain, and then we have no need of learning it, or the idea is not already present in the brain, and then we cannot learn it either, for even if it should happen to enter from outside, we could not recognize it. This argument is clearly analogous to the argument for a selective mechanism for antibody formation, in that the immune system could not recognize the antigen if the antibody were not already present. Socrates concluded that all learning consists of being reminded of what is pre-existing in the brain.[34]

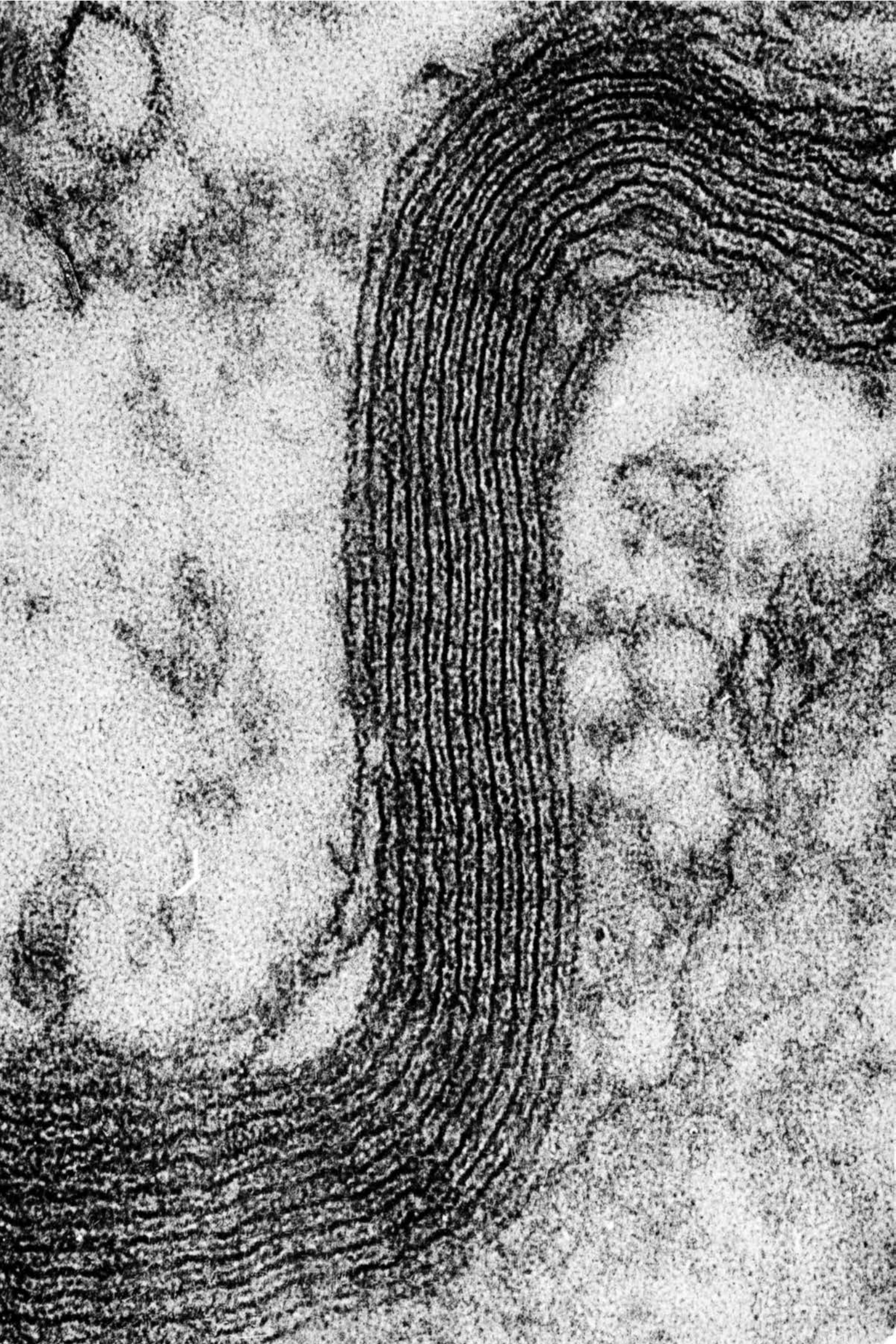
Summary

In concluding this analysis, it would seem that selection refers to a mechanism in which the product under consideration is already present in the system prior to the arrival of the signal, and is thus recognized and amplified.

Each system that is capable of receiving a signal, however, is subject to instruction by this signal. Thus, at the level of an entire system, all such processes are instructive, whereas all instructive processes at a lower level imply selective mechanisms. In learning, and in all processes resembling learning, a discussion of instruction versus selection serves only to determine the organizational level of the elements upon which selective mechanisms operate.

During recent years the belief that antigen plays an instructive role in antibody formation by intracellular guidance of the formation of the tertiary structure of globulin molecules has been replaced by the idea that antibody formation is based on a selective process in which antigen selects pre-existing patterns and causes molecules representing these patterns to be produced at increased rates. The logical arguments for selection have been enforced by experimental evidence showing that the general mechanism of protein biosynthesis also applies to antibody production, that primary polypeptide structure determines antibody specificity, and that plasma cells can produce antibody in the absence of intracellular antigen. The antibody response appears to depend on multiplication of cells of the immune system. Attempts are being made to describe the cellular dynamics involved and to understand the nature of the antigenic stimulus.

The replacement of instructive by selective theories appears to be a general trend in the development of biology. A number of analogies are drawn between the central nervous system and the immune system, and the question is posed whether a selective mechanism may also underlie the learning process. An analysis of this question leads to the conclusion that the terms instruction and selection can apply to descriptions of the same process at different levels. Each system that is capable of receiving a signal is subject to instruction by this signal. Thus, at the level of an entire system, all such signals are instructive, whereas all instructive processes at some lower level imply selective mechanisms, through which products that were already present in the system prior to the arrival of the signal are selected and amplified. In learning, as in all processes resembling learning, a discussion of instruction versus selection serves only to determine the organizational level of the elements upon which selective mechanisms operate.



MOLECULAR BIOLOGY OF BRAIN CELLS

"*The molecular organization of* [*brain*] *cells and the myriad nets they contrive constitute a field of study,* molecular neurobiology, *that is basic to brain and behavioral science.*" SCHMITT, PAGE 209.
The bridge between morphological and biophysical study of the nervous system is illustrated by this electron micrograph, taken at 320,000 magnification by H. Fernández-Morán, of a myelin sheath segment in a frog sciatic nerve, PAGE 283.

INTRODUCTION

FRANCIS O. SCHMITT

Molecular Neurobiology in the Context of the Neurosciences

THE STUDY OF brain cells, neurons, and glia occupied a focal position in the 1966 Intensive Study Program. The molecular organization of these cells and the myriad nets they contrive constitute a field of study, *molecular neurobiology*, that is basic to brain and behavioral science.

Neurobiology stands between neurophysiology, which provides bioelectric data concerning the function of neural nets and brain regions, and the more phenomenological and behavioral of the neurosciences. The molecular sciences, on a lower level, may provide the biophysical and biochemical clues necessary eventually to construct a truly self-consistent, theoretical neuro-behavioral science. As indicated graphically in Figure 1, the molecular sciences and the neural, e.g., electrophysiological, sciences by themselves represent intellectual "flatlands," as it were, each lacking a dimension represented by the biology of brain cells. It is in the properties of brain cells that each of these

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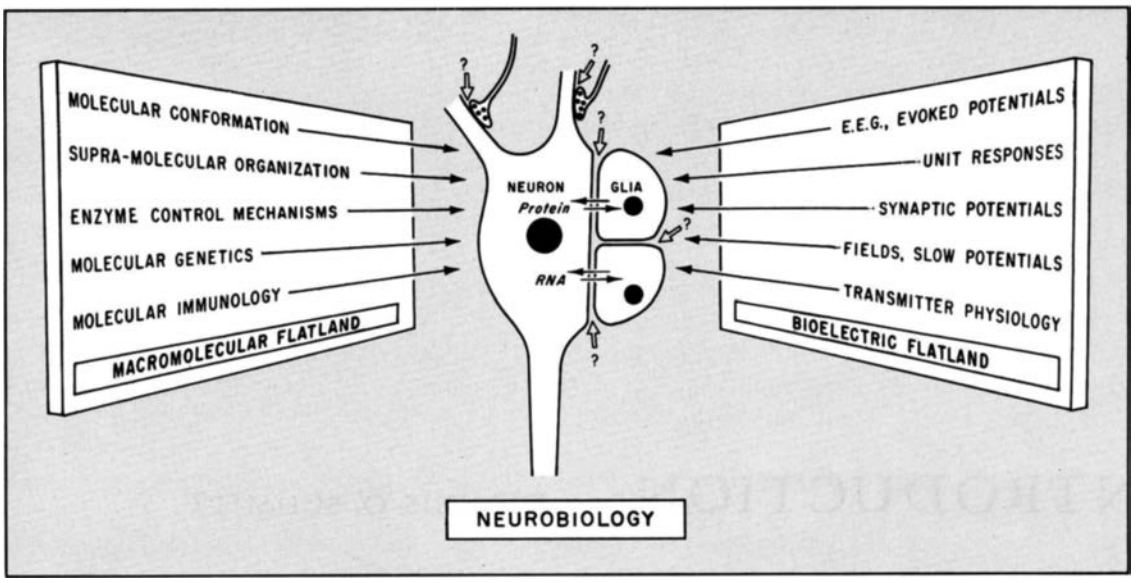

FIGURE 1 Graphic illustration emphasizing the centrality in the neurosciences of the study of the biological properties of brain cells, neurons, and glia. The problem of the nature of intercellular molecular traffic is indicated by arrows.

disciplines seeks answers to the phenomena at the lower and the higher levels of complexity. Neurobiology also provides a basis on which scientists from other fields, particularly physicists, chemists, and mathematicians, may gain broad introductory experience preliminary to formulating their individually conceived research programs in the neurosciences.

The ability of neurons to differentiate, to form specific sequential tracts, and, through self-organization, in due course to structure the brain of man represents one of the greatest problems to which science can address itself. The processing of psychological information, which in its totality constitutes the mind and which, it may be surmised, depends on the ability of macromolecules to encode information in a retrievable form, nevertheless rests on emergent properties unique to brain cells and their nets. The study of brain cells as individual, living, multipotential entities is thus fundamental to a biologically based neurology or psychology. To understand the manner in which the intracellular molecular assemblies and organelles conspire through electrical means to produce a fast-reacting nervous system requires knowledge of the control of gene expression, bioenergetics, biophysical cable theory, membrane properties, and the like—a formidable task indeed.

This chapter sketches a few of the salient concepts of molecular biology applied to brain cells, to a consideration of physiological properties that result from the aggregation of cells into the complex network of the brain, and to certain psychological processes such as learning and memory. No attempt is made at systematic coverage of the subject; rather the aim is to provide a commentary on the area dealt with in those lectures of the Intensive Study Program concerned with brain phenomena at the cellular level.

Molecular biology of brain cells

In this section will be briefly considered a few selected properties of neuronal and glial cells that lend themselves to study at the molecular level. The definitive papers on these subjects are those of M. V. Edds,[1] J. D. Ebert,[2] and L. Levine.[3]

A. NEURONS *Metabolic Dynamics in Neuronal Development and Maintenance* Essential to the organizational logic of neurons is a cell body capable of generating and maintaining a long cylindrical outgrowth, the axon. Message-bearing action waves travel over this channel, which may be very long compared with cell dimensions, and the channel volume may be many times the volume of the cell body. Functional synaptic junctions with other neurons thereby form complex networks from an aggregation of individual, highly asymmetric cells.

After differentiation, neurons rarely divide, yet their cells continue to synthesize neuroplasm and transport essential components cellulofugally. Data concerning the rate of this axonal flow, discovered byWeiss,[4] the gaseous metabolism, cellular ultrastructure, and histochemistry, all

indicate that the neuron is one of the most actively biosynthesizing cells in the animal body. This is true not merely of neurons in the process of generating or regenerating an axon, but also of adult neurons. The living neuron thus differs markedly from the static entity likely to be inferred from anatomic textbooks or from the observations of physiologists whose primary concern is with bioelectric parameters. Dynamism is the leitmotiv of neuronal function.

What happens to the synthesized neuroplasm remains obscure. Weiss believes the protein is catabolized as it passes down the axon. Yet protein structures such as the neurofilaments and neurotubules appear essentially similar near the ending of fibers as they do near the somata. It is possible that components of the neuroplasm escape at the endings or at Nodes of Ranvier (Figure 2D). It is conceivable that a component of the flow goes also into the dendrites and that material may escape into the intercellular space from dendrite tips. The arborization of dendrite structure is usually explained teleologically as the structure best adapted to receive maximal synaptic input. However, it is also the structure to be expected of an actively synthesizing cell having a plastic surface membrane able to adjust to the flow of synthesized neuroplasm. The important, yet unanswered, question is: Are neuroplasmic components passed from dendrites into intercellular space?

The radioautographic method using tritiated precursors lends itself well to the determination of the fate of axoplasm moving cellulofugally, both down the axon and possibly out along the dendrites. Large cells, such as those of *Aplysia,* would seem particularly advantageous for investigating this problem.

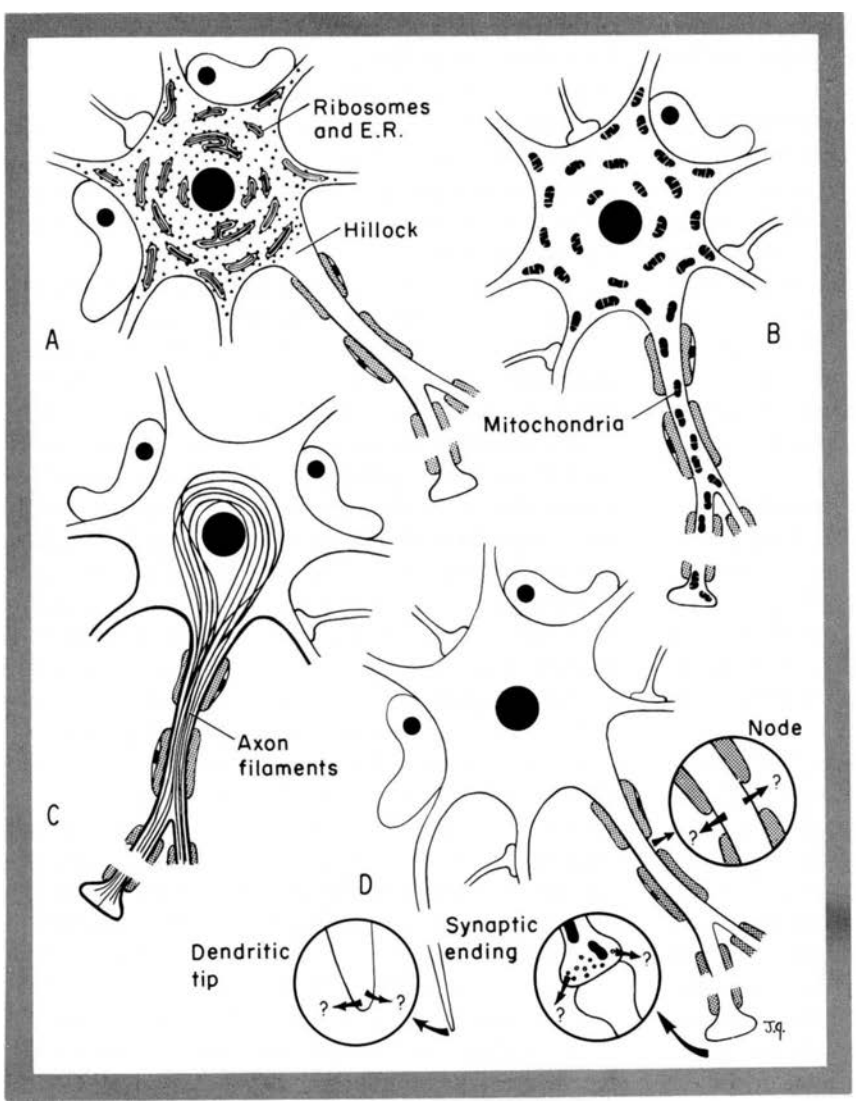

FIGURE 2 Distribution of organelles within the neuron.
A. Ribosomes and endoplasmic reticulum (E.R.).
B. Mitochondria.
C. Axon filaments.
D. Possible points of egress of constituents synthesized in the cell center and translocated peripherad.

As illustrated graphically in Figure 2A, ribosomes, even those not attached to cytoplasmic membranes, do not pass down the axon, but are limited to cellular regions above the axon hillock. In view of the dynamic flow of synthesized products, some fairly rigid, cross-bonded cytoskeleton must characterize the cytoplasmic structure of the neuron.

Mitochondria arising in the cytoplasm of the nerve cell, perhaps by a self-regenerative process, seemingly pass freely down the axon and are observable all the way to the terminals (Figure 2B). The role of mitochondria is presumably energetic, to supply adenosine triphosphate for the work of the axon and its endings. The possible role of the DNA in mitochondria (Lehninger[5]) remains to be clarified.

Neurofilaments—100 Å wide, smooth-edged structures coursing undivided from the soma, where they are synthesized, down the axon to the terminal twigs (see Figure 2C)—are ubiquitous in nerve fibers, yet their function remains unknown. This neuronal organelle is discussed by P. F. Davison.[6]

Neurofilaments, as microtubules generally (Porter and Tilney[7]), may serve as stable reservoirs of protein molecules required for particular physiological processes (one of which may be vesicle formation). Like certain microtubules, neurofilaments may also have contractile properties. Important clues concerning the function of neurofilaments may be found from their behavior under various experimental and pathological conditions. Thus in neurolathyrism and in various kinds of degenerative diseases there is marked hypertrophy of neurofilaments causing neurons to be packed with a fibrous neuroplasmic protein (Ule[8]).

Amacrine, Silent, and Short-Axon Cells Sometimes important scientific conclusions are deducible from a consideration of objects or phenomena that seem to contradict currently favored theories or at best to be poorly assimilable in them. The amacrine, "silent," and short-axon cells may well belong in this category. They are included here in the hope that stimulation of research on their role may result.

Amacrine cells have a typical neural soma with "busy" cytoplasm and a highly developed dendritic tree richly endowed with terminal boutons of presynaptic inputs, but possessing *no axon*. Gallego and Cruz,[9] working on mammalian retinas, describe cells that have no axons but that are organized into a plexus extending across the whole retina. Is the output of such neuronal oddities of neuron dogma primarily in the nature of "field" changes resulting from alteration of charge, of the secretion of potent neurohumors, or of what? It would seem that these cells would repay histochemical, biophysical, biochemical, and electrophysiological (particularly single unit) study.

Similarly the so-called "silent cells" encountered by microelectrode exploration of cortical regions would probably repay investigation. Observations of electrical potential changes indicate that the electrode has impaled a cell, yet stimulation evokes no response (Phillips[10]). What is the role of these cells?

Short-axon cells possess axons which may be so short and have such architectonics as to suggest that firing of spike waves is not their major characteristic function. These cells have been regarded by some as intermediate between neurons and glia (not actually either) and as tending to form extensive nets in which, because of the shortness of axons, the somata preponderate, according to Gallego.[11] It is interesting that Ramon y Cajal considered that short-axon cells are "condensers of nervous energy" and the "storehouse of memory."[12]

Determination of Cell Shape The shape and dimensions of the nerve cell body and dendritic tree vary enormously, from the ovoid perikaryon of monopolar neurons to the cells with extraordinarily developed arborization. Each dendrite apparently adapts to the function it subserves in the network. An excellent example of pleomorphic variability is seen in the "printed circuit" of the cerebellar net. What biophysical and biochemical processes specify such a profusion of shapes?

The most significant factor in determining cell structure is probably the membrane, though elongate, fibrous, or gelled neuroplasmic contents may also play a contributing mechanical role. The interfacial tension of most cell membranes is close to zero. The bimolecular layer of mixed lipids composing the inner part of the membrane's sandwichlike structure constitutes a smectic mesomorphic system unlikely to produce specific geometric patterns at the microscopic level. Membrane *proteins* are much more likely to be determinative of such structure. If the monolayer of structure protein, lining the inner surface of the lipid bilayer, were composed of a molecular type susceptible of forming many variants, the two-dimensional structure produced by packing these variants in various ways might well lead to the wide range of neuronal cell types observed. Langmuir (Schaefer[13]) long ago showed that different soluble proteins, when spread and deposited under low pressures as monofilms, form expansion patterns characteristic of the kind of protein molecules spread. Perhaps each characteristic cell shape is produced by a particular variant of a general type of protein employed as a structure protein in the cell membrane. Little is known in detail about such structure proteins, though progress is being made in the study of mitochondrial proteins (Lehninger[14]; Green and Perdue[15]). Other fundamental neuronal functions may also depend upon the incomprehensibly large number of vari-

ants possible in large molecules such as proteins.

Development of Specific Neural Nets and the Brain Levi-Montalcini and her colleagues have isolated a protein which, in 10^{-11} gram amounts, selectively stimulates the growth of sympathetic neurons.[16] Treatment with antisera against this protein inhibits or prevents growth of sympathetic neurons; indeed, animals lacking sympathetic innervations have been produced by treating embryos with such antisera. Insulin stimulates neuronal growth with similar high activity, but lacks the specificity of the nerve growth factor (NGF). Levi-Montalcini suggests that the action is at the level of DNA readout, the NGF derepressing synthesis of proteins essential for neuronal growth. However, it is probably too early to be certain about the actual molecular mechanism involved; J. D. Ebert[2] considers this matter in more detail.

Two alternative explanations of the phenomena suggest themselves. If such systems are determinative for neurons during development, it may be anticipated that specific proteins (or other derepressors) may be elaborated by each neuron type, secreted into its microsurround, and, upon reaching and entering a neuroblast of appropriate type, may specifically derepress its synthetic mechanisms, causing an axon to grow out that would presumably make stable synapse only with neurons of the type that produced the derepressor. The *target* cell for innervation might thus also be the *evocator*. Embryonic brain tissue would then be characterized by concentration gradients of numerous protein variants of specific derepressors of neuronal growth, each destined to evoke outgrowth, hence eventual innervation by specific neurons.

Alternative to this view, and at first sight seemingly much simpler, is the idea that as neuroblasts differentiate into neurons the axons grow out more or less probabilistically. When in their wanderings they make contact with a cell of a type manifesting molecular recognition, a specific chemical affinity presumably stabilizes such junctions in preference to other possible ones.

Highly relevant to the problem of specificity of development of neurons and their interconnections are the experiments of Weiss and of Sperry, which are dealt with in detail by M. V. Edds.[1]

Hormones are known to have a strong effect on gene expression in certain types of cells and might therefore be expected to influence developmental processes in the brain. Determination of neuronal nets in the development of the brain need not depend exclusively on macromolecular protein derepressors such as the nerve growth factor; small molecules such as steroids may also participate.

B. Glial and Schwann Cells Glial cells, like neurons, become differentiated into several different types of highly variable size, form, and function (see chapters by S. L. Palay[17] and D. Bodian[18]). The tendency to form fibrous and lamellar outgrowths is probably related to the ability of these cells to synthesize membrane material prolifically. This ability is most strikingly illustrated in the formation of the myelin sheath. In peripheral nerves the neural crest cell—precursor of the Schwann cell—wanders until it meets outgrowing axons of developing neurons. Presumably, due to a high affinity between the surface membranes of Schwann cell and axon, the Schwann cell engulfs the axon, rapidly synthesizes membrane material, and wraps its double membrane-limited protoplasm round and round the axon, resulting, after compaction of the near-concentric layers, in the finished myelin sheath (Geren[19]). This process provides rich grist for analysis by the molecular neurobiologist. What kind of molecular recognition between satellite cells and axons produces firm adhesion between them? Yet, when sections through these cells are viewed with the electron microscope, one sees the customary 100- to 200-Å space between the apposed membranes, rather than a tight membrane type of adhesion or some kind of structure bridging the gap. Perhaps current electron-microscopic technique is not yet adequate to the task of revealing molecular structures such as those that provide the molecular recognition between these two types of cell membranes or bridge the gap between them.

What triggers the hyperactive membrane synthesis in glial or Schwann cells, and how do the newly formed molecules become organized into membrane? That is, do the protein and lipid components synthesized inside the cell self-organize into plasma membrane that expands by intussusception of the components, or does plasma membrane biosynthesize itself by topochemical reactions in situ?

The properties of the glial membrane are physiologically crucial and may differ from those of neurons and most tissue cells in that permeability to solutes, particularly ions, is probably high. According to one view, the astrocytes, one type of glia, interpose themselves between blood capillaries and neurons, thus forming the so-called "blood brain barrier" (perhaps better: "blood brain pathway") and monitoring the materials that get into neurons.

Glia are frequently considered merely the "soup kitchen" for the neurons, hence are of little specific neurophysiological or psychological importance. Hydén[20] has demonstrated mutual interchange of vital substances, particularly nucleotides. Consistent with Galambos's view[21] of a determining role of glia in psychological processes, Hydén considers that interchange of nucleotide bases and possibly RNA may be of significance for the molecular processing of learning and memory.[22]

As satellites, glial or Schwann cells cover most of the neuronal surface. They may serve as impedance modulators relevant to neurophysiological and psychological processes, as suggested by Adey.[23] Neurophysiologists seldom consider the possible physiological role of current flow through the glial membrane and inside the glial cell during the action wave; attention is usually paid exclusively to the 100- to 200-Å space between neuron and glia as a pathway for Na^+ and K^+ ions and the flow of action currents. Electrotonic spread over distances of many microns is believed to occur in the dendro-somatic system to excite the axon at the region of the hillock, but such spreading currents, electrotonic or self-regenerating, are seldom portrayed as influencing glial cell processes. Perhaps this possibility for transduction of bioelectric into biochemical parameters within glial cells should be examined more carefully. Adey's data suggest that glia, by changing impedance loads on neurons through subtle changes of shape, perhaps by alteration of the width and conductance of the intercellular conducting channel, may meaningfully modulate the ability of neurons to fire under specified kinds of barrages of input signals. So far as molecular information processing is concerned, the central biophysical problem may well be the transduction of weak bioelectric signals into stable chemical changes, the engram, or permanent trace of the memory. This process may involve glia as well as neurons, particularly because the ion permeability of the former is apparently higher than that of the latter.

The ribosomelike, doublet appearance of the neuronal–glial complex stylized in Figure 1 is meant to emphasize the integral relation between neurons and glia. It may well be that the true unit of the nervous system is not the neuron, but the neuron–glia complex to which no descriptive term has yet been applied.

C. Fractionation of Neuronal and Glial Cells Modern molecular biology abounds with examples of the fruitfulness of fractionation, isolation, and characterization of partial systems and organelles; the example of mitochondria suggests itself immediately. Application to brain cells of similar methods may prove profitable.

If methods could be found to separate neurons from glia without substantial damage to either, important analytical problems might find a basis for solution. Thus far the least damaging treatment is that routinely used by Hydén and his colleagues, in which the glia are scraped from the neurons by microknives and pools of several dozen neurons and several hundred glia are used for microanalytical studies. Methods of gross separation from bulk macerates have been developed by Satake and Abi,[24] and milder methods, which leave the two kinds of cells still capable of performing certain relatively normal physiological and biochemical functions, have recently been developed by Rose,[25] using density gradient sediment in "Ficoll"-potassium chloride-phosphate media.

Conceivably, as methods are improved, cellular subfractionation might be accomplished, perhaps with the use of free-flow electrophoresis. Preliminary experiments in collaboration with A. L. Rubin and K. H. Stenzel, using the free-flow electrophoresis device developed by K. Hannig (and executed with his expert technical assistance), offer promise. Antisera might be prepared against individual neuronal or glial types and their functions determined by observing physiological or psychological alteration resulting from intracisternal injection with antisera or by other appropriate treatment.

Molecular neurophysiology

The essential neuronal functions concerned are those of input, transmission via axon, and output. This section will deal with these—insofar as it is now possible—at the molecular level and will start with the "simplest" approach experimentally most accessible to biophysical and biochemical as well as electrophysiological investigation: the propagated impulse and membrane changes subserving this dynamic bioelectric process.

A. Impulse Propagation The brain is the most dynamic of all tissues. It is always active, in sleep as well as in wakefulness. It has been facetiously suggested (M. A. Arbib) that we stay awake in order to allow neurons that are highly active during sleep to get some rest! Neurons fire up to 1000 times per second. This high neuronal activity is reflected by biophysical and biochemical dynamism at the molecular level.

Although nerve theory (e.g., Hodgkin[26] and Huxley[27]) has dealt exhaustively with the flow of ions in explaining how inorganic electrochemical changes may subserve bioelectric processes, an equally fundamental problem is that of molecular membranology, i.e., the way in which macromolecular and paucimolecular changes within, upon, and through the membrane subserve function. These problems will be discussed by P. F. Davison,[6] E. P. Kennedy,[28] R.Whittam,[29] R. E. Taylor,[30] and A. Katchalsky.[31] Included are the questions whether "pores" specific for the permeability and transfer of Na^+ and K^+ exist in the membrane and, if so, whether specific macromolecular conformation, presumably protein, is responsible for the specificity or is due to cooperative molecular properties in which many molecules, probably lipid as well as protein, participate. Some highly competent investigators do not believe that specific pores exist; rather, they suggest that ions traverse the nonpolar bilayer of the membrane by forming

complexes with membrane lipids; D.W. Woolley[32] espoused this view. A promising possibility, based on evidence from perfused giant fibers of the squid, is that a specially differentiated "electrogenic" protein valves ion flow through the membrane by conformational changes at the tertiary or quaternary level (see Davison[6]). Macromolecular conformational changes may be very fast (10^{-6} to 10^{-9} seconds), compared with bioelectric phenomena (10^{-3} to 10^{-4} seconds), and enzymatic action triggered by the altered conformational state may have high turnover, as required for bioelectric phenomena.

Artificial membranes consisting of a bimolecular layer of certain lipids may simulate cell membranes (Mueller, Rudin, et al.[33]; Huang and Thompson[34]), but display properties of axon membranes, e.g., excitability and rectification, only after the addition of protein, such as the excitability inducing material (EIM) referred to by Mueller and Rudin.[35] They are inclined to attribute these properties primarily to the protein (still uncharacterized chemically), rather than to the lipid bilayer; the latter can be widely varied in properties and composition without loss of the excitation characteristic provided when protein with EIM-like properties is present.

B. Synaptic Transmission Biophysical, biochemical, physiological, and anatomical aspects of synaptology are dealt with in detail by other authors in this volume. In this section are included chiefly items meant to point to areas of uncertainty or complete ignorance.

Nature of the Transmitter Ingenious experiments have elucidated the biophysical and electrophysiological aspects of humoral transmission by acetylcholine across the neuromuscular junction (Katz[36]). Acetylcholine is known to act as a transmitter in *certain* parts of the central nervous system, but the tendency to assume that neuromuscular-type transmission applies to junctional transmission *generally* in the nervous system is unwarranted. Gamma-aminobutyric acid, norepinephrine, dopamine, serotonin, and other biogenic amines are thought to act as the transmitters in adrenergic neurons, but these are also ruled out as significant in cortical synapses.

Actually little is known about the humoral transmitter concerned in synaptic transmission in the cerebral cortex and in other brain regions. The usual cholinergic and adrenergic types may be ruled out. Metabolites and regulators of protein synthesis, particularly acidic amino acids, seem implicated (Curtis and Watkins,[37] Krnjević[38]), a fact that will be considered in relation to the role of neuron cell metabolism in processing input signals. Whether any such substance is declared a transmitter depends on how rigidly the term is defined. Under the definition proposed by Curtis,[39] probably none would qualify.

Though esterases have been localized at synaptic membranes and may actually contain receptor sites, hydrolysis of neural transmitters such as acetylcholine may actually not accompany, or functionally cause, depolarization and excitation.[40,41] Clearly the whole process of transmission across synapses, especially in the cortex—in which a highly active organic small molecule can alter the polarization of the postsynaptic membrane, leading to excitation or inhibition—awaits clarification. Clearly such small-molecule transmitters are not information-bearing or engrammatic, although mechanisms that valve their flow or participate in microcybernetic systems whose output is transmitter material may well be engrammatic.

Role of Vesicles Transmitter molecules are generally believed to be packaged in vesicles, 0.05 to 0.1 μ in diameter, found in profusion in presynaptic terminals. In adrenergic axons and endings, vesicles manifest varying degrees of density in electron micrographs; vesicles can be traced from their site of synthesis in the cell body and down the axon to the endings from whence they are liberated by nerve excitation. Adrenergic tracts in the brain have been traced by fluorescence histochemistry of the biogenic amines (Dahlström and Fuxe[42]).

The site and the mechanism of synthesis of acetylcholine and its enclosure within the vesicles of cholinergic endings are unknown. A recent speculation (Aidley[43]) relates vesicle protein to the engram and suggests that the protein may be liberated from the ending and associated with the postsynaptic mechanism. The physical process by which the transmitter is released on arrival of the impulse in the presynaptic ending also remains completely unknown. It seems strange that a process so basic and so routinely assumed by physiologists should not have generated more hard physical and chemical investigation; here is an excellent opportunity for contributions in molecular neurology.

Role of Molecular Specificity in Determination of Synaptic Connectivity In "wired-in" neuronal circuits where, like a doorbell circuit, afferent signals are automatically followed by specific efferent outputs, intermediate synapses must be morphologically and chemically conditioned in order to leave no choice or alternatives of firing patterns. However, to execute patterns of innate behavior and to store memory patterns on the basis of which learning occurs require a high degree of plasticity or selectivity coupled with permanence of memory patterns once acquired. How is this specifiable plasticity of synaptic connectivity accomplished?

The view presently favored in neurophysiological theory, representing a combination of the Cajal neuron doctrine plus the Loewi–Dale neurohumoral hypothesis, suggests that neurons are discrete, not interconnected at junctions, and are fired by liberation of quanta of neurohumor-

al transmitter substances at presynaptic junctions. No specificity is invoked; the presynaptic endings must merely be within transmitter diffusional range of the postsynaptic membrane. The transmitter substance, excitatory or inhibitory, is digital in nature, exciting or inhibiting according to the concentration of substance liberated per unit of time. Variability and control of such a synaptic system might occur by central action of the cell or by local action at the synapse. The former might be brought about by the action of macromolecular material elaborated in the cell body and passed down the axon to the synaptic ending.

Local action occurring at the synapse, which might modulate connectivity across the synapse, may be either nonspecific or specific. Concerning the former, one might postulate that rate of synthesis or activation of transmitter material might valve transmission. If this is reflected in numbers of vesicles, no statistical electron microscopy supports the view. Another type of local effect might be postulated to retract presynaptic endings or remove them from active diffusional range of postsynaptic endings. This view seems to be favored by physiologists.[44] So far as the author is aware, electron-microscopic studies reveal no range of synaptic cleft width, up to a micron or more; recognizable synaptic clefts all fall in the width range of approximately 100 to 200 Å. Finally, some chemical adhesive, for example some constituent of the interstitial ground-substance such as mucopolysaccharide or glycoprotein, might bind presynaptic and postsynaptic membranes together to form functional synaptic units; this assumption is frequently made by morphologists.

Some electron microscopists, describing various kinds of structures observed in presynaptic endings, in the synaptic cleft, and in postsynaptic structures, suggest that organic, largely fibrous, connections bridge the synaptic gap (De Robertis,[45] Gray,[46] Taxi,[47] Hama[48]). Other equally competent electron microscopists are conservative about such structures, which they view as possibly artifactitious (Robertson)[49].

If mere liberation of a transmitter does not suffice for synaptic excitation and some kind of organic connection between presynaptic and postsynaptic elements is required, the connecting material may be highly specific, e.g., protein–antiprotein in nature (corresponding to an antigen–antibody type of system), in which molecular recognition provides the key to connectivity. Such a system might have the same breadth of plasticity as the antigen–antibody system and might be called a specific molecular "synaptic solder" theory of engram formation. Such a molecular solder might be digital (on-off switchgear) or analog. Like gamma globulin, one portion of the molecule might serve molecular recognition purposes, while the other portion might be the chemical or physiological effector portion. Presumably only the latter would correspond to a true molecular (selectional) coding of experiential information (engram).

How would such a specific protein theory of synaptic connectivity be testable? Intact axons are not electromotorily susceptible to proteases externally applied. However, if a connectivity protein exists at the synapse, proteases should remove them.

Another line of investigation might utilize the techniques of immunochemistry. If a connectivity-activating synaptic protein exists, one should be able to make an antibody against it by using the whole tissue as antigen. Experiments have touched on the problem tangentially. Bornstein and Crain[50] found that antiserum against experimental allergic encephalitis antigens and serum from multiple sclerosis patients abolished evoked potentials and net firing in tissue cultures of explants that had formed synapses and possessed net-firing characteristics.

Mihailović and Janković[51,52] found that antiserum to caudate nucleus of the cat, injected into brain ventricles, abolishes firing and physiological functioning of the caudate. Apparently a high degree of specificity or molecular recognition is manifested in these experiments. They suggest the important role of immunochemistry in searching for evidence for molecular recognition (specific synaptic solder) at synaptic junctions in the brains of mammals.

C. Role of Intercellular Material Electron micrographs show channels 100 to 200 Å wide between brain cells, as between most tissue cells. It is through such channels that ion currents required by the Hodgkin–Huxley theory are assumed to flow and to determine impedance changes accompanying neurophysiological and psychological function as investigated by Adey.[53,54] Considerable uncertainty attaches to the actual value of the width of the intercellular space in the living state caused by indeterminacies associated with fixation artifacts (Schmitt[55]). On the other hand, Karlsson,[56] Van Harreveld,[57] and Nevis and Collins[58] claim that fixation does not substantially alter the semipermeability of neuronal membranes. In such small volumes, values based on statistical or colligative properties, such as *p*H, osmotic pressure, and ionic strength, have little meaning; changes in cell activity involving ionogenic processes might therefore quickly alter local channel thickness between cells. Changes in impedance, which Adey observed, reflect primarily changes in interstitial (that is, channel) space, and to some extent glial alterations, but few neuronal alterations: the interstitial changes may result from reversible sol-gel alterations, possibly of mucopolysaccharides or of acid glycoproteins of the extracellular ground substance present in the channels. Calcium ions, essential for neuronal membrane changes accom-

panying excitation, also shift colloidal changes of state in the direction of gelation, which, in narrow intercellular channels, might well decrease impedance of the bulk neural tissue. This is consistent with his experimental findings during certain behavioral performances of the cat. These global impedance changes merit detailed analysis in terms of molecular alterations that might be involved. As Adey[54] has suggested, the interface between neurons and glia may play a specialized and critical role in neurophysiological and psychological processes. The question whether intercellular space contains macromolecules capable of selective modulation of electrical activity deserves careful investigation.

Enzymological reactions in intercellular space are also important, yet poorly understood. Into this space, it is thought, are poured quanta of transmitters with each impulse delivered to presynaptic endings. If frequency and coding by temporal sequential ordering are not to lose their specific physiological and psychological significance, transmitter molecules not utilized at postsynaptic membranes with each impulse must presumably be deactivated as by hydrolysis or oxidation. Therefore esterases, oxidases, and other enzymes might be expected to be active within the channels to deal with acetylcholine and the biogenic amines, proteases for proteins, ribonuclease for RNA, etc.

D. The Dendro-Somatic Complex as Transduction "Computer" Between Input and Output In the doorbell circuit types of reflexes the neurons are linked in a "wired-in" fashion, i.e., the relation of input to output is one-to-one. Other more complicated neuronal circuits, particularly in the sensory, e.g., visual, circuits, of the central nervous system appear also to be "wired in." In special cases, such as in the innervation by the climbing fiber of the cerebellar Purkinje cell, whose output is modulated by several kinds of inhibitory inputs, a basic firing pattern is assured by multiple (as many as 20 to 30) synapses of the climbing fiber upon individual Purkinje cells.

The tacit assumption is made by many nonspecialists in neurophysiology that connectivity determines most transactions in the central nervous system, including the cortex, and that, once specified, connectivity is not plastically alterable by innate or experiential inputs; this thesis is certainly open to question. Another oversimplified view is that after deliverance of a quantum of transmitter to an axo-dendritic or an axo-somatic synapse, the postsynaptic membrane is automatically depolarized and a regenerative action potential courses over the cell surface, followed by firing of a spike in the axon. This is far from characteristic of most input–output relations in the central nervous system, particularly in the cerebral cortex. The input may be on dendritic regions remote from the soma and may suffice only to contribute to hypopolarization or excitatory postsynaptic potential (EPSP) or to hyperpolarization or inhibitory postsynaptic potential (IPSP). Through electrotonic spread, membrane depolarizations in dendrites or soma may excite the axon at its point of origin at the axon hillock, causing an action potential to be propagated down the axon. On some cells both excitatory and inhibitory inputs impinge; the resultant output thus depends on spatially and temporally complex membrane processes occurring over the entire dendro-somatic complex.

Many neurophysiologists tend to extrapolate the sodium-potassium ionic hypothesis descriptive of the inorganic electrochemistry of perfused squid axons directly to the membrane of dendrites and somata of cortical neurons, with chloride occasionally included in the simple membrane hypothesis. The justification for this practice can be questioned.

It is also frequently and tacitly assumed that, as in the case of the axon, the surface membrane of the entire dendro-somatic complex contains all the molecular equipment necessary to account for changes of polarization with excitatory and inhibitory inputs (e.g., with EPSP's and IPSP's) and to fire the axon when net depolarization reaches a certain value, and also for carrying out the computerlike data processing that determines when firing shall occur, although the kinds and amounts of inputs are very complex indeed. The actively metabolizing cell nucleus and cytoplasm, like axoplasm in axons, is viewed as playing a role merely in maintaining the "vitality" of the neuron, but not as being an active participant in neurophysiological and psychological data processing. Such an oversimplified view might be testable if, in an appropriate preparation of neuron cell body and dendrites (perhaps the giant cells of *Aplysia*), it would be possible to remove the cell nucleus and cytoplasm, as a squid giant axon that has had its neuroplasm removed by lavage can still conduct impulses. It would be interesting to determine to what extent such a devitalized cell could carry on the normal input–output computing. Presumably the extent to which such a devitalized neuron fails to duplicate the normal processes of the neuron would be attributable to cytoplasmic and nuclear metabolism and to coupling of that metabolism with membrane processes.

On several grounds it seems unrealistic to exclude cell metabolic processes from the molecular mechanisms that determine the transduction from input to output in a typical, very busy, neuron. First, axon perfusion studies have demonstrated the importance of a reactive "electrogenic protein" in mediating action waves in axons.[59,60] The sulfhydryl groups in this protein, or in some other membrane component necessary for action-wave propagation, gate

excitability sensitively; reagents blocking -SH groups block action potentials.[60] This is one of the commonest biochemical functions of cell metabolism; it seems highly probable that biosynthetic and redox reactions of the neuron may intereact with membrane molecules, thereby altering membrane bioelectric properties. In this connection, dicarboxylic amino acids, particularly glutamic acid, are excitatory to cortical neurons,[37] and these same substances inhibit protein synthesis in brain slices (Orrego and Lipmann[61]), as does electrical stimulation. One wonders to what extent the neuronal biogenetic apparatus is also involved with the highly biogenic amines, hormones, and psychopharmacological drugs.

Second, if neurons mediate innate and experiential memory patterns in learning, whether through molecular coding or through synaptic modulating macromolecules, there must be some kind of feedback between neuronal DNA and its RNA and protein readout products and sensitive neuronal surfaces, especially postsynaptic structures. Although many metabolic reactions are probably slow compared with time constants of membrane polarizations and excitation, neuronal cytoplasm is a highly ordered structure (e.g., the subsynaptic web described by De Robertis[62]), which may speed biochemical–bioelectric coupling. A rich field of investigation concerning metabolic–bioelectric coupling awaits investigation by teams including experts on cellular biosynthesis and on electrophysiological, particularly single unit, technique.

E. Control of Gene Expression (Repression-Inducer Action) in Neurons Molecular biological methods of investigation, which have immeasurably expanded our knowledge of bacterial physiology and which are now being applied to differentiated vertebrate tissue, should prove richly applicable to investigation of brain tissue. Since there is a bipartite cellular population, one part of which does not divide, the cell will undoubtedly show special features of homeostatic and epigenetic control over which bioelectric modulation would have to apply.

Memory, learning, and other psychological processes viewed from the molecular neurobiological level

No detailed discussion will be presented here concerning the chemical and engrammatic bases of memory and learning; these will be provided by G. C. Quarton,[63] B. W. Agranoff,[64] and H. V. Hydén.[22,65] It is the purpose of this paper to document the molecule-cell: brain-mind hierarchical dilemma, particularly the reason for the embarrassment of the cellular neurobiologist in trying to deduce the essential role of brain cells *as cells* in subserving psychological function, which depends on the integrated action of many cells.

The highlights of the physical basis of learning may be presented in truncated form as follows:

At sensory endings there is a transduction from physical modalities of the environment into processible bioelectric signals in varying temporal and spatial order (action potentials, synaptic potentials, and cell potentials). When information to be learned is processed, electric signals pass over thousands, possibly millions, of cells and their junctions, which are organized in complex nets. No purely electrophysiological theory (e.g., the reverberatory circuit hypothesis) suffices to explain storage, transfer, and retrieval of memory, learning, motivation, drive, conditioning, and other psychological functions; the electric phenomena are evanescent and do not qualify as permanent memory traces.

Permanent storage requires transduction from bioelectric to some chemical form (the engram) having high stability (probably that of covalent bonds). This poses a problem of energetics: how to transduce 0.1 volt signals into 25 to 50 kilocalorie bonds. Some form of amplification and triggering seems necessary, utilizing cellular energy presumably through ultrastructural devices of macromolecules or assemblies of them mounted on surface membranes. Possibly the high field across such paucimolecular films is also utilized, especially if the reaction is a fast one, as in triggering the conformational change of a protein molecule, which in the altered conformation acquires enzymatic activity or triggers an enzymatic action.

Storage in chemical form is first temporary, then permanent. Synthesis of macromolecules such as RNA or protein seems essential for such storage. The engram may be truly coded, i.e., codons sequentially ordered may bear a one-to-one relation to the information stored. This possibility is viewed with disfavor by molecular nonneuroscientists who prefer to think of engrammatic determination as occurring "at the cellular level." Another possibility is that of synaptic "solder," i.e., the activation of particular nets by macromolecules, presumably proteinaceous, manifesting molecular recognition for the neuronal junctions involved, hence activating them to fire.

The embarrassment of the cellular neurobiologist stems from the following: According to Hydén, the RNA base ratios in brain cells change when learning occurs, but not when the same neurons fire in a nonlearning situation. How do individual cells "know" when learning is taking place as distinguished from other sensory-motor activity? Learning is presumably a function of nets, systems, and the organism, not of individual cells, yet the products of activity of the individual cells provide the physical basis of the engram. Possibly the macromolecular products of

many individual cells may be joined in supracellular macromolecular nets, but only during learning does this extracellular union of engrammatic subunits occur or remain stable. If there were any substance in such a suggestion, extraction of brain material for injection into a naive animal in a transfer-of-learning experiment would have to be carried out under exceedingly gentle chemical manipulation, lest such a giant polymeric code be partially depolymerized, destroying the coding ability. Perhaps unextracted brain tissue from learned donors should be implanted into the brains of naive receptor animals in testing the transfer hypothesis.

It seems obvious that, as intracellular recording opened up new vistas in neurophysiology by virtue of the development of appropriate microtechniques, so may microanalytical, electron-microscopic, biochemical, and immunochemical techniques applied to individual neurons and glia or pools of them reveal processes that could never be discovered by use of bulk brain material. Particularly interesting is the coupling between bioelectric and biosynthetic processes, not merely in effecting changes in membrane potential and the firing of action waves, but also in the alteration of the neural-glial metabolism that leads to engram formation. If a novel, coded molecule such as protein or RNA is formed by the neuron–glia complex, is the molecule arrayed on the cell exterior, secreted at dendritic or axonal endings, or otherwise delocalized? Is the molecule stable; is it replicable? These and similar questions may find answers when the full armamentarium of molecular science is brought to bear on the problems of the neurosciences in developing the field of molecular neurobiology.

Summary

The study of the molecular organization of neurons, glia, and the neuronal nets of the central nervous system comprises a field, *molecular neurobiology*, that is basic to brain and behavioral science. If learning, memory, and other psychological products of higher nervous activity are to be explained in chemical, molecular terms, it will be profitable, and probably necessary, first to seek at the cellular level for processes known from modern molecular biology to characterize the storage, recall, and homeostatic processing of biological and chemical information vital to the life of cells generally, micro-organisms, liver cells, or neurons. We may then be able to inquire fruitfully how such processes have been adapted to nervous tissue in which information processing (at the neurophysiological and psychological levels) is effected bioelectrically. Computation of input information at the neuronal dendro-somatic level and effector output as action waves in motor fibers all involve transductions between metabolic, biogenetic, and bioelectric parameters. In this chapter some current views regarding the status of this science of molecular neurobiology are briefly presented.

Immunochemical Approaches to the Study of the Nervous System

LAWRENCE LEVINE

THE ENORMOUS CAPACITY of antibodies to recognize various specific chemical structures has been stressed by Drs. Nossal,[1] Edelman,[2] and Jerne,[3] in their chapters dealing with the possible relationships in recognition mechanisms between the antibody of the immune system and the engram of the nervous system. This chapter deals with the ways in which these antibodies can advantageously be used in studying the nervous system. Because this approach may not be familiar to the neurophysiologist, I first concentrate on a few examples that illustrate the capacity of the antibody to recognize molecular structure. The uses of antibodies (1) to probe the structure of a protein unique to nervous tissues; (2) to localize this protein in nervous tissue; and (3) to study the biological properties of nervous tissue are reviewed. Finally, some limitations of the immunochemical approach are discussed.

Effect of antigen conformation on the antigen–antibody reaction

The problems the immunochemist encounters when he attempts to describe the antigenic site(s) in native globular protein are similar to those that the enzymologist encounters in elucidating the structure of the active site in enzymes. The active centers, both antigenic and enzymic, may depend on the over-all conformation of the molecule.

The primary structure of bovine pancreatic ribonuclease is known, i.e., the amino-acid sequence[4] and disulfide bridge positions,[5] and attempts have been made to describe its antigenic structure.[6] The molecule consists of a single polypeptide chain of 124 amino-acid residues, with the eight half-cystine residues linked I to VI, II to VII, III to VIII, and IV to V, respectively. These disulfide bridges are the major bonds that restrict the configuration of the molecule. Physical studies have shown that, within the structure, the disulfide bridges impose additional forces that serve to confer a tightly compact configuration to the native protein. The requirement of intact sulfur-sulfur (S-S) bridges has been demonstrated for the serologic activity of ribonuclease.[7] Under suitable conditions of reduction and reoxidation, the correct disulfide bridges may be re-formed, followed by a return of serologic activity.[8] However, not all three-dimensional structures can fulfill the requirements for antigenic specificity. Reduced ribonuclease, reoxidized under conditions that allow the disulfide bridges to re-form incorrectly, do not cross-react with antisera to native ribonuclease, although such molecules differ only in three-dimensional structure.[9]

The dependence of serologic activity on the integrity of the disulfide linkages is shown with two other protein-immune systems (Figure 1). When pepsinogen and pancreatic deoxyribonuclease are treated for one hour at 37° C with 3 per cent 2-mercaptoethanol in the presence of 8 M (moles per liter) urea at *p*H 8.1, both protein antigens lose their capacity to react with their homologous antisera. Treatment with 8 M urea alone at *p*H 8.1 for one hour does not affect antigenic activity.

Exposure of proteins to extremes of *p*H or to reagents such as urea and guanidine can alter the secondary and tertiary structures of proteins. The data in Figure 2 show the effect of treatment at *p*H 12.4 on the serologic activity of pepsinogen and pancreatic deoxyribonuclease. At this hydrogen ion concentration, charges on amino-acid residues are altered and noncovalent bonds, essential for maintaining the protein in its native configuration, may become labile. After 15 minutes at 37° C, the serologic activity of pepsinogen is completely lost and that of deoxyribonuclease is decreased. After 60 minutes, deoxyribonuclease no longer reacts with antideoxyribonuclease by complement (C′) fixation. Figure 3 shows the denaturation of chick-heart lactic dehydrogenase by urea as measured enzymatically, serologically, and by polarization of fluorescence. After removal of the urea by dialysis, polarization of fluorescence measurements shows that refolding of the molecule has occurred. The enzymatic and antigenic activities, however, do not return; incorrect refolding has probably taken place.

It is likely that the determinant groups of the majority of proteins are unique to the molecular conformation imposed by the amino-acid sequence, disulfide bridges,

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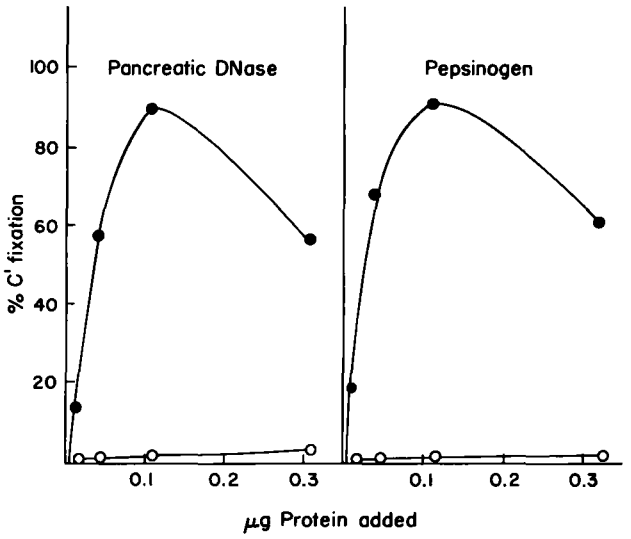

FIGURE 1 C′ fixation of pancreatic deoxyribonuclease and pepsinogen with their homologous antisera before (●) and after (O) treatment with 3% 2-mercaptoethanol in 8 M urea for 60 min. at 37°.

hydrogen bonds, and hydrophobic forces. While this dependency of antigenic activity on native configuration makes structural studies of the antigenic sites difficult, it offers unlimited advantages to the immunochemist for recognition of specific conformations.

PEPSINOGEN–PEPSIN IMMUNE SYSTEMS Antibodies to the proenzyme pepsinogen and to pepsin have been prepared in rabbits. The homologous immune systems are specific as measured by direct complement (C′) fixation. Using the two antisera and measuring direct C′ fixation,

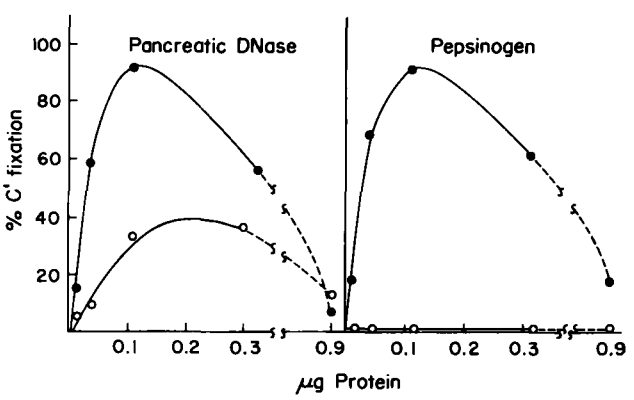

FIGURE 2 C′ fixation of pancreatic deoxyribonuclease and pepsinogen with their homologous antisera before (●) and after (O) treatment with NaOH (*p*H 12.4) for 15 min. at 37°.

the autocatalytic conversion of pepsinogen to pepsin can be followed immunologically. During conversion, pepsinogen loses its antigenic activity when assayed with antipepsinogen. Concomitantly, antigenic activity increases when measured with antipepsin.[10]

The antipepsin has been used to detect the unmasking of a pepsinlike configuration that results from treating pepsinogen by a variety of physical and chemical procedures.[11] If pepsinogen is equilibrated for 10 minutes at various temperatures, rapidly diluted in chilled veronal buffer, and assayed with antipepsinogen and antipepsin for serologic activity, a series of C′ fixation curves are obtained. With antipepsinogen, these C′ fixation curves are identical for equilibrium temperatures of 25°, 30°, 35°, and 40°. Then the curves shift progressively toward the region of higher antigen concentration at 45°, 50°, and 55°. However, the curves for pepsinogen heated at 55°, 60°, and 70° are identical. The curves of pepsinogen samples treated at temperatures above 70° undergo a progressive shift toward the region of higher antigen concentration, but with an accompanying decrease in peak height, until, at 100°, the pepsinogen no longer displays any reactivity with its homologous antiserum. The lateral displacement of the C′ fixation peak was used to estimate the amount of immunologically unaltered pepsinogen in the reaction mixture that was exposed to each temperature for 10 minutes. The percentage of unaltered pepsinogen that remained is shown in Figure 4. Because the changes in pepsinogen at 70° to 100° resulted in decreased maximal C′ fixation, pepsinogen could not be estimated quantita-

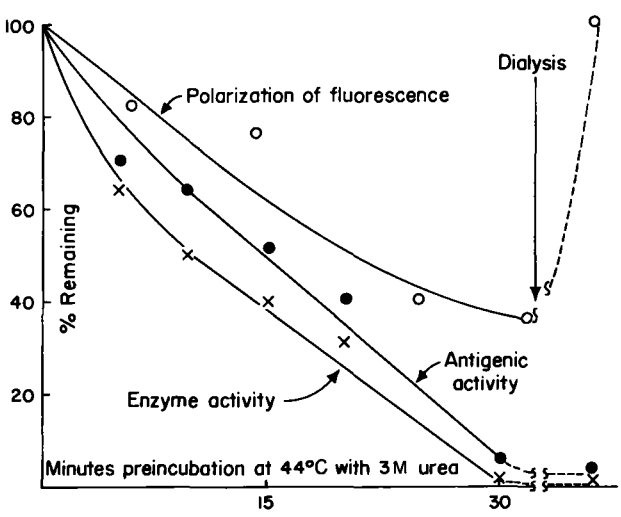

FIGURE 3 Effect of time of incubation of chicken-heart LDH with 3 M urea at 44°; O = polarization of fluorescence; ● = antigenic activity; X = enzymatic activity.

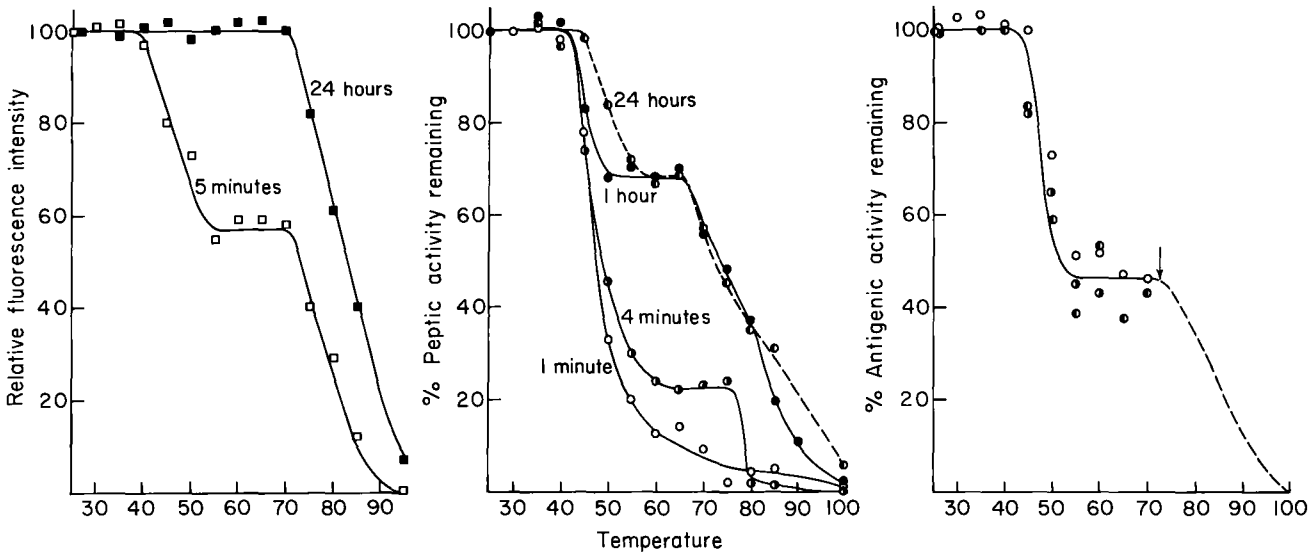

FIGURE 4 Temperature profile of pepsinogen assayed with antipepsinogen. Symbols represent values obtained in three different experiments. The per cent antigenic activity remaining was calculated from the lateral shift of the curves. The verticle arrow represents the point at which the peak height of the curves progressively decreased. Thus, estimation of remaining antigenic activity after this point could only be approximated. Relative fluorescence intensity of pepsinogen equilibrated at various temperatures, diluted into buffer and determined within 5 minutes (□) or after 24 hours at 0° (■). Potential peptic activity of pepsinogen, heated at various temperatures, and diluted into cold buffer. The samples were activated at *p*H 1.4 at 1 minute, 4 minutes, 1 hour, and 24 hours after dilution into buffer. (From Gerstein, Van Vunakis, and Levine, Note 12)

tively. The variation in the intensity of fluorescence of pepsinogen exposed in the same manner to elevated temperatures displays a profile quite similar to that obtained from the immunologic data.[12] The peptic activity of pepsinogen activated after heating followed this same profile and a "plateau" occurred within the same temperature range. Such a diphasic temperature profile was first obtained by Perlmann and Harrington in their studies on the optical rotation of pepsinogen.[13]

If these samples of heat-treated pepsinogen are reacted with antipepsin, the C′ fixation curves shown in Figure 5 are obtained. Pepsinogen incubated at temperatures from 25° to 55° does not react with antipepsin to any significant extent. Pepsinogen heated at temperatures between 60° and 80° becomes increasingly more reactive with antipepsin. Presumably this reflects an "unmasking" of previously protected antigenic sites on the pepsin portion of the pepsinogen molecule. At 90°, a reversal of this trend begins, probably reflecting a structural disorganization of the previously unmasked pepsin moiety.

HEMOGLOBIN AND MYOGLOBIN IMMUNE SYSTEMS

Since the heme moieties of mammalian hemoglobins are identical, differences in immunochemical specificity, absorption spectra, and function with respect to oxygen and carbon dioxide equilibriums reflect structural differences in the globin moieties. The structure of globin differs from that which it possesses in a conjugated protein. Human globin is roughly half the size of hemoglobin, has a different shape with significantly more asymmetry, is more labile to heat, and has a different electrophoretic mobility than has the intact heme protein.[14,15] Optical rotatory dispersion studies of the globins of myoglobin and hemoglobin indicate a lower helical content than in the conjugated proteins.[16] The appearance of 2 to 3 groups has been observed in sperm whale apomyoglobin, which are in hydrogen ion equilibrium in the neutral *p*H range as compared to the conjugated protein.[17] Dr. Blout has presented data[18] showing the decreased optical rotary dispersion properties of apomyoglobin when compared to myoglobin, and the restoration of the original optical rotary dispersion properties upon the addition of heme.

Antibodies to hemoglobin and myoglobin can also recognize the conformational differences between these heme proteins and their apoproteins.[19] In Figure 6 are seen the C′ fixation curves obtained when hemoglobin and globin react with antihemoglobin. Globin is the less effective serologically, as judged by the decrease in maximum fixation and the increased quantity of protein required for this maximum. The extent of C′ fixation (at peak) with

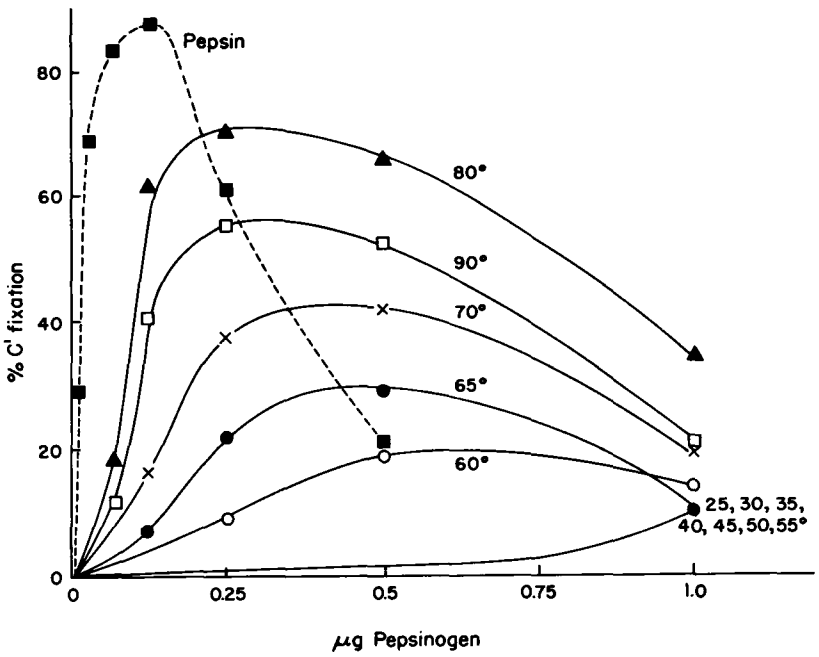

FIGURE 5 C′ fixation curves of pepsinogen incubated at various temperatures and assayed with antipepsin. (From Note 12)

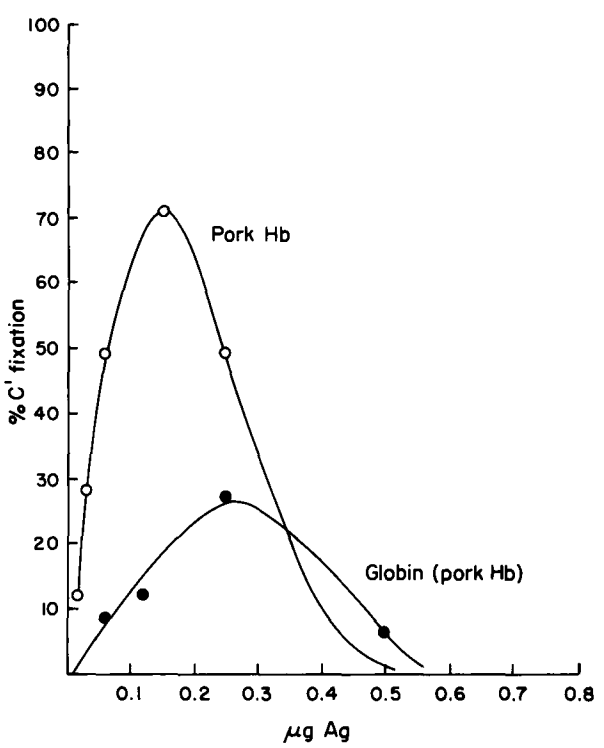

FIGURE 6 Fixation of C′ by increments of porcine hemoglobin (O) and porcine globin (●). (From Reichlin, Hay, and Levine, Note 19)

globin varies with different preparations, but is always significantly less than that of the hemoglobin. Furthermore, peak fixation is always reached with higher concentrations of globin. That the globin and hemoglobin are reacting with the same population of antibody was demonstrated by inhibition experiments in which a large excess of globin completely inhibited the C′ fixation reaction of hemoglobin–antihemoglobin. The possibility that hematin is an antigenic determinant is unlikely, as in 30,000 molar excess hematin fails to inhibit the reaction of hemoglobin–antihemoglobin. The restoration of the original serologic activity of pork hemoglobin is complete after addition of 3.0 moles of heme bound per 66,000 grams protein.

Studies similar to those performed on hemoglobin were also carried out with myoglobin. The difference in reactivity of myoglobin and apomyoglobin is shown in Figure 7. The same characteristics are exhibited in the myoglobin systems as in the hemoglobin system. Apomyoglobin fixes less C′ at peak and requires a higher antigen concentration to reach peak fixation. As observed with globin prepared from hemoglobin, the apomyoglobin also exhibited variation in reactivity with antimyoglobin. Heme was not inhibitory when tested with 100,000 molar excess, whereas globin in high excess completely inhibited the homologous system. Titration of the apomyoglobin revealed that 17,000 grams of protein bound

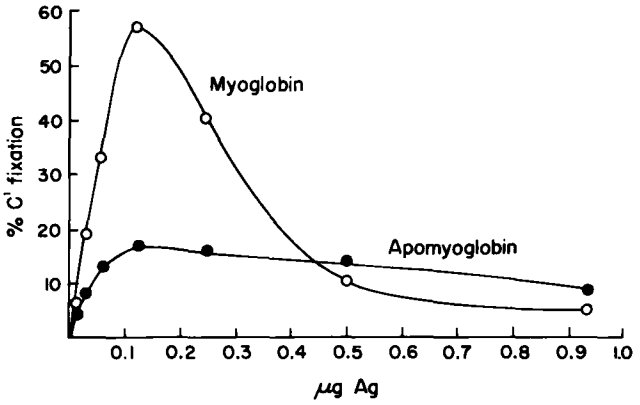

FIGURE 7 Fixation of C′ by increments of myoglobin (O) and apomyoglobin (●). (From Note 19)

1.0 mole of heme as measured by hyperchromicity at 405 millimicrons. Immunological experiments on the same reaction mixtures revealed that full serologic activity was not reached until each monomeric myoglobin chain had bound one molecule of heme (Figure 8).

Hydrolysis by carboxypeptidase A of the two C-terminal amino acids from the β-chains of hemoglobin ($\alpha_2^A\beta_2^A$) results in a conformational change of the molecule as judged by the loss of its characteristic oxygenation properties.[20] On the other hand, removal of the C-terminal amino acids of the α-chains by carboxypeptidase B does not affect the oxygenation properties of hemoglobin. The C′ fixing properties of these molecules[21] are shown in Figure 9. Hydrolysis by carboxypeptidase A results in a decrease in serologic activity, while hydrolysis by carboxypeptidase B is without effect. Removal of the C-terminal amino acids by carboxypeptidase A does not affect the C′ fixing capacity of hemoglobin A (β_4^A), a molecule that exhibits no heme–heme interaction. A decrease in serologic activity has also been observed[22] when comparing oxygenated and deoxygenated hemoglobins molecules differing only in the distance between their β-chains.[23]

Aspartate transcarbamylase immune systems

This enzyme[24] is composed of two types of subunits: two molecules of a catalytic unit of 90,000 molecular weight and four molecules of a regulatory unit of 30,000 molecular weight.[25] The regulatory units bind effectors, while the catalytic units bind substrate. The macromolecules can be dissociated into subunits by treatment with *p*-hydroxymercuribenzoate (*p*HMB), heat, or urea. Antibodies directed toward the intact macromolecular con-

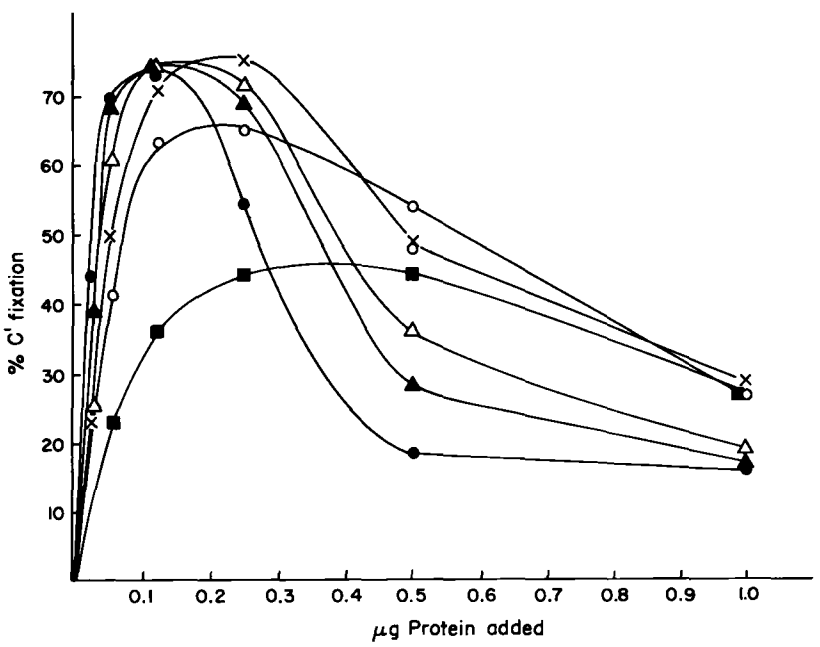

FIGURE 8 Fixation of C′ by increments of Mb_1 globin, Mb_1 myoglobin, and partially reconstituted myoglobin. Mb_1 globin (■); 0.25 mole hematin added (O); 0.50 mole hematin added (X); 0.75 mole hematin added (△); 1.0 mole hematin added (▲); Mb_1 myoglobin (●). (From Note 19)

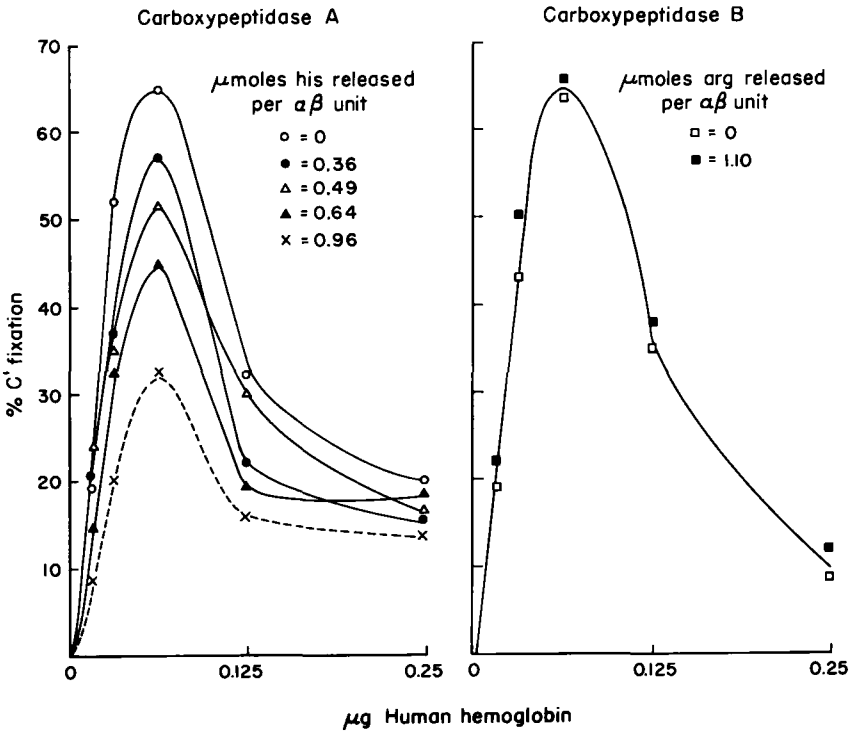

FIGURE 9 Fixation of C′ by increments of A_1 hemoglobin and carboxypeptidase-treated hemoglobin. (From Reichlin, Hammerschlag, and Levine, Note 21)

formation and antibodies directed toward the catalytic unit have been used to measure dissociation of aspartate transcarbamylase.[26] The C′ fixation curves shown in Figure 10 are obtained when varying quantities of *p*HMB are incubated with the enzyme and the reaction products are analyzed serologically.[27] At a concentration of 4×10^{-5} M *p*HMB, the serologic activity of the intact enzyme decreases to a level obtained with the purified catalytic unit. Concomitant with this decrease in activity with anti-aspartate transcarbamylase is the increase in serologic activity of the reaction products with anticatalytic unit. Similar data have been obtained after heating the enzyme. Thus, the ATCase–antiATCase reaction is dependent on the macromolecular conformation of the intact ATCase. Reagents that dissociate the enzyme into its subunits decrease its serologic activity. These immune systems have been used to investigate the effect of substrates and effectors on the conformation and stability of the enzyme.[27]

MITOCHONDRIAL MALATE DEHYDROGENASE IMMUNE SYSTEM The "impossibility" for proteins that possess the same amino-acid sequence to exist in different conformations has been discussed in preceding chapters. Just such an impossibility has been recently detected in the malic dehydrogenase (MDH) of chicken mitochondria.

Kitto, et al.[28] have examined the structure of the multiple forms of MDH after separating the different forms by ion-exchange chromatography. These forms cannot be distinguished on the basis of catalytic activities or amino-acid composition. However, it is possible to interconvert the various enzyme forms by reversible dissociation in acid, and to measure them by optical rotatory dispersion. By using antibody directed toward one of the isolated forms of MDH, it was also possible to measure interconversion of these "conformers" after reversible dissociation in acid. The ability of antibody to distinguish the proteins that have the same amino-acid sequence but different conformations is shown by the C′ fixation curves in Figure 11.

Immunological studies of S-100 protein

A protein unique to nervous tissue was recently detected by B. W. Moore in a number of vertebrate species (rats, rabbits, guinea pigs, chickens, catfish, alligators, cattle, monkeys, and humans), and the protein (S-100) from beef brain was isolated and purified.[29] In September 1964, a conference was held at the Neurosciences Research Program Center attended by B. W. Moore, F. O. Schmitt, P. F. Davison, B. Smith, and L. Levine to initiate co-

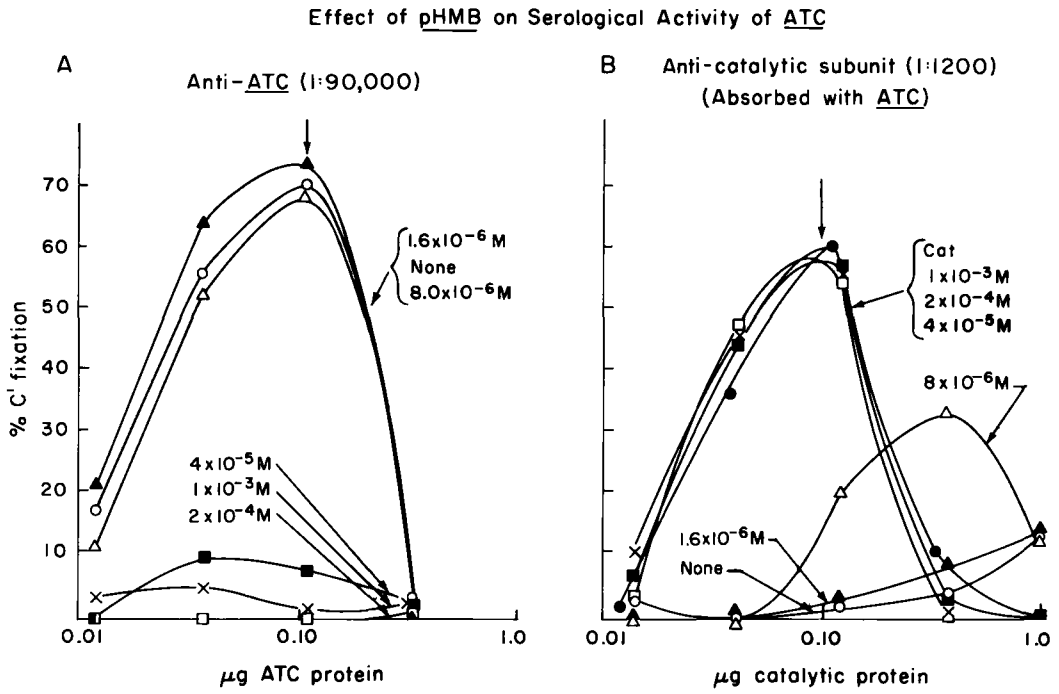

FIGURE 10 Fixation of C′ by ATCase and ATCase after incubation with varying concentrations of *p*HMB. (From Bethell, Jones, and Levine, Note 27)

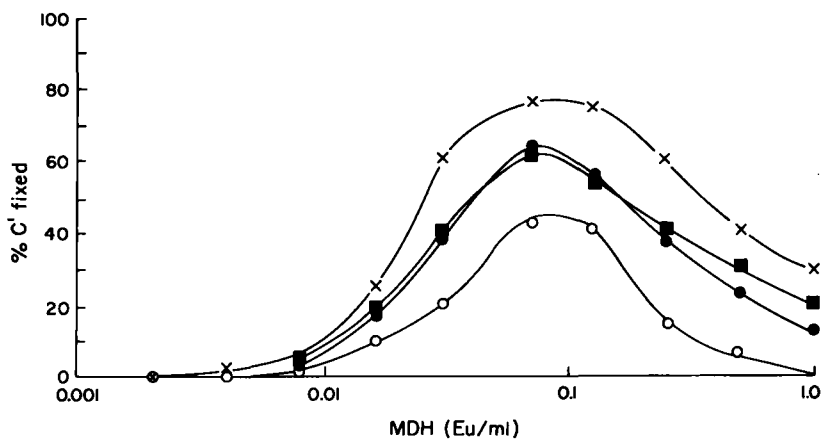

FIGURE 11 An immunological comparison using complement fixation of the reactivity of unresolved enzyme (●), Band A (x), Band E (O), and a sample of reversibly acid dissociated Band E (■), with a rabbit antiserum directed against Band A. (From Kitto, Wassarman, and Kaplan, Note 28)

operative immunological studies on the protein in the hope of elucidating its neurophysiological role.

MOLECULAR EVOLUTION OF S-100 PROTEIN In many immune systems the quantitative antigen–antibody reaction reflects the conformation of the antigen. Thus, a sensitive serologic method such as micro-C′ fixation can be used to measure structural changes during evolution.[30] The changes accompanying enzyme evolution in the conformation of lactic dehydrogenase (the heart type), lactic dehydrogenase (the muscle type), triosephosphate dehydrogenase, glutamic dehydrogenase, aldolase, and hemoglobin are shown in Table I. Nonmolecular evidence indicates that the degree of change, as measured by the increment of antiserum required for a reaction equivalent to that observed with homologous chicken proteins, is related to phylogeny. In a similar study with the primate growth hormones and rabbit-, rat-, human-, and guinea pig-antiserum to human growth hormones, the molecular changes accompanying primate evolution were measured.[31,32] In marked contrast to the rates at which the above proteins evolved, there is a relative lack of change of S-100 among the species studied (Table II). With S-100 from the bullfrog brain, only 2.5 times as much antiserum was required to get the same activity as that obtained with the homologous beef brain S-100. As can be seen in Table I, 14 to 40 times more antiserum was required with bullfrog antigen to get activity equal to the homologous chicken proteins.

TABLE I

Complement fixation with antisera to pure chicken proteins

Species	Antiserum Concentration Required for 50% C′ Fixation*					
	H_4LDH	M_4LDH	TPD	GDH	Aldolase	Hemoglobin
Chicken	1.0	1.0	1.0	1.0	1.0	1.0
Turkey	1.4	1.2	1.0	1.0	1.0	1.0
Duck	1.5	4.3	1.2	1.2		2.2
Pigeon	2.3	2.0	1.3	1.3		3.6
Ostrich	1.9	3.1	1.3	1.4	5.0	
Caiman	3.3	4.2	3.8	4.0	6.5	
Painted turtle	4.0	5.2	4.2	4.0		6.5
Bullfrog	14	40	30	19	18	
Sturgeon	80	20	12		25	
Halibut	†	>200	>50		>100	
Dogfish	>100	>200	>50			
Lamprey	†	30	>50			
Hagfish	>100	>100				

*Data presented are based on the use of several anti-chicken H_4 LDH sera, two anti-chicken M_4 LDH sera, and one each of the other anti-chicken protein sera. † Halibut and lamprey tissues contain no detectable H_4 LDH. (From Wilson, Kaplan, and Levine, Note 30)

STABILITY OF S-100 PROTEIN Earlier I described how antibodies to pepsinogen could be used to measure structural changes induced by temperature (Figure 5). A similar approach has been used to study the stability of S-100. Figure 12 shows the serologic activity of S-100 protein after incubation of 10 micrograms of S-100 per milliliter (0.01 M Tris, 0.14 M NaCl at *p*H 7.4) at varying temperatures for 10 minutes. It should be pointed out that the reaction is diluted into nine volumes of cold diluent after incubation at the various temperatures, and any rapidly reversible conformational changes would not be measured by the anti-S-100.

This acidic protein undergoes irreversible changes in structure even after incubation for 10 minutes at 40°. A number of reagents alter this thermal stability profile. The presence of 1×10^{-4} M 2-mercaptoethanol during incubation of S-100 at varying temperatures stabilizes its structure. A similar stabilization was observed when 10^{-4} M of ethylene diamine tetraacetate was present during the incubation. On the other hand, the loss in serologic activity at elevated temperatures is accelerated in the presence of Mg^{++}, Cu^{++}, Mn^{++}, and Ba^{++} ions. In contrast to the destabilization effects of the above cations, Ca^{++} ions stabilize S-100 structure almost as effectively as 2-mercaptoethanol.

LOCALIZATION OF S-100 PROTEIN The validity of quantitative estimations of proteins, polysaccharides, and single-strand deoxyribonucleic acid by immunological methods is well established. Once purity of the immune system is rigorously demonstrated, the antibody response with purified antigen can be used as a calibration curve to estimate the quantity of that antigen in crude material. Such an approach was used in an attempt to localize the S-100 in white or gray matter of the brain. Electrophoretic

TABLE II

Complement fixation with antiserum to beef brain S-100 protein

Brain Extracts From	Relative Antiserum Concentration Required for 50% Fixation	Brain Extracts	Relative Antiserum Concentration Required for 50% Fixation
Beef	1.0	pig	1.8
Rat	1.0	Rabbit	1.9
Sheep	1.0	Human	2.0
Guinea pig	1.2	Chicken	2.2
Mouse	1.3	Pigeon	2.3
		Bullfrog	2.5

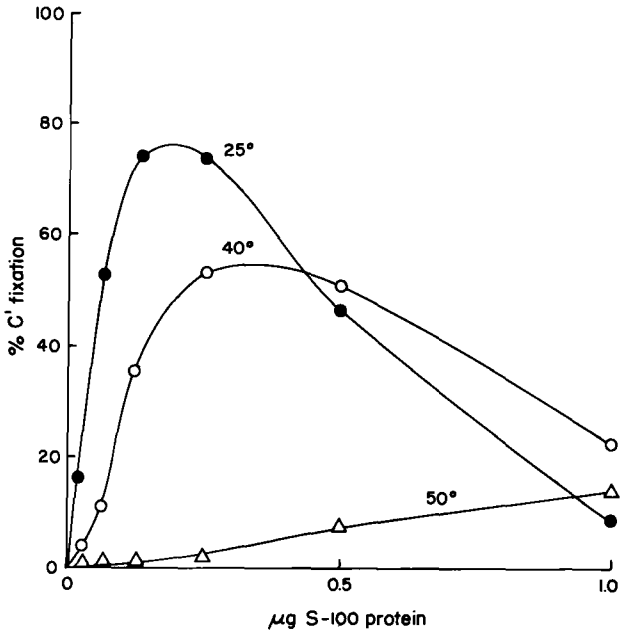

FIGURE 12 Serologic activity of S-100 protein after incubation of S-100 protein (10μg/ml, in 0.01M Tris, *p*H 7.4, 0.14M NaCl at varying temperatures for 10 minutes.

analyses originally revealed that S-100 was found in roughly equal concentrations in white and gray matter and in the spinal cord. The data in Table III show the amount of S-100 protein in micrograms per milligram total extractable protein in various areas of the brain as measured immunologically. It can be seen that S-100 protein is present in higher concentrations in the white than in the gray matter. Almost 24 times more S-100 protein per milligram total extractable protein was found in the frontal white matter than in the frontal gray matter.

Hydén and McEwen[33] have attempted to localize the S-100 protein using both fluorescent antibody methods and gel precipitation procedures. With extracts of 50 Deiters' nerve cells, 50 glial cells, and 50 cells from a brain-stem homogenate, precipitation in agar was observed with extracts of the glial cells and brain-stem homogenate. This demonstrates that S-100 protein was present in higher concentrations in the glia than in neurons. Studies with the fluorescent antibody "sandwich" techniques demonstrated that S-100 protein was present around the nuclei of oligondendrites and scattered throughout the glial membrane system. In addition, S-100 appeared to be present in the nuclei of the neurons.

TABLE III

Quantitative estimation of S-100 protein in various areas of the brain

Area	μg S-100 protein per mg total extractable protein
Thalamus	3.1
Cerebellum	10.5
Frontal white matter	24.0
Frontal gray matter	1.1
Occipital gray matter	2.4
Corpus callosum (white)	11.3

Alteration of biological properties of nerve tissues by antibody

Antibodies can combine with biologically active antigens and alter their activities in different ways. Although combination between an antibody and a biologically active molecule may lead to loss of activity, this does not necessarily represent a direct interaction of the antibody with the active site. For example, antibody to ribonuclease affects the ability of this enzyme to hydrolyze substrates of varying molecular weights in different ways. It markedly inhibits hydrolysis of the large substrate, ribonucleic acid; it inhibits hydrolysis of the dimer of cytidylic acid to a lesser extent and does not inhibit hydrolysis of cyclic cytidine phosphate.[34] The relatively large antibody can attach to areas on the surface of the enzyme and sterically prevent the hydrolysis of the large substrate while permitting hydrolysis of the smaller substrate. For enzymes that change conformation during biological activity, the combination with antibody may prevent the transient conformational change needed for biological activity by "freezing" the original structure.

Further, instances have been found in which reaction with antibody can enhance the biological activity of the antigen. The pharmacologically active polypeptide bradykinin normally has a transient existence, but combination with its specific antibody sustains its activity.[35] Antibody may combine with the peptide in such a way that the destruction of the peptide by proteolytic enzyme is reduced.

Thus, it is quite conceivable that antibody to neural material can work in several ways: (1) it can neutralize biological activity because of its size, although it might not be directed toward the active site(s); (2) it might prevent the antigen from undergoing conformational changes; or (3) it may give the appearance of activating the antigen by exerting a protective effect upon it.

The effect of antibody on biological properties of the brain has been studied.[36,37] The investigators prepared rabbit antisera directed toward homogenates of the caudate

nucleus and hippocampal regions of the cat. The gamma globulins of the appropriate unabsorbed antisera, as well as gamma globulin preparations of normal rabbit sera, were introduced into the lateral ventricle of the cat, and the electrical activity of various cerebral structures was recorded. Intraventricular application of the immune gamma globulin, in contrast to normal gamma globulin, was followed by an alteration of electrical phenomena. Furthermore, alteration was observed only in that region of the brain from which the immunogenic material had been prepared. For example, rabbit anticat caudate nucleus homogenate modified the electrical activity of the caudate nucleus but not of the hippocampus, while rabbit anticat hippocampus homogenate altered the bioelectric properties of the hippocampus but not of the caudate nucleus. This specificity of the caudate nucleus and hippocampus homogenate antisera was also reflected in the increase in histaminelike substances in the regions of the brain from which the immunogenic material was obtained.

The effects of intraventricular injection of rabbit antimonkey caudate nucleus or rabbit antihippocampus homogenate on delayed alternation and visual discrimination were also measured. A sustained decrease in the ability of monkeys to perform the delayed alternation test was observed in the animals injected with anticaudate nucleus homogenate, while their visual discrimination was variable. Injection of normal gamma globulin was without effect, while injection of antihippocampal gamma globulin resulted in impairment in the delayed alternation test only during the injection days. No impairment of visual discrimination was observed after injection of normal globulin or immune globulin to hippocampus homogenate.

The specificity of rabbit antimonkey caudate nucleus and antihippocampus was also demonstrable after intercerebral injection of the immune and normal gamma globulins. In these experiments, the identical structures in the two sides of the brain permitted comparison of the effects of simultaneously injected gamma globulin preparations of different specificity. For example, intercerebral injection of antihippocampus gamma globulin into the hippocampus of one side of the brain produced localized irritative abnormalities that developed into a long-lasting epileptiform discharge, while simultaneous injection of normal or anticaudate nucleus gamma globulins into the hippocampus of the other side of the brain had no such effect.

The effects of immune serum on the membrane potentials of lobster axons have also been measured.[38] When compared with normal gamma globulin, axons immersed in immune gamma globulin solutions lost their electrical activity much earlier. Blocking of the action potential was always preceded by a decrease of the resting potential. The alteration of bioelectric activities of the squid axon by antibody is described in detail by Davison.[39]

The sera of animals affected with experimental allergic encephalomyelitis and sera from human patients obtained during acute exacerbations of multiple sclerosis have been tested for their effects on the electric activities of cultured fragments of cerebral or spinal cord tissue.[40] These sera, in contradistinction to sera of normal animals and humans, produced rapid modification in complex, evoked, bioelectric (synaptic) response. This alteration of bioelectric activity was reversible and was mediated by the complement system.

Limitations of the immunochemical approaches

Two potentially informative approaches to the study of the nervous system have been presented. One approach, characterization and localization of an antigen unique to nervous tissue, is limited by the availability of pure immunogens. Davison[39] has discussed the progress being made in the purification and characterization of proteins unique to nervous tissue. One hopes that in the next few years more proteins will become available to the immunoneurologist. If any of these purified antigens have a physiological function, the second approach—alteration of biological properties by specific antibody—can be investigated. However, the ability of the specific antibody to reach its antigen in vivo must be assessed, because the cell wall may prove to be an obstacle for the antibody directed toward an intercellular antigen. If the antibody were directed toward antigens at the synaptic surface, the biological effect could be determined directly, as intracellular penetration would pose no problem.

While alteration of biological properties of the nervous system by antibody directed toward purified antigens represents the ideal experimental situation, an approach of immediate use has been taken by Mihailovic and Jankovic,[36-38] i.e., alteration of biological properties by antisera to all of the immunogens in extracts of defined areas of the brain. It is unlikely that the antibodies can penetrate living cells, so another antibody to a cell membrane antigen may, through the mediation of complement, change the permeability of the cell in such a way as to permit cellular penetration. Thus, an antibody population of varied specificity may be useful in the initial assessment of alteration of biological properties, and may provide an assay system for purifying the active antigen. For example, if antisera to caudate nucleus alters biological activity, the active antigen from the caudate nucleus will absorb and neutralize the antibody responsible for this alteration.

Summary

Antibodies directed toward native pancreatic deoxyribonuclease, porcine pepsinogen, chicken lactic dehydrogenase, porcine and human hemoglobins, horse heart myoglobin, chicken malate dehydrogenase, and *E. coli* aspartate transcarbamylase detect alterations in conformation of these molecules. How antibodies have been used to study molecular evolution of several proteins is reviewed. Antibodies directed toward bovine S-100 brain protein have been used to study the molecular evolution, localization, and the structure of S-100 protein. The effects of antibodies directed toward homogenates of various areas of the brain and/or nervous tissue on the bioelectric properties of nervous tissue are reviewed. Some limitations of the immunochemical approach to the study of the nervous system are discussed.

Neuronal Specificity in Neurogenesis

M. V. EDDS, JR.

DURING EMBRYONIC DEVELOPMENT neurons, and perhaps neuroglial cells as well, develop highly refined affinities and disaffinities for one another and for peripheral tissues. They reveal these properties by entering selectively into specific cellular associations that become the interknit tracts and nuclei of the adult nervous system and by selectively re-establishing some of these associations during nerve regeneration. The capacity of developing cells generally to enter complementary groupings lies at the basis of all embryonic events at cellular and higher levels. The origin of these abilities and their nature are largely unknown.

In the nervous system, the large number of morphologically different cell types joined by a bewildering variety of interconnections might, at first glance, appear too complexly organized to arise from selective cellular recognition and association at the level of individual neurons. Yet the experimental facts point strongly in this direction. Even if selectivity does not extend to the individual neuron, however, it certainly extends to small groups of cells that form larger associations in strict accord with their specific properties.

In this chapter, some of the key questions about the origin of neural pathways are characterized by discussing a few especially pertinent examples. In an effort to present the essential concepts as clearly as possible for a diverse audience, a selective approach seems justified. Those readers who pursue further any of the issues raised here will, in resorting to the original literature, quickly become aware of the subtle qualifications, uncertainties, and disagreements with which the subject abounds. For such readers, reference is made to reviews by Gaze,[1] Hamburger,[2] Sperry,[3] Jacobson,[4] Székely,[5] and Weiss.[6] Especially valuable earlier treatments are to be found in Detwiler,[7] Harrison,[8] Sperry,[9] Hamburger,[10] and Weiss.[11]

Initial developmental events

In all vertebrate embryos, the first visible sign of the future nervous system is a thickening plate of cells lying dorsally in the surface ectodermal layer. This neural plate forms toward the end of gastrulation as a result of an extensively studied but still little understood set of interactions with the underlying notochord and mesoderm.[12] The plate gradually folds into a tube that extends along the anteroposterior axis of the embryonic body and is enlarged anteriorly, foreshadowing the brain that is to come. During the folding, the entire neural tube sinks beneath the surface ectoderm. From the neural tube and from associated cellular masses deriving from the neural crest (plus other ectodermal patches in the head region), the entire nervous system is developed. The neural crest components form the outlying ganglia—clusters of neurons from which arise sensory and autonomic axons. The peripherally directed somatic motor and preganglionic sympathetic axons, the intracentral neurons of second and higher orders, and the neuroglial cells are all derived from cells lying within the

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neural tube. For the details of these early events, the reader should refer to such accounts as that of Weiss[11] or of Hamburger and Levi-Montalcini.[13]

Before and during the period when the embryonic neurons first send out their axonal and dendritic processes toward the periphery or to some intracentral station, four distinct but interwoven cellular events contribute to the emergence of the typical orderly pattern of the young nervous system. These events, which occur with unusual clarity in the chick embryo,[14] include: mitotic cell divisions; migrations of individual and small groups of cells; changes in the shape, size, and content of individual cells; and the death and disappearance of already partly differentiated cells. Occurring in closely timed and precisely interlocking patterns distributed differentially along the anteroposterior and dorsoventral axes of the developing spinal cord and brain, these events collectively produce a recognizable neural axis with appropriately segregated areas of future white and gray matter, with the rudiments of cranial and spinal nerves arising as cables of fibers from many individual neurons, with enlargements, foldings, and outpocketings that already foretell both the final gross form and the inner detail of localized cell grouping—with, in short, all the major features that finally characterize the nervous system and distinguish one part of it from another. The key point is that the final unity emerges from the coordinated activity of thousands of individual cells, each behaving as if it knew its place in the final product, yet subordinating itself to that product.

There is a large body of evidence, summarized in the sources cited, that these patterned cellular events within the nerve centers are partly determined by regionally localized factors and thus occur autonomously, without reference to the state of their embryonic surroundings. In addition, however, they depend on obscure influences from their surroundings, especially from the peripheral tissues to be innervated, or from other parts of the nervous system. The same differentiative events are also known to be influenced by hormonal substances, such as the nerve growth factor discussed elsewhere in this volume by Ebert. Interactions with peripheral tissues have been most fully studied; some influences, such as those affecting the extent of cell division, are exerted before any direct contact is established between the young neurons and the peripheral tissues; others, such as those affecting the number of differentiated nerve cells that will survive once their axons reach the periphery, depend critically on contacts with the periphery. In either case, so fine a numerical balance develops between the nerve centers and the periphery that the numbers and kinds of neurons accumulated in particular locations are appropriate to the size and nature of the periphery to be innervated.

Axonal outgrowth in embryos

The developmental origin of some of the simpler fiber tracts and nuclei can be followed with precision in the embryo.[15] But the extension of axonal and dendritic processes in all directions from myriad differentiating neurons soon makes it difficult or impossible either to trace out the origin and destination of individual processes or to unravel the factors influencing their specific courses. Additional information must then be sought by experimental intervention. As a case in point, consider Hibbard's study[16] of the axons exiting from the two large Mauthner cells of the salamander medulla. While still within the hind brain, each axon crosses over to the other side of the center line; after so decussating, it runs the length of the spinal cord. Hibbard transplanted to the hind brain of early, tailbud embryos an extra segment of medulla containing a pair of Mauthner neurons. The implant was positioned either in normal or in reversed anteroposterior orientation.

Subsequent histological study revealed, as Figure 1a indicates, that the axons of the supernumerary neurons in normally oriented grafts followed courses closely paralleling those of the host's own Mauthner cells. In implants

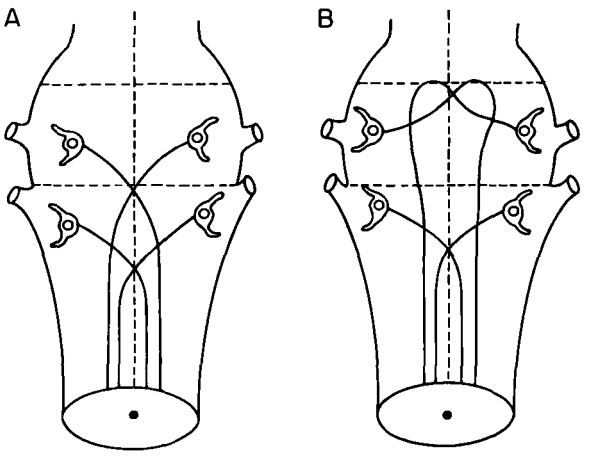

FIGURE 1 These diagrams show the courses followed by the axons of supernumerary Mauthner cells transplanted to the medulla of the salamander in embryonic stages. In both A and B, an extra segment of medulla containing two Mauthner cells was transplanted just anterior to the host medulla, as indicated by the dotted lines. In A the antero-posterior orientation of the graft was retained; the axons of the transplanted cells decussate with and run parallel to those of the host. In B the graft was implanted with its antero-posterior axis reversed; after crossing the midline, the axons of the transplanted cells first course anteriorly as far as the original posterior border of the transplant, then turn posteriorly to join the host axons. (Redrawn after Hibbard, Note 16)

with reversed orientation, however, the extra axons first grew out to the old posterior surface of the graft (now, of course, facing anteriorly in its new location). Then, as though recognizing the inappropriate orientation of the more anterior levels of the host brain, the axons turned in their course, ran in reverse through the graft, and entered the spinal cord along with the host axons (Figure 1b). Hibbard concluded that "it is not the polarity of the axon which determines its course, but the polarity of the segment of the medulla in which the axon finds itself."

It is instructive, and especially relevant to the interpretation of examples to be considered later, to recall that the results of an experiment of this type depend critically on the developmental stage of the embryos used. Had the operation been performed somewhat earlier—for example, late in neurulation before the axial polarity of the medulla was fully established—the implanted segment would have developed as though it had been grafted in normal orientation; the Mauthner axons of the graft would then have pursued a posterior course from the beginning.

From this and comparable cases, then, one may conclude with Hamburger[2] that: "directional forces with wide spread rather than specific narrow pathways guide [advancing nerve fibers]. We have to postulate in all these cases a biochemical conditioning or infiltration of the substrate over a considerable range, perhaps in a gradient fashion."

Embryogenesis compared to regeneration

Faced with the limitations imposed by working with the embryo, investigators of neurogenesis have often turned to older organisms with the fortunate capacity of regenerating amputated nerve fibers. The basic premise is that the interrupted and regenerating axons will reveal by their pathways and reconnections something of the means whereby their previous courses and terminations were achieved in embryonic life. While the premise is acceptable, it is hazardous to overlook the obvious differences between embryonic and regenerating systems—for example, the length and directness of the pathway, the specific cells encountered by the advancing axon tip, the state and interconnections of the cell bodies of origin, and the developmental status of the end organs that become re-innervated.

With these precautions in mind, let us now consider the questions: What is the evidence that even structurally and functionally similar neurons become specifically different from one another, and when and how does this occur?

Specification of peripheral sensory neurons

The nerve fibers that grow out from the cell bodies of the segmental sensory ganglia are guided to their end organs by the physical configuration of their surroundings, according to the principle of contact guidance elaborated by Weiss.[11] Contact guidance finds expression, however, in the context of specific preneural pathways marked out as subtly but as truly as the invisible route pursued in the preceding example by Mauthner axons. Presumably, in following these pathways nerve fibers are revealing their properties of cell-to-cell and cell-to-substrate recognition and acceptance or rejection. Although the basic mechanism underlying these critical events is obscure, the evidence for their occurrence is substantial.[2,11]

Once sensory fibers have reached the skin, a new type of interaction begins between neuron and periphery. The consequence is the emergence in each of the previously indifferent nerve fibers of specific properties reflecting the precise zones in which they terminate. The fibers become modulated, to use Weiss's term, and thereby acquire their local sign properties.

Support for these general assertions may be drawn from several sources, but perhaps most rewardingly from Miner's[17] study of the acquisition of local sign specificity in frogs with rotated skin grafts. The experiment began with tadpoles midway through the larval period (Taylor and Kollros Stages X to XIII) at a time when the sensory nerve fibers were as yet unspecified, although they had reached the skin. Strips of flank skin a centimeter wide were excised and then replaced after a 180° rotation of their dorsoventral axes. Since the cutaneous nerve fibers were interrupted by the operation, reinnervation was totally dependent on nerve regeneration. The regenerating fibers were led back into foreign territory, axons that previously ended in dorsal skin now penetrating ventral skin and vice versa (Figure 2).

After several weeks of further development and postmetamorphic growth, tests were made on the behavioral responses of the young frogs to localized irritations of the skin. Identification of the rotated skin patches was easy because their pigment patterns had self-differentiated. Stimuli to the normal areas of skin yielded entirely different responses from those to the grafts. An intact frog will correctly localize a cutaneous stimulus and wipe at it with a foot. But in the experimental frogs, stimuli to the pigmented dorsal areas of the graft (now located ventrally on the host flank) caused wiping reflexes aimed at the back. Similar misreferral and dorsoventral reversal of the responses followed stimulation of the unpigmented ventral area of the graft. Although the animals were observed for up to ten months, no correction ever occurred in these stereotyped response patterns as a consequence of experience. To check the possibility that the regenerating dorsal nerve fibers might have coursed ventrally under the graft and thereby re-established their original connections with

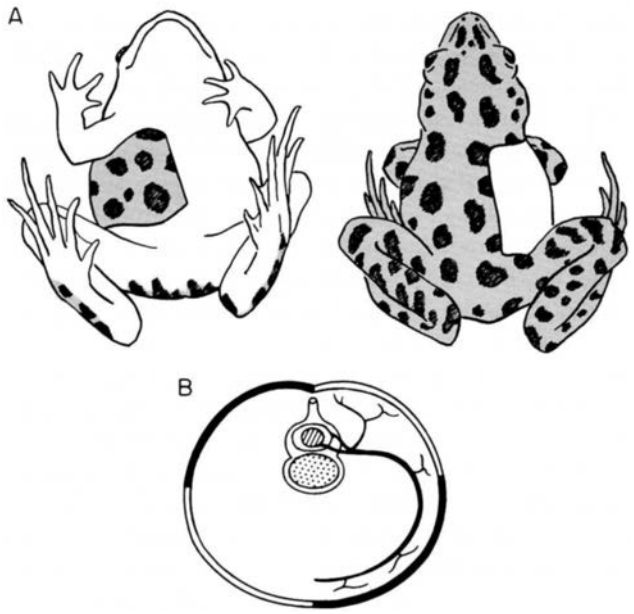

FIGURE 2 Miner's experiment with rotated patches of frog larval skin. Such grafts develop their innate pigment patterns after metamorphosis, as shown in the dorsal and ventral views of adult animals in A. Following the rotation, the interrupted nerve fibers in the dorsal rami of the spinal nerves regenerate into ventral skin, and vice versa, as indicated in B. When the skin grafts are stimulated, behavioral responses are dorsoventrally reversed. That is, the central associations formed by the sensory axons entering the grafts are appropriate to the local skin area rather than to the general topography of the body surface. (Redrawn after Sperry, Note 9)

dorsal skin, or vice versa, the dorsal and ventral halves of the grafts were surgically separated immediately before the skin was stimulated. The reversed responses persisted.

These remarkable findings have become generally accepted as cogent evidence that the regenerating axons were modulated by their new endings and that, in accordance with their new properties, the parent neurons made functionally appropriate adjustments with central neurons, both sensory and motor. Whether these adjustments involve a reshuffling of synaptic connections or only a selective opening or closing of previously established synapses remains moot.[3,6] But in either case, one must infer the existence within the nerve centers of complementary patterns to which the modified peripheral neurons became matched.

In concluding the consideration of Miner's experiment, the importance of the developmental stage should be emphasized again. The same experiment on an adult frog (although apparently never actually performed), after the sensory axons have been definitively specified, should yield normally directed response patterns. Moreover, the organism chosen for the experiment is also crucial. For instance, mammals apparently specify their peripheral sensory neurons relatively early, certainly before birth. Thereafter, if forced to make new connections, no respecification is possible and permanently misreferred sensations result.[18] Finally, different neurons in the same organism may become fixed and unmodifiable at different times. Even in adult frogs and rats, for example, the gustatory axons innervating the taste buds of the tongue apparently can have both their chemical sensitivities and their action potentials modified in accordance with the specific taste buds in which they are forced to terminate. There is no evidence, however, of grossly detectable behavioral changes relating to new taste preferences.[19,20]

Specification of peripheral motor neurons

Somatic motor axons issuing from cell bodies in the neural axis are guided to and become specified by their developing motor end organs according to the same principles just set forth for sensory neurons. The most cogent evidence in support of their specification by the muscles they innervate comes from Weiss's exhaustive investigations of transplanted limbs in larval salamanders.[6,21-23] The experimental animal in these studies was usually an axolotl of some 4- to 12-cm body length whose limbs had been functioning for weeks or months. Just posterior to the normal forelimb of such an animal, a supernumerary forelimb was implanted in any of several possible orientations. The site of implantation lay close to the plexus of spinal nerves 3, 4, and 5, which normally innervate the muscles of the shoulder girdle and the forelimb (Figure 3). One of these spinal nerves, usually the fifth, was cut and its proximal stump diverted in order to send a quantitatively restricted supply of regenerating axons, both motor and sensory, into the graft. After reinnervation of the transplanted limbs was complete, their movements were observed over many months and recorded cinematographically. Finally, anatomical and electrophysiological analyses were made of the origin and the distribution of nerve fibers in the grafts.

The essential observation made by Weiss, and since confirmed by others, was that the transplanted limb moves precisely in phase, muscle for muscle, with the adjacent normal forelimb, as though the transplant had simply tuned in an on-going pattern of neural activity of the brachial spinal cord that programs coordinated limb movements. The results were the same whether or not the movements were functionally relevant. A limb with re-

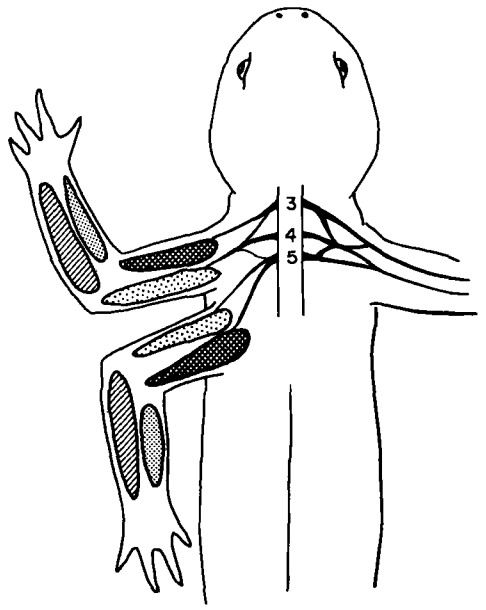

FIGURE 3 Diagram of the anatomical relations between the brachial nerve plexus and the original and transplanted limb in Weiss's demonstration of myotypic response. (For details of innervation patterns, see Weiss[22].) The right limb is innervated by branches of spinal nerves 3, 4, and 5. The normal left limb receives axons principally from spinal nerves 3 and 4, spinal nerve 5 having been diverted into the transplanted limb. Muscles of the same name are shaded alike. Each muscle of the transplant contracts in unison with the homonymous muscle in the adjacent normal limb. The two limbs therefore move in duplicate despite the obviously maladaptive effect on locomotion. (Redrawn with modifications after P. Weiss, 1952. Central versus peripheral factors in the development of coordination, *Res. Publ. Assoc. Nerv. Ment. Diseases,* Vol. 30, pp. 3–23)

versed orientation, as in Figure 3, would move in opposition to the normal limb; even such hopelessly maladaptive responses were never corrected with experience. Other tests revealed that the sensory fibers entering the transplant had no bearing on the outcome of the experiment, which could be repeated even in animals from which the appropriate spinal ganglia had been removed.[23]

Because the recorded limb motions were produced by individual muscles contracting in phase with homonymous muscles of the normal leg, Weiss called the phenomenon a homologous, or myotypic, response. So clear-cut, yet so difficult to explain if a complete reshuffling of nerve-muscle connections had indeed occurred, the myotypic response led other investigators to wonder if an explanation might not better be based on the selective ingrowth of specific motor axons to the particular transplant muscles with which they are normally associated. Such an explanation seemed reasonable because, in the normal animal spinal nerves 3, 4, and 5 each supply nerve fibers to all the forelimb muscles, although in different proportions.

In a detailed test, however, Weiss[22] apparently disposed of this explanation. Although some of his evidence against selective axonal ingrowth is today less cogent, at least one set of facts still seems incontrovertible. Instead of allowing one of the spinal nerves to innervate the transplant, the inferior brachial nerve—which supplies only the flexor muscles of the wrist and hand—was diverted into the extra limb. Despite the reduced size of the nerve fiber source and its restricted qualitative composition, functional nerve connections were re-established in the entire limb musculature. During limb movement, the same myotypic responses were recorded. This observation, unless explicitly contradicted or explained away, would seem to rule out the relevance of a recent challenge to Weiss's argument raised by compelling demonstrations of selectivity in the reinnervation of muscles of the eye and pectoral fin of adult teleost fish.[24,25]

The conclusion has seemed unavoidable that muscles confer unique qualities on the motor axons that enter them and that the modulated neurons then effect new structural and/or functional relations with precisely matching neuronal patterns in the nerve centers. It remains largely a matter of taste whether one imagines a mechanism based on a physical reshuffling of synaptic relations,[3] or one based on functional adjustments within neuronal nets so that pre-existing but nonfunctioning channels become transmissive, while previously open channels are closed.[5,6] Either alternative depends on specific changes in the peripheral neurons, on the retrograde passage of those changes from the end organ, and on the induction or prior existence of strictly matching qualities in the nerve centers.

Finally, this discussion of motor specificity should not end without a reminder that, like sensory axons, motor axons cannot be remodulated once they have been specified definitively. Thus, adult amphibian limbs would not display myotypic responses following transplantation. And the literature on muscle transpositions and on nerve regeneration, if critically reviewed,[26] supports the conclusion that motor specification in mammals occurs before birth and cannot be modified thereafter.

Intracentral specificities

In the preceding pages, several allusions have been made to the necessity of inferring the existence in the central nervous system of specificities that are complementary to peripheral neuron specificities, but that develop independently of the periphery. We now inquire: Do such complementary patterns also influence the specific connections

that develop between two centers within the brain or spinal cord? The answer is affirmative, and the evidence has been best worked out in the visual system of fish and amphibians. This is the only example that will be considered here; Sperry[3] has reviewed supporting information from vestibular, cutaneous, and other systems.

THE VISUAL SYSTEM The main structural features of the amphibian visual system are shown diagramatically in Figure 4. Axons from the ganglion cells of each retina (which arises embryologically as a derivative of the forebrain) extend through the optic nerve to the contralateral optic tectum; decussation is complete, but some impulses reach the ipsilateral tectum by secondary pathways.[1] There are about a half-million axons in the frog, mainly small ($<1\mu$) and unmyelinated.[27] The axons reach their endings in the tectum by running across its surface and finally turning inward to synapse with one or more of the tectal neurons, most of which have dendritic arbors limited in diameter to 50 to 80μ. The cortex of tectum, in whose superficial layers the terminal synapses lie, comprises nine layers of complexly arranged cells and fibers. As Figure 4 illustrates, the tectum not only receives other sensory modalities but also, as a main coordinating area of the brain, provides connections to outgoing motor pathways. These include axons to centers of the cord and brain involved in various visuomotor responses.

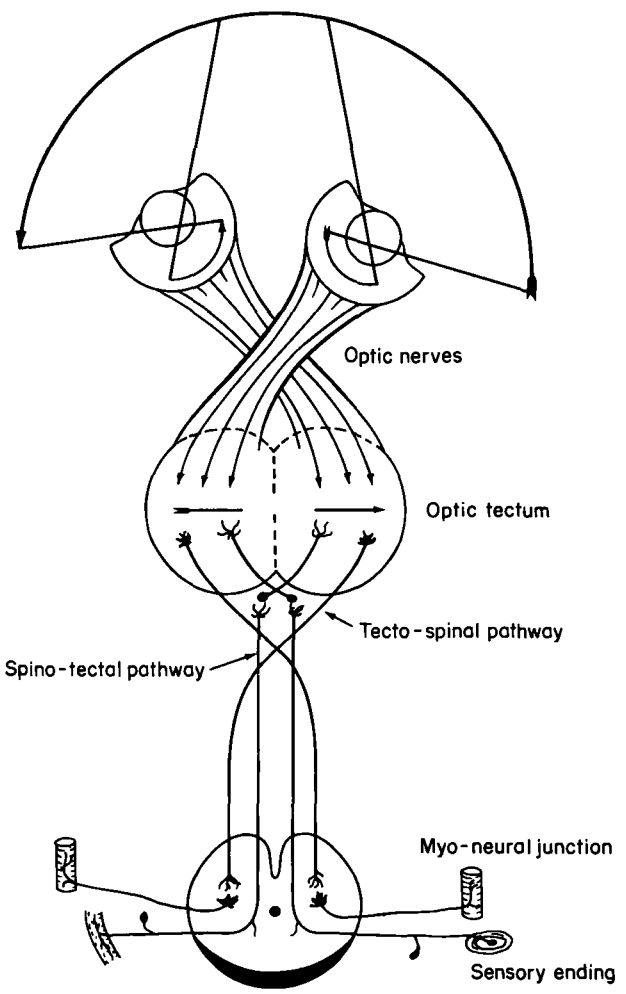

FIGURE 4 Diagram of the anuran visual system showing the complete decussation of optic nerve fibers and their termination in the contralateral optic tectum. The midbrain is a major coordinating center in the frog, and the axons shown leaving it provide for various motor responses to visual stimulation. (Redrawn after S. Ramon Cajal, 1955. Histologie du Systeme Nerveux de l'Homme et des Vertèbres. Traduite par L. Azoulay, Madrid, Instituto Ramon y Cajal, Vol. 2, Figure 243)

The projection of optic axons on the tectal surface, which may be analyzed either by histological or electrophysiological methods, is extremely precise in all vertebrates. As is true of sensory projections on the nervous system generally, the projection of optic fibers inverts the retinal map. Thus, fibers from the dorsal retina terminate ventrally in the tectum; the ventral retina connects with the dorsal tectum. The same inversion occurs in the naso-temporal (anteroposterior) axis, so that nasal fibers end posteriorly in the tectum and temporal fibers end anteriorly. These relations are reviewed in detail by Gaze.[1]

OPTIC NERVE REGENERATION It has long been known that, following section, the optic nerves of amphibians and fish, unlike those of higher vertebrates, will regenerate with good visual recovery. The early studies on urodeles were extended to several anuran and teleost species by Sperry,[28,29] thereby achieving an important technical advantage, because the superior visual acuity of these animals permits more exacting tests of visual recovery. The amphibian eye has another remarkable capacity. Not only will retinal axons regenerate, the entire retina can be reconstructed from its pigmented epithelial layer.[30] A completely new set of optic nerve fibers will then be regenerated and excellent visual performance will be re-established.

The recovery both of movement perception and the ability to localize and respond accurately to visual stimuli obviously implies that, after optic nerve regeneration as before, the excitation of small retinal areas leads to unique patterns of evoked activity in the optic tectum and its motor output. Since local visual sign depends first of all on the anatomical orderliness of the retinal representation in the tectum, the various retinal points apparently become selectively reassociated with their original end stations in the tectum, even though during regeneration the

advancing axons become hopelessly scrambled in crossing the scarred region between the nerve stumps. The only alternative would be an initially chaotic, random reconnection followed by some sort of central readjustment in which the animal would have to relearn its visual field.

A series of classical experiments by Sperry[3,9] has strongly supported the first alternative. Sperry first[31] exploited the distorted and permanently misoriented behavioral responses that occur in amphibian species after the adult eye is rotated. The optokinetic reactions of such animals are reversed, and their responses to a lure presented in a localzied portion of the visual field are inverted to the same degree as the eye has been turned. No correction of these patterns ever occurs, unless the eye is rotated back to its original position; normal reactions are then restored immediately. A diagramatic representation of the structural basis of these phenomena appears in Figure 5.

If the adult eye is rotated and the optic nerve cut and allowed to regenerate, the visuomotor responses after recovery will be reversed or inverted in proportion to the amount of rotation. That is, the functional projection of the retina upon the optic tectum is restored as though the eye were still in its normal position. The result is a hopelessly maladaptive behavior pattern, which, again, is never corrected by experience.

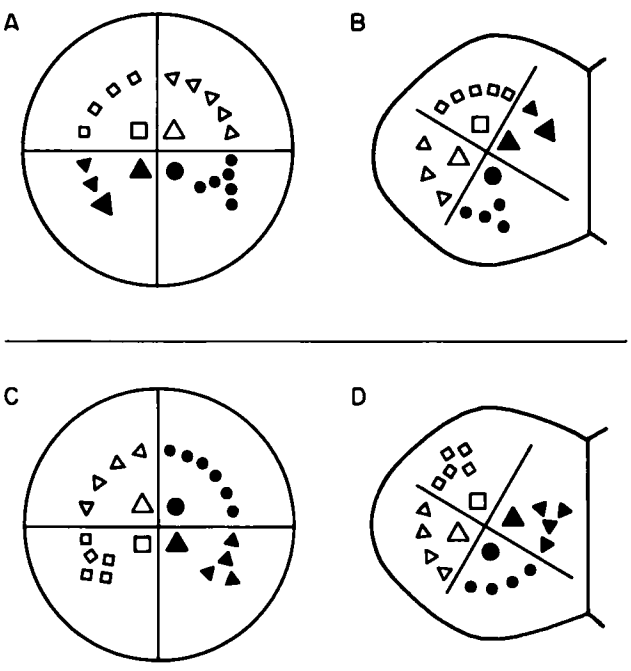

FIGURE 5 The image of a curved arrow on the normal anuran retina in A is inverted during the projection to the optic tectum in B. If the adult eye is rotated 90°, indicated in C by the counterclockwise displacement of the retinal symbols, the image of the arrow will fall on different retinal quadrants, and its representation on the optic tectum will be correspondingly rotated, D. After section and regeneration of the optic nerve, the retinotectal projection is unchanged, and the tectal representation of the arrow remains rotated. (Redrawn after Gaze, Note 1)

Sperry[9] made similar observations in a series of elegant variations on the theme. He cut both optic nerves and crossed them so that each retina became connected with the ipsilateral tectum. Or he transplanted eyes from one orbit to another, and even from one species to another. In all such cases, the stereotyped visuomotor responses that were recovered indicated that the retinotectal projections were selectively re-established.

ELECTROPHYSIOLOGICAL EVIDENCE The demonstration was incomplete, however, in that it gave no certain information about the details of the recovery process. Do the regenerating optic fibers seek out particular pathways provided by residual neuroglial cells in the distal nerve stump? Or do they enter the tectum in disarray and only then start the matching process? Is matching achieved by a secondary selection among initially random endings, or do fibers go directly to appropriate terminals? Or do all fibers end indiscriminately, leaving the specificity to a functional selection of impulse patterns? Such questions require electrophysiological and histological answers.

Several workers have collected evidence of the physiological type.[32-35] Mapping of tectal responses evoked in the adult frog by stimulation of discrete retinal areas of varying size and shape was accomplished by microelectrode recording. The essential findings fully confirmed Sperry's observations. After optic nerve regeneration, the normal retinotectal projection was recovered. Maturana and his associates, however, added a critical new dimension to the story. In mapping the retinotectal projection of normal frogs, they learned that the function of the retina "is not to transmit information about the point-to-point pattern of distribution of light and dark in the image formed on it." Rather, "its function is mainly to analyze this image at every point in terms of four qualitative contexts (standing edges, curvatures, changing contrasts, and local lessening of light intensity) and a measure of illumination, and to send this information to the colliculi, where these functions are separated in the four congruent layers of terminals."[33] That is, the tectum actually has a fourfold map, and its layers are superposed and in register. Following nerve regeneration, the fourfold map was restored without error or confusion; any possible randomness was too fine to detect with the methods used.

Yet, numerous variations and anomalies in the post-regenerative projection patterns were noted in exceptional cases, especially in the study by Gaze and Jacobson[35] who

have speculated on the relation of these patterns to normal embryogenesis. The same authors,[36] however, did not encounter comparable variations in a more recent analysis of the goldfish, and the significance of abnormal projections early in recovery remains to be clarified. Of perhaps greater interest was the finding that after regeneration in the frog the areal representation on the tectum of particular receptive fields had increased from two to four times.[37] Apparently the new nerve terminals were indeed larger than normal.

HISTOLOGICAL EVIDENCE Despite the encouraging confirmations gained through these electrophysiological studies, many uncertainties remain. Some of these have found answers in Attardi and Sperry's[38] neurohistological study of the regenerated optic nerve and tectum of the goldfish.[3,39] No trace could be found of an early, nonselective phase of regeneration. Even fibers en route through the distal nerve stump followed their original courses, entering, as appropriate, either the lateral or medial branch of the primary nerve trunk. By ablating large sectors of the retina at the time of nerve section (e.g., the entire dorsal half or all but the most central cells), Attardi and Sperry were able to trace fibers of known retinal origin to their tectal endings. A representative example is illustrated in Figure 6. In this and similar cases, regenerating axons selectively bypassed inappropriate denervated zones to reach their original stations.

These histological results have meanwhile been supported[36] in the electrophysiological analysis of the goldfish already mentioned. By hemisecting the optic nerve and then crushing the entire nerve just proximally, it was possible to study the projection of the crushed but uncut axons that got back to the tectum first. The normal projections of these axons led the authors[36] to a concept of a "fixed system of place specificities." Attardi and Sperry, in turn, concluded that their "results would appear to dispell any remaining doubts that the growing fibers are destination bound."[38]

Developmental origins of retinal and tectal specificity

The question now arises as to when and how these remarkable patterns of neuronal specificity emerge during embryonic life. Unlike the peripheral neurons discussed earlier, which receive their conforming properties in some unknown fashion from the end organs they encounter, intracentral specificities seem to develop from exclusively intrinsic factors. As Weiss[11] put it, "modulation plays no constructive part in the design of central coordination patterns." The over-all polarity and the gross functional and structural differences between parts of the central nervous system are already inherent, although invisible, in the organization of the folding neural plate. Subsequent segregation and interaction of neural parts are believed to contribute to the elaboration of finer details, but our knowledge is woefully incomplete.[11]

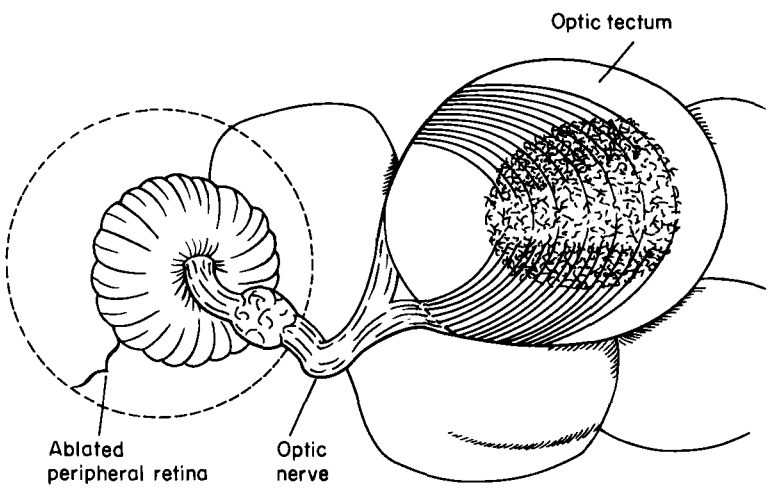

FIGURE 6 Diagramatic reconstruction of the courses and terminations of axons regenerating from the central retina of the goldfish. The peripheral retina was destroyed and the optic nerve sectioned. During regeneration of the severed nerve, axons entered both its medial and lateral branches, then reached the central tectum by growing past the denervated tectal areas where fibers from the peripheral retina normally terminate. (Redrawn after Sperry, Note 3)

POLARIZATION OF THE EYE The developing amphibian eye becomes irreversibly polarized early in its developmental history. That is, for a brief period immediately after the optic placode evaginates from the forebrain wall, it can be rotated to any extent without affecting subsequent visual performance. However, at about the time when the placode is being converted to a cup and the lens is forming, the axial properties become firmly fixed; thereafter, rotations of the eye cup lead in the adult to intractably inverted motor responses to visual stimuli. The unknown events responsible for polarization occur well before the cytological differentiation of the future retinal cells, to say nothing of the outgrowth and connection of their axons.

The development of retinal polarity in the salamander *Amblystoma* has been extensively analyzed.[30] Both the anteroposterior and the dorsoventral axes appear to fix simultaneously within a few hours at Harrison State 36 (mid-tailbud embryo). In the salamander *Triturus* and the toad *Xenopus* the anteroposterior axis is fixed before the dorsoventral.[4,5,40] In *Xenopus* embryos between Nieuwkoop–Faber Stages 30 and 32 (early tailbud), eye-cup rotations produce visual inversions in one axis but not the other. The retinotectal projection of an adult *Xenopus* eye, studied by Jacobson[4] after rotation at State 30 is shown in Figure 7. Only the anteroposterior axis is inverted. After State 32, the dorsoventral axis is also fixed.

The development of polarity in the optic tectum is much less well known. The tectal rudiment in *Amblystoma* can be rotated without any subsequent effect on visual orientation, at least through Stage 35, and regeneration of a functionally adequate tectum will occur following excision of its rudiment through Stage 39.[41] Tectal polarization must set in shortly thereafter, however, since cytodifferentiation is about to begin, and ingrowing optic axons will soon arrive.

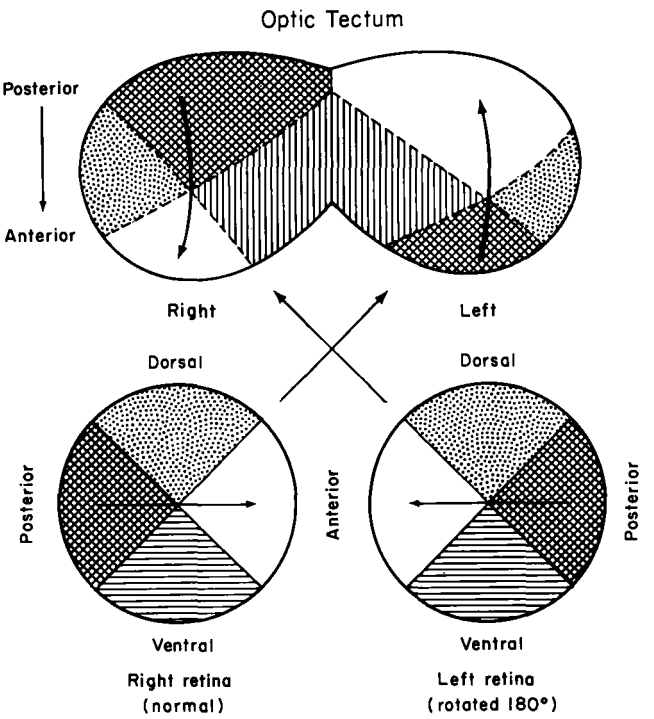

FIGURE 7 The tectal projection of the retinal quadrants in the adult toad, *Xenopus*, following 180° rotation of the left optic cup at embryonic Stage 30. The map of the right retina on the left optic tectum is normal and shows the usual inversion of both anteroposterior and dorsoventral axes. The projection of the rotated left retina, though correct with respect to the dorsoventral axis, is reversed anteroposteriorly (cf. arrows). Thus, e.g., ganglion cells of the now anterior quadrant of the rotated retina had already developed irreversible posterior qualities before the eye was turned. Despite the subsequent anterior location of their cell bodies, optic axons entered a portion of the tectum suited for posteriorly arising fibers. The dorsoventral polarization, being still labile at the time of the operation, developed later to match the new surroundings. (Modified from Jacobson, Note 4)

POLARIZATION AND NEURONAL SPECIFICITY It is probably necessary to assume, although there is no direct evidence either for or against it, that the fixation of retinal polarity is causally associated, or at least proceeds simultaneously, with the acquisition of individual specificities by the retinal neurons. This question is brought into critical focus by studies of Gaze, Jacobson, and Székely (reviewed in Jacobson[4]) on the retinotectal projection of compound *Xenopus* eyes. The eyes were surgically compounded in Stages 30 to 32, the critical period when the anteroposterior but not the dorsoventral axis has been laid down. Embryonic eye cups were bisected to separate their anterior from their posterior halves. Two anterior or two posterior halves were then combined along their cut edges to reconstitute a single eye cup. One of the halves retained its original orientation, but the operation unavoidably reversed the anteroposterior axis of the other half. After metamorphosis, retinotectal projections were established electrophysiologically.

Although a few exceptional experimental animals, for obscure but perhaps significant reasons relating to axial fixation, had essentially normal projection maps, the predominant result was that the projection from each half-retina covered the entire tectum! The two projections were each well organized along both axes, but they were mirror images of one another. Each point on the tectum, then, received impulses from two different retinal positions. The evidence strongly suggests that the axons in

these cases spread into and thereby filled "foreign" regions of the tectum. Other conceivable explanations, such as differential hypertrophy or atrophy of, respectively, innervated and noninnervated parts of the tectum, were explicitly ruled out.

It is instructive to reflect on the results that might be expected were this experiment to be repeated in embryos younger than Stage 30 or older than Stage 32. Presumably, the former would yield normal retinotectal projections, because the still unpolarized compound eyes could undergo full regulation. Indeed, this may be the explanation of the exceptional normal projections. Embryos operated on after Stage 32, however, would probably still give duplicate and mirror image projections until the tectum became fully polarized. Then the two half-retinas should project in duplicate, but with the maps in register, to either the anterior half or the posterior half of the tectum, as appropriate. These predictions should be tested.

Jacobson[4] has discussed the early state of the tectum, and the reason for the duplicate connection of optic fibers with it, in terms of quantitative "gradients of specificities," which ultimately give way to the "fixed system of place specificities" referred to above. It will be of great interest to learn from future investigations whether such conjectures are meaningful.

SPECIFICITY IN BIRD NEUROGENESIS The difficult nature of these problems, as well as another direction in which new answers may be sought, may be illustrated by an analysis of developing retinotectal connections in the chick embryo.[42] The experiments bear on the question: Is there a point in development at which a limited group of optic axons, given the opportunity, will no longer spread throughout the tectum but will proceed instead only to their normal adult stations? The answer seems to be that there is such a point and that in the chick embryo it falls somewhere between three and four days of incubation. Thus, if a retinal quadrant is destroyed by electrocautery at three days, a normal eye with a histologically normal retinotectal projection results. If the same defect is imposed at four days or later, however, localized tectal zones, corresponding to the retinal defect, will lack terminating optic fibers. However, it is impossible to decide from the available evidence whether these results are to be explained by some lability of three-day retinal cells (lost at four days), permitting them to regenerate a complete retina, or by some initial inability of the tectum, corrected within a day, to coapt selectively with arriving axons.

The problem is compounded because the normal entrance of optic axons into the chick embryo tectum extends over a period of several days, starting at day 6, when fibers first reach the ventral tectum, and ending at day 12, when the dorsal pole of the tectum is finally supplied.[42] Unfortunately, we do not know whether the specification of individual tectal areas (or the development of tectal polarization) follows or does not follow this spreading pattern in space and time. However, an unpublished study by DeLong of small retinal patches transplanted to the tectal surface suggests that the tectal surface is already specified by day 6 when the first axons reach it. DeLong eliminated the optic fibers to one tectum of the host embryo by removing the contralateral eye on day 4. Two or three days later a small piece of retina (0.5–0.8 mm square) from another four-day embryo was implanted in the pia-arachnoid tissue immediately overlying the host's denervated tectum. In a series of cases, the grafts were taken from all parts of the retina and put in various tectal locations. They were left in place for four or five days, then examined histologically.

As the optic axons emerged from the transplants through the mesenchyme covering the tectal surface, they grew out for varying distances (up to 2.5 mm, or 2/3 the greatest dimension of tectum) following relatively straight courses. Finally, they turned inward and entered the tectum, apparently to terminate locally. The course of the fibers from a given graft, and the point of termination, were by and large functions of the specific source of the graft and its initial position over the tectum. That is, the fibers tended to go to what would have been their normal end stations, even though they were arriving from abnormal directions and had gone through tissues not ordinarily a part of their pathway. Is the pia-arachnoid provided with a duplicate tectal map that can be traced by advancing axons? Is DeLong's experimental method applicable to the analysis of complementary specificities in other parts of the central nervous system? Efforts to answer these and related questions are now very much in order.

Conclusion and Summary

The foregoing discussion points clearly in one direction. Although it reveals nothing about how the finished nervous system works,[3,6] it demonstrates that neurogenesis depends heavily on those properties of neurons that enable them to enter selectively into functionally critical groupings and interconnections. The developmental events are flexible and dynamic rather than static, but a high degree of fixed structural order is attained.

Early in development, all cells of the embryo—neural and nonneural—become different from one another, different in ways not ordinarily visible, and involving refined, graded variations in some basic properties of their cell surfaces, their contents, and/or their synthetic capacities. The peripheral tissues of the embryo are already

highly differentiated in these respects when the first outgrowing axons reach them. End organs such as individual muscles and small areas of skin then confer upon the invading axons a set of equally specific, individuating properties corresponding to the topography of the terminal stations. These modulating events occur at different relative times during development, depending on the animal species and on the part of the nervous system involved. Once completed, they irreversibly alter the character of the affected neurons and determine the nature and extent of their structural and functional relations with other cells within the nerve centers of the spinal cord and brain.

Simultaneously, and independently, corresponding and equally precise properties arise within the central nervous system. They affect the ability of cell groups, and probably individual cells, to recognize other cells and to enter or not enter into synaptic and transmissive relations with them. Once set, these central specificities, like those of the periphery, are irrevocable, and they become independent parameters in subsequent functional adjustments, regenerative events, and the like. The final interrelations between the central and peripheral neurons arise in ways still obscure, but clearly based on a selective matching of their respective properties. Whether the selective affinities thus revealed are expressed mainly by the establishment of specific synapses, or mainly through functional conformance of activity patterns in widely interconnected neuronal nets, or through both, remains debatable and, for the moment, is largely a matter of taste in methods of interpreting the experimental evidence.[3,5,6]

Also still a matter of taste, and highly conjectural, is the mechanism underlying neuronal specificity. Sperry, for example, has often argued in favor of graded properties based on subtle chemical differentials of individual cells and their surfaces. His position seems unexceptionable. But the real question is what these molecular differentials amount to. The "long range of properties running from one extreme to the other with many graded intermediate steps, each one of which is precisely controlled and precisely replicated from within a given cell"[3] pose awesome problems for the molecular neuroscientist. But there is no reason to push aside the phenomena just because they are difficult to explain. Readers of this volume will find no shortage of possible mechanisms, ranging from differential gene activation through sequential enzyme inductions[43] to conformational changes in macromolecules. The problem is not to find *an* explanation; it is to find the right one.

In the context of a treatise devoted to exploring broadly the functions of the nervous system, it is tempting to conclude, as Sperry[3] has, that the mechanisms of learning and memory may be "distinct and removed from those processes one deals with in development and maturation. It is also possible, and not at all improbable, that the changes imposed by function are similar to, or a direct derivative of developmental features, and perhaps best understood in those terms."

Molecular and Cellular Interactions in Development

JAMES D. EBERT

ONE OF CLIFFORD GROBSTEIN'S recent articles was entitled, "What we do not know about differentiation."[1] I shall not attempt to catalogue what we do not know about mechanisms of development of the nervous system. Instead I shall call attention to crucial questions and promising directions, drawing extensively from my more comprehensive article, "The keys to change: factors influencing differentiation."[2] That article and its companions in the volume resulting from the Twenty-fifth Anniversary Symposium of the Society for Developmental Biology[3] provide an up-to-date assessment of most of the major problems of development.

As Sussman[4] has emphasized, development differs significantly from physiological modulation in being characterized by the existence of an over-all regulatory program which insures that each sequence of developmental alterations occurs in invariant chronological order; that these alterations are spatially restricted both within cells and within specific cells of multicellular assemblies; and that they occur to fixed extents. Differentiation is defined here as a change in a cell's biochemical repertoire, which results from the release of information encoded in one-dimensional sequences. Morphogenesis encompasses the processes concerned with the shaping of three-dimensional structures by folding and aggregation of one-dimensional gene products, or by aggregation or redistribution of cells.[5,6]

This paper is also concerned with intra- and extracellular factors that impinge upon the nucleus and regulate the flow of information; with the controls that must operate in differential replication; and with differential release of information.

I have emphasized,[2] along with Pavan[7] and Schultz,[8] that there is no direct proof that a full genome is maintained in differentiated cells. Is the total content of information per nucleus in all cells in a given species equivalent? Have stringent requirements for establishing differences or identities in information content been met?

The arguments for DNA constancy are well known. The diploid progenitor of the haploid gamete must have all the genes present in the antecedent zygote. In appropriate environments, some of the cells in specialized tissues of higher plants are capable of giving rise to a whole organism.[9-11] The plants are only slightly more versatile than animals in some of the lower phyla in which the whole organism regenerates from a small fragment.

By comparison, in the nematode worm *Ascaris* and the fungus gnat *Sciara* differentiation is associated with a regular loss of parts of chromosomes,[12,13] and in mammals one of the X-chromosomes becomes genetically inactive and cytologically compact at about the time of gastrulation.[14,15] Moreover, there are examples of disproportionate replication of DNA; thus far, these examples are limited to insects and some amphibians, and for most of them the exact mechanisms remain to be analyzed. Nevertheless, the questions raised are compelling, and studies in depth should be undertaken.

Some of the key examples may be enumerated: In interphase or prophase nuclei of salivary-gland cells in *Drosophila,* large segments of chromosomes stain darkly while other segments of the same chromosomes stain lightly or not at all. Thus, we are able to distinguish cytologically between two kinds of chromatin, termed euchromatin and heterochromatin, respectively. Although the lightly stained heterochromatin may be located at various places along the arms of chromosomes, it is normally found adjacent to the centromere (or kinetochore), the chromosome's organ of movement and orientation on the spindle. In the development of these nuclei the euchromatic regions replicate, but the heterochromatic regions around the centromeres do not. However, there is evidence for differential replication even within the heterochromatic regions: the nucleolar organizer is located in the heterochromatic region of the X-chromosome. Salivary-gland nuclei do have nucleoli. Thus, even within the heterochromatin, the nucleolar organizer region must have replicated.

There are replications of DNA in which the DNA in specific regions is increased, e.g., in the giant chromosomes of *Rhynchosciara* and *Sciara,* in which compacted chromosomal bands are extended into giant puffs in which there

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is a disproportionate synthesis of DNA.[7] In these insect salivary-gland nuclei there are no organized nucleoli; possibly the puff DNA serves the same function as the nucleoli of other cells in the synthesis of ribosomal RNA. Schultz[8] calls for a comparison of this DNA with that of nuclei in the malpighian tubules of the same insects, tissues in which the nucleolar organizer is functioning. In the oocytes of some amphibians there is no single large nucleolus; rather, there are over one thousand nucleoli in *Triturus,* and from 600 to 1200 in *Xenopus.* These nucleolar bodies do contain DNA,[16,17] which presumably functions in the synthesis of ribosomal RNA.[18,19]

The evidence for differential change in information content is clear; however, it is frequently overlooked. As I observed on an earlier occasion, the evidence for differential release of information has captured the interest of all of us because it fits so readily into the messenger hypothesis. The classic examples—the lampbrush chromosomes and puffs in giant polytene chromosomes—have been reviewed so often that it would be redundant to tell the story here.[7,8,16,20,21] In contrast to *Rhynchosciara* and *Sciara,* in which there is an exceptional synthesis of DNA in puffs, there appears to be no exceptional DNA synthesis related to puffing in either *Chironomus* or *Drosophila.*

Also widely known is the evidence bearing on the complementary questions: are there compactions of specific regions of chromosomes and are they signs of inactivity or restriction in the release of information? An example of the stability of chromosome differentiation is provided by studies of the production of the enzyme glucose-6-phosphate dehydrogenase in human cells. Studying cell cultures of heterozygotes for the sex-linked locus controlling that enzyme, Beautler, Yeh, and Fairbanks[22] and DeMars and Nance[23] found that cultures were mosaics: cells were phenotypically of one character or the other. This example emphasizes the correlation between compaction of regions of the chromosome and synthetic activity. The more compact the chromosome, the less its activity in synthesis.[24] Thus, before considering possible control mechanisms, let us examine the relations between replicative and transcriptive functions.

DNA and RNA synthesis during oogenesis and early development

In oogenesis and early development, these functions tend to be compartmentalized in time. Brown[25] has emphasized that ribosomal RNA (rRNA), 4S RNA (which presumably includes transfer RNA), and DNA-like RNA (dRNA) are synthesized at rates characteristic of the developmental stage of the embryo. Although to date no studies have unequivocally demonstrated protein synthesis within the oocyte,[26] a major event in amphibian oogenesis is the synthesis of rRNA. In fact, the unfertilized egg is endowed with an excess of ribosomes. In *Xenopus,* these ribosomes are conserved and function during early development, interacting with the progressively increasing amounts of dRNA and 4S RNA that are synthesized. New ribosomes are not formed in significant numbers (i.e., there is no net increase) in *Xenopus* until hatching. Moreover, anucleolate embryos develop normally until the tail-bud stage. These facts add weight to the argument that all of the protein synthesis in early embryogenesis is accomplished on ribosomes formed during oogenesis and stored for later use. Thus, 4S and dRNA synthesis following cleavage, during gastrulation and neurulation, and up to hatching proceed in the absence of rRNA synthesis.[27-30] During this time, the ribosomes are seen to aggregate increasingly and it is suggested that new ribosomal synthesis is initiated only after all, or most, ribosomes have been aggregated or "fixed" into polyribosomes by increasing amounts of dRNA.[25]

Briggs and Cassens[31] have studied the recessive gene (o) of the axolotl, which offers interesting possibilities for revealing direct gene products. This gene, discovered by Humphrey,[32] exerts a maternal effect, modifying the cytoplasm during oogenesis and leading to a cessation of development during gastrulation. The injection of normal cytoplasm improves development. Prior to maturation, the corrective component is concentrated in the nuclear sap, later being dispersed in the cytoplasm. Briggs and Cassens offer, as one possible interpretation, the idea that the component may be one of the RNA's that is produced and stored during oogenesis.

In summary, DNA is replicated during oogenesis. Much of this is highly specialized as nucleolar DNA, which functions in the synthesis of rRNA. Some 4S RNA and dRNA are formed. DNA replication is the principal synthetic activity during cleavage. Fertilization is interposed between maturation and cleavage. Despite the synthesis of dRNA and proteins immediately after fertilization in some animals—the sea urchin is an example—the synthesis of dRNA and 4S RNA is generally accomplished only after substantial DNA synthesis has occurred.

In oogenesis and early development, then, the transcriptive events, at least those in which bulk syntheses are involved, follow DNA replication.

DNA and RNA synthesis during cytodifferentiation

Many tissues go through a period of rapid cell division prior to specialization. Students of the development of cartilage, muscle, and the ocular lens all have stressed the

mutual exclusivity of DNA synthesis and differentiation.[33-35] As a burst of cell division ceases, cytodifferentiation and the formation of tissue-specific proteins begin. It is during proliferation that actinomycin D inhibits the subsequent appearance of specialized products, as in the exocrine pancreas[36] and in blood-forming regions of the early chick embryo.[37] Brown and Gurdon[27] have, in fact, suggested that the transition from labile to stable messenger RNA, implied by the loss in sensitivity to actinomycin D as proliferation ceases, may involve or require a change in size of the RNA, high-molecular-weight polygenic RNA being reduced to mRNA of the size required to code for individual protein subunits. This change would occur only in cells at terminal stages of differentiation and might result in a special ribosome-messenger complex that would be protected from degradation.

Instead of emphasizing mutual exclusivity, however, I would ask whether new transcription may depend upon an immediately preceding replication. In short, is the concept of differential gene expression to be thought of as a "steady stream"—a continuous flow of genes being activated or inactivated; or does the concept at least include the possibility that development may proceed in part by "bursts" of gene activity? If the latter, development would encompass a replication of DNA (must it invariably be the whole genome?), a new transcription of a gene or a sequence of genes, followed by a series of differentiative steps at other levels: translation, the interaction of protein subunits, changes in molecular conformation, etc. Then, before a further transcription could occur, another replicative step would perhaps be required.

Two especially pertinent lines of evidence may help clarify the problem. One, the requirement for cell division preceding antibody formation is treated elsewhere in this volume by Nossal and Jerne. I have considered the second, the observation that neoplastic transformation requires induction of cellular DNA synthesis, in detail elsewhere.[2] My colleagues and I[2,38,39] have shown that embryonic skeletal myoblasts and fibroblasts are equally susceptible to Rous sarcoma virus (RSV); both undergo morphological transformation and produce infectious virus. A similar transformation has been observed in mass cultures of infected iris epithelium.[40,41] The results of our fluorescent antibody experiments[39] supplement the transformation and virus-production data by proving that myoblasts can synthesize viral antigen and by showing that infected cells can take part in myotube formation. That antigen is localized either directly on the surface or closely associated with it is in accord with earlier studies in vivo showing RSV antigens to be localized on or near the sarcolemma of chick wing muscle three days after infection.[42] Since myotubes form by fusion of myoblasts,[43,44] viral particles could infect the myotubes in two ways—by direct penetration of the myotube membrane, or by entering the myotube after fusion with an infected myoblast. The weight of evidence favors the second explanation.[2,38,39,45,46]

The development of muscle involves the fusion of myoblasts into multinucleate fibers in which there is no further division or DNA synthesis. This raises an interesting question: Can a cell which, in the course of its differentiation, has lost the ability to divide remain susceptible to infection by RSV? Neoplastic transformation by a DNA virus, polyoma, requires induction of cellular DNA synthesis.[47-51]

In studies with another oncogenic DNA virus, SV 40, two stages in the transformation process have been found: (1) fixation of the transformed state in the infected cell; and (2) the expression of the transformed state during subsequent cell divisions. Both events require cell growth (presumably cellular DNA synthesis). One cell generation appears to be sufficient for fixation of the transformed state, while several generations are required for its full expression.[52] Bader[53] has concluded, on the basis of experiments employing inhibitors of both the function and synthesis of DNA, that DNA synthesis is also required for transformation of RSV-infected chick embryo fibroblasts and for viral synthesis as well.

This evidence adds weight to the argument that myotubes may be infected by the incorporation of infected myoblasts. The possibility remains that mature myotubes may contain immature virus particles or virus-associated antigens derived by a more direct route.

Thus, although it is clear that multinucleated myotubes contain virus-associated antigens, the question of whether myoblasts and myotubes differ in their susceptibility to infection can only be resolved by experiments using isolated myotubes.

Factors regulating differentiation

INTRINSIC CONTROLS I have emphasized the necessity of keeping an open mind on whether most differentiated cells maintain a full genome. Although evidence is available for irreversible differentiation at the chromosomal level in some forms, there are also examples of differentiated cells whose ability to regenerate appears to demand the continued presence of a full genome, any part of which is capable of expression. We know next to nothing about the area between the extremes; however, the operon hypothesis for the regulation of gene action in micro-organisms is so attractive that most students of development believe that selective gene regulation underlies much, if not most, of differentiation.[54] It would be redun-

dant to state the operon hypothesis,[55-57] because the model has been reviewed in this context repeatedly.[6,26,58] There are, however, possible sites of effectors of differentiation. In the operon hypothesis, a repressor is bound to the operator locus and RNA synthesis on that operon is prevented. Assuming that the hypothesis is correct, and also assuming the correctness of the current speculation that the repressors are proteins, how are they bound to DNA? The nature of DNA-protein interactions remains obscure. It is clear that, compared to DNA, chromatin is a poor primer for RNA synthesis in vitro. When chromatin is used to prime RNA synthesis, the rate of synthesis is much lower and the base ratio of the synthesized RNA is different from that which occurs when DNA is used as primer. Several authors have postulated that only a restricted range of DNA sequences is available for transcription in chromatin. Recently, Paul and Gilmour[59] have concluded that (1) hybridizable RNA formed after chromatin priming in vitro has the same sequences as the RNA formed in vivo; and that (2) in higher organisms, there are some sequences of DNA in different organs that are common and readily available for the synthesis of non-organ-specific RNA. In addition, there are some restricted DNA sequences that are available for the synthesis of organ-specific products.

Because the majority of template-bound histones do not turn over in nondividing cells, it has been postulated that cellular differentiation involves a specific pattern of masking and unmasking of DNA during embryonic development. Histones are known to inhibit DNA replication and the synthesis of DNA-dependent RNA in vitro. However, the amounts and types of histones within animal cells are relatively constant, even in cells that vary widely in their tissue of origin, age, character, and physiologic state. It now appears unlikely that histones embody the level of specificity required to make them a mechanism capable of effecting the control of differential gene action in vivo.[38,60] Recent findings on histone-bound RNA in the chromatin of several systems suggest that the masking of DNA by histones may be mediated by RNA through a hydrogen-bonding mechanism.

Thus far, I have centered attention on gene control at the level of transcription. Davis[6] and Atwood[58] have discussed the evidence for regulation at translation in microorganisms. Elsewhere in this volume, Stent has extended his earlier hypothesis[61] of gene control at that level. The idea is appealing, especially because evidence on regulation in differentiating cells is negligible.

EXTRINSIC CONTROLS Although I have emphasized the cell's inner controls,[26,62,63] the differentiation of a given cell often depends upon extrinsic factors, especially the influences of effectors produced by neighboring cells.[64-66] However, we remain ignorant of the nature of these effectors and of their initial site of action. We do not know if they impinge directly upon the genetic and regulatory circuits or operate through intermediaries within the cell or at its surface.

It has been suggested repeatedly that differentiating cells may influence each other by exchange both of small molecules and of direct gene products.[67,68] Nevertheless, there have been no definitive experiments, no generally applicable and verifiable findings.[26,69,70] In attempting to single out the most convincing evidence for the role of an RNA as an intermediary, one turns to the interactions between macrophages and lymphocytes. Yet, since the subject was last reviewed in this context[26] the meaning of the evidence that RNA isolated from antigenically stimulated macrophages can evoke specific antibody formation in lymphocytes in vitro has been questioned.[71] We must now ask whether the RNA is truly informational or whether it combines with traces of antigen to form a "superantigen."

There is also evidence that the course of differentiation may be influenced by effectors acting at the cell's periphery. It now appears that collagen or materials associated with it may play a role in regulating many diverse differentiative events.[72,73] By empirical modification of the methods of Puck and his associates,[74] Konigsberg[75] was able to demonstrate the growth and histiotypic differentiation of single myoblasts isolated from embryonic chick skeletal muscle. The success of this method depended initially upon the use of a properly "conditioned" medium that had been in contact for a time with a nongrowing population of fibroblasts. It was then established[73] that collagen is present in such a conditioned medium; the use of a collagen substratum replaces the requirement for a conditioned medium. The implication of these results is that the development of muscle cells requires the presence of a product of an associated cell type—the fibroblast. The development of a number of cell types has been shown to be dependent upon the close proximity of connective tissue elements.

I have selected this example both because it illustrates the action of a macromolecule in permitting differentiation, and because it points up the possible advantages in clonal methods of analysis in studying neuronal differentiation and the interactions of neurons and glia.

Earlier studies have emphasized the difficulties in, and crucial importance of, studying the interactions of animal cells of known origin and constitution at precise times during the course of differentiation. Until recently, such investigations have been hampered by the lack of a suitable system in which to carry them out. What attributes should a system have in order to render it useful for this

kind of study? While our experimental approaches must ultimately be directed toward an explanation of processes operative in the intact organism, the primary event takes place at the level of the individual cell. The populations of cells under study should be (1) homogeneous with respect to cell type, and (2) capable of yielding single-cell suspensions by appropriate dissociation methods. Such cells should be able to grow in vitro—ideally in clonal culture—and undergo specific, easily detected, characteristic differentiative changes.

Other workers, encouraged by the successful cloning of skeletal muscle,[75] were able to extend this approach to other cell types of the chick embryo. To date, cardiac muscle,[76] retinal pigment cells,[77] and cartilage cells[78] have also been cloned and subcloned, showing that these differentiated phenotypes are stably inherited through many generations. Each of these cell types has clearly recognizable features that permit its ready identification in the differentiated state. In these systems, too, the cumbersome requirement for "conditioned" medium has been eliminated. High concentrations of chick embryo extract reduce both plating efficiency and the fraction of differentiated colonies. A very simple method was employed to separate the deleterious factors from those that promote growth and differentiation.[79] When the embryo extract was fractionated by gel filtration, it was found that the low-molecular-weight fraction promoted higher plating efficiencies and expression of differentiation in clones of cartilage and pigmented retina, whereas the high-molecular-weight fraction, while stimulating growth in low concentrations, inhibits phenotypic expression.

These pioneering clonal analyses should provide new approaches to the study of cell interactions during differentiation. They suggest an explanation for the apparent paradox of "dedifferentiation" in mass cell cultures and the proved heritability of the differentiated state. There is evidence[78] that these earlier observations could have been caused by the sensitivity of log-phase cells to crowded conditions and to the inhibitory factors present in embryo extract.

Retinal pigment cells have been grown as clones from single cells while retaining their pigmentation and epithelial morphology.[77] Pigmented cells have been subcloned four times (over 50 cell divisions) and have remained pigmented. Cartilage cells have retained their differentiated phenotype through at least 35 cell generations.[78] On the other hand, myogenesis seems to require a progenitor cell. Is the fusion of myoblasts to form multinucleate myotubes an irrevocable event? If so, through how many cell generations can descendants of a myoblast remain myoblasts? What is the life expectancy of such a cell line?

Virtually nothing is known about the mechanism through which the interaction of a molecule like collagen with an inductive interface may alter the course of differentiation. Yet the phenomenon is real. Grobstein[1] has remarked that in several inductive systems a "morphogenetic event," such as a change in shape in a cell collection, precedes the first recognizable differentiative changes within the cells. I have cited[2] the examples proviedd by the observation by Kocher-Becker, et al.,[80] that one of the first effects of implanting a highly purified mesodermal factor into the blastocoele of the early *Triturus* gastrula is spreading of endoderm over ectoderm, suggesting a change in cell affinities.

If the analysis of morphogenesis is confined to the level of the cell group, can it lead to meaningful distinctions? Perhaps an effector must activate a gene before morphogenesis can be expressed. Thus, a differentiative step would precede morphogenesis, and one should search for factors regulating, say, synthesis of a new surface protein. However, I would re-emphasize my earlier conclusion that if it can be shown that an effector acts *directly* in altering the conformation—the folding and aggregation of a specific protein—then it would be meaningful to accept a primary role of morphogenesis in effector systems.

Nerve growth factor

It is against this background that I would consider the nerve growth factor (NGF). Note, please, that I say "against this background" rather than "in this context." I have been discussing effectors of differentiation; despite recent suggestions to the contrary,[81] it is not clear that NGF controls differentiation. Its mechanism of action is obscure, making it difficult to categorize. It is a very real phenomenon, and one cannot help being impressed by the serendipity, ingenuity, and insight that have marked its discovery and characterization. The isolation and biological effects of the factor and its neurocytotoxic antiserum have been reviewed extensively,[69,81] notably in Levi-Montalcini's recent Harvey Lecture.[82]

It was initially discovered that the transplantation of a fragment of mouse sarcomas 37 or 180 into the chick embryo resulted in massive enlargement of sensory and sympathetic ganglia and invasion of the viscera of the embryo by a dense network of sympathetic fibers that even penetrated the blood vessels. These findings led to further experiments, which established that the effects were attributable to an agent released by the tumor into the circulation. Fragments of these tumors explanted near sensory or sympathetic ganglia in vitro elicited the outgrowth of a halo of nerve fibers, providing evidence for a direct effect of the agent and a convenient bioassay.

The effects of the tumor in vitro were then duplicated by a heat-labile, nondialyzable protein purified from sarcoma 180, and in the course of further purification, it was accidentally discovered that snake venom is a potent source of NGF. According to Levi-Montalcini, NGF is present in venoms of all poisonous snakes and in that of Gila monsters. Snake venom is also a protein, with a molecular weight of about 20,000. It is destroyed by acid (0.1 N) but stable to alkali (0.1 N) and to 6 N urea. NGF derived from snake venom produces the same effects as the factor derived from the sarcomas, whether tested in the intact chick embryo or on ganglia in vitro. Again, the activity of the snake-venom factor is destroyed upon incubation with proteolytic enzymes, and is inhibited by specific antisera produced by rabbits in reaction to it.

Snake venom is secreted by a modified salivary gland, so other salivary glands were then examined. In the male mouse, the most active NGF is formed in the submaxillary gland. This factor is active not only in vitro and in the chick embryo, but also when injected into newborn mice. It is similar, but not identical, to the factor isolated from snake venom. Its molecular weight is about 44,000, leading to the speculation that the factor from the mouse may be a dimer of a basic unit of about 20,000.

The principal effects of NGF in vivo may be summarized as follows. (1) In the chick embryo it evokes hyperplasia and hypertrophy in both sympathetic and sensory systems. However, the sensory effects are limited to cells innervating exteroceptive peripheral fields. Hyperinnervation of proprioceptive peripheral fields is not observed. (2) Neither in newborn nor adult mice does NGF elicit overgrowth of sensory ganglia. Only the sympathetic ganglia are affected. The response of the sympathetic ganglia varies considerably with the particular preparation of NGF used; however, the following generalizations appear to be warranted. During the first nine days after birth, NGF results in an increase in mitotic activity, and the total cell population in superior cervical ganglia in treated animals is two to three times as large as those in controls. At nine days, mitotic activity ceases in both treated and control animals; thereafter, NGF calls forth only an increase in the size of individual neurons.

At the biochemical level, NGF elicits an increase in acetate incorporation and an increase of uridine uptake into RNA, and stimulates the incorporation of amino acids. Actinomycin D blocks uridine uptake and only slightly affects amino acid incorporation; puromycin blocks amino acid uptake and has little effect on uridine incorporation. Insulin produces similar biochemical effects on both brain and spinal-cord explants but does not elicit nerve outgrowth. Levi-Montalcini and her associates believe the findings to date are "indicative of an effect at the genetic level." (See Note 82, p. 255)

Does NGF play a role in the normal growth of the sympathetic chain? The most compelling evidence is that the injection into newborn mice, rats, rabbits, and kittens of antisera produced by rabbits in reaction to NGF results in the near-total destruction of sympathetic cells without adverse effects on other tissues. The dissection of the sympathetic chain ganglia for months and even years after the treatment, shows that no regeneration occurs in these "immunosympathectomized" animals. Levi-Montalcini[82] argues that NGF can be inferred to be part of the basic growth-control mechanism of responsive nerve cells because it is normally present in these cells, and has proved to be essential for their survival. However, NGF appears to be present in nerve cells, as in serum, only in trace amounts, as contrasted with its concentration in the salivary gland and in granuloma tissue.

In the mouse, the salivary gland begins to accumulate NGF in its convoluted tubules at puberty, but there is no evidence to prove that the factor is produced there. Extirpation of the salivary glands produces no adverse effects on sympathetic nerve cells. Moreover, there is no evidence of whether antisera to NGF binds the factor in nerve cells or on their surface, or whether they exert their effect only by inhibiting NGF in the circulation and in the salivary gland. Injection of the antiserum into mice has been reported to result in degenerative changes in the salivary-gland tubules.

Epidermal growth factor

A second factor has been isolated from the submaxillary gland of the male mouse. As described by Cohen,[69,83] this epidermal growth factor (EGF), originally detected as a "contaminant" in NGF preparations, is a protein devoid of lysine and phenylalanine and with a minimal molecular weight of 15,000. Upon injection into the newborn mouse or rat, EGF elicits precocious eyelid separation and tooth eruption. These phenomena are secondary to enhancement of epidermal keratinization and an increase in thickness of the epidermis. EGF also stimulates epidermal proliferation and keratinization in vitro. Thus far, there is no evidence for a role of EGF in normal development.

Perspectives

Fluorescent antibodies have revealed the presence of NGF in the salivary gland. Thus far, comparable evidence has not been advanced for the localization of NGF in, or on the surface of, nerve cells or their axons. It is possible that the factor is not sufficiently concentrated to make possible its detection by fluorescent antibodies. In view of the

need to know if antibody does react with NGF in neurons, every effort should be made to apply more critical methods—for example, the combination of ferritin-labeled antibodies and electron microscopy. An important lead to the action mechanism of NGF might be provided by knowledge of the localization of the factor and its antibody viewed against what is known of the compartmentalization of function in the cell body and axon.

Other questions are raised by the compartmentalization of function in the neuron, e.g., the restriction of nuclear DNA and nuclear-derived RNA's to the cell body, and the virtual absence of the latter from the axon. We urgently need research in depth relating DNA structure to specific synthetic events, especially in clonally derived neurons. The possibilities for cloning nerve cells in association with glial products must not be overlooked. In particular, it is important to perfect techniques of mapping specific regions of the genome in neurons. The molecular hybridization techniques, especially the hybridization of DNA and RNA, should permit a "molecular mapping" of the genome.[84]

Although there is said to be no translocation of the nucleic acids of the cell body down the axon, mitochondria do participate in axonal flow. The replication and development of such cytoplasmic structures as mitochondria and chloroplasts, which contain DNA, appear to involve the reading of an autonomous code,[85,86] but as Lehninger has emphasized earlier in this volume, the manner in which this information is regulated, especially in relation to the circuitry that regulates chromosomal heredity, is completely obscure.

Information is needed from different cell types in diverse animal species before we can assess the general significance of mitochondrial DNA. We know that the bulk of the cytoplasmic DNA of both *Xenopus laevis* and *Rana pipiens* is mitochondrial. After confirming that the eggs of these species contain 300 to 500 times more DNA than their somatic cells, Dawid[87] demonstrated in an elegant study that the cytoplasmic DNA is high-molecular-weight, double-stranded material, complementary in sequence only to a small proportion of liver DNA of the same species. He has now presented direct evidence that the bulk of this egg DNA is mitochondrial.[88]

Dawid's earlier findings had excluded the well-known hypothesis that the cytoplasmic DNA might function as a reservoir of stored material for the rapid assembly of chromosomes during early development. It must now be asked if the patterns of mitochondrial and ribosomal synthesis during oogenesis and early development are similar. Is mitochondrial synthesis, like that of ribosomes, intense during oogenesis, only to cease until much later in development? Can mitochondrial replication be observed during cleavage and early development?

Do these self-perpetuating structures provide a key to the central problems of differentiation? An unequivocal answer is not yet possible; too many pieces of the puzzle are missing, especially those dealing with their control mechanisms. The role of autonomously replicating organelles may be to conserve patterns, rather than create new patterns during differentiation.[6] It does appear, however, that the bulk synthesis of mitochondria during oogenesis offers an especially attractive system in which to approach the following question: How many of the mitochondrial proteins (structural proteins and associated enzymes) have their origin in situ, and how many are produced elsewhere and assembled on the mitochondrion? Many of the egg constituents are known to have their origin outside the ovary,[26] and this may facilitate an examination of the question.

The highly specific interactions between neurons, and between neurons and peripheral tissues, described by Edds in the preceding chapter, emphasize that neurons must have mechanisms for regulating synthesis and function that depend on information coming from the environment. As Dulbecco[89] put it, the cell surface has the role of a "sensor" that receives information from the environment and presumably transmits it to a regulatory site.

How do specific modifications of a cell surface influence genetic and regulatory mechanisms? Again the phenomenon is real.[33,52,89-92] The precise relations between cell contact and position in the cell cycle[93,94] must be explained.

Summary

In some developing systems, intra- and extra-cellular controls that impinge on the nucleus must operate in regulating the content of DNA (differential loss or replication). In other systems, regulation occurs in the release of information. Emphasis on examples of the latter has resulted in the prevailing hypothesis of differential gene expression. In considering the nature and sites of action of effectors of differentiation, attention is called to the differentiation of a given cell, which often depends upon the influences of effectors produced by neighboring cells. Cloning techniques are useful for studying cell differentiation and cell interactions. Against this background, the nature and possible roles of the nerve growth factor, and more briefly, the epidermal growth factor, are described.

RNA in Brain Cells

HOLGER HYDÉN

THIS CHAPTER has four sections. The first concerns the biosynthesis of nucleic acids, mainly RNA, in parts of the mammalian brain and in isolated neurons and glial cells, in relation to age. Examples are also given to demonstrate that RNA is produced in brain cells as a result of sensory and motor stimulation, and that it has essentially the same characteristics as ribosomal RNA. Second, some micromethods of analysis are described briefly. Such methods are a *sine qua non* to obtain pertinent information on biosynthetic processes in defined, small areas in the brain, and in neurons as compared to neuroglia. Third, biochemical data are presented on the relationship between glia and neurons. These data show that the neuron and the glia immediately surrounding it constitute a metabolic and functional unit in the brain. Fourth, evidence is given for a possible transfer of RNA from glia to neurons.

The article will also serve as a background to my discussion of macromolecular changes during learning that appears later in this volume.

It is evident that today any discussion of biochemical and functional properties of brain cells must include the glial cells. The present view is that the astroglia may mediate ion transport and be part of the blood-brain barrier.[1-4] The oligodendroglia, on the other hand, constitute primarily auxiliary metabolic appendices to neurons. This last is not a new idea based on recent findings. In 1901, Holmgren[5] published a paper on the structural relationship between ganglion cells and satellite cells. Figure 1, an illustration from that paper, shows a spinal ganglion cell exposed to osmic acid fumes. Channel-like processes from the satellite cells can be seen invaginating the perikaryon. A gradient of small granules from the satellite cells is indicated. Holmgren concluded that this demonstrated how satellite cells supplied the nerve cells with nutritional material.

Merely as an example of idea flow, I would like to mention a book by Schleich that appeared in 1916: *Vom Schaltwerk der Gedanken.*[6] The central theme was that neuroglia constitute the controlling cells of the brain and that they perform their function by massaging the neurons, thereby altering their metabolism. In 1951, Pomerat[7] used those very words—"massaging of the neurons by the neuroglia"—to describe facilitation of the transport of material in the axons. This, however, was based on his careful observations of glial pulsations in cell cultures.

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DNA and proliferation of neurons

Altman deals more extensively with this problem in his chapter in this book. He used ^{3}H-labeled thymidine and autoradiography to indicate cell proliferation,[8-10] and found that such proliferation actually occurs in hippocampal neurons of adult and young cats and rats, although on a very limited scale.

The recent observations of Altman and Das[11] (see my other chapter in this volume) demonstrate that small neurons may develop from primordial cells remaining in the adult tissue, mainly in the hippocampal region.

It seems safe to state that, in general, adult neurons do not divide. By contrast, neuroglia do, at a low rate of proliferation. In damaged areas, as has been well known for a long time,[12] a lively glial proliferation takes place. There is no indication that polyploidy occurs in neurons of mammals, but glial nuclei may be polyploid.[13] It has also been known since the turn of the century that there is a loss of neurons during the life cycle, especially in the Purkinje cells of the cerebellum; this phenomenon has been accepted as an event in physiological aging.[14]

A few biochemical studies have been performed on the distribution and amount of DNA in the brain. The total in the rat brain was found to increase up to the age of 14 days, after which it stabilized.[15-18] With the exception of the cerebellum, the DNA determinations have not corroborated the observation of neuronal losses.[19] These studies have, however, not taken into account the compensatory role of the glial nuclei, or that the glia are by far the most numerous cells. In several regions, the neurons constitute only 5 to 10 per cent of the area in question. The rest are glia.

Biosynthesis of RNA in brain cells

The medium-sized and large neurons and the glia are rich in RNA. As a matter of fact, the neurons have no competitors among somatic cells as RNA producers. They contain 20 to 2000 micromicrograms of RNA per cell, i.e.,

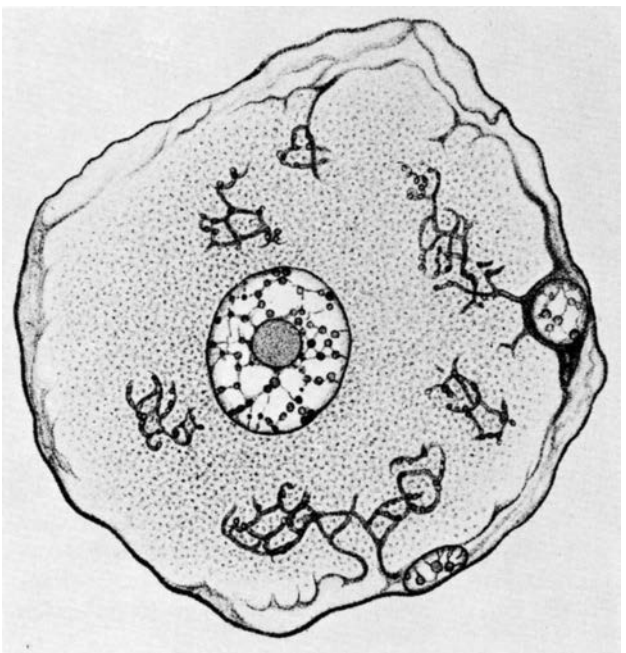

FIGURE 1 Section through spinal ganglion cell, calf, exposed to osmic acid fumes. Rays of granules from the satellite cells are invading the perikaryon-like channels. (From Holmgren, Note 5)

5 to 10 per cent of their dry weight. For the most part this is cytoplasmic RNA and has the characteristics of ribosomal RNA. The guanine and cytosine values are high. The nucleolar RNA of neurons has the same characteristics.

The nuclei of nerve cells are usually small in comparison with the bulk of the cytoplasm. Deiters' nerve cells from rats, for example, contain 30 $\mu\mu$g of RNA compared to the 650 $\mu\mu$g of cytoplasmic RNA. This nuclear RNA has the following base ratios: A 21.4, G 26.2, C 31.9, and U 20.5.[20] Presumably the nuclear ribosomes and the nucleolar RNA, which is of the ribosomal RNA type, dominate.

Very little RNA is present in the dendrites, and none has been found with certainty in the axons of mammalian nerves. Miani and his colleagues[21] have tried selectively to label axonal RNA of the hypoglossal nerve and have described the presence of RNA with sedimentation characteristics of ribosomal RNA. The possibility remains that this RNA originates from the myelin sheath or the Schwann cells, although the evidence points to its being axonal in origin. Glial RNA constitutes around 10 per cent of the neuronal RNA.[25,26]

Edström, et al., have found axonal RNA in small concentrations in Mauthner neurons of goldfish.[22,23] Despite the volume of this large neuron, it was found that the axon contains four times more RNA than does the cell body. The axon of the lobster sensory stretch receptor was also found to contain RNA.[24]

BIOSYNTHESIS OF NUCLEAR AND CYTOPLASMIC RNA IN BRAIN The first example is taken from a study in our institute.[27] Rabbits were given 150 microcuries of ^{3}H-labeled orotic acid through permanent cannulas inserted into the fourth ventricle, in pulses lasting from 15 to 180 minutes. At the end of this time, 0.2 to 0.4 gram of the vestibular area of the brain stem was removed and homogenized. The RNA was extracted and subjected to sucrose density gradient centrifugation, and the specific activity was determined as cycles per minute per microgram of RNA. RNA from *E. coli* was used as carrier. Figure 2 demonstrates that after a 15-minute pulse the *nuclear* RNA shows primarily low-molecular-labeled RNA up to 16S. After a 30-minute pulse, the sedimentation profile shows larger species—12S to 30S—of RNA molecules labeled. The specific activity had increased from 27 to 123 counts per minute per microgram of RNA. This shift toward larger, more heterogeneous species was found after a 60-minute pulse and the specific activity had increased to 335 counts per minute per micromicrogram of RNA. There is good reason to believe that the 8S to 12S labeled RNA is a messenger RNA. For this to be proved, however, template activity studies and hybridization with DNA are required.

The sedimentation study of *cytoplasmic* RNA showed the presence of 8S to 12S, labeled RNA only after a 30-minute pulse. Thus, the same type of highly labeled RNA as that in the sedimentation of nuclear RNA was found in the cytoplasmic RNA after a phase shift of 15 minutes. This agrees with other work, and is deduced from studies of different types of mammalian cells. It is taken to indicate a flow of nuclear RNA into the cytoplasm, although this is not yet completely established. These data agree with those of Jacob and his collaborators,[25,26] who found a high amount of radioactivity in the 10S to 45S of the nuclear RNA from rat brains 30 minutes after intracisternal injections of nucleosides.

Whole-brain analyses of RNA have been made in adult rats.[28,29] Also, 4S, 17S and 28S RNA fractions have been separated, and small but significant differences have been found in the base composition.[26,30]

Microchemical fractionation of nerve-cell RNA has shown that a considerable fraction consists of RNA with a low G + C/ A + U ratio (0.85)—a DNA-like base composition.[31] This may be a cytoplasmic messenger RNA. To look for structural correlates, isolated, fresh nerve cells were cut open by free-hand microsurgery and the cellular content was emptied on electron-microscope grids and photographed.[32] A striking finding was the

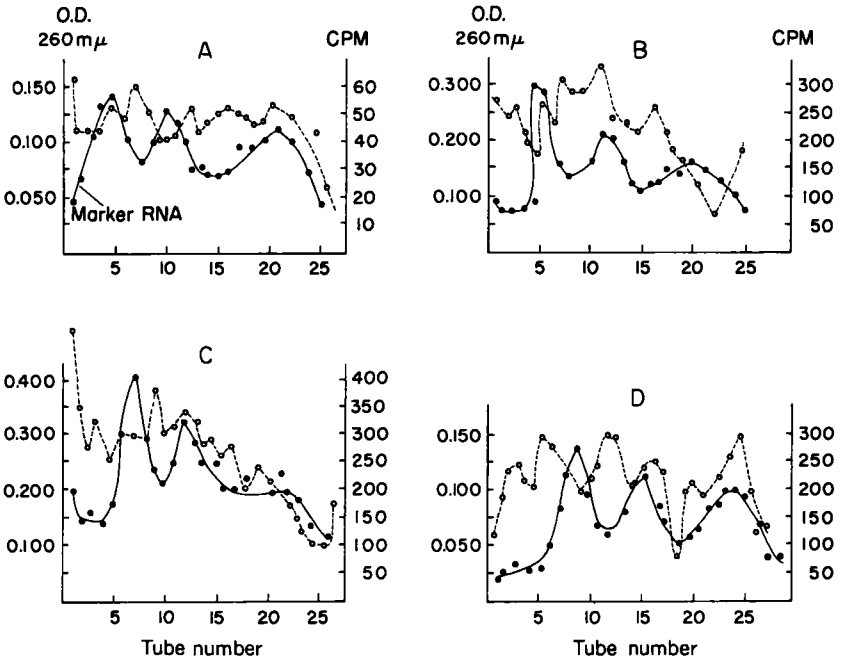

FIGURE 2 Sucrose gradient centrifugation of nuclear RNA from the vestibular area of the brain stem, rabbit. A. 15 min. pulse given through a cannulus inserted into the 4th ventricle. B: 30 min. pulse. C: 60 min. pulse. D: 180 min. pulse. (From Egyházi and Hydén, Note 27)

large polysomal structures, some of them containing more than a hundred ribosomes (Figure 3).

In the following section, it will be shown that a 30 per cent increase of RNA content can occur in neurons within one hour, with concomitant changes in guanine and cytosine values. One would like to assume that this is caused by an excessive biosynthesis of nuclear RNA flooding the cytoplasm, because there is no evidence so far that a mechanism exists for RNA synthesis in the cytoplasm. The possibility cannot, on the other hand, be ignored.

RNA changes in the brain with age

Let us proceed to the question of RNA changes in neurons during a life cycle in rats. As has been pointed out, the rat —the animal that has been used for such studies—is immature at birth, and the greatest changes in the chemical composition of the brain occur about two weeks after birth.[18] At this time the mature pattern of electrical activity appears.[33] The anaerobic part of the metabolism begins to decrease in relation to the aerobic part, and the animals begin to be more sensitive to anoxia.[34] At two weeks of age their eyes open, the thermoregulation becomes stabilized, and the motor activities are co-ordinated. The total amount of RNA increases sharply between the first and second week. In an adult rat that weighs 200 grams, the ribosomal RNA content of the cortex has decreased somewhat from that of the three-week-old animal.[19,35] All these data are interesting, but they apply only to the rat brain.

Adams[35] has shown a difference in the rate of RNA synthesis between newborn rats and adults. Incorporation of RNA precursors into nuclear RNA proceeds at a similar rate in both cases, but in the newborn animal there is little incorporation in the ribosomal RNA during the first hour; then it increases. In the adult, the incorporation proceeds linearly from the very beginning (Figure 4). Adams believes that it is more likely that the ribosomal RNA in the adult brain has become self-replicating than that precursors are flowing from the nucleus to the cytoplasm at a greatly increased rate. During the first half year, base ratio changes involving adenine and guanine also occur in rat-brain RNA.[36]

During the maturation period of the important first two weeks and of the following eight months, the rat brain differentiates morphologically and biochemically. A pertinent problem is whether gene areas successively become activated and available for transcription according to the program. Can key factors in the environment act as triggers, causing the system to react in new ways, also as a result of experience? In highly differentiated neurons and glia, as in other somatic cells that are highly differentiated, the percentage of active DNA can be expected to be low —around 10 to 20 per cent.

If experience can modify species of RNA in brain cells, it should be reflected in changes in the RNA composition of individual neurons. To test this possibility, Ringborg[37] in our laboratory chose the pyramidal nerve cells of rat hippocampus because of its great importance in learning. He used rats living with the other members of the litter.

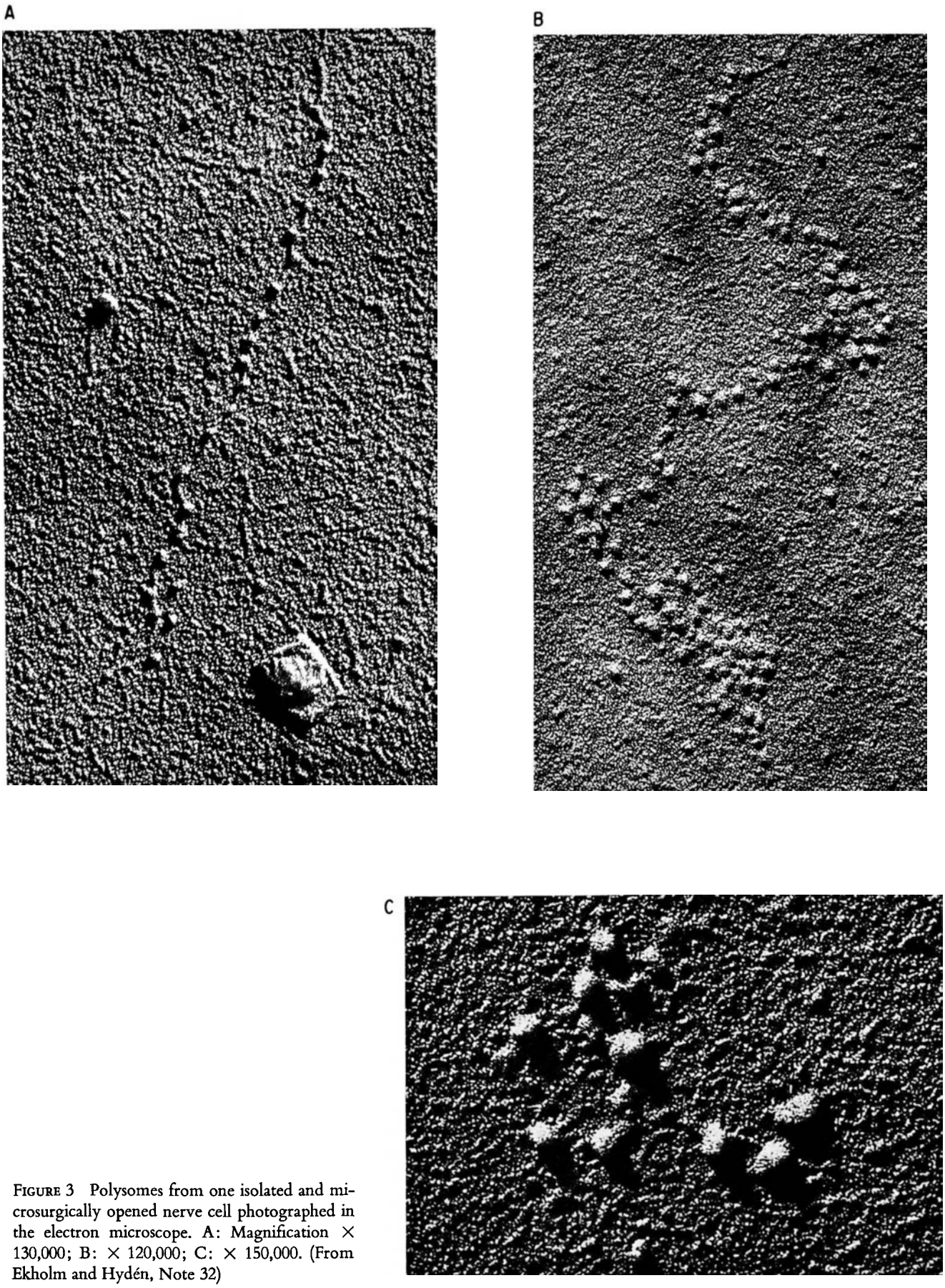

FIGURE 3 Polysomes from one isolated and microsurgically opened nerve cell photographed in the electron microscope. A: Magnification × 130,000; B: × 120,000; C: × 150,000. (From Ekholm and Hydén, Note 32)

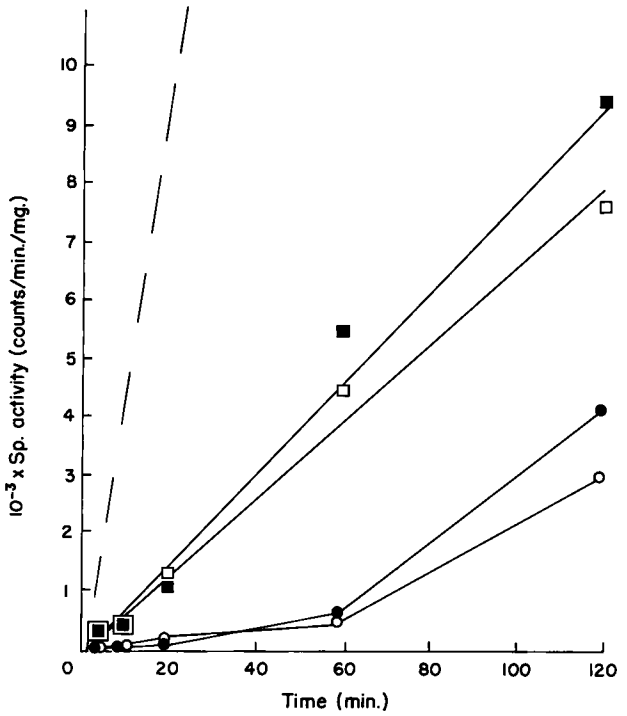

FIGURE 4 Incorporation of [^{14}C] orotic acid into the ribosomal and microsomal RNA of rat cerebral cortex over a 5 to 120 min. period. Incorporation into nuclear RNA is represented by the broken line. Adult rats: ■, ribosomal RNA; □, microsomal RNA. Four-day-old rats: ●, ribosomal RNA; ○, microsomal RNA. (From Adams, Note 35)

TABLE I

Content of RNA in single pyramidal cells of the hippocampal CA_3-zone at different ages

	pg RNA/cell	N
Foetal rats	19 ± 3	4
New-born rats	24 ± 1	4
Adult rats	110 ± 10	4
Old rats	53 ± 1	2

Results are mean values ± SEM.; N = number of animals. (From Ringborg, 1966)

TABLE II

Base composition of RNA from pyramidal cells of the hippocampal CA_3-zone at different ages

	Foetal rats	New born rats	Adult rats	Old rats
A	20.9 ± 0.8	21.0 ± 0.7	17.7 ± 0.8	14.9 ± 1.7
G	26.2 ± 0.5	27.6 ± 0.8	24.5 ± 2.0	29.0 ± 1.6
C	30.8 ± 1.2	31.0 ± 1.2	37.8 ± 2.0	36.9 ± 0.2
U	22.1 ± 0.5	20.8 ± 0.3	20.0 ± 0.5	19.3 ± 0.3
$\frac{G+C}{A+U}$	1.33 ± 0.05	1.41 ± 0.04	1.66 ± 0.04	1.95 ± 0.13
N	4	4	4	2

A = Adenine, G = Guanine, C = Cytosine, U = Uracil. The results are mean values ± SEM.; N=number of animals. (From Ringborg, 1966)

Table I shows that there is a steep increase in the amount of RNA in cells from birth to adult age. In old rats, the RNA content per cell has decreased significantly. Table II shows that during maturation a significant change in the RNA base ratios takes place.

Altman and Das[10] found that rats reared in an enriched environment had a larger number of glial cells in the cortex compared with rats in an impoverished milieu.

All these findings may reflect differentiation of the brain. Do they also reflect changes resulting from experience? This will require further studies involving, for example, analysis of brain cells from animals living in an impoverished or an enriched environment.

In the following section, several examples are presented of RNA production effected by physiological stimulation or chemical substances. It should not be forgotten, however, that a cyclic change occurs in the RNA content of neurons during the animal's lifetime, as was demonstrated in the hippocampal cells.

Table III gives some figures from a study of motor neurons in the spinal cord of man. The samples were taken

TABLE III

Total RNA content in motor nerve cells from homo (spinal cord, C_5, nucleus ventralis lateralis)

Age in years	RNA in μμg/cell
0–20	402 ± 28
21–40	553 ± 38
41–60	640 ± 55
61–80	504 ± 31
over 80	420 ± 30

from neurologically sound persons from 3 to more than 80 years of age who died in traffic accidents.[38] The RNA content increases up to the age of 40, when it reaches a certain average level that obtains for two decades. After age 60, the RNA content falls rapidly. This life-cycle change is superimposed upon the short-lasting, reversible fluctuations in RNA content that result from increased functional demands.

Some microchemical methods used for brain cell analysis

These results on brain cells have been obtained by both macrochemical and microchemical methods. It may seem strange that efforts have been made to develop and apply micromethods for the analysis of 10^{-10} grams of RNA and proteins, especially in brain cells. Why should not ordinary biochemical methods be successful, as they are in the liver? The answer, after 15 years of experience with such methods, seems simple—the brain is a highly complex structure. Micromethods are a prerequisite for the analysis of a small number of a defined category of neurons. For instance, it may be 500 neurons that, including their processes, constitute only 10 per cent of an area under study. Furthermore, neurons and glia can react with inverse biochemical changes, as will be demonstrated. An analysis of the whole region, even if it constitutes only half a milligram, will give average results; any differences will be leveled out. The straightforward method was to separate glia from neurons mechanically—not just any glia within the area, but those immediately surrounding the nerve cell bodies to be analyzed. It was necessary to determine their dry weight per sample for comparison with neurons. These neuronal glia are of special significance to their neurons as compared to those situated around capillaries, perhaps 200-μ away.

MICRODISSECTION As I have pointed out, the mechanical separation of neurons and glia has proved to be the preferred method if the purity of the sample is of importance. Nerve cells, including the basal part of the dendrites, comprising 30 to 100 microns, and defined samples of glia are removed from fresh brain sections. Free-hand dissection is preferred, using microtools made from stainless steel, and a stereomicroscope with a X 80 to X 160 magnification.[39] Figures 5, 6, and 7 give examples of neurons so isolated.

How vital are these isolated neurons? We inserted microelectrodes and found, under visual inspection, that they maintained membrane potentials between 40 and 70 millivolts. In a nitrogen atmosphere the potential dropped, but increased again to around 40 millivolts when an oxygen atmosphere was reintroduced.[40] One must therefore assume that the cell surface closed at the points where it was damaged during the isolation of the cell—for example, at the end of the broken dendrite processes. Furthermore, the isolated neurons phosphorylated.[41,42] A study of the endogenous respiration has found that when the oxygen

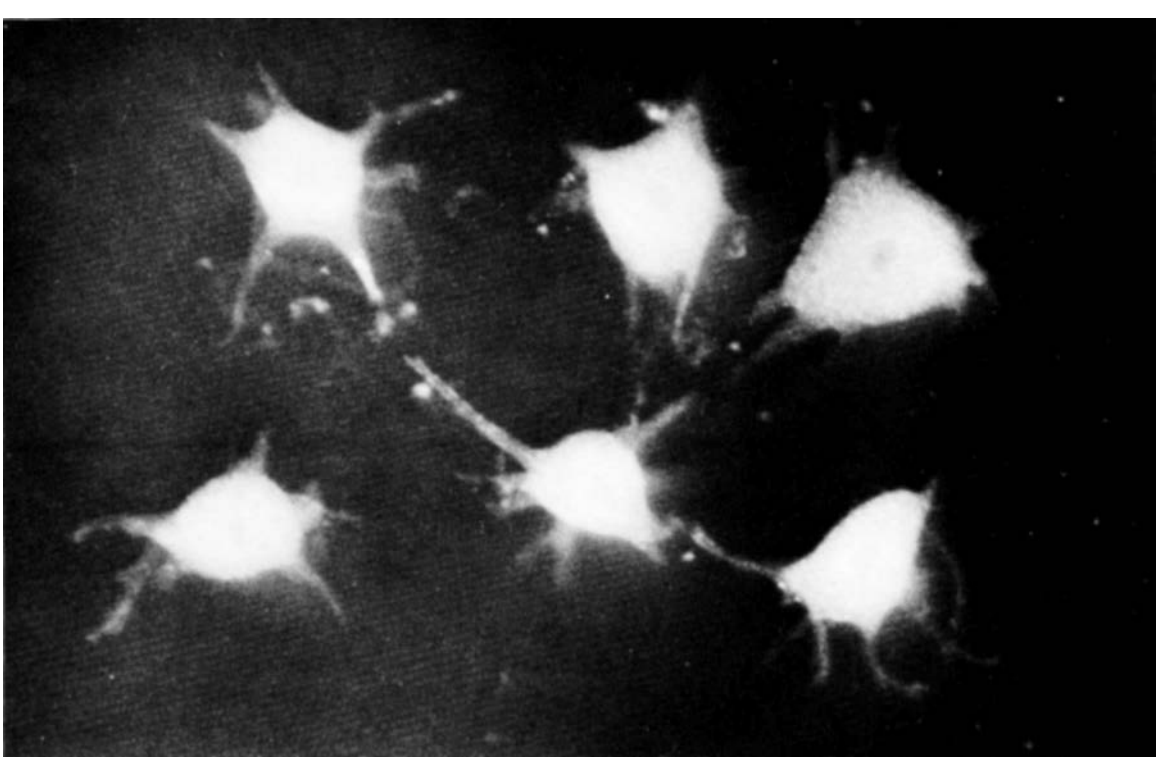

FIGURE 5 Isolated neurons from the lateral vestibular nucleus, rabbit, photographed in incident light. Magnification × 250.

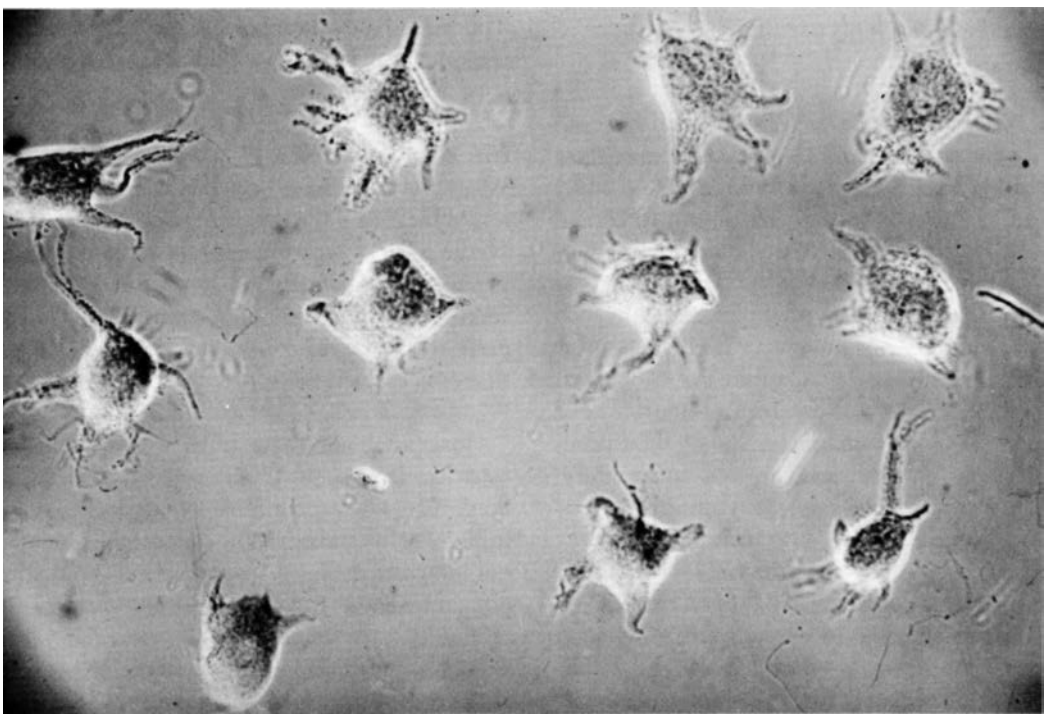

FIGURE 6 Isolated neurons from the lateral vestibular nucleus, rabbit, photographed in the phase-contrast microscope. Magnification × 250.

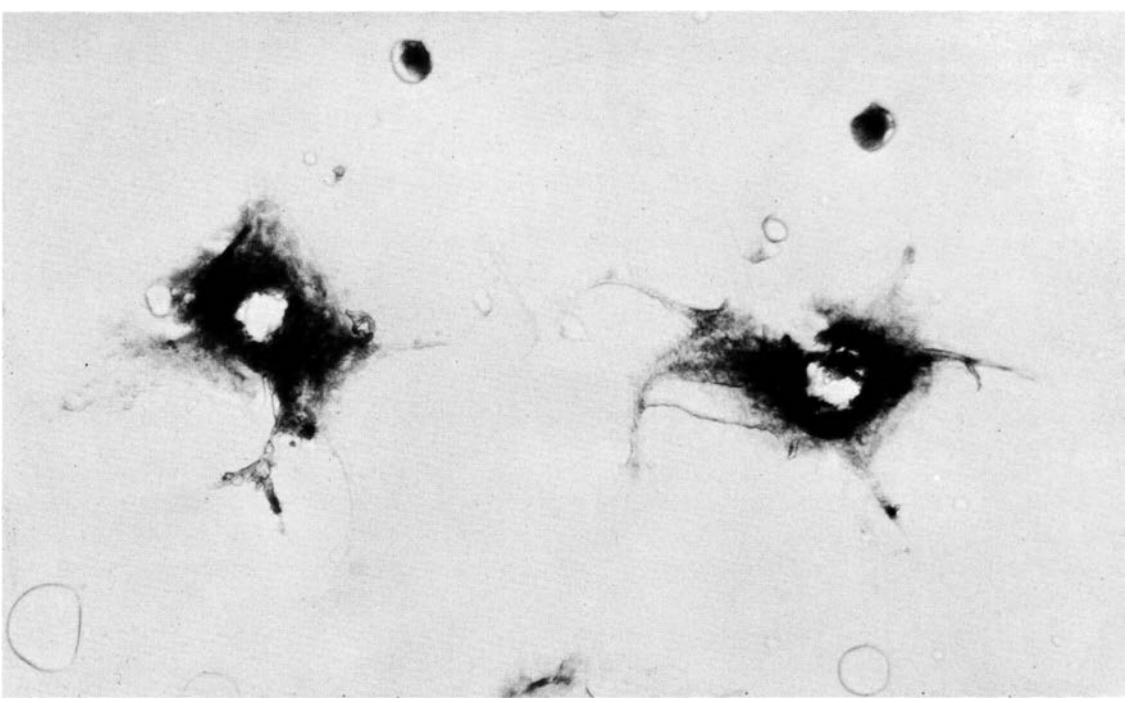

FIGURE 7 Two isolated nerve cells from which the nuclei have been removed, precipitated with cold perchloric acid. Above the nerve cells are seen two nuclei. Photographed at 2570 Å.

consumption of the isolated neuron had decreased to zero levels after 180 minutes in a medium without substrate, and glucose was added, the oxygen consumption rose to high values.[43] Isolated neurons have also been found to grow processes in cultivation.[44] Fresh, isolated neurons are therefore satisfactory as material for biochemical analyses.

We dissected precipitated, embedded, and sectioned brain material with a de Fonbrune micromanipulator under a phase-contrast microscope. In some cases only nerve cell nuclei were used for RNA analysis (Figure 7). For each analysis, 25 to 30 nuclei were isolated by microdissection. Briefly, the technique is as follows. The isolated nerve cells are placed on a glass slide and treated with cold phenol-saturated water for 15 minutes, by cold absolute ethanol for 10 minutes, and are then covered by paraffin oil. This treatment causes the nuclei to contract slightly (which is hardly noticeable at a magnification of X 600). The nucleus from each nerve cell can then easily be removed with the micromanipulator. Twenty-five nuclei were used for each RNA analysis. It was found that the cold phenol treatment precipitates all the RNA in nerve cells.

QUANTITATIVE AND QUALITATIVE RNA ANALYSIS The micromethods developed at this laboratory were used in both the quantitative and qualitative analysis of RNA. A detailed technical paper describing the microelectrophoretic procedure has recently been published.[23] For each analysis, 500 to 700 micromicrograms of RNA were used. The random error in the determination of the RNA in single nerve cells was found to be 4 per cent. The average variation coefficient of the microelectrophoresis of the analytical results was 5 per cent for nerve cell RNA and 7 per cent for yeast RNA. In one case it was possible to compare the result of the microelectrophoretic separation of hydrolyzed RNA from biological material with that obtained by conventional macrochemical electrophoretic separations. The analysis of the nucleolar and ribosomal RNA in mature starfish oöcytes gave the same results with both methods.[45] In model experiments on purified samples of RNA, the correspondence between macro- and microelectrophoresis is clear.[23]

The advantage of microelectrophoresis over macroelectrophoresis is that samples can be analyzed at the cellular level. This has proved to be of prime importance for nervous tissue, because its two cellular components, neurons and glia, differ in the amount and composition of RNA they contain.

The neuronal and glial RNA were determined on the same dry-weight basis as that determined by quantitative X-ray microspectrography[46] by using a scanning and computing densitometer.[47,48]

RNA METABOLISM At the cellular level, RNA metabolism has been determined by a new micromethod.[49,50] Total, labeled RNA extracted from nerve cells or glia, or the individual bases obtained on a microscopic cellulose strip after electrophoresis, are combusted in glass capillaries at 650° in a single step. The capillaries contain potassium perchlorate and zinc particles, and the organically bound tritium is transformed into hydrogen gas. The activity is determined in a modified Geiger-Müller tube.[49] This method combines the high efficiency in tritium detection (>70 per cent) and a low background (<5 cycles per minute) and permits an accurate assay of low levels of radioactivity. The RNA metabolism of nerve cells and glia has been compared by means of these micromethods after electrophoretic separation of bases.[50] The conversion relations between the RNA purine precursor pools and the pyrimidine precursor pools were the same in nerve cells and glia, although the labeled RNA in the two types of cells differed in composition. One example, taken from the paper of Brattgård and collaborators, is given in Table IV.[50] The synthesis of RNA was twice as rapid in the glia as in the nerve cells.

Egyházi[31] has devised an RNA fractionation method at the cellular level with special regard to isolated neurons and glia. Nerve cells are extracted with phenol at 3°, which leaves 50 per cent of the RNA. Subsequent extraction with phenol at 45° removes from the remaining RNA a fraction with high adenine and uracil values. Similar treatment of the glia gave a ribonuclease-resistant RNA with a G + C/A + U ratio of 0.77. This microfractionating method was combined with incubation of neurons and glia in low concentrations of ribonuclease.[27] The material was taken from animals that had received ^{3}H-orotic acid through cannulas permanently inserted into brain ventricles. A small RNA fraction was obtained with a very high specific activity—more than 10,000 cycles per minute per microgram of RNA. The base ratios were characterized by relatively high adenine and uracil values.

TABLE IV

RNA base composition of hypoglossal nerve cells and glia from rabbit (Mean values of molar proportions in per cent of the sum, ± SEM)

Base	Nerve cell	Glia	P
Adenine	21.1 ± 0.63	28.1 ± 1.30	0.001
Guanine	24.8 ± 0.60	23.5 ± 1.47	
Cytosine	31.9 ± 0.53	21.8 ± 1.15	0.001
Uracil	22.2 ± 0.53	26.6 ± 1.83	0.05

PROTEIN SEPARATION AND INCORPORATION STUDIES AT THE CELLULAR LEVEL Recently, a new micromethod has been devised for analysis of proteins in isolated brain cells.[51] A short description of the method might be of interest because a few results have been obtained in the ultimate task of characterizing the end products of the activity of the mechanism for macromolecular synthesis in brain cells.

Soluble proteins extracted from fresh, isolated neurons or glia are analyzed by disk electrophoresis on polyacrylamide gel in capillaries whose diameters are 200 to 300 microns.[51] The necessary manipulations are carried out freehand. The sample contains around 10^{-8} grams of protein. The cells are homogenized in a capillary with a twisted loop of steel wire 28 microns in diameter driven at 12,000 revolutions per minute. Separation current is 1.5 microamperes for 3 to 4 hours. The protein pattern is obtained by amido-black staining and is scanned with a microdensitometer. The biosynthesis of proteins is studied on material from animals that received ^{3}H-labeled amino acids. The individual bands in the 300-micron-diameter gel are cut out under a microscope, combusted in capillaries, and counted as described above.

For the localization of the acidic protein in the various types of brain cells, single diffusion agar precipitation was performed in glass capillaries 300 microns in diameter, using antiserum against the acidic protein. To localize intracellular details of this protein the multiple layer method of Coons was applied.[52] This technique requires cyrostat sections of the tissue, and the antigens are identified by fluorescence.

ACIDIC PROTEINS SPECIFIC FOR THE BRAIN Kinetic analysis of whole-brain protein has led to the conclusion that lifetimes of brain proteins vary from a few minutes to many months (for a review, see Lajtha[53]). Proteins with a low turnover may comprise only a small fraction of brain proteins. Most have an average half-life of 10 to 20 days.[54] Moore and his co-workers[55] have recently isolated and characterized an acidic protein, called S100 protein. This was found to be specific to nervous tissue and makes up 0.5 per cent of the brain's soluble proteins. Protein from a number of vertebrate species showed cross-reaction to antiserum for S100. This finding has given a new impetus to studies of brain proteins.

At this laboratory we have studied the protein by macro- and microelectrophoretic and immunological methods[56,57] with the future aim of correlating protein reactions with the extensive RNA synthetic processes that occur in brain cells. The protein band containing the S100 protein could be separated into more than three components. Of the three acidic proteins, two attained very high specific radioactivities within 30 minutes after ^{3}H-leucine injection. The specific activities for the two proteins—one of which consisted only of the S100 protein—declined rapidly between 6 and 12 hours (Figure 8). This suggests that the newly synthesized proteins are turned over rapidly. A third acidic protein belonging to the original S100 protein had a low specific activity.

Rubin and Stenzel[58] have reported that 15 per cent of the radioactive protein formed a by cell-free system from rabbit brain can be precipitated by anti-S100 serum. Their observations suggest that a considerable part of the protein-synthesis capacity of the brain is devoted to the synthesis of this protein. The observations at our laboratory imply that this large capacity is necessary because of the high turnover of the acidic brain proteins.

The detailed localization of the S100 protein was determined by single-diffusion agar precipitation using antiserum and antigen extracted from isolated neurons and glia, and by fluorescent antibody technique.[57] We found that S100 was localized mainly in the cell bodies and membranous system of oligodendrocytes, but was absent from their nuclei. By contrast, the protein was present in the nuclei of the big neurons, but not in the cytoplasm. A perti-

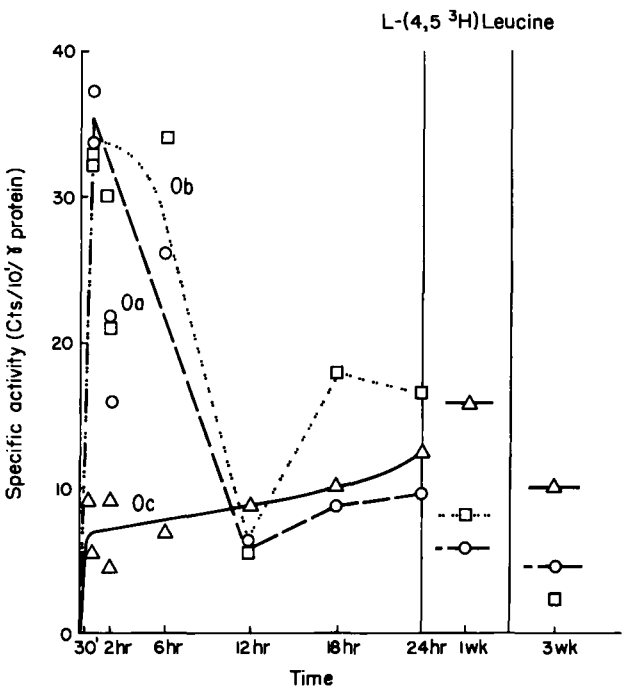

FIGURE 8 Specific radioactivity of bands Oa, Ob, and Oc separated on 11.2 per cent polyacrylamide gels as a function of time between isotope injection and sacrifice. Radioactivity was determined after combustion of slices of the polyacrylamide gels by liquid scintillation counting. Isotope: L-(4,5^{3}H) leucine. (From McEwen and Hydén, Note 56)

nent question is where this protein is synthesized—in the glia, in the neuron, or in both? Is it transferred after synthesis from one cell type to the other?

There is also a cell regulatory question to consider. Histones seem to regulate the activity of genes. Considering the acidic nature of the S100 proteins, perhaps they can react with the histones in the nuclei of the neurons and control gene activity. In other words, the acidic proteins in the brain might control the controllers.

RNA response of neurons to physiological stimulation

If physiological stimulation is used to sustain an increased neural activity, the amount of RNA per nerve cell increases. The first data leading to this conclusion were obtained by using ultraviolet microspectrographic measurements on sections through nerve cells.[59] Microchemical methods that allow the analysis of RNA extracted from isolated nerve cells have opened new possibilities for studies of the RNA response in defined categories of neurons. To judge by the base ratio composition and incorporation studies, *the RNA synthesized at increased physiological, motor, and sensory activity has the characteristics of ribosomal RNA.*

Inverse RNA changes in neurons and the surrounding glia have been observed from these types of stimuli. This finding, together with studies of protein and enzyme changes, have demonstrated the existence of a regulatory mechanism between the glia and the neuron that form a functional unit. This raised the question whether transfer of RNA occurs between glia and neurons.

By contrast, the RNA content increases in both neurons and glia during learning processes, and the RNA synthesized in both types of cells have highly specific base ratios with high adenine and uracil values, high uracil-to-cytosine ratio, or high adenine-to-uracil ratio. (See my later chapter in this volume.)

In a series of experiments Giacobini and his collaborators[60] have used crayfish stretch receptor neurons to study the correlation between metabolic events and the impulse activity. The cell body, part of the axon, and a muscle bundle with dendrites were isolated by dissection and placed in a microdiver for manometric determination of oxygen consumption. Respiratory inhibitors decreased both oxygen consumption and impulse activity. A marked effect of glycolytic inhibitors on impulse activity, and an increased level of glycolytic intermediates after impulse activity suggested that glycolysis played a major role in the mechanism that maintained the impulse activity in the crustacean neuron.

Below are some examples of the RNA response of neurons at increased neural activity. Other such examples are given in a 1960 review article.[61]

INCREASED MOTOR ACTIVITY The first example is taken from experiments with fish.[62] Because fish are cold-blooded their metabolic changes usually are slower than are those of mammals. It was therefore surprising that increased motor activity of fish was followed by extensive RNA changes in motor nerve cells. Sixty-centimeter barracudas from the Caribbean Sea were motorically exhausted by swimming for 20 to 30 minutes in an open sea-pool. The motor nerve cells in the upper part of the spinal cord were analyzed for the amount of RNA per cell. Figure 9 demonstrates that the RNA content had increased significantly after 30 minutes of activity and continued to increase for the 4.5 hours during which the animals were motorically inactive. A possible explanation for this phenomenon is that the biosynthetic mechanism in the neurons of the fish responds like a system with inertia.

INCREASED SENSORY ACTIVITY Recently, cold- and warm-water irrigation of the ear has been used for functional stimulation of the Purkinje nerve cells in certain

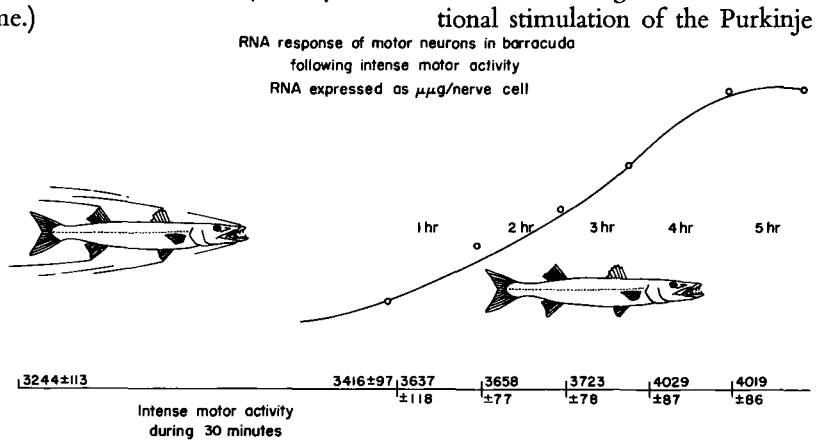

FIGURE 9 RNA response of motor neurons in barracuda at intense motor activity.

parts of the cerebellum.[63] In this way the RNA response could be used as a functional index for a topographic study of the cerebellar cortex.

When cold-water irrigation was used, the vermian and lateral part of lobulus centralis, contralateral to the irrigated ear, showed a significantly higher amount (30 per cent) of RNA per Purkinje cell than had the corresponding cells on the ipsilateral side (Figure 10). Warm-water irrigation gave higher RNA values for the Purkinje nerve cells on the ipsilateral side distributed as indicated in the figure. In these experiments no base ratio changes were found in the newly synthesized RNA.

These results have extended the knowledge about the extent to which changes in RNA content in defined nerve cells can be used as a functional index. Furthermore, they demonstrate how important it is to know where to look. The topographically localized RNA changes would certainly have been drowned in the bulk of RNA had the analyses been carried out on whole pieces of the cerebellar cortex.

To give an over-all stimulation of the vestibular nuclei, both horizontal and vertical rotation through 120° was used with a change in direction every 2 seconds. The so-called Deiters' nerve cells in the vestibular nucleus of the brain stem and nerve cells from the reticular formation increased their RNA content by 5 to 25 per cent (Table IV), but no significant change of the RNA base ratios could be observed. The composition remained typical for the cytoplasmic, high-polymer ribosomal RNA, which for Deiters' nerve cells in the rat is: A 20.5, G 33.7, C 27.4, and U 18.4. This type of cytoplasmic RNA has a slow turnover.[50]

INCREASED HORMONAL ACTIVITY The RNA content of supraoptic neuron in rats increases when stimulation increases production of antidiuretic hormone (Table V). For a month the animals were given an increased concentration of sodium chloride (1.5 per cent) in their drinking water. They showed increased diuresis but no weight loss. The supraoptic neurons showed an 80 per cent RNA increase.[64] Thirst for seven days also doubled the RNA content of the supraoptic neurons.[45] No base ratio changes could be found in the neurons with increased amounts of RNA.

It can be stated in summary that RNA newly formed in

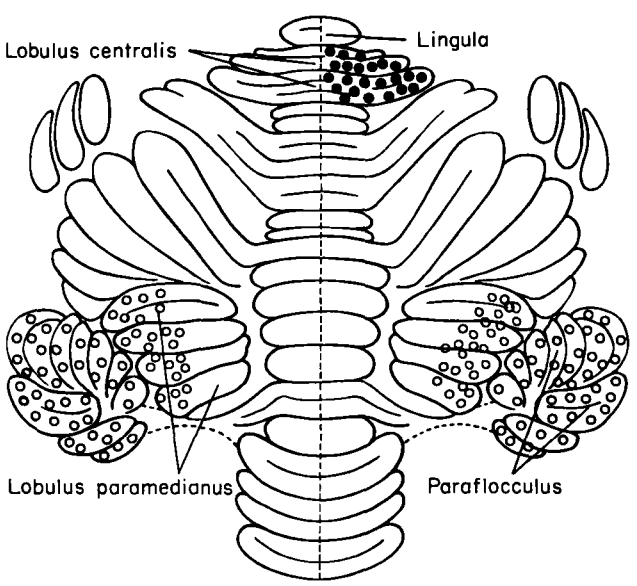

FIGURE 10A Rabbit cerebellum unfolded in one plane. Surface view. (Redrawn after Brodal, 1940)

● Areas with *unilaterally higher* Purkinje cell RNA content after 30 minutes of *cold* water irrigation in the left outer ear.

○ Areas with *bilaterally similar* Purkinje cell RNA content after cold water irrigation in the left outer ear.

In this figure and in 10B, RNA is expressed as micro-micrograms per nerve cell. (From Jarlstedt, Note 63)

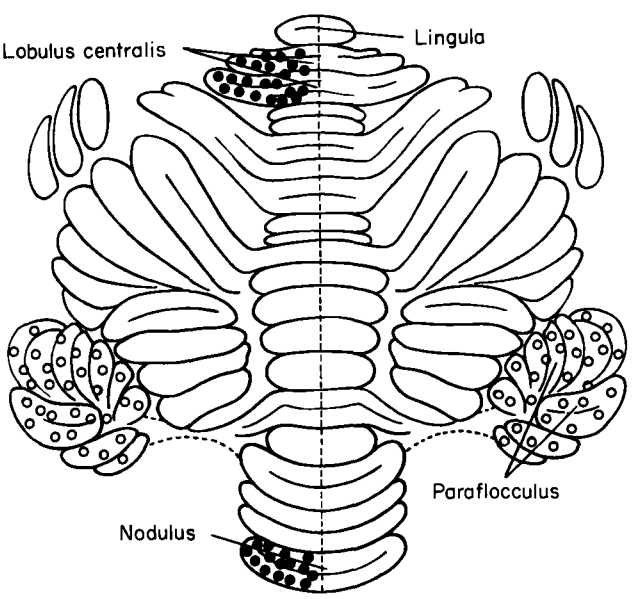

FIGURE 10B Rabbit cerebellum unfolded in one plane. Surface view. (Redrawn after Brodal, 1940)

● Areas with *unilaterally higher* Purkinje cell RNA content after 30 minutes of *warm* water irrigation in the left outer ear or after right-sided vestibular neurotomy.

○ Areas with *bilaterally similar* Purkinje cell RNA content after warm water irrigation in the left outer ear or after right-sided vestibular neurotomy. (From Jarlstedt, Note 63)

TABLE V

RNA response of neurons to increased sensory stimulation

Stimulus	Cell type	RNA increase in μμg per cell	%	P
Intermitt.[1] horizont.rot. 25 min/day, 7 days	Deiters' neurons rabbit	1550 ⟶ 1750	10	0.01
Intermitt.[2] horizont.rot. 25 min/day, 7 days	Deiters' neurons rat	680 ⟶ 750	10	0.02
Intermitt.[2] vertical r[illegible] 30 mi[illegible]	Deiters' neurons rat	680 ⟶ 850	25	0.001
Sodium chloride[3] 1.5% 30 days	neurons of N.supraopticus rat	68 ⟶ 121	80	0.01
Thirst[4] for 7 days	neurons of N.supraopticus rat	52 ⟶ 129		0.001

1. Hydén-Pigon, 1960[81]
2. Hydén-Egyházi, 1963[104]
3. Edström-Eichner, 1960[64]
4. Edström-Eichner-Schor, 1961[105]

nerve cells as a result of physiological stimulation has the same base composition as the original RNA and is of the ribosomal RNA type.

The neuron–glia unit

As I have pointed out, the neuron and its immediate surrounding of glia—mainly oligodendroglia—constitute a biochemical and functional unit. This is reflected in, among other phenomena, inverse biochemical changes between glia and neuron at changed functional equilibrium.

Data are accumulating[4,65] on differences in structural and biochemical properties between neurons and the glia surrounding them. The main part of glial cells consists of thin, convoluted membranes and processes enveloping the neuronal structures except where the synaptic structures adhere. The glial dry material has a lipid-to-protein ratio of around 80-to-20. By contrast, the corresponding value for the large neurons is 20-to-80.[61] The RNA base composition of glia differs significantly from that of their neuron[66,67] and shows inverse guanine and cytosine values. The carbonic anhydrase activity is more than 100 times higher in glia than in neurons, which has led Giacobini[68,69] to assume that CO_2 is released from the neurons and hydrated to carbonic acid in the presence of carbonic anhydrase. The enzyme systems for hydrolysis of adenosine triphosphate differ in the two types of cells. On the inner side of the neuronal membrane is an adenosine triphosphatase activity stimulated by Na^+ and K^+ with a maximum of activity at *p*H 7.4 (see Whittam, this volume). There is no such activity on the outside of the neuronal membrane. The adenosine triphosphatase activity of the glia increases from adjacent capillaries toward the neuron, is stimulated by K^+ alone, and is maximal at *p*H 8[41]. From data obtained at various laboratories it can be concluded that the oxygen consumption is five to ten times higher in neurons than in glia, while the capacity for anaerobic glycolysis is higher in glia, which also show a dominance of glucose shunt metabolism. Furthermore, oligodendroglia have more RNA and proteins, and some studies show that they exhibit higher respiration and activity of enzymes than do astrocytes.[70-73]

Friede, using histochemical staining methods, has found an inverse relationship between oxidative enzyme activities of axons and their fascicular glia. This suggests that glial enzymatic activity is dependent on a symbiosis with axons. In this connection, Friede has made the interesting observation that the longer the axon, the larger the number of satellite cells surrounding the neuron.[74]

Working with isolated samples of glia, Hamberger[75] found that the capillary glia showed higher succinate, α-ketoglutarate and glutamate oxidation per volume and hour than did the neuronal glia, which have a preponderance of oligodendrocytes.

In a recent review, Galambos[65] discussed various aspects of the glia, including electrical properties and glial response in myelination processes. The reader is referred also to the article by W. R. Adey in this volume.

Svaetichin and collaborators[76-79] have used the fish retina as a model system and have made intracellular recordings to study the glial–neuron interrelationship. In experiments with light influx they found reciprocal electrical excitation-inhibition between the glial and neuronal elements in the retina. The amplitude of the neuronal potential was inversely related to the height of the glial membrane potential. The membranes of these non-neural cells were electrically inexcitable, and membrane activity depended on excitation spread from cell to cell. The membrane potential of the satellite cells was electrically inexcitable and was reduced by NH_3 and increased by CO_2. The opposite phenomenon was obtained from the neurons of the retina. Adey and collaborators[80] found a fall in resistance and rise in capacitance in, for example, the hippocampus, if animals breathed CO_2. Svaetichin considers CO_2 to be a regulatory substance influencing the respiratory activity of the neuron and itself being regulated by the carbon anhydrase

in the glia, which thus should act as modulators of the neuronal activity.

INVERSE NEURON–GLIA CHANGES AT SENSORY STIMULATION The first example is the result of a series of studies on the amount of RNA, total proteins, and enzyme activities in the vestibular neurons of the brain stem through intermittent rotatory stimulation.[75,81-83]

Figure 11 shows that the amount of RNA, proteins, and respiratory enzyme activities increase in the neurons as a function of stimulation. In the neuronal glia, the changes were inverse with RNA and respiratory enzyme activities. By contrast, the anaerobic glycolytic activity, measured as production of CO_2 per nerve cell or glia sample, decreased in the neurons and increased in the glia. This may reflect a Pasteur effect in the neuron and a Crabtree effect in the glia.

The enzyme activities were expressed on a dry-weight basis. The results indicate, therefore, an increased enzyme production and a greater activity of the electron-transporting system in the neuron. Whether this depends on an activation reaction or on release from inhibition is a problem for future studies. Hamberger,[75] using the micromanometric technique, has found similar inverse enzyme changes in neurons and glia after stimulation. Pevzner used electrical stimulation and found that the content of RNA increased in the neuron and decreased in the glia.[84]

A kinetic study[85] permitted a discussion of the neuron–glia relationship from the point of view of energy relationships. After six days of intermittent stimulation (for 25 minutes per day), the reaction rate of respiratory enzyme activities of the neurons was twice that of the glia. This shows that the electron-transporting system of the neuron has the greater capacity. At medium hypoxia, the glia increased their capacity for anaerobic glycolysis, but did not increase their respiratory enzyme activities.[83] On the other hand, the neurons did. Thus it seems that with increasing functional demands the neuron has priority over the glia in its high-energy requirements. Furthermore, the rate of enzyme reaction in the glia had no relation to the duration of the stimulation.

A study of the endogenous respiration of isolated neurons and glia showed that the oxygen consumption rate of the Deiters' neuron exceeded that of the glia by at least ten times. The neuron contained five to ten times more glucose, corresponding to a 70-millimolar glucose solution, than did the same dry weight of glia.[70]

The interpretation of this data is that there exists *an energetic coupling between the neuron and its glia* in which the neuron is the dominant part in energy utilization.

Not all the glia in a specific area respond with inverse biochemical changes in relation to the neuron. So far, that

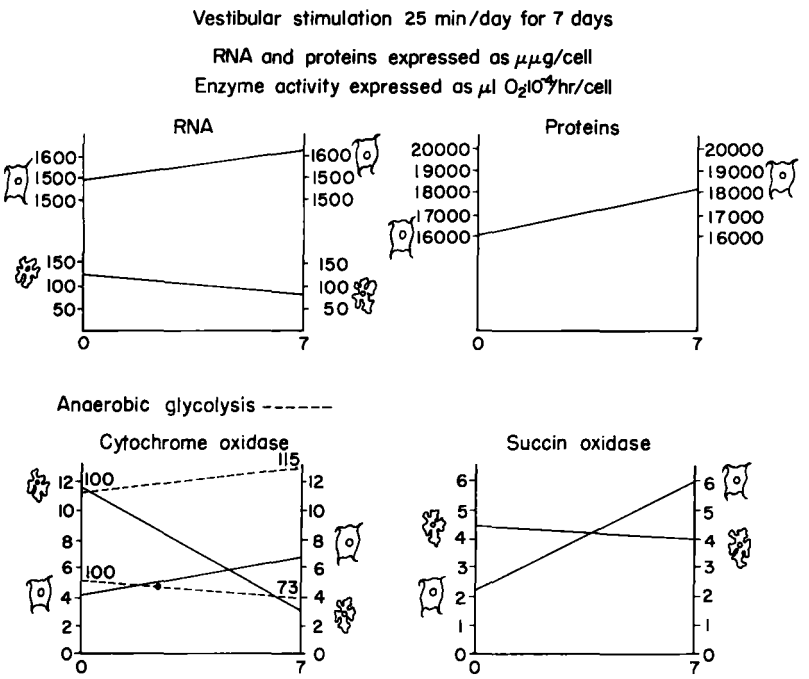

FIGURE 11 Survey picture of quantitative macro-molecular changes in vestibular nerve cells following stimulation.

has been observed only in the neuronal glia. The capillary glia, unlike the neuronal glia, did not respond to vestibular stimulation with a decrease in enzyme activities.[75]

INVERSE NEURON–GLIA CHANGES DURING SLEEP The advantages of using microdissection of neurons and glia and microchemical methods is clearly demonstrated in two studies on sleep. Inverse rhythmic enzyme activity changes occur at the cellular level in a small, defined area of the brain stem.[86,87] Rabbits were taught to sleep for five hours per day in tailor-made boxes, and EEG curves were recorded for control. On the twelfth day the animals were killed during sleep and the succinoxidase activity of neurons and glia were determined as 10^{-4} microliters of dioxide per sample and hour. During sleep, the enzyme activity was high in the neurons and low in the glia in the caudal part of the reticular formation (nucleus reticularis gigantocellularis). During wakefulness this situation was reversed (Table VI). In the oral part of the reticular formation, a short distance from the caudal part, changes occurred only in the neurons. No rhythmic, inverse changes took place in the cells from the control areas, one of which was situated very close to the caudal reticular formation. There were no inverse enzyme changes in neurons and glia during barbiturate sleep. The analytical values were, over-all, depressed.[88]

The findings during physiological sleep were taken to mean that the neuron–glia units of the caudal part of the reticular formation reflect, through metabolic changes, the biological clock behind the sleep rhythm. In oscillatory systems there is usually a phase shift responsible for the rhythmic phenomena. Whether this involves rates of reactions or transfer of molecules is a problem for future research.

TABLE VI

Succinoxidase activity of neurons and glia isolated from rabbits. The results are expressed as 10^{-4} µl of oxygen per sample per hour
Group 1, sleep; group 2, wakefulness; group 3, cage controls.

	Nerve cells		Glia	
Group	Activity (10^{-4} µl O_2)	No. of analyses	Activity (10^{-4} µl O_2)	No. of analyses
	Nucleus reticularis giganto-cellularis			
1	3.41 ± 0.51	29	2.34 ± 0.18	25
2	1.30 ± 0.25	24	3.06 ± 0.24	28
3	2.74 ± 0.21	39	2.16 ± 0.18	33
	Nucleus reticularis pontis oralis			
1	6.38 ± 0.58	35	3.50 ± 0.30	19
2	4.01 ± 0.52	15	2.94 ± 0.21	17
3	5.41 ± 0.39	32	3.72 ± 0.58	12
	Nucleus trigeminus mesencephalicus			
1	2.68 ± 0.29	9	1.46 ± 0.23	8
2	3.08 ± 0.83	5	1.11 ± 0.12	5
3	3.21 ± 1.16	5	1.15 ± 0.15	13
	Nucleus hypoglossus			
1	1.03 ± 0.17	13		
2	0.83 ± 0.08	13		
3	0.68 ± 0.13	3		

When wakefulness was compared to sleep, the difference between the enzyme values in nucleus reticularis giganto-cellularis proved to be significant both for nerve cells ($p < .001$) and glia ($p < .02$). Values for group 1 compared to those for group 3 were not significant. The difference between the values for the nerve cells in the nucleus reticularis pontis oralis of groups 1 and 2 was significant ($p < .01$).

INVERSE NEURON–GLIA CHANGES DURING NERVE REGENERATION If severed, a peripheral nerve can regenerate repeatedly, provided the damage to the axon does not occur too close to the perikaryon. Favorable mechanical conditions are also needed to allow the outgrowing axon to find the path given by the bridges of proliferating Schwann cells in the peripheral part of the original neurite. During the first week of regeneration in the hypoglossal nerve, the nerve cell bodies increase in volume through uptake of water and the concentration of RNA decreases, although the amount remains the same. The protein concentration decreases, but the amount of protein increases slightly (Figure 12).[89]

The incorporation of ^{3}H-adenine and ^{3}H-cytidine into the RNA and its composition of regenerating hypoglossal neurons and glia was studied by Brattgård, et al.[67] After one week's regeneration, the specific activity of RNA adenine had decreased by 30 per cent. On the other hand, there was a 30 per cent increase in the other three RNA bases. This indicates that an increased ribosomal RNA synthesis occurs early during nerve regeneration, but that there is a concomitant decrease in the synthesis of an adenine-rich RNA fraction.

Watson,[90-91] using labeled RNA precursors and autoradiography, found indication of an increased formation and breakdown of RNA in hypoglossal nerve cells during the first week of regeneration. Sjöstrand,[92] using the same type of cell material and a similar technique, found a hypertrophy of the astrocytes surrounding the regenerating nerve cells, beginning on the third day and showing a maximal reaction at the ninth day. He also found a hypertrophy of the microglia surrounding the capillaries in the area during the same period. From the second to the fourth day of regeneration the microglia proliferated markedly. Hamberger and Sjöstrand[93] measured and compared the

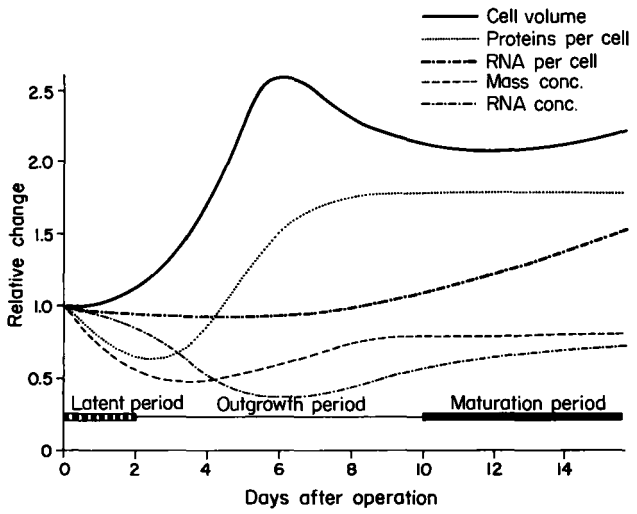

FIGURE 12 RNA and protein changes during nerve regeneration. (From Brattgård, et al., Note 89)

succinoxidase activity of regenerating hypoglossal nerve cells with that of the neuronal glia. The glial enzyme activity had increased more than three-fold on the sixth day of regeneration. After two weeks, when the neurons had a peak of enzyme activity, the glial succinoxidase activity had decreased.

Although regeneration presents a special case, this neuronal–glial response also constitutes an example of inverse changes of the neuron–glia unit. Future studies may determine if the rapid glial response supports the nerve cell metabolism before the neuron has adjusted to changed equilibrium.

INVERSE NEURON–GLIA CHANGES AT CHEMICAL INDUCTION OF RNA SYNTHESIS Within one hour after tricyano-amino propene (triap) has been administered to animals at 20 milligrams per kilogram of weight, there is an increase in the amount of RNA per nerve cell, the respiratory enzyme activities, and the total dry weight, including proteins. The base ratios of the total nerve cell RNA changes significantly.[94] The quantitative increase was 570 micromicrograms of RNA in addition to the original amount of 1550 micromicrograms per nerve cell. Table VII shows that the effect of the chemical agent significantly changed the guanine and cytosine values of the total RNA per neuron.

A more informative insight into the changes in the RNA composition is obtained if the base ratio alterations are related to the change of the amount of RNA (subsequently called the "RNA-fraction" or "ΔRNA") and not to the final amount of RNA. For instance, if the RNA content increases and there is a simultaneous change of the base ratios (as in the case above, after injection of tricyano-amino propene), the chemical change of RNA is described as a synthesis of a newly formed amount of RNA (ΔRNA) with a different base ratio composition than the original RNA. In the same way, a decrease in the amount of RNA, with a simultaneous change in base ratio composition, can be considered as a loss of an RNA fraction (ΔRNA); the composition of the ΔRNA removed differs from that of the original RNA.

Table VII shows that the increase of the guanine and decrease of the cytosine value is characteristic for the final RNA. This is more accentuated if the changes are computed on the newly formed ΔRNA, as described above. The ratios G + C/A + U in both cases (final RNA and ΔRNA) do not differ from 1.7 in the control nerve cell; they are 1.6 and 1.4, respectively. This means that the newly synthesized RNA is of the ribosomal type.

As was pointed out above, the amount of RNA in the nuclei of these large nerve cells is only a fraction of the RNA total—55 μμg of 1550 μμg, or 3.5 per cent. Considering, however, the importance of the nucleus in cellular protein synthesis, the nuclear RNA composition was analyzed in control nerve cells and in the cells after induction of RNA synthesis by triap. We did this by microdissection; the nucleus was removed from each cell and the RNA collected from 25 nuclei was pooled for each electrophoretic analysis.

An average of 16 per cent of RNA had been lost from the nucleus. When this loss was computed it was found to

TABLE VII

Microelectrophoretic analyses of the composition of the RNA in Deiters' nerve cells from rabbits

Treated with 20 mg/kg of tricyano-amino propene and killed 1 hour later. Purine and pyrimidine bases in molar proportions in percentages of the sum. Total amount of RNA increased significantly from 1550 (±78) μμg/cell to 2120 (±106) μμg/cell.

	Controls	Tri-a-p	Δ RNA fraction
Adenine	19.7 ± 0.37	20.5 ± 0.31	22.5 ± 1.61
Guanine	33.5 ± 0.39	34.6 ± 0.28	37.7 ± 1.66
Cytosine	28.8 ± 0.36	26.7 ± 0.24	21.0 ± 2.03
Uracil	18.0 ± 0.18	18.2 ± 0.20	18.8 ± 0.91
$(G + C)/(A + U)$	1.65 ± 0.023	1.58 ± 0.018	1.42 ± 0.090
A/U	1.09 ± 0.021	1.13 ± 0.021	1.20 ± 0.103
No. of animals:	5	11	
No. of nerve cells:	490	1200	

From Egyházi-Hydén, Note 94

have an unusually high amount of cytosine and uracil. The ratio G + C/A + U is 0.73 compared to 1.34 for the control nuclear RNA and 1.52 for the total nuclear RNA of the triap nerve cells.

In using phenylcyclopropylamine, a powerful inhibitor of monoaminooxidase, at 0.3 milligram per kilogram of body weight, a 30 per cent increase of the neuronal RNA per neuron was obtained in one hour; glial RNA decreased by almost 50 per cent. Significant base ratio changes involving guanine and cytosine were also found. Moreover, the cytochrome oxidase activity increased in the neurons by 250 per cent and decreased in the glia by 30 per cent. We also gave actinomycin D to rabbits intravenously in doses of 0.3 mg three to seven hours before injection of phenylcyclopropylamine. We found that actinomycin both inhibits the RNA changes and produces a loss of RNA by 100 $\mu\mu$g per cell. The base composition of this lost RNA fraction was: A 25.7, G 43.3, C 23.0, and U 8.0. The conclusion drawn from these experiments was that the RNA production induced by the chemical is immediately DNA-dependent. Furthermore, it is interesting that a loss of RNA actually occurs through the influence of actinomycin, the base composition of which is highly asymmetric.

The cytoplasmic RNA newly synthesized after both physiological stimulation and induction by a chemical agent has the base ratios of a ribosomal RNA.

To summarize at this point, inverse RNA and enzyme activity changes have been found in neurons and glia as a result of various types of physiological stimuli and processes. The RNA content increased in the neuron and decreased in the glia, and electrophoretic separation and incorporation studies showed it had the characteristics of ribosomal RNA. The kinetics of the enzyme reactions show an energetic coupling between the neuron and its glia. The neuron is the dominating part with respect to energy utilization in this unit. The interpretation of such changes is that the neuron and its glia form a biochemical and functional unit in the nervous tissue.

The time sequence and pattern of neuron–glia changes in a pathological case

When considering the evident morphological and biochemical relationship between neurons and glia, one can suspect a functional relationship. Such a relationship could be studied in Parkinson's disease.

In biopsy material from the globus pallidus of patients suffering from Parkinson's disease, the large nerve cells and the surrounding glia were analyzed for RNA.[95] At a very early stage of the disease, a highly aberrant RNA was found in the glia. The adenine value was greatly increased and those of guanine and uracil were decreased, and the changes persisted. The neuronal RNA was less changed. During the development of the disease, from the time the overt clinical symptoms emerged and progressed, similar but not identical changes were also characteristic for the neuronal RNA. Thus, to judge from the time sequence, the biochemical error first develops in the glia, which then seem to influence their neuron both biochemically and functionally. The amount of RNA in nerve cell and glial samples was significantly increased, averaging 145 micromicrograms of RNA per nerve cell (control 116) and 34 micromicrograms of RNA per glial sample (control 17). These striking base ratio changes and quantitative RNA changes were found in the biopsies whether the patient had been ill for one or twenty years. Due regard was paid to the time relationship between clinical symptoms and biochemical changes in the neurons and glia in the two sides of the same brain. These analyses indicated that a biochemical error arises in the glia at a very early stage of Parkinson's disease and involves the synthesis of polynucleotides. RNA changes in the nerve cell were probably secondary to their nature.

Let us discuss the characteristics of the RNA changes in the glia during the course of the disease. In Table VIII, the observed base ratios of the controls and the biopsies are seen in the second and third columns. The change of the base ratios are calculated on the increased amount of RNA (ΔRNA, column 4). Clearly an RNA of a highly asymmetric type has been formed in the glia. Especially striking is the unusually high increase in adenine. The ratio G + C/A + U is 0.79 compared with 1.69 for the control glia.

TABLE VIII

Microelectrophoretic analyses of the composition of the glial RNA in globus pallidus of six cases of Parkinson's disease

Purine and pyrimidine bases in molar proportions in percentages of the sum. The amount of RNA increased from 17 ($\pm$ 1.7) $\mu\mu$g/glial sample (controls) to 24 ($\pm$ 2.2) $\mu\mu$g/glial sample (PARKINSON).

	Controls	Parkinson	Δ RNA fraction
Adenine	19.0 $\pm$ 0.78	30.8 $\pm$ 0.70	42.4 $\pm$ 3.19
Guanine	29.1 $\pm$ 0.15	20.3 $\pm$ 0.90	11.5 $\pm$ 2.75
Cytosine	33.7 $\pm$ 0.72	33.2 $\pm$ 1.70	32.8 $\pm$ 3.45
Uracil	18.2 $\pm$ 0.36	15.7 $\pm$ 0.60	13.3 $\pm$ 1.25
$(G + C)/(A + U)$	1.69 $\pm$ 0.044	1.15 $\pm$ 0.047	0.79 $\pm$ 0.093
A/U	1.05 $\pm$ 0.048	1.96 $\pm$ 0.087	3.18 $\pm$ 0.384
No. of analyses:	23	32	
No. of cells:	4600	4800	

From Gomirato-Hydén, Note 95

The A/U ratio is 3.18 compared to 1.05 for the control of glial RNA.

In the neurons of globus pallidus, the RNA base ratio changes are less striking (Table IX). If the composition of the total RNA is considered, a significant increase of adenine and a decrease of guanine is apparent. If the change in composition is related to the increased amount of RNA (ΔRNA, column 4), analagous to the treatment of the glia data above, this neuronal RNA is characterized by a decreased G + C/A + U ratio and a high adenine value, as was also the case with the glial RNA. However, the neuronal changes are less conspicuous.

All data indicate that in Parkinson's disease the glia are the brain cell type in which the change in RNA formation begins and which have the most pronounced RNA changes. An explanation for this may be that the glia have less RNA content relative to that of the neuron. Therefore, a change in the nuclear RNA formation is more easily traced in the glia.

It may be that Parkinson's disease represents a type of disorder in which environmental factors (e.g., infections) at a crucial period of the life cycle initiate the release of undesirable genomic activities that lead to biochemical error in the glia. A more doubtful explanation, for which at present there is no evidence, is that ΔRNA-fraction reflects a virus infection.

RNA transfer between glia and their neuron

The results above led to the conclusion that during physiological and chemical stimulation RNA is formed both in glia and in neurons with the same base ratios as those constituting the main part of the cytoplasmic RNA already present in the cell and having characteristics of ribosomal RNA. In two types of learning experiments, on the other hand, the RNA synthesized during the course of learning was of an asymmetric, adenine-rich type, probably a chromosomal RNA, to judge by the base ratio composition. (See my later chapter, this volume.)

This finding leads to the problem of the relationship between the neuron and its surrounding glia at a molecular level. That the morphological relationship between them is most intimate is well established. Glial membranes can even invaginate the nerve cell cytoplasm.[94] Furthermore, all parts of the neuronal surface not covered by synapses are covered by glial membranes. It has already been pointed out that, from such biochemical observations as those of enzyme activities,[75,82,83,85] the conclusion has been reached that the neuron and its glia are coupled energetically and that they form a functional unit of the central nervous system.

Following physiological and chemical stimulation, the

TABLE IX

Microelectrophoretic analyses of the composition of the RNA in nerve cells in globus pallidus of six cases of Parkinson's disease

Purine and pyrimidine bases in molar proportions in percentages of the sum. The amount of RNA increased from 116 (± 5.3) μμg/nerve cell (controls) to 145 (± 6.3) μμg/nerve cell (PARKINSON).

	Controls	Parkinson	Δ RNA fraction
Adenine	18.3 ± 0.42	20.7 ± 0.65	30.3 ± 5.00
Guanine	30.5 ± 0.44	28.8 ± 0.25	22.2 ± 3.53
Cytosine	35.3 ± 0.60	34.4 ± 0.79	30.8 ± 5.02
Uracil	15.9 ± 0.36	16.1 ± 0.38	16.7 ± 2.16
$(G + C)/(A + U)$	1.92 ± 0.038	1.72 ± 0.042	1.13 ± 0.185
A/U	1.15 ± 0.037	1.29 ± 0.051	1.82 ± 0.380
No. of analyses:	31	39	
No. of cells:	310	300	

From Gomirato-Hydén, Note 95

RNA and protein content and the respiratory enzyme activities were found to increase significantly in the neuron, but to decrease in the glia. The study of the kinetics of enzyme reactions demonstrated that the neuron is the demanding and dominating partner of the functional unit.[85] At increased function, it rapidly doubled the rate of oxygen consumption. The glia did not change in this respect, but resorted partly to anaerobic glycolysis to cover their energy demands. Based on these measurements, it was not difficult to hypothesize the existence of a mechanism that transfers macromolecules between the glia and the neuron. Speculations on a transfer of RNA between neurons or between glia and neurons have been published recently.[97-99]

The discussion of such a mechanism is based on the data obtained from induced RNA synthesis. We have chosen the results of studies on vestibular nerve and glial cells after triap was administered to rabbits.[20,94] The animals were killed one hour after the intravenous injection of 20 milligrams of triap per kilogram of body weight. Quantitatively, an increase of 570 μμg of RNA was found to the existing 1500 μμg per nerve cell, and a decrease of 55 μμg per glial sample from the control value of 123 μμg. These are dramatic changes and, from a quantitative point of view, are sufficiently large to serve as a basis for discussion.

As I have pointed out the synthesized ΔRNA fraction was ribosomal in character. In Table X the data from both the glia and the neurons are listed, including the calculated composition of the corresponding ΔRNA fractions.

First, the ΔRNA fractions of the neurons and glia are of the same ribosomal type.

TABLE X

Microelectrophoretic analyses of the composition of the RNA in nerve cells and in glia of the Deiters' nucleus from rabbits

Treated with 20 mg/kg of tricyano-amino propene and killed 1 hour later. Purine and pyrimidine bases in molar proportions in percentages of the sum. Total amount of nerve cell RNA increased significantly from 1550 (± 78) μμg/cell to 2120 (± 106) μμg/cell. Total amount of glial RNA decreased significantly from 123 (± 6.2) μμg/sample to 68 (± 3.4) μμg/sample.

	Neuron		Glia		Neuron	Glia
	Control	Tri-a-p	Control	Tri-a-p	Δ RNA fraction	Δ RNA fraction
Adenine	19.7 ± 0.37	20.5 ± 0.31	20.8 ± 0.28	20.1 ± 0.74	22.5 ± 1.61	21.6 ± 1.11
Guanine	33.5 ± 0.39	34.6 ± 0.28	28.8 ± 0.64	21.9 ± 2.15	37.7 ± 1.66	37.8 ± 3.19
Cytosine	28.8 ± 0.36	26.7 ± 0.24	31.8 ± 0.27	38.6 ± 2.40	21.0 ± 20.3	23.0 ± 3.33
Uracil	18.0 ± 0.18	18.2 ± 0.20	18.6 ± 0.55	19.4 ± 0.65	18.8 ± 0.91	17.6 ± 1.49
$(G + C)/(A + U)$	1.65 ± 0.023	1.58 ± 0.018	1.54 ± 0.030	1.53 ± 0.090	1.42 ± 0.090	1.55 ± 0.138
A/U	1.09 ± 0.021	1.13 ± 0.021	1.12 ± 0.058	1.04 ± 0.051	1.20 ± 0.103	1.23 ± 0.122
No. of animals:	18	20	9	12		
No. of nerve cells:	720	800				
No. of glia samples:			50	62		

(From Hydén and Lange)

Second, the amount of glial RNA is about one-tenth of that per nerve cell, determined on the basis of the same volume and dry weight. The amount of ΔRNA fraction determined in the glia is also about one-tenth that of the neuronal ΔRNA fraction. The volume relationship of glia–neurons within the lateral vestibular nucleus is not known with certainty, but the following may be said. The large Deiters' cells used in our studies are situated some hundred microns apart. We are concerned with the so-called neuronal glia that enclose the perikaryon of each neuron. Using figures the size of territories of individual nerve cells as calculated by Sholl[100] and Schadé, et al.,[101] a volume of 10^6 cubic microns for the neuronal glia related to each Deiters' nerve cell seems reasonable. Based on this figure, and with the volume of each nerve cell about 10^5 cubic microns, the volume ratio of nerve cell to glia would be 1 to 10. It is not unreasonable, therefore, to assume that the loss of 55 × 10 μμg of glial RNA corresponds to the determined increase of 570 μμg of RNA in each neuron (Figure 13). These experimentally found values could reflect a transfer of RNA from glia to neurons.

Third, a support for this assumption is supplied by the calculated base composition of the glial and neuronal ΔRNA fractions (Table X). The values are identical. However, experiments using labeled RNA, which at the present time cannot be carried out, will be decisive in proving RNA transfer from glia to neurons.

It is possible that the mechanism of RNA transfer between glia and neurons, which expresses itself in inverse RNA changes, is linked to and regulated by the level of neural function. In learning, on the other hand, the establishment of the new functional response releases genomic activities. These express themselves in their primary RNA

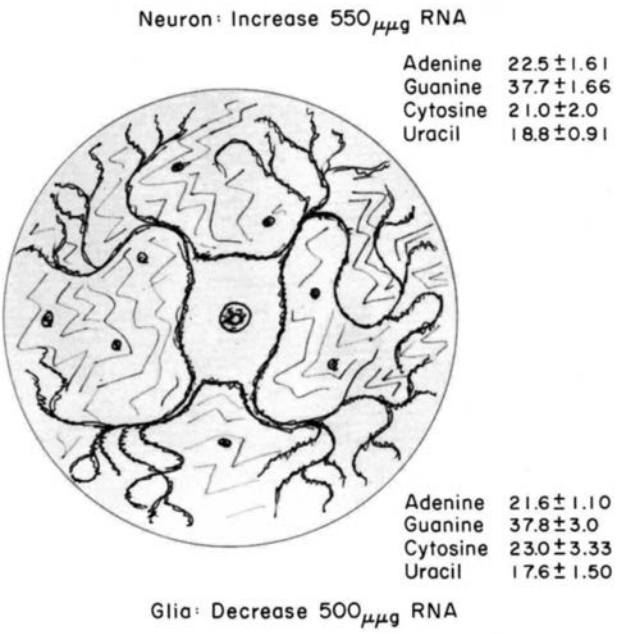

FIGURE 13 Schematic representation of concomitant changes in the glial and neuronal compartment at induced RNA changes, indicating transfer of RNA from glia to neuron. (Modified after Hydén and Langè, Note 70)

products, which are formed in both neurons and glia, and consequently the RNA content will increase in both.

A concluding remark may be made at this point with respect to the difference in response of neurons and glia in learning and during stimulation. The RNA content increased in both glia and neurons during learning (see my later chapter, this volume) in contradistinction to the response during stimulation, in which case they were inverse.

Summary

During the last ten years, there has been an increasing interest in properties and biochemical response of glial cells. Based on data presented in this article, it seems possible to express some definite view about the relationship between glia and neurons and some of the functional aspects of the glia. On one hand, the viewpoints represent the subjective view of the author; on the other hand, they may hopefully serve for planning and discussion of future experiments.

First, the biochemical response of neurons and the surrounding glia at changed functional equilibrium shows that these intimately connected cells form a functional unit within the nervous system. They represent a kind of collaboration between two cell types, metabolically and functionally. The rate of biosynthesis of the two parts can swing between two levels. This constitutes a stable system from a cybernetic point of view. The glia seem to be metabolic stabilizers of the sensitive neurons.

Second, the loss of a glial RNA fraction with a certain base composition as a result of stimulation, and a concomitant increase in their neuron of an RNA fraction with a similar base composition and the same order of magnitude, indicates a transport of RNA from glia to neuron. This would mean that glia program the synthesis of macromolecules in neurons.

The assumption of a transport of RNA from glia to neurons is supported by the presence of nonribosomal-bound RNA in the axoplasm of Mauthner nerve cells.[22] The base ratios of the axonal RNA agreed with that of the RNA in the myelin sheath and the Schwann cells, but was not similar to the RNA of the nerve cell body. Another observation may be pertinent in this connection. In newts, the pattern and time sequence of injected, labeled histidine in nerves indicates a transport of the amino acid from the Schwann cell nuclei through the myelin sheath into the axon.[102] Such inverse neuron–glia changes, which involve enzyme activities and quantitative changes of an RNA with ribosomal RNA characteristics, are taken to be the characteristic response of a functional unit of the nervous system: the neuron–glia unit.

Third, the neuronal glia could act as a modulator of the electrical properties of the neuron. One possible mechanism would be for the glia to change the electrokinetic potential and alter the membrane properties of the neuron, as Elul[103] has suggested occurs at synapses.

Protein of Nervous Tissue: Specificity, Turnover, and Function

PETER F. DAVISON

THE MANNER in which the cells of the central nervous system interact to transduce transient electrical activity into integrated motor responses, modify reactions as a result of experience, and manifest higher nervous functions, such as prognostication, is a problem that demands all the experimental resources available. One approach is through a study of the usual cellular effectors, the proteins. In the review of the proteins of nervous tissue that follows, certain topics are emphasized and others mentioned only briefly; for supplemental reading that provides a fuller coverage, the reader is referred to two recent publications reporting conferences on brain proteins.[1,2] Reference to these reviews is also made in the present text.

Brain tissue may be differentiated histologically into glia and neurons. The glia are of several types; some enwrap the neuronal axons and soma and in many instances give rise to the myelin sheaths; some interpose themselves between the neurons and the blood capillaries in a way that has suggested that they function as intermediaries in assimilating and transferring cellular metabolites; and others appear to serve the role of cellular scavengers similar to phagocytes. In many cases the neurons can be seen to possess distinct structural features that presumably correspond to distinct functions; the dendrites and soma apparently receive and integrate the incoming signals from external receptors and other neurons; the axon transmits the non-decremental action potential; and the synaptic regions chemically or electrically transduce the excitation to succeeding neurons or effector cells. Organized behavior and higher nervous functions arise, in some manner, from the conspiracy of these nets of neurons and glia. Clearly the search for structural correlates to specialized functions of neurons and glia could best be served by an analysis of the separated classes of cells or even, in the case of neurons, by analysis of the separated parts of the neurons. Unfortunately a partial resolution of neurons and the glial populations suitable for macroscopic analytical chemistry has only recently been achieved, and little analytical data has been published.[3-5] For the most part, analytical studies on brain and nervous tissue have used homogenates or slices of brain. It follows that the ascription of cellular components to their cells of origin has in most instances not been feasible. Resolution of neurons and glia has been achieved only by sensitive microtechniques, by histochemistry (e.g. Friede,[6]) or by the analysis of small samples of cells procured under microscopic dissection (e.g. Lowry,[7] Hydén[8]).

The subject of the enzyme distribution in the brain will not be discussed in this review, not only because of the author's incompetence, but also because the roles of the enzymes cannot yet be divided to separate those pertaining to normal cellular metabolism from those relating to the specific functions of the tissue.

Perhaps the most comprehensive program of analysis of total soluble proteins derived from human and, more recently, from animal brains is that of Bogoch and his collaborators. These analytical results have prompted the speculation that one way in which information may be coded into brain structure is through the addition of polysaccharide appendages to form families of glycoproteins from a common parent protein.[1] A similar hypothesis has been advanced by Dische.[2]

Several groups of investigators have studied proteins that comprise major fractions of brain extracts. Among these proteins may be mentioned the proteolipid studied by Folch-Pi and his associates.[1] This protein is normally strongly associated with lipid, but it may be isolated in a water-soluble form; it is identified as a constituent of myelin and other cellular membranes, and as such is not specific to brain. A second protein component of myelin, a basic protein, has been studied by Kies and her associates.[1] This protein, largely restricted to brain, upon injection into guinea pigs promotes an immune allergic encephalomyelitis, which results in a demyelination of the animal's nerves and eventual death. Bornstein and Crain[9] have studied in tissue culture the effects of immune sera that provoke a similar demyelinating reaction, but the chemical characterization of this immunogen is incomplete. It may be presumed that the proteolipid is in some way related to membrane structural protein[10] and that the basic protein must also be present in the cell membranes together with enzyme complexes, but a comparison of

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neuronal and glial membrane composition has yet to be made.

Heald[1] and Rodnight[2] have studied a phosphoprotein, again a protein present in brain but not specific to brain. They have suggested that this protein has a role in ion pumping and possibly in energy transduction, as runs of serine-phosphate residues have been detected.

Moore and McGregor[11] have surveyed a variety of mammalian tissues by electrophoretic and chromatographic techniques and have selected for further study a number of brain-specific proteins. The first of these, the so-called S-100 protein, has been shown by specific immunologic techniques to be a protein apparently little changed in evolution from amphibians to man.[12] Moore, by ultramicrocomplement fixation techniques (private communication), and Hydén,[1] by micro-Oudin techniques, have shown that the protein occurs primarily in glia, although McEwen and Hydén[13] have reported the presence of the protein in the nuclei of neurons. The appearance of this protein in embryologic development of the chick has been studied by Wenger.[1] Studies of the rapid metabolic turnover of this S-100 protein, or of the components into which it may be split by special electrophoretic techniques, have been reported[13] but the function of this protein remains unknown. Meanwhile, other brain-specific proteins isolated by Moore are under study. Independently of these experiments, Edelman and McClure[1] have initiated a search for brain-specific protein immunogens obtained from brain homogenates. Certain brain-specific proteins have been found, but their metabolic role remains unknown. A similar study of brain-specific antigens has been reported by Freidman and Wenger.[14]

Another protein specific to nervous tissue, and in this case detected in all neurons (with the possible exception of those in some of the lowest phyla), is the neurofilament. Numbers of these 100Å-diameter, threadlike structures appear to follow parallel paths uninterruptedly from the nerve soma to terminal synapses and on occasions they form a toroid in the synaptic boutons.[15] They have been isolated for physical and chemical study from the giant axons of squid.[16] In this animal the nerve grows to a diameter of approximately one millimeter, and several centimeters length of axon may be obtained. Such axons from the squid *Dosidicus gigas* may be cleaned of the greater part of the surrounding sheath of connective tissue, and axoplasm may be extruded to provide the investigator with a small quantity of pure axonal contents. Such preparations are highly viscous and contain some particulate material that may include mitochondria. Low-speed centrifugation of squid axoplasm has provided an opalescent solution for study. Both the low[17] and the high[16,18] molecular weight constituents of the axoplasm have been analyzed. The neurofilament protein has been of central interest. The protein contains 25 per cent mole residues of dicarboxylic acids and hence is an acidic protein. No nucleotide bases or lipids have been detected in it, but the 1 per cent phosphorus content raises the possibility that this is an energy-store similar to the protein, phosvitin. The neurofilament appears to be formed from the helical packing of a globular protein subunit which shows a pronounced tendency to aggregate.[16] The functional role of this protein structure in the neuron is unknown. In this regard it may be significant that in a certain number of pathological conditions a hyperplasia of neurofilaments has been found.[19,20] Whether these filaments are identical with those in normal neurons is uncertain; in the case of Alzheimer's disease the long-recognized bundles of argyrophilic fibrils observed by light microscopy relate at the electron-microscope level to structures like neurofilaments, but Kidd[21] has shown these intracellular filaments appear as paired helices so that the identity with neurofilaments may well be questioned.

One of the most outstanding features of brain metabolism is the high rate of protein synthesis and turnover observed in this tissue. Lajtha[22] has recently published studies showing that only a small percentage of the brain proteins has a half-life longer than 20 days (see also the review by Waelsch and Lajtha.[23] On the other hand, Piha, et al.,[2] have studied the turnover of histones in the brain and found a long half-life; however, these proteins constitute only a small fraction of the brain protein. The reason for the high protein turnover is unknown, because the neurons do not replicate and the glia probably do so rarely, and because the tissue does not excrete any known protein product (except in a few specialized regions such as the pituitary body, the hypophysis). It is sometimes suggested that the high metabolic turnover of proteins in a neuron is correlated with the necessity to replenish the enormous volume maintained in the extended axon. This point could only provide an explanation of high protein turnover were the turnover measured as a function of cell number, but in terms of cell weight a high protein turnover must imply a high rate of production and catabolism of one or more proteins for a role that is at present a mystery.

Possibly the high rate of protein production does correlate with the apparently continuous export of protoplasm from the neuronal soma to the axon, and protein is somehow consumed at the synapse. Centrifugal transport of axoplasm was reported and has been confirmed by Weiss and his collaborators, by Droz, and by Mania (see Note 1). On the other hand, Hebb[2] and Lubinska[1] have pointed out that with nerve transection certain enzymes appear toward the cut ends at increasing concentrations—

findings that could be interpreted to show the distal synthesis of protein. This finding could also be explained, however, by the unmasking of latent proteins at these injured sites. The problem of axonal flow has been recently reviewed by Lubińska.[24] I have concluded that axonal flow occurs at a rate between 1 and 3 mm per day, at least in many axons, but it is also apparent from studies of time-lapse photography of neurons that a much faster transport of particulates, and probably also solutes, occurs in both directions along the axon, a movement presumably superimposed upon the flow from the cell.

The relatively enormous length of the average axon of a neuron raises a considerable problem with respect to cellular control of replenishment and maintenance in distant parts of the axon and the synapses—particularly in the context of modern molecular biological theories involving feedback control through the repression or activation of genetic expression. Most histochemical analyses have shown consistently that RNA is restricted to the cytoplasm of the cell and is not found beyond the axon hillock. Some recent microchemical analyses by Edström[25] have shown that some RNA is, in fact, detectable in the axon of the Mauthner cell of the goldfish, but in view of the highly specialized nature of this cell, the generality of this observation cannot be taken for granted. Independent work by Koenig[26] has indicated the presence of small quantities of RNA in demyelinated facial nerves of cats. The quantity, however, was less than a hundredth of that in a corresponding volume of normal cells; but there must remain a question of how efficiently the myelin and satellite cells may be removed from the neuronal membrane prior to analysis. Simpler analytical studies may be made on the axoplasm obtained from the squid. Analysis of dialysed axoplasm by ultraviolet spectroscopy has shown no detectable absorption peak at 260 mμ, which indicates that nucleic acids must be less than 0.1 per cent of the concentration of protein, if they are present at all (Huneeus-Cox, Lusted, and Davison, unpublished).

Thus, at least within the squid axon, protein catabolism must be balanced by supply from the perikaryon, and the same situation may hold for the synapse, although there the presence of mitochondria, with their lately recognized content of DNA and RNA, provide the possibility of locally directed protein synthesis. The squid giant axon is as uncommon a structure as the goldfish Mauthner cell, and again these findings cannot yet be safely generalized, although it is probable that most axons contain little, if any, nucleic acids.

A number of authors have attempted to show a direct relationship between changes in metabolic activity or functioning of neurons or brain cells and physical or chemical changes in the proteins. Shooter and Boycott[1] attempted but failed to find differences in brain proteins of active and hibernating lizards. Altman,[2] using autoradiographic techniques with tritiated leucine as a protein precursor, studied protein synthesis in rat brains under selected conditions in which nervous activity was inhibited or solicited. It appeared, however, that nonspecific stimulation of the animal caused protein uptake, particularly in neurons, which concealed any more subtle effects that could be ascribed to learning or activity. Ungar[1] has studied protein conformation changes resulting from electrical stimulation of nervous tissue but, in view of the nonphysiologic nature of the stimulation, the relevance of these changes to those occurring normally in excitable tissue remains to be demonstrated.

One of the most promising lines of research to relate functional changes to physicochemical changes in the brain is in a study of the so-called synaptosomes, the pinched-off synaptic endings of neurons that may be isolated by differential centrifugation from homogenized brain tissue.[27] If "cementing" of pre- and postsynaptic membranes occurs to facilitate synaptic transmission, as has been postulated frequently, some evidence of such changes should be observable in appropriately selected preparations.

A number of experiments have been reported that deal with the inhibition of higher nervous function by the effects of antimetabolites interfering with RNA synthesis or transcription. Both of these subjects will be dealt with more fully by other authors in this volume.

A recent series of experiments by Huneeus-Cox, Fernandez, and Smith[28] have probed those molecular mechanisms in the axon membrane that mediate the action potential. By appropriate manipulations, the axoplasm may be flushed out of the squid giant axon, and so long as the appropriate salt and osmotic conditions are maintained inside and outside the axon, electrical excitability may be maintained while a variety of chemical probes are introduced into the perfusing solutions. No metabolic energy source appears necessary for the demonstration of the action potential so long as a transmembrane ion gradient is maintained by perfusion. It was found that the action potential could be reversibly blocked by the application of a number of sulfhydryl-blocking reagents without gross change in the integrity of the membrane as evidenced by the largely unchanged resting potential. A similar blocking could also be obtained by external application of the sulfhydryl-blocking reagents; the action potential may be restored by the internal or external application of sulfhydryl-containing reagents (e.g., cysteine or β-mercaptoethanol). Block could be induced also by the internal or external application of fluorescein mercuric acetate, a reagent that cannot penetrate the membrane. These results

imply that the molecular mechanism upon which depends the selective ion diffusion occurring in the action potential requires the presence of free sulfhydryl groups on both the inside and the outside of the membrane, or that the vulnerable groups are in a membrane pore that is accessible from either side.

Earlier experiments (see, for example, Rojas[29]) have shown the susceptibility of the action potential mechanism to internal perfusion by protease. Moreover, the action potential mechanism may be blocked, without change in the membrane resting potential, by perfusion (under critically selected conditions) with antibodies directed against the axoplasmic proteins (Huneeus-Cox and Fernandez, unpublished). This result would indicate that the ion-selective sites cross-react immunologically with, or are constructed from, a soluble protein of the axoplasm, the nature of which is under investigation.

In view of these experiments implicating protein and sulfhydryl groups, and since no oxidative energy supply appears necessary, it has been postulated that the gating of ions is mediated by a protein or proteins embedded in a pore in the membrane, and these proteins, by a change of interaction with their neighbors or of shape, induced perhaps by depolarization of the membrane or by a change in ion concentration mediated by current adjacent to the membrane, provide apertures to permit the transient flow of selected ions (Figure 1). (See also Note 1, pp. 55–76)

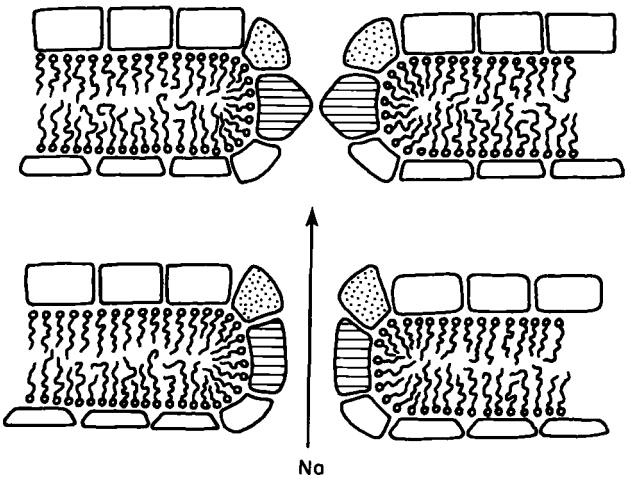

FIGURE 1 Hypothetical representation of an axon membrane in which are embedded pores selective for certain ions. The upper diagram shows a cross-section through a closed pore: the lower shows the same structure after a postulated conformation change in two species of proteins (dashed and stippled) lining the pore has occurred, triggered by the local depolarization of the membrane. With the opening of the pore a transient flux of sodium ions (Na) occurs. The membrane is shown as a lipid bilayer surrounded by an electrostatically associated protein sheath. Recent findings that lipid binds strongly to membrane proteins suggests that the latter may penetrate and perhaps traverse the lipid, interacting with it through hydrophobic groups, so the lipid may be less extensive than in the diagram, and the "gating" proteins may comprise the whole of the postulated pore.

Summary

There has been described, or reference has been given to, a wide variety of investigations into proteins and protein metabolism in brains and the nervous systems of higher organisms. In no case has it yet been possible to correlate the physical or chemical properties of one pure protein with metabolic functions in vivo, and no relationship to higher mental function has been demonstrated except possibly through the experiments of Flexner and Agranoff (see Agranoff, this volume). However, the mounting tempo of attack upon these problems gives hope of a widening of our understanding. There is much to be learned on the subject of brain proteins, and it is to be hoped that further studies will give us insight into the functioning of neurons and brain.

Some Recent Developments in the Biochemistry of Membranes

EUGENE P. KENNEDY

PROBLEMS OF MEMBRANE structure and function are of central importance to neurobiology. Studies of the role of the membrane in the generation of bioelectric potentials and the propagation of the nerve impulse form an essential part of classical neurophysiology. These aspects of membrane function in the central nervous system are reviewed in this volume in chapters by Taylor, by Whittam, and by Grundfest. However, there is increasing biochemical documentation for other fundamental functions of membrane systems, which in the long run must also prove of considerable significance to the experimental attack on problems of the function of the central nervous system. Some of these may be mentioned briefly here.

Recent studies on the role of cyclic adenylic acid (3′5′-AMP) in mediating the interaction of certain hormones with target tissues suggest that the primary action of these hormones may take place at the cell membrane. The fundamental work of Sutherland[1] and his colleagues led to the recognition that cyclic AMP is the chemical mediator in the mechanisms by which the catecholamines control the activity of glycogen phosphorylase in the liver and other tissues. It now appears that not only the catecholamines but also many other hormones, including glucagon, ACTH, and perhaps insulin may similarly act by mechanisms affecting the intracellular level of cyclic AMP. Cyclic AMP is formed in the reaction catalyzed by the enzyme adenyl cyclase indicated in Equation (1):

$$\text{ATP} \rightarrow 3',5'\text{-AMP} + \text{P—O—P} . \qquad (1)$$

Adenyl cyclase appears to be localized in the cytoplasmic membrane of most tissues.[1] Thus, the direct effect of the hormones that act through mechanisms involving cyclic AMP may be at the membrane, leading to stimulation or inhibition of the adenyl cyclase reaction. According to this view, cyclic AMP functions as a cytoplasmic or "second messenger," which interacts directly with the enzyme systems to be controlled. Much further work remains to be done in this area, but if these general concepts prove to be valid, it is clear that the membrane plays a vital part in mediating the interaction of at least some hormones with target tissues. Furthermore, the second-messenger concept has obvious relevance to mechanisms of neurohumoral transmission. Needless to say, the second messenger may not always be cyclic AMP.

Another important type of membrane function may lie in the area of DNA duplication and cell division. Jacob and his collaborators[2] have suggested that the duplication of DNA takes place while the bacterial chromosome is fixed to the membrane. The exact structural features by which DNA is attached to the membrane have not as yet been determined. It is postulated that the synthesis of the membrane itself is an essential part of the mechanisms by which parent and daughter cells each receive equivalent copies of the newly duplicated DNA during the process of cell division. Studies of cell division in *Bacillus subtilis* with the electron microscope offer support for these views.[3] It would obviously be advantageous to exploit the great power of bacterial genetics to the fullest in exploring such membrane functions. However, mutations leading to a serious disruption of the structure of the membrane might be lethal in a large percentage of cases. Here it may be possible to take advantage of "conditional lethal" mutations such as those that lead to an increased temperature sensitivity of the proteins that are controlled by the mutated genes. It is possible to select mutants that will grow at room temperature, but not at higher temperatures, because of the increased temperature sensitivity of some essential enzyme or other protein. Jacob and his collaborators[4] have recently described some properties of a large number of such conditional lethal mutants in *E. coli*, some of which exhibit grossly altered morphology. A systematic study of these mutants might reveal the presence, for example, of temperature-sensitive enzymes involved in lipid biosynthesis, the study of which might throw some light on the specific role of such lipids in the membrane.

The function of membranes in the organization of complex enzyme systems, especially those of the mitochondrion and endoplasmic reticulum, is now under intense study. Developments in this rapidly moving field are reviewed by Lehninger elsewhere in this volume.

These few examples may perhaps serve to show how

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problems of membrane biochemistry lie at the heart of very diverse fields of cell biology. We may, therefore, be justified in attempting a survey of current research in certain selected fields of membrane biochemistry, with the hope that ultimately some of the developments to be considered may prove relevant to the study of the central nervous system.

More comprehensive reviews of membrane biochemistry may be found in the monograph by Kavanau[5] and in reviews by Green,[6] Hokin and Hokin,[7] Csáky,[8] Vandenheuvel,[9] Benson,[10] and Wilbrandt,[11] and in recent symposia.[12-14]

Lipid–protein interactions in membranes

Membranes throughout nature appear to be made up of two principal kinds of building blocks—proteins and lipids. The models put forward by Danielli and Davson[15] and by Robertson[16] to explain the arrangement of these structural units in membranes have gained widespread acceptance. The Robertson model, which represents an extension and development of the previous model of Davson and Danielli, is shown in Figure 1. The phospholipid component is represented as the familiar bimolecular leaflet with the long-chain hydrocarbon moieties of the fatty acids interacting hydrophobically, and the ionized hydrophilic "head groups" pointed outward in a palisade arrangement. The lipid layers constitute the essential barrier between the cytoplasm and the external medium. In keeping with this idea, fat-soluble substances generally penetrate membranes readily, while the membrane is relatively impermeable to most hydrophilic substances. Support for this aspect of the Robertson model also comes from the studies on model membranes, such as those carried out by Mueller and Rudin and their co-workers,[17] and by Thompson and his collaborators.[18] The latter workers have prepared membranes from highly purified phosphatidylcholine and n-tetradecane. The dimensions of these membranes, measured by optical methods, correspond roughly to those expected for bimolecular leaflets of the type shown in Figure 1. Model membranes of this kind exhibit some of the most striking properties of cellular membranes, such as a high electrical resistance together with a rather high permeability to water. In the studies carried out by Mueller and Rudin and their collaborators it has been possible also to simulate some of the bioelectric properties of natural membranes, including electrical excitability.

Robertson has suggested that membranes throughout nature may have structures based on the "unit membrane" shown in Figure 1. However, there is a growing body of experimental evidence, both morphological and

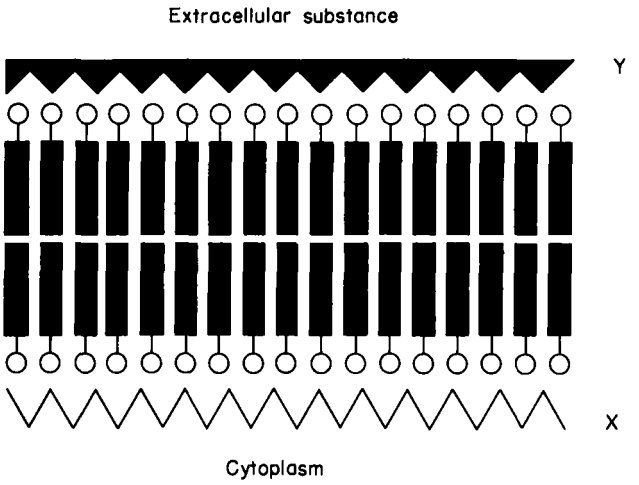

FIGURE 1 The unit membrane model proposed by Robertson, Note 16.

biochemical, which indicates that some biologically active membranes, such as the mitochondrial membrane and the cytoplasmic membrane of bacteria, must possess a higher degree of complexity than the model shown in Figure 1. In this connection, Green[19] has pointed out that much of the evidence for Robertson's formulation has been derived from studies of the structure of myelin, and may not be relevant to other membranes with a much higher rate of metabolic activity. Green has also criticized the Robertson model on the grounds that it directs attention primarily to ionic interactions between proteins and the hydrophilic head groups of phospholipids in the binding of protein to lipid. While ionic forces undoubtedly contribute to the stability of membranes, the primary importance of hydrophobic interactions (lipid–lipid, protein–lipid, and protein–protein) is becoming increasingly evident both in the organization of the mitochondrial structure and in the binding of specific transport proteins (such as the lactose transport protein in *E. coli*) to cytoplasmic membranes. Although Davson and Danielli[20] also considered the possible interaction of amino acid side chains of proteins with the lipids in the membrane, they visualized this interaction as involving fully extended films of protein at the lipid surface.

The enzymic and transport functions carried out by membranes leads the biochemist to conclude that these structures cannot be monotonous. Highly specific proteins must be localized at certain sites in the membrane structure. Models of the Robertson type offer the difficulty that the proteins are depicted as being localized at the membrane surface rather than as an intrinsic part of the barrier between the cytoplasm and the external medium. On the other hand, Benson[10] has recently suggested that

certain proteins, because of their genetically determined primary structure, may assume conformations with a high specific affinity for membrane lipids and may be intercalated directly into the membrane, as shown in Figure 2. Benson's formulation has the merit of depicting the protein itself as constituting part of the membrane barrier. It is not necessary that the entire membrane structure be made up of lipoprotein units of the kind depicted in Figure 2. Indeed, as Vandenheuvel[9] has pointed out, if the membrane were uniformly of such a structure, it would be difficult to account for its high (20 to 30 per cent) content of lipid. A large percentage of the area of a given membrane may, in fact, have a structure fundamentally similar to that shown in Figure 1. However, if specific proteins associated with enzyme function or with transport may be intercalated, as suggested by Benson, it would be much easier to understand the function of such transport proteins as the membrane protein of the lactose system of *E. coli*. Thermal agitation might expose the sugar-binding sites of such a protein alternately to the exterior and cytoplasmic surfaces of the membrane, thus enabling it to function in mediated transport.

The function of lipids in membranes

The importance of lipids as structural components of membranes is reflected in the gross chemical composition of the brain, a tissue very rich in membranes. About 50 per cent of the total solids of mammalian brain consists of lipid. The lipid content of isolated myelin fractions is even higher.

The lipids of brain may be classified into three major groups: glycerophosphatides, sphingolipids, and sterols. In attempting to decide the fundamental function of lipids in membranes, it may be useful to inquire which of these three groups is always associated with membrane structures throughout nature. Although sphingolipids and

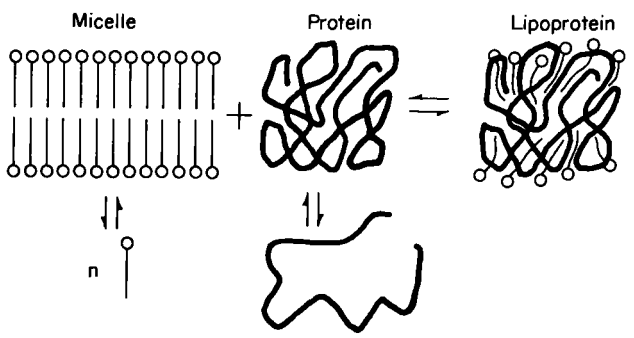

FIGURE 2 A possible mode of interaction of protein and lipid in membranes, suggested by Benson, Note 10.

sterols are widely encountered in higher plants and animals, bacteria appear to contain neither of these types. Bacterial membranes, however, contain a characteristically high content of glycerophosphatides, which are in fact major components of membranes in every type of living cell thus far examined.

This finding might suggest that glycerophosphatides are indispensable components of membrane structure. Some doubt is cast on this conclusion by the work of Van Deenan and his collaborators,[21] who have studied the relative lipid composition of the erythrocyte membranes in several mammalian species. Presumably, the erythrocyte membrane carries out closely similar functions in each of these species, but the relative proportions of glycerophosphatides vary widely. Phosphatidylcholine, for example, which is a major component of erythrocytes from nonruminants, is present only in small amounts in erythrocytes from ruminants. However, the sum of phosphatidylcholine and sphingomyelin approaches a much more nearly constant value in the species studied.[21] Because both lecithin and sphingomyelin are choline-containing phospholipids, it might appear that what is really necessary for a functional erythrocyte membrane is not the specific lecithin structure but rather a phospholipid of certain general physicochemical properties that can be exhibited either by sphingomyelin or by lecithin. These may include the possession of the phosphorylcholine head group and the ability to interact with cholesterol to form a tightly packed complex. Models of cholesterol, lecithin, and sphingomyelin made by Vandenheuvel[22] reveal that the cholesterol molecule can interact strongly by van der Waals forces with the hydrocarbon chains of lecithin or of sphingomyelin. The molar ratio of cholesterol to total phospholipid in the mammalian erythrocyte is roughly unity, which also appears to be the case in isolated myelin fractions. It has been suggested that the tight packing of cholesterol and phospholipid contributes considerably to the stability of the membrane.

Because these considerations might point to the conclusion that choline-containing phospholipids may be essential components of the erythrocyte membrane, it is again of interest from the comparative biochemical point of view to note that choline-containing phospholipids are found only rarely in the true bacteria. For example, choline is completely lacking from the lipids of *E. coli*. This organism contains phosphatidylethanolamine and phosphatidylglycerol as its principal phospholipids. The one truly ubiquitous glycerophosphatide appears to be phosphatidylglycerol, which has been detected in every kind of living tissue examined, although not always in abundance.

Returning to the lipids of the brain, one may note that although cholesterol is the sole sterol found in appreciable

quantities, a wide degree of variation is found within the families of glycerophosphatides and sphingolipids. The glycerophosphatides vary not only in the nature of the hydrophilic head group but also in the type and position of the long-chain fatty acid residues. In certain of these phosphatides, an ester bond may be replaced with an ether bond, which may be saturated or of the vinyl ether (plasmalogenic) type. A similar high degree of elaboration is encountered in the sphingolipid family. Thus, at least ten gangliosides have been recognized as constituents of beef and human brain,[23] four of which have been well characterized. In addition to the gangliosides, the sphingolipids of the brain include sphingomyelin and the cerebrosides, which also possess a number of different molecular structures.

Bloch has pointed out[24] that the vast structural diversity of lipid structures, so well exemplified by the brain lipids but encountered also in bewildering variety throughout nature, poses a real challenge to the lipid biochemist who seeks to correlate structure and function. In seeking a rationale for such diversity, it seems inevitable that research workers must turn more and more to the comparative biochemical approach.

DYNAMIC THEORIES OF LIPID FUNCTION Theories of the function of phospholipids in membranes can be divided into two general categories—dynamic and structural. We may arbitrarily consider as *dynamic* all hypotheses which require that the phospholipid itself undergo an active metabolism in a specific membrane function, such as active transport. *Structural* theories, on the other hand, do not require that the phospholipids undergo active metabolism. That phospholipids are important elements of membrane structure follows, of course, directly from an examination of the chemical composition of such membranes, but in addition phospholipids may contribute to membrane function in more specific fashion by interacting with and thus modulating the activity of protein components of the membrane.

Support for the idea that phospholipids are dynamically involved in membrane function comes from studies of the rate of turnover of phospholipids in higher organisms. These lipids are renewed at a high rate in metabolically active tissues, such as intestinal mucosa, kidney, and liver. Two theories of phospholipid function in membrane systems consistent with such high rates of turnover have recently attracted considerable attention. The first of these is the phosphatidic acid hypothesis put forward by Hokin and Hokin.[25]

These workers have suggested that phosphatidic acid may be an essential part of the mechanism for the specific transport of sodium (and perhaps other metabolites) across cell membranes. Some features of the scheme are indicated in Figure 3. Phosphatidic acid is postulated to interact with a protein in such a manner that the phosphatidic acid–protein complex has a high affinity for some metabolite to be transported (indicated as X in the diagram). In the specific case of sodium transport, X may represent one or more sodium ions. In the operation of the cycle, the protein–phosphatidic acid–sodium complex, formed on the inner side of the cytoplasmic membrane, may move through the membrane, and as a result of the action of a specific phosphatase may be converted at the external side to a diglyceride–protein complex, which has little affinity for sodium ions. This leads to the extrusion of sodium ions. The diglyceride–protein complex then moves back through the membrane and is rephosphorylated in a reaction catalyzed by the enzyme diglyceride kinase, forming the phosphatidic acid–protein complex once again. In this fashion, sodium ions (or other metabolites) may be pumped through the membrane in continuous cyclic fashion, utilizing energy released by the breakdown of ATP.

Perhaps the most striking evidence advanced by the

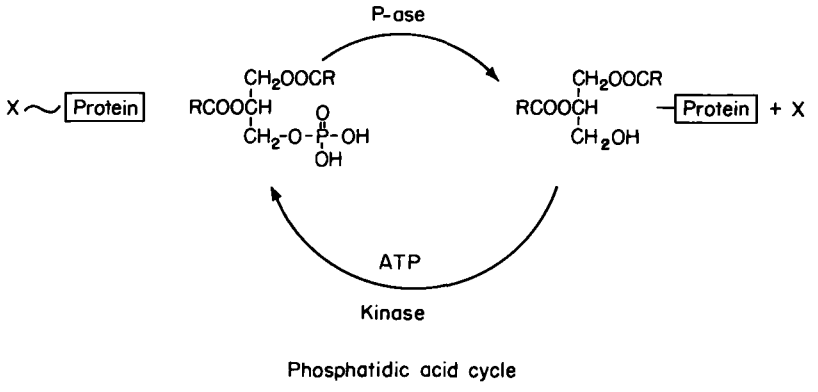

FIGURE 3 Postulated function of phosphatidic acid in active transport.

Hokins to support their theory comes from studies on sodium secretion by the salt gland of certain marine birds. The salt gland, which is controlled by cholinergic innervation, has the ability to secrete highly concentrated solutions of sodium chloride. Slices of the gland when treated in vitro with acetylcholine are apparently stimulated to secrete sodium chloride. When the rate of incorporation of ^{32}P into the phospholipids of the slice was measured in such experiments, it was found that the administration of acetylcholine caused a marked increase in labeling. This "phospholipid effect" was most marked in the phosphatidic acid fraction. Experiments of this kind have been interpreted as indicating an increased rate of turnover of phosphatidic acid associated with the cycle of transport shown in Figure 4. However, phosphatidic acids are also known to be central intermediates in the biosynthesis of phospholipids,[26] and it is not easy to dissociate effects on the biogenesis of phospholipids from those ascribed to the cycle postulated by the Hokins to be involved in sodium transport.

Similar effects on phospholipid labeling in slices of sympathetic ganglia treated with acetylcholine have also been reported.[27] This led to the hypothesis that the phospholipid effect in synaptic tissue is concerned with increased activity of the sodium pump.[27] However, this suggestion has been criticized by McIlwain[28] on the grounds that the demonstrated levels of activity of diglyceride kinase are insufficient to account for the rapid flux of sodium in neural tissues.

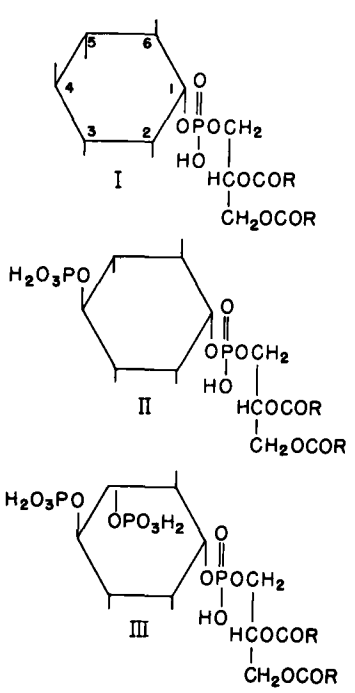

FIGURE 4 Structures of the phosphoinositides of the brain.

Recent advances in our understanding of the molecular basis of the sodium pump resulting from the fundamental work of Skou[29] are reviewed elsewhere in this volume. It has been possible to demonstrate that cell-free extracts of various tissues contain an adenosine triphosphatase dependent upon both sodium and potassium ions for maximal activity. Such an enzyme can also be demonstrated in cell-free homogenates of the salt gland of the herring gull. A study of ^{32}P incorporation into the lipids of such homogenates, carried out by Hokin and Hokin,[30] failed to reveal the presence of an active pool of phosphatidic acid such as would be required by the scheme shown in Figure 4. This finding, together with recent evidence that the actual intermediate in the sodium–potassium-activated adenosine triphosphatase has the properties of an acyl phosphate, makes it unlikely that phosphatidic acid metabolism is directly involved in sodium transport in the manner suggested by the Hokins.

Another theory of phospholipid functions draws attention to the high rate of metabolism of inositol-containing phospholipids in animal tissues in general, and in the brain in particular. It has long been known that the rate of renewal of the inositol-containing phospholipids in the brain of living animals is very high.

Thanks largely to the work of Ballou and his collaborators,[31] the structure of the three inositol-containing phospholipids of the brain have been completely worked out and are indicated in Figure 4. There is now considerable evidence that the di- and triphosphoinositides of brain (II and III, Figure 4) are derived from phosphatidylinositol by a sequential process of phosphorylation with ATP as the donor of the phosphate residues. However, the di- and triphosphoinositides undergo rapid dephosphorylation. Colodzin and Kennedy[32] have described an enzyme in brain that catalyzes the phosphorylation of phosphatidylinositol to form the diphosphoinositide (Figure 5). Brain extracts also contain an active phosphatase, capable of cleaving the phosphate from the 4-position of the diphosphoinositide, thus regenerating the monophosphoinositide. This cycle of reactions (Figure 5) constitutes an adenosine triphosphatase system. Why ATP should be cleaved at a high rate by this series of reactions is at present unknown, but there have been various theories implicating the inositol phosphatides in transport function.[33] In this connection it should be pointed out that it was believed for a long time that di- and triphosphoinositides were present only in brain tissue. As a result, however, of the experiments of Garbus, et al.,[34] and of Hawthorne and his collaborators,[35] it has now become clear that many oth-

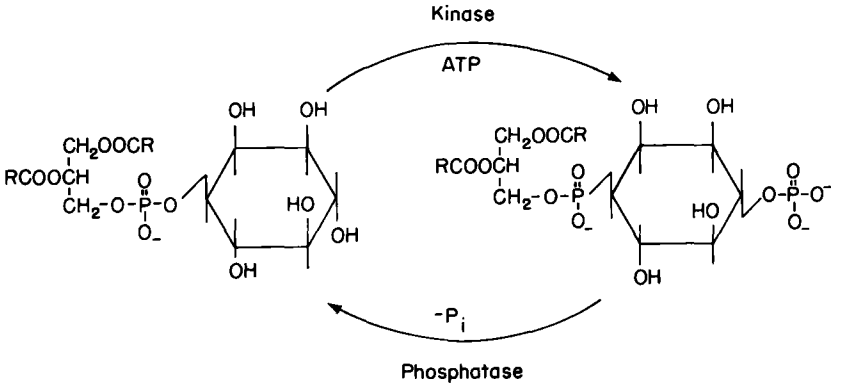

FIGURE 5 Phosphorylation and dephosphorylation of phosphoinositides as part of an adenosine triphosphatase system.

er tissues catalyze the phosphorylation of phosphatidylinositol to diphosphoinositide and probably to triphosphoinositide. Colodzin and Kennedy[32] reported that the enzyme catalyzing the phosphorylation of phosphatidylinositol (Figure 5) was widely distributed in tissues of the rat. Presumably, the action of phosphatases is so rapid in tissues other than the brain that the di- and triphosphoinositides do not accumulate in appreciable amounts.

One may ask whether high rates of turnover of phospholipids, presumably associated with dynamic functions, are universally observed. To answer this question we turned some years ago to the study of the phospholipid metabolism of *E. coli*. The principal glycerophosphatide of this organism is phosphatidylethanolamine. It was found[36] in pulse-labeling experiments that phosphatidylethanolamine is perfectly stable in rapidly growing cultures of *E. coli*, in marked contrast to the rapid rate of renewal of phospholipids in animal tissues. Once formed, phosphatidylethanolamine is not broken down during the course of several generations. Whatever the function of phosphatidylethanolamine may be in this organism, it clearly does not involve continuous metabolism. Perhaps it should be mentioned that in contrast to phosphatidylethanolamine, phosphatidylglycerol does reveal a dynamic pattern of labeling in pulse experiments. This, however, is probably related to the role of phosphatidylglycerol as an intermediate in the biosynthesis of other macromolecular constituents of the cell.

Theories that hydrophilic metabolites must be converted to lipid-soluble intermediates in order to traverse the cytoplasmic membrane are abundant in the literature, but in general have been poorly supported by experimental evidence. Recently, however, Strominger and his collaborators[37] discovered that the muramyl-pentapeptide units, which are building blocks for cell wall structures in *Staphylococcus aureus*, must be converted to lipid-soluble intermediates before their incorporation into the wall material. The biosynthesis of the muramyl-pentapeptide leads to the formation of the uradine diphosphate (UDP) derivative. In a reaction with a lipid, the full structure of which has not yet been elucidated, the muramyl-pentapeptide is transferred to the lipid, with release of uridylic acid (UMP). The lipid thus replaces UMP as the "handle" to which the reactive subunit is attached. Because the wall structure is external to the cytoplasmic membrane, it is suggested that conversion to a lipid is needed for the precursor subunits to pass through the membrane to the actual site of assembly of the wall. Apparently, a similar situation is encountered in the biosynthesis of the O-antigen groups of *Salmonella typhimurium*, in which lipid-linked di- and trisaccharide intermediates have been detected.[38,39]

THE STRUCTURAL ROLE OF LIPIDS IN MEMBRANES As suggested above, the function of the lipid components of membranes cannot be limited to the formation of a water-insoluble diffusion barrier, as might be concluded from a naive interpretation of the model shown in Figure 1. The specific interaction of lipids with proteins in the membrane may profoundly alter the catalytic and other properties of the proteins.

The highly organized enzyme reactions of the mitochondrial membrane are disrupted by the removal of lipid, either chemically or enzymically. This is true not only for the multienzyme complex itself but also for individual purified enzymes extracted from mitochondria. Jurtshuk and his collaborators[40] purified the β-hydroxybutyric dehydrogenase of mitochondria and found that the purified enzyme was completely inactive in the absence of phospholipid. Upon the addition of lecithin, however, the enzyme was fully reactivated. The requirement for lecithin

is highly specific; other phospholipids may not be substituted for it. The type of fatty acid in the phospholipid structure is also important. Lecithins containing only fully saturated, long-chain fatty acids are inactive; those containing unsaturated fatty acids are active. This was interpreted as indicating that a certain kind of micellar structure of phospholipid is essential for activating the enzyme. It is well known that *cis* double bonds in the fatty acid chains of a phospholipid profoundly modify its solubility and other characteristics. It seems reasonable to suppose that the lecithin does not participate directly in the reaction catalyzed by the enzyme, but rather forms a complex in which the enzyme assumes a conformation required for activity.

Many cases are now known in which a phospholipid is required for the catalytic function of an enzyme. In an interesting example, Rothfield and his collaborators[41] recently have shown that one of the enzymes involved in the biosynthesis of the lipopolysaccharide complex of *Salmonella* has a specific requirement for phosphatidylethanolamine. The phospholipid is apparently required for the binding of substrate to enzyme. These observations, taken together with the necessity of converting lipopolysaccharide precursors to a lipid form as discussed above, lead to the conclusion that lipids play a crucial role in the control of cell-wall synthesis.

Role of structural protein in the organization of the membrane

In 1961 Green and his collaborators[42] reported the isolation from beef heart mitochondria of a protein devoid of enzymic properties and thought to be an essential structural component of the mitochondrion. This structural protein may account for about half of the total protein of beef heart mitochondria. At low *p*H, the protein exists in the form of a highly aggregated complex, but at *p*H 11, or in the presence of anionic detergents, another more soluble form can be demonstrated that is apparently that of a subunit, with a molecular weight of about 22,000. The structural protein forms specific complexes with cofactors such as ATP and DPNH as well as with other proteins (particularly enzymes of the electron transport system) and with lipids. Other properties of the structural protein and its postulated role as a component of the repeating unit of membrane structure have been reviewed.[19]

Recent studies by Woodward and Munkres[43] support the view that the structural protein has an important general function in mitochondrial organization throughout nature. These workers have isolated the structural protein from the mitochondria of *Neurospora* and found that it has properties surprisingly similar to those of the protein derived from beef heart mitochondria. The *Neurospora* protein has a molecular weight of 23,000 in comparison to the reported value of 22,000 for the beef heart protein, its pattern of solubility is similar, and it also has a high affinity for lipids and for other proteins.

Woodward and Munkres[43] also studied mutant forms of the *Neurospora* mitochondrial structural protein and found that in some mutants mitochondrial function was severely affected. In one such mutant, the replacement of a single cysteine residue by tryptophane led to the accumulation of cytochrome *c* in the nonmitochondrial, soluble fraction in amounts about 16-fold higher than in wild-type organisms. Clearly, the structural protein must play a highly specific role in the organization of the mitochondrial structure.

In an important further contribution, Munkres and Woodward[44] obtained evidence indicating the importance of *locational sites* in the evolution of mitochondrial enzymes. Certain mutations in *Neurospora* have given rise to strains that cannot metabolize malate. Some of these mutants contain an altered form of malate dehydrogenase, which when extracted and purified exhibits only a slightly reduced affinity for malate. However, when the mutant enzyme is tested in the presence of mitochondrial structural protein, its affinity for malate is greatly decreased. Malate dehydrogenase from wild-type *Neurospora,* on the other hand, has essentially the same K_m for malate when tested in the presence or absence of structural protein.

These results make it seem likely that enzymes and other specific proteins localized in membranes must be the products of rigorous selection during the course of evolution. Such proteins must possess not only efficient catalytic binding and control sites, but also sites that assure specific interaction with structural protein and with lipid as essential steps in the self-organization of the membrane. Mutations in the locational site that bring about unfavorable conformational changes when the enzyme is associated with structural protein may lead to an enzyme with little activity in the living cell. Because the purified enzyme may not reveal this altered property, insufficient attention may have been paid in the past to this important feature of protein structure.

Experimental approaches to the investigation of membrane function in bacteria

It is by now commonplace to observe that bacterial systems offer special advantages in the study of some fundamental cellular processes. In particular, the study of specific transport processes in bacteria is enormously aided by the fact that many such processes are under specific genetic control. Although some of the problems of special interest

to neurobiologists cannot be studied in bacteria, principles of membrane function may be discovered in these microorganisms that may shed light on problems encountered in higher organisms.

It would take us too far afield to undertake a detailed review of bacterial membrane function. The following discussion is confined to recent work on three bacterial systems that give promise of yielding information on the fundamental chemical basis of specific transport across membranes. Emphasis is placed on the experimental approaches currently employed, in the hope that some of these methods, suitably modified, may find application in neurobiology.

LACTOSE TRANSPORT IN *Escherichia coli* The brilliant contributions of workers at the Institut Pasteur on the genetic control of the system for the utilization of lactose in *E. coli* have been comprehensively reviewed by Stent elsewhere in this volume. Among the important accomplishments of these workers was the demonstration of a genetically controlled transport system mediating the uptake of lactose in this organism. Considerable previous evidence, provided by Doudoroff and his collaborators,[45] and by others, had indicated that the bacterial cell might not be freely permeable to sugars by a process of simple diffusion, but it remained for Monod and his co-workers to establish clearly and unambiguously the essential role of a specific transport system in the utilization of lactose.

The term *permease* was introduced by the Parisian workers to describe the lactose transport system, and other similar systems in bacteria. In several models put forward to explain the biochemical basis of the transport system for lactose and other β-galactosides, the word permease has also been used rather ambiguously to designate a hypothetical enzyme thought to play an essential part in the transport process. In the early model proposed by Cohen and Monod,[46] the accumulation of β-galactosides within the cell against a concentration gradient was thought to be the result of a process in which the inward flow of sugar is catalyzed by such an enzyme, while the outward flow is the result of simple diffusion. When the intracellular concentration reaches a level at which the outward leakage is equal to the inward flow, equilibrium would be attained.

It soon became clear that the simple "pump-vs.-leak" model cannot explain all the experimental facts. The exit of sugar from the cell must also be a mediated process. More detailed models, which attempt to account for the later experimental findings while still retaining the idea of a permease-catalyzed reaction, have been put forward by Kepes[47] and by Koch.[48] These models invoke a second entity, the *transporteur* or transport substance, which acts as a vehicle for the sugar in its passage through the membrane.

In the model proposed by Kepes, an energy-rich activated form of the *transporteur* combines with the sugar to be transported in a reaction postulated to be catalyzed by the hypothetical permease:

$$\text{R} \sim \text{T} + \text{G} \xrightarrow{\text{Permease}} \text{T—G} + \text{R}. \qquad (2)$$

Because reaction (2) is thought to be virtually irreversible, it must proceed with a considerable release of free energy. An important consequence of this postulate is that there should be a continuous expenditure of metabolic energy in the permease-mediated entry of sugars into the cell. However, the experiments of Koch,[48] of Winkler and Wilson,[49] and of Luria and his collaborators[50] suggest that the entry of sugars into the cell, mediated by the Lac transport system, does not require the expenditure of metabolic energy, although *accumulation* of sugars against a concentration gradient is energy dependent.

As pointed out by Kepes in his 1964 review,[51] it has proved very difficult to obtain direct experimental support for those models of lactose transport that postulate a permease-catalyzed reaction. Despite diligent search, no evidence for such an enzyme has been found. Furthermore, it is difficult to imagine how a reaction such as that shown in Equation (2) can proceed without the continuous expenditure of metabolic energy. These considerations led us to attempt to reformulate the problem in the hope that other models could be devised which would suggest new lines of experimental approach. One such model currently under investigation in our laboratory is shown in Figure 6.

In this formulation, the process of *facilitated entry* is

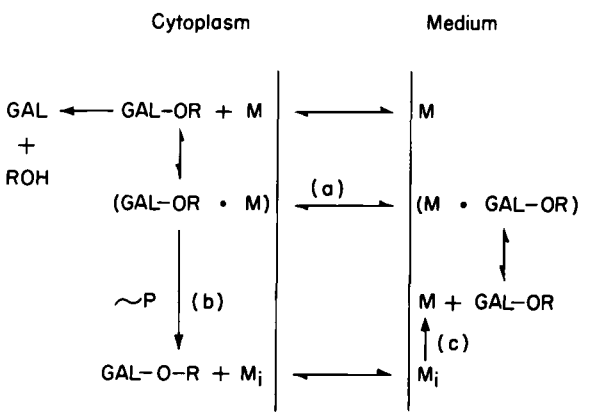

FIGURE 6 Simplified working model of the β-galactoside transport system.

sharply distinguished from the process of *accumulation against a concentration gradient*. A molecule of lactose (or other β-galactoside) combines with a component M, a specific protein localized in the membrane. This combination is of the Michaelis–Menten type and does not necessarily involve the catalysis by the protein of the formation of any covalent bond. The galactoside–protein complex may move through the membrane to the internal or cytoplasmic aspect. Here the complex may undergo one of two fates. It may simply dissociate, and if the galactoside is a substrate for the enzyme β-galactosidase, its breakdown to galactose and aglycone may lead to a substantial flux of sugar from the medium into the cell. This flux is driven by the hydrolysis of the galactoside, which does not accumulate as such in the cell.

Alternatively, and especially if the substrate is a thiogalactoside that cannot be attacked by β-galactosidase, the complex may undergo reaction (b), linked to the utilization of metabolic energy. In reaction (b) the protein undergoes a transformation, leading to the formation of a modified protein, indicated as M_i. This form has a greatly reduced affinity for the galactoside. M_i may then move through the membrane to the outer face, where it can be reconverted in a reaction not requiring metabolic energy, to the M form, with high affinity for β-galactosides.

A more detailed comparison of this model of the lactose transport system with the previous formulations has been given elsewhere.[52] Here we may consider the experimental approaches by which biochemical identification of the components of such a system can be achieved. The membrane protein, according to such a model, need not possess enzymatic activity, so we cannot rely for its detection upon its presumed ability to catalyze some chemical transformation of the substrate. Instead, it is necessary to identify the protein by some stoichiometric principle, such as isotopic labeling. Furthermore, in order to be certain that we are dealing with a component of the transport system as it exists in the living cell, ideally it would be very useful to label the protein while it is present in the living cell. Accordingly, a search was undertaken for inhibitors of the lactose transport system of the kind that function by irreversible attachment to proteins. One such inhibitor that proved useful is N-ethylmaleimide. The transport process in the intact cell is inhibited by N-ethylmaleimide,[53] and this inhibition is not caused by a reaction of the inhibitor with either β-galactosidase or thiogalactoside transacetylase, the two previously characterized proteins of the Lac operon. The N-ethylmaleimide-sensitive component is, in fact, a protein, hitherto unrecognized, located in the membrane fraction.

A great technical advantage arose from the observation[53] that the membrane protein is protected against reaction with N-ethylmaleimide when it is saturated with thiodigalactoside, one of the substrates for this transport system. This fortunate property, together with the fact that the proteins of the Lac system are found in wild-type (i^+) cells in significant amounts only after exposure of the cells to an inducer, led to the development of the general procedure for the specific labeling of the membrane protein outlined in Figure 7.

Cells of strain ML-30, which are $i^+z^+y^+a^+$, are divided into two portions. One-half of the culture is induced by exposure to isopropylthiogalactoside, which induces the Lac system without itself undergoing further metabolism. The control culture is not induced and therefore lacks the proteins of the Lac system, including the M protein. Both portions of cells are now separately incubated with unlabeled N-ethylmaleimide in the presence of thiodigalactoside. This step is necessary in order to reduce the level of proteins which react with N-ethylmaleimide. The M protein, because it is fully saturated with thiodigalactoside, is specifically protected from the inhibitor. Unfortunately, however, because of the irreversible nature of the inhibition, if the incubation is carried out for a very prolonged period, then all of the M protein will eventually also react with the inhibitor. The conditions of incubation and its timing must therefore be carefully controlled. At the end of the incubation the cells are washed to remove unlabeled N-ethylmaleimide and thiodigalactoside. The induced cells are then incubated with ^{14}C-labeled N-ethylmaleimide in the absence of thiodigalactoside, thus allowing the unprotected M protein to combine with the labeled inhibitor. A similar step is carried out with the uninduced cells using ^3H–N-ethylmaleimide. The cells are now combined and fractionated. The distribution of labeled proteins in the mixture is indicated in Figure 7. There will be a considerable variety of tritiated proteins (A, B, C, etc.)

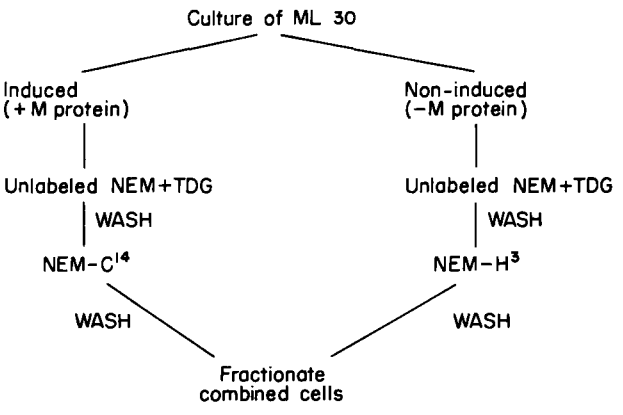

FIGURE 7 Plan of the double-label experiment.

derived from the uninduced cells. Each of these proteins is also present in the induced cells, labeled with ^{14}C. In addition, however, the induced cells contain the M protein for which there is no corresponding tritiated equivalent. Upon fractionation of the proteins, those fractions that do not contain M protein will have a $^{14}C/^{3}H$ ratio of unity, while those enriched in M protein will have an increased ratio of ^{14}C to ^{3}H.

This procedure has led to the direct chemical identification of the membrane component of the lactose transport system. Some of its properties have been described in recent publications.[53-55]

Although the great advantage of having a genetic control over the presence of a given transport system cannot easily be achieved in work on mammalian systems, other aspects of the experimental approach, outlined in Figure 7, perhaps may be applicable to the study of membrane function in brain and nerve. The possibility that specific pharmacological blocking agents could be used in the "protection step" deserves consideration.

PROLINE UPTAKE IN CELL-FREE MEMBRANE PREPARATIONS FROM *E. coli* In contrast to the intensive study of the sodium potassium-activated adenosine triphosphatase in the membranes of higher organisms, relatively little has been learned about the biochemical coupling of metabolic energy to active transport in bacterial systems. In an important recent contribution, Kaback and Stadtman[56] have demonstrated that cell-free preparations of *E. coli*, consisting apparently of vesicles formed from the spheroplast membrane, retain the ability to concentrate the amino acid, proline. These workers have provided convincing evidence that the accumulation process under investigation is not caused by the remaining intact cells. The process of proline accumulation is inhibited by anaerobiosis and by inhibitors that block oxidative phosphorylation. Added glucose stimulates the base level of accumulation of proline. These facts suggest that the membrane preparations still retain a complex set of enzymes and coenzymes necessary for the metabolism of glucose with the production of metabolic energy and also are able to couple the energy so obtained to the transport process. Such membrane fractions also accumulate lactose, as well as glycine and lysine. These investigations offer the hope that the link between metabolic energy and active transport can be explored in cell-free systems, with all the advantages that can be derived from the present state of development of bacterial genetics.

SULFATE TRANSPORT IN *Salmonella* Strains of *Salmonella* possess a system for the uptake of inorganic sulfate ions. Pardee and co-workers[57] have recently identified a sulfate-binding protein, apparently devoid of enzymatic activity, which appears to be a component of this transport system.

They have utilized an ingenious assay for the measurement of this protein in cell-free fractions. Dowex-1 ion exchanger is saturated with radioactive sulfate. The sulfate-binding protein is then added, and the protein and the resin compete for radioactive sulfate. The resin may then be removed by centrifugation or filtration and the activity of the displaced sulfate measured directly. With the use of this ingenious assay the sulfate-binding protein has been extensively purified. It will be of great interest to determine the specific function of this protein in the transport process.

These few examples of current investigations on the molecular mechanisms of membrane function in bacteria may suffice to indicate the great promise of this experimental approach for a solution of the problems of membrane function in general. Some of the methodology employed may be directly applicable to problems of membrane function in brain and nerve. Where this does not prove to be possible, the elucidation of general principles of membrane function may lead to the formulation of specific hypotheses that then can be confirmed or denied by direct experimentation on neural tissue.

Summary

After a long and frustrating lag period, during which it appeared that the biochemist could make little contribution to our understanding of membrane function, it now appears that the next decade should see a rapid development of membrane biochemistry. As is so often the case in science, developments in several apparently unrelated fields were needed to facilitate progress in this area. Recent advances in electron microscopy, in protein chemistry, in lipid chemistry and metabolism, in bacterial genetics and physiology, and in neurophysiology and neuropharmacology, all have set the stage for a full-scale attack on the molecular basis of membrane function, which is certain to be vigorously pursued in laboratories throughout the world.

Membrane Ultrastructure in Nerve Cells

HUMBERTO FERNÁNDEZ-MORÁN

MEMBRANES AND their derivatives play a key role in all biological systems, constituting about 40 per cent to 90 per cent of the total mass in different cell types. They represent a major structural component of nervous tissues, ranging from individual limiting membranes of the wide variety of cells to multiply folded or invaginated systems, which are often disposed in "paracrystalline" arrays.[1-3] Attention has focused on these specialized lamellar systems, including mitochondria and photoreceptors, as prototypes of organized membranes that carry out energy transduction functions with remarkable efficiency. The highly ordered structural framework of these cell membranes provides relatively stable "modular floor-space," thereby ensuring periodic arrangement of specialized macromolecular enzyme complexes and related assemblies.[4-6]

It is possible, in the light of accumulating evidence, that these membrane systems may operate equally well as information transducers. In fact, the neuron—that unit of the nervous system invested with the primary function of information transfer—possesses membrane-bound terminal endings with specialized synaptic structures that functionally interlink specific cell groups.[7] The suggested location of subcellular or molecular switch gears at such synaptic junctions manifests their integral association with the processes of information storage, transfer, and readout. According to Schmitt,[7] the formation of the exceedingly complex neuronal network of the human brain is the primary role of molecular recognition of coded information stored in macromolecules of the brain cells. Some of the most sophisticated behavioral patterns of the instinctual type primarily involve molecular recognition of coded information stored in DNA-RNA-protein macromolecular systems. The underlying molecular specificity in the key junctional regions of the nervous system must ultimately be taken into account in any attempts to explain the singular capacity for accurate storage of the near-infinite number of memories that span a lifetime.

The pioneering polarized light[8] and X-ray diffraction investigations of the nerve myelin sheath[9] provided a standard of reference for the study of cell membrane ultrastructure in general. However, the task of directly visualizing individual cell membranes, whose thickness is in the order of 100 Å to 200 Å, remained for the higher resolving power of the electron microscope.[10-11] Ultrastructural analysis is now progressing beyond the initial stage of delineating the membrane's lipoprotein framework.

Based essentially on the paucimolecular theory of membrane structure proposed by Davson and Danielli,[12] Robertson has propounded a unit membrane hypothesis.[13] After demonstrating that plasma membranes of all cells and the membranes of their organelles show certain common structural features, he proposes that biological membranes are comprised of a phospholipid bilayer system, 40 Å to 60 Å thick, which is coated on both sides with monolayers of macromolecular material, presumed to be proteins.

This unit membrane concept of Robertson serves as a useful working hypothesis, which must now be extended and modified according to the large body of evidence provided by correlated electron microscopic and biochemical investigations during the past few years. These studies have revealed specific macromolecular functional repeating units and the counterpart structural elements in mitochondria,[14-18] chloroplasts,[19,20] retinal rod outer segments,[21-23] and in a large number of membranes from different cell types.[24-27]

Investigations of nerve membranes and related structures were recently carried out by high-resolution electron microscopy, using improved preparation and instrumentation techniques, which have already proved successful in the analysis of virus fine structure and other biological systems. Increasing emphasis has been placed on the study of the detailed organization of the enzyme and multienzyme complexes intimately associated with cell membranes as the molecular componentry ultimately responsible for the highly specific energy and information transduction functions.

Only salient aspects can be given here of the available evidence bearing on organization within the plane of the layers, and the existence of macromolecular repeating units in membranes.

Organization of myelin membranes

Elucidation of the general submicroscopic organization of the nerve myelin sheath may be regarded as the corner-

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stone of membrane ultrastructure research. Nearly two decades ago, F. O. Schmitt and his associates[9,28] gave the first detailed account of the highly ordered, layered structure of the myelin sheath, as deduced from their classic small-angle, X-ray[9] diffraction studies of living nerve and earlier polarized light analysis.[8,28] The postulated regular, concentric arrangement of the myelin sheath layers was subsequently verified by direct observation in the electron microscope.[10,11,14,30] Schmitt and Geren[29,31] then showed that the myelin sheath derives from invaginated Schwann cell membranes wrapped multiply around the axon of embryonic peripheral nerve fibers. In the morphogenetic process of myelination, leading to the formation of this condensed, "liquid crystalline" membrane system, the constituent lipid and protein components in the limiting membranes of both axons and Schwann cells (or glia in the central nervous system) play specific roles.

The X-ray diffraction pattern of fresh intact nerve, which serves as a standard of reference for the study of myelin structure, features a number of well-defined, low-angle reflections. These reflections exhibit a characteristic alternation of intensities in the even and odd orders, and can be accounted for as the first five orders of a fundamental spacing, varying from about 170 Å in amphibians to 178 Å (Figure 1C) to 184 Å in mammalian peripheral nerve.[9,32,33] The low-angle X-ray diffraction patterns recorded from normal nerve furnish general information on the dimensions and approximate distribution of scattering groups in the radial direction of the myelin sheath. Earlier studies of physical and chemical modifications in the myelin sheath established a correspondence between the layer spacings observed in the electron micrographs (Figgure 1A) and the fundamental radial repeating unit indicated by the low-angle X-ray diffraction patterns (Figure 1C).

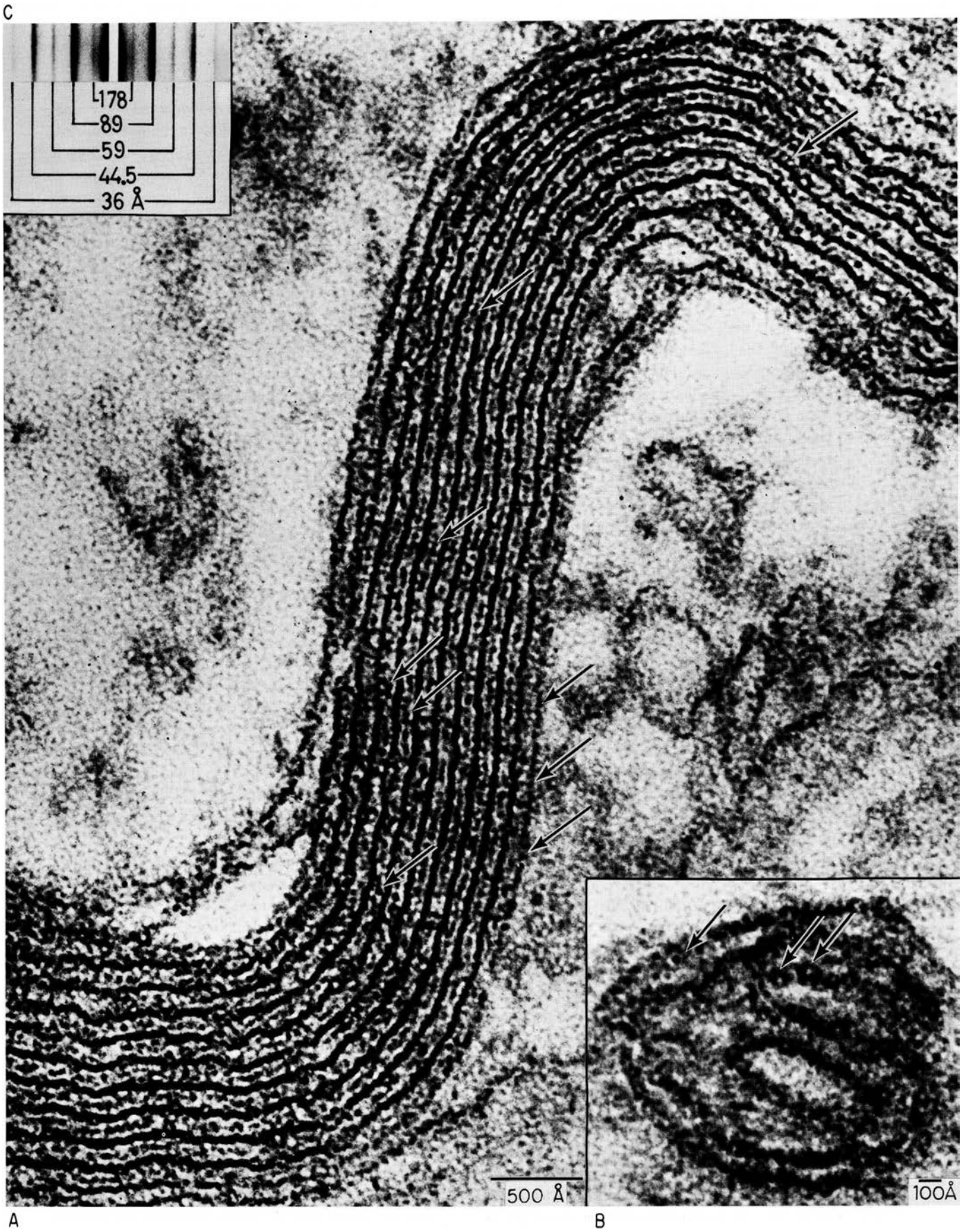
It should be pointed out, however, that the concentric laminated structure observed in electron micrographs of thin nerve sections is simply a pattern of the selective deposition of osmium or other reagents at certain sites, and it cannot be interpreted in terms of specific regions containing lipids, lipoproteins, or protein constituents of the myelin sheath. Moreover, extensive modifications are introduced by complex fixation, dehydration, and embedding artifacts that must be taken into account in a critical evaluation of the fine structures observed in thin sections.[33,34] With these reservations in mind, it is assumed that the framework of the fundamental radial unit of the sheath is formed by two adjacent bimolecular layers of mixed lipids about 60 Å in thickness; each bilayer is coated with sheets of hydrated protein or other polysaccharide, non-lipid, macromolecular material. The two layers are distinguished by a "difference factor," which can be explained by assuming that in the process of myelin formation the asymmetric wrapping of the Schwann cell membrane around the axon will produce a symmetry difference in successive layers.

Lipid composition of myelin

Relatively little is known about the composition of the major lipids and their fatty acids in myelin, because this component is difficult to isolate reliably and in sufficient quantity. Recently, however, J. S. O'Brien and associates[35] have reported the results of their efforts to determine the composition of lipids and fatty acids obtained from carefully isolated myelin, gray matter, and white matter in frontal lobes of normal humans of various ages. The lipid content of myelin, ranging between 78 per cent and 81 per cent of the dry weight, was found to be much higher than the lipid content of gray or white matter. According to the pioneering studies of Folch-Pi[36] and other earlier workers, myelin contains much higher molar proportions of cerebroside sulfate and plasmalogenes and slightly higher molar proportions of cholesterol than does gray matter. It is noteworthy that in myelin the content of sphingolipids, containing very long-chain fatty acids (19 to 26 carbon atoms), is proportionately ten times that of any other membrane structure analyzed. Also, one in 17 fatty acids is polyunsaturated in the myelin group of lipids, while in the gray matter group the corresponding value is one in 5.

The recent molecular model of myelin proposed by Vandenheuvel[37] serves to illustrate certain features characteristic of myelin lipids, which could account for the highly stable membrane structure. Lipids in myelin are not covalently bound. The three major forces holding these molecules within the membrane result from: (1) electrostatic interactions between polar groups of lipids and op-

FIGURE 1 A: High-resolution electron micrograph of myelin sheath segment from transverse thin section of frog sciatic nerve, showing concentric array of dense and intermediate layers. Particulate subunit structures (arrows) are regularly found within the plane of the layers in these well-preserved, osmium-fixed, low-temperature preparations. × 320,000. B: Axonal mitochondria from a similar preparation, demonstrating globular subunits in the dense layers of the cristae. × 400,000. C: Low-angle X-ray diffraction pattern of fresh rat sciatic nerve featuring a fundamental period of 178 Å, with typical alternation of the intensities of the even and odd orders.


C
178
89
59
44.5
36 Å
500 Å
100Å
A
B

positely charged groups in adjacent proteins; (2) hydrogen bonding between oxygen and nitrogen atoms in lipids and adjacent proteins; and (3) London-van der Waals dispersion forces between CH_2 pairs in hydrocarbon tails of adjacent lipid molecules. The latter may be the major force holding such molecules together, since the total force resulting from the additive interactions of the large number of CH_2 pairs is appreciable. On the basis of steric hindrance, it can be expected that lipids containing polyunsaturated acids will not be held as rigidly in a bimolecular leaflet, thus leading to a more free and less stable structure. The higher proportion of saturated glycerophosphatides present in myelin will therefore impart greater stability, as the lipid molecules can be more closely packed than in the membrane structures of gray matter, which have a significantly higher content of polyunsaturated glycerophosphatides. Finally, the sphingolipids containing long-chain fatty acids of the type found in myelin can form interdigitated cholesterol-sphingo-myelin complexes as proposed by Vandenheuvel. All of these new data make it possible to account for the closely packed and highly ordered myelin membrane structure. It may also help explain the remarkable degree of metabolic inertness of myelin lipids, which have been derived from turnover studies with labeled lipids.[38,39] Myelin is thus considered to be the most stable membrane known, lasting the lifetime of the animal.

All of these conclusions relate mainly to the lipid composition, but our knowledge about the structure, composition, and function of other constituents of myelin, including proteins, water, polysaccharides, and salts is still very meager. Recent experiments by M. Singer and M. Salpeter[40] indicate that tritium-labeled L-histidine is transported through the Schwann cells and myelin sheath into the axon of peripheral nerves. Further studies along these lines should help clarify the way in which incorporated amino acids traverse the myelin sheath.

Schmitt[41] has emphasized that the unique properties of the axon surface membrane appear to be determined by the inner protein component, which is considered to be different from the bimolecular lipid phase and the external non-lipid layer. The inner monolayer of globular protein molecules, forming a continuous, mosaic sheet, may be capable of responding to electrical and ionic changes with fast conformation changes of the protein molecules. Such dynamic changes of protein molecules in relation to the adjoining lipid bilayer may significantly alter the net fixed charge and, consequently, the membrane or action potential. Schmitt and co-workers have given the name "electrogenic protein" to this membrane-associated protein. Identification and localization of the electrogenic protein may eventually be possible by combining the immuno-neurological experiments of Huneeus-Cox, et al.,[42] with appropriately labeled antibody techniques for electron microscopy, possibly using cytochrome-C, which is smaller than the usual ferritin. The typical combination of the antibody or -SH reagent with the electrogenic protein may also make it possible to introduce selective labeling with heavy metals for high-resolution electron microscopy.

Subunit structure of myelin membranes

Earlier investigations[10,11,14,30,33] had shown that the layers of the myelin sheath tend to dissociate into granular or rod-shaped particles of about 60 Å thickness. This transformation occurs following a wide variety of controlled physical and chemical treatments, which include enzymatic digestion, freezing and thawing of nerve, and low-temperature fixation and embedding techniques (Figure 1A). Correlated electron microscopic and X-ray diffraction data also indicated the possibility of a regular organization within the plane of the layers, probably involving units of 60 Å to 80 Å.[33] In vitro nerve degeneration studies[4,6] have disclosed various forms of granular dissociation in the lamellae that closely resemble structures observed after enzymatic digestion. It was determined from low-angle, X-ray diffraction patterns that modifications of the X-ray scattering power within the radial unit corresponded with the rate of degeneration, and were also consistent with the presence of a granular structure in the dense and intermediate layers of the myelin sheath. These results and consistent electron microscopic findings indicate a regular fine structure within the concentric arrays of the dense and intermediate layers (Figure 1A) that warrants careful consideration.

Our investigations made use of cryofixation techniques, which are based on rapid freezing of fresh or glycerinated specimens with liquid helium II at 1° to 2° K, or with undercooled nitrogen.[4,6] This is followed by freeze-substitution and embedding in plastics at low temperatures. Conditions are maintained that minimize ice-crystal formation and artifacts of chemical fixation, extraction, and embedding. These and related techniques for thin sectioning have consistently yielded better morphological and histochemical preservation of all types of lamellar systems and membrane derivatives than have the standard procedures.[4,21,34] As shown in Figure 1A, particulate subunit structures of 60 Å to 80 Å are regularly found, mainly within the plane of the intermediate layers, but also in the dense layers of the well-preserved concentric laminated structure. This type of structure was seen uniformly in all kinds of peripheral and central optic nerve, mainly in frog and rats. It is detected in cross sections, longitudinal sections, and

especially in tangential sections revealing face views of the plane of the layers.

Special techniques were also applied in preparing ultrathin, frozen sections of fresh tissue. They were cut in a cryostat with a special microtome and diamond knife at −30 to −180° C.[6,11,15] The specimens often were examined directly without thawing by embedding them in vitrified heavy metal layers. A liquid nitrogen-cooling device was used in conjunction with appropriate low-temperature electron microscopy techniques. Ultrathin frozen sections of fresh frog sciatic nerve, negatively stained with phosphotungstate (Figure 2B), show characteristic repeating particulate units of about 50 Å to 60 Å. These units, localized mainly in the intermediate layers and attached to the dense myelin layers, were consistently found in the negatively stained, unfixed specimens. Bearing in mind inherent artifact possibilities (e.g., myelin figures), these and related methods are now being further investigated in an attempt to examine the fine structure of the myelin sheath under conditions closely approximating its native hydrated state.

Recent work by Branton[44] and Moor,[45] using the freeze-etching technique, show that the fracture faces of completely myelinated membranes generally appear smooth and relatively free of particles, in contrast to the inner faces of all other membranes, which show distinct particulate components. These findings are of considerable significance, because no heavy metal staining is involved. How-

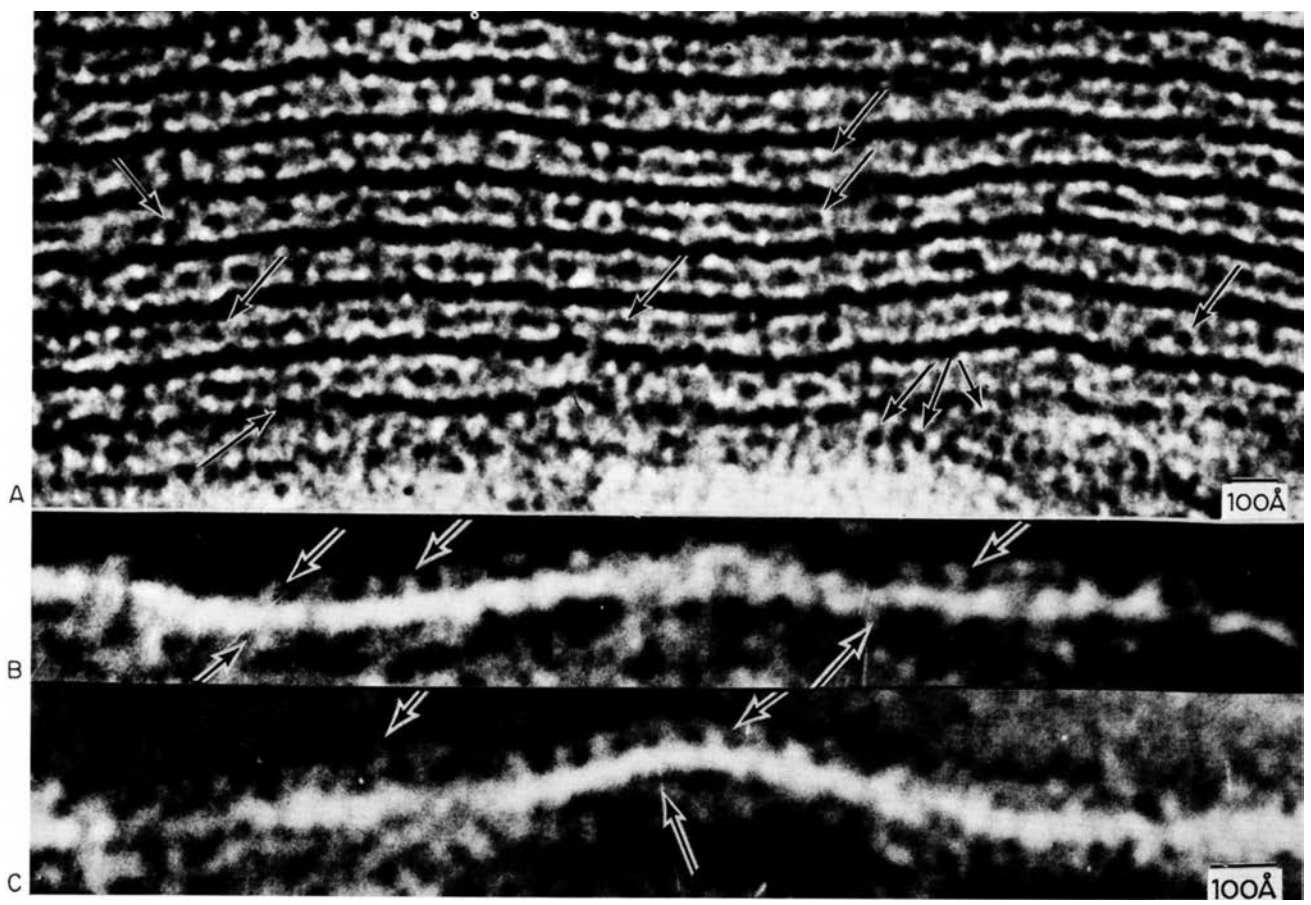


FIGURE 2 A: High-resolution micrograph of thin myelin sheath segment from transverse section of frog sciatic nerve, fixed and embedded at low temperatures. The characteristic particulate fine structure (arrows), detected mainly in the dense, intermediate layers, is also revealed by other techniques yielding improved preservation of the highly regular laminated sheath structure. × 600,000. B,C: Ultrathin frozen section of fresh frog sciatic nerve negatively stained with phosphotungstate and examined by low-temperature electron microscopy. These repeating particulate units (arrows) attached to the dense myelin layers have consistently been found only in negatively stained, unfixed specimens. Bearing in mind the inherent artifact possibilities, their possible relationship to the subunit structures, already shown by other methods, is now being further investigated. × 870,000.

ever, this involves pretreatment of nerves in 20 per cent glycerol, followed by freezing, which is seen in our earlier low-temperature, X-ray diffraction studies[4,6] to produce characteristic modifications in the hydrated lipoprotein constituents, often with underlying drastic alterations of the hydrated matrix permeating the entire sheath. In view of the limited level of resolution and the degree of possible artifacts inherent in freeze etching when dealing with such fine structures, further work is clearly indicated.

Organization of membranes in photoreceptors

Membranes of the vertebrate photoreceptors comprise "unit disks," which are stacked in regular order in the outer segments. Earlier polarized light studies[8] indicated that the rod outer segments consist of thin, transversely arranged protein layers that alternate with longitudinally oriented layers of lipid molecules. Electron microscopy has confirmed this concept by revealing the fine structure of the outer segment and its composition of several hundred unit disks about 150 Å to 200 Å thick.[2,4,30,49]

Improved low-temperature electron microscopy techniques revealed an intermediate layer between the dense layers and, within the layers, electron-dense structures that appear as globular subunits (Figure 3A).[6,21] These subunits look well organized in dark-adapted outer segments, and may be related to the presence of the photopigment complexes, as suggested by collateral data from polarized light analysis and experimental modifications. Rhodopsin represents about 40 per cent of the dry weight in frog retinal rod outer segments, so it may be regarded as a principal structural component of the photoreceptor membrane system.

Because of its inherent uncertainties, however, electron microscopical data on the fine structure of membranes in vivo cannot be considered reliable unless confirmed by an independent technique such as X-ray diffraction analysis. Previous studies of this type[50] demonstrated a spacing of about 320 Å, which was tentatively correlated with the repeating units observed along the axis of the rod outer segments in osmium-fixed preparations. Although these low-angle, X-ray diffraction patterns revealed layering of the membranes and the intermembrane spacings, they did not yield any specific information on the structure within the plane of the membranes. Recent low-angle, X-ray diffraction studies by Blasie and Dewey, Blaurock and

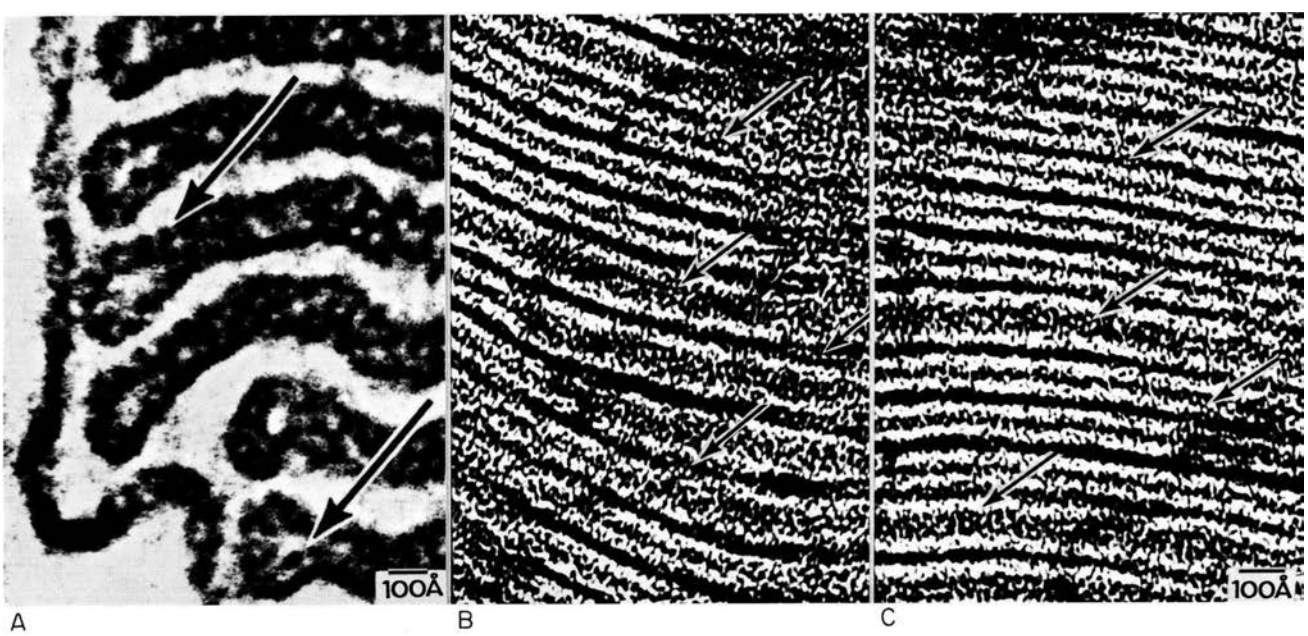


FIGURE 3 High-resolution electron micrographs of thin sections of frog retinal rod outer segments. A: Osmium-fixed, low-temperature preparation of dark-adapted retina showing arrays of dense particles about 40 Å in diameter (arrows) within the compound membrane of the unit disks. × 500,000. B,C: Ultrathin sections of fresh, unfixed, light-adapted rod outer segments prepared without embedding by improved techniques, and stained with uranyl formate. The ordered spherical particles with indications of substructure of 10–15 Å (arrows) can be detected in the dense and intermediate membrane layers. The presence of an ordered substructure within these membranes is substantiated by recent X-ray diffraction studies. × 600,000.

Worthington[51] have substantiated the presence of an orderly, globular substructure within isolated outer segment membranes of photoreceptors in frog retina.

The X-ray diffraction patterns gave reflections that are consistent with a square array of particles with a unit cell size of 70 Å within the membranes. Low-angle, X-ray diffraction showed periodicities in isolated outer segments to be identical with those of intact outer segments from the retinal receptors. The authors also observed an orderly arrangement of particles about 40 Å in diameter within negatively stained preparations of outer segment membranes. However, our limited knowledge of the chemical composition of photoreceptors precludes interpretation of these particulate subunits in terms of specific photopigments or associated multienzyme components.

We have used special preparation techniques and low-temperature electron microscopy to examine outer segments of the frog retinal rod. Fresh, ultrathin sections of light and dark-adapted segments were prepared without fixing and without embedding. The specimens were kept frozen or dried, and were stained negatively or positively with buffered phosphotungstate, uranyl formate, and other electron-dense reagents. Additional structural detail can be detected in these well-preserved preparations (Figure 3B, C). The globular subunits (60 Å to 70 Å), which are prominent in the dense and intermediate layers, appear to be built up of minute particles, about 10 Å to 15 Å, regularly arranged in clusters or rows. Symmetrical alignment of these particles is usually maintained, even in certain regions where the dense layers are split.

These preparation techniques, which combine the advantages of thin sectioning with the enhanced preservation of negative staining applied to native, unextracted specimens, are particularly suitable for correlated biochemical studies.[6,15] Moreover, when used in combination with enzymological assays of isolated retinal outer segments,[52] it should be possible to obtain essential data on the localization of the visual pigments, with their associated lipoprotein components and specific enzyme systems.

Invertebrate photoreceptors

Invertebrate photoreceptors are essentially composed of highly ordered tubular compartments, which are considered differentiated membrane extensions, or microvilli, of the retinal cell membrane. Closely packed arrays of these tubules (*ca.* 400 Å to 1000 Å in diameter) comprise the radially disposed rhabdomeres of the retinula cells in each ommatidium of the insect compound eye.

Earlier electron microscope studies[53-55] and more recent electrophysiological investigations by Waterman, et al.,[56] point to the complex arrangement of photopigments within the plane of the membranes as a key to an integral understanding of the photoreceptor system. For example, the remarkable capacity of the insect eye for analyzing polarized light depends essentially on the dichroism of the photopigment molecules, which must possess a highly ordered "paracrystalline" arrangement within the microvilli in order to provide the molecular basis for differential sensitivity to linearly polarized light. The precise orientation of the many thousands of closely packed tubular compartments in a single rhabdomere comprise a unit dichroic analyzer at the cellular and subcellular levels. Recent data by Waterman and Horch,[56] which includes a model for a two-channel polarization analyzer, indicates that one ommatidium suffices for detecting small e-vector orientations.

The differentiated, membrane-bound components in these photoreceptors display a highly ordered and symmetrical arrangement throughout the various dimensional levels of organization. Ultimately, however, this exquisitely built, compact system owes its ability to extract optimum information, through selective interaction with incoming light signals, to the precise three-dimensional arrangement of the photopigment molecules within the membranes.

Macromolecular repeating units of structure and function in mitochondrial membranes

A large body of evidence has accumulated during the past decade[4,6,15-18] showing that the electron transfer system, the respiratory transformations, and certain other systems of the mitochondrion are built up from characteristic macromolecular complexes. Large-scale isolation of stable mitochondria, under conditions that do not impair their main enzymatic activities, plays a significant role by making these membraneous organelles and their constituents available for biochemical analysis.

Correlated electron microscopic and biochemical studies[6,15] have resulted in the detection of a macromolecular repeating particle associated with the cristae and the inner membrane of the external mitochondrial envelope. By using negative staining and other preparation techniques, many thousands of these particles (*ca.* 10^4 to 10^5) are found disposed in regular arrays in a single mitochondrion. The repeating particle, designated as the mitochondrial elementary particle (EP), consists of three parts: a spherical or polyhedral head piece, 80 Å to 100 Å in diameter; a stalk (*ca.* 50 Å long); and a base piece (40 Å x 110 Å). As shown in Figures 4A and 4B, the base pieces of the elementary particles form an integral part of the outer dense layers of the cristae. These characteristic repeating particles are particularly well demonstrated in negatively stained specimens of mitochondria *in situ*, of isolated mitochondria,

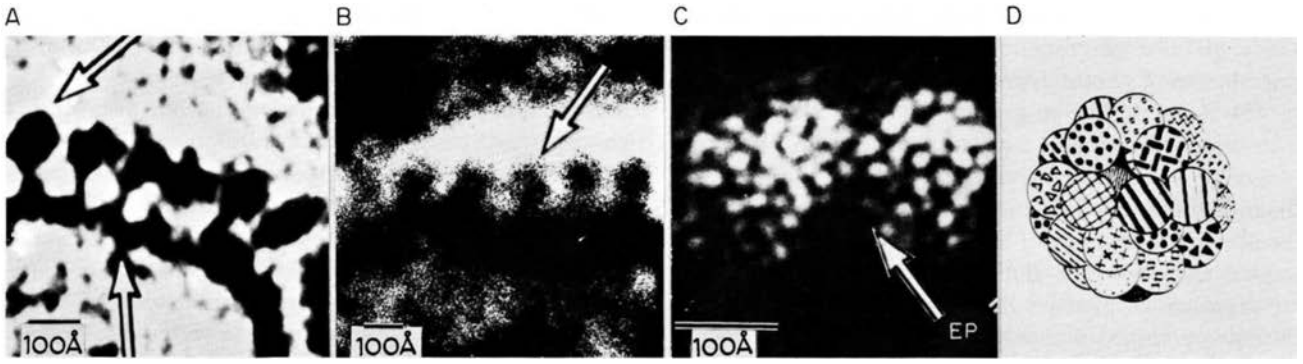


FIGURE 4 Electron micrographs of (A) enlarged segment of crista from negatively stained beef heart mitochondrion, demonstrating three parts of the elementary particle (EP): head piece, stalk, base piece, × 700,000. (B) Segment of crista from thin mitochondrial section showing arrays of similar particles, × 500,000. (C) Particulate units of approximately the same size as the EP, containing the electron transfer chain, isolated from beef heart mitochondria by Dr. D. E. Green, et al., × 1,330,000. (D) Diagramatic illustration of a macromolecular assembly containing many species of macromolecular components. (Courtesy of Professor F. O. Schmitt)

and of submitochondrial membrane fragments with a complete electron transfer chain.

Following my first observations,[6,14,15] numerous investigators[6-18,60,61] confirmed the existence of the repeating particles and the underlying subunit organization of mitochondrial membranes through the use of a wide variety of techniques, including the improved freeze-etching method applied by Moor and Mühlethaler.[62] This technique operates on native frozen specimens and does not involve the use of chemical fixatives or stains, so the successful demonstration of globular repeating units in mitochondria and other membrane systems has effectively disposed of tentative suppositions[63] that these characteristic units might be preparative artifacts. It is, therefore, now generally agreed by most workers[61,64-66] that the mitochondrial subunits exist, although the observed structural details depend on the preparation techniques and experimental conditions.

Detection of these subunits suggested the existence of counterpart macromolecular functional units. A particulate unit containing a complete electron transfer chain was isolated from beef heart mitochondria.[15,59] The repeating unit has a molecular weight of about 1.3×10^6 (corresponding to approximately 40 molecules of protein and 400 molecules of phospholipids). The experimental evidence obtained from our studies was consistent with the notion that the isolated (Figure 4C) and reconstituted particles could be identified with the elementary particles visualized *in situ*. It was also assumed that the isolated elementary particles contained the complete electron transfer chain.

Although subsequent studies by Green and Perdue[17] indicate that there may be multiple species of mitochondrial elementary particles, the basic concept of a macromolecular repeating unit of mitochondrial structure and function is now generally accepted. The elementary particle of the mitochondrion is believed to be a prototype of a class of functional particles or macromolecular assemblies found in association with membranes generally.

Macromolecular repeating units in chloroplast membranes and other membranes

Investigations by Park and Pon and others[19,20] have led to the identification in the chloroplast membranes of repeating units called quantasomes that may be the morphological expression of the physiological photosynthetic unit. X-ray diffraction work by Kreutz also indicates that the chloroplast lamellae are built up of repeating units about 71 Å in diameter. The molecular weight of a single quantasome is 2×10^6. The interesting relationship between the quantasome of chloroplasts and the elementary particle of mitochondria has been pointed out by Park, et al.[20] Both are associated with the electron-transport system in membranes, and both contain certain cytochromes, substituted benzoquinones, and non-heme iron. Morphological differences have been revealed, however, in that four or more subunits appear in the quantasome. Branton and Park[46] show a second type of subunit, 110 Å in average diameter, which forms part of a matrix around the larger 175 Å quantasome units. It is concluded that the chloroplast lamellae consist of a matrix within which are densely packed subunits that form the major constituents of the photosynthetic membrane.

Plasma membranes of liver cells,[26,27] microvilli,[25] microsomal membranes,[45] bacterial membranes,[17] and synaptic membranes[47] are among the different types in which repeating units have been observed. In general, there is evidence that such units appear in all membranes and either make up the entire membrane or comprise a major part of the membrane framework in close association with the bilayer lipid structure. Although these repeating units are of variable shape and size, with a range of molecular weights from 50,000 to several million, all have significant features in common with the macromolecular assemblies conceived by Schmitt[48] as distinctive hierarchial units of structure and function (Figure 4D) invested with unique properties.

It has been shown that enzymes constitute a large proportion of the total protein, particularly of specialized, energy-transducing, mitochondrial membranes. This catalytic protein may therefore be regarded as the key determinant of the structural and functional organization of membrane systems. In all such systems, including nerve membranes, specific enzymes or enzyme complexes are attached to, or are otherwise intimately associated with, the basic membrane framework in repetitive patterns of exquisite, three-dimensional, stereospecific configuration.

Therefore, the concept of macromolecular repeating units or assemblies in membranes can be regarded in many ways as an extension of the unit membrane hypothesis of Davson, Danielli,[12] and Robertson,[13] which represents a necessary and logical transition consistent with present biochemical and physicochemical evidence.

Macromolecular organization of enzyme systems

PYRUVATE AND α-KETOGLUTARATE DEHYDROGENATION COMPLEXES A unique opportunity to correlate functional properties with ultrastructure has recently been provided by biochemical and electron microscopic studies of the pyruvate dehydrogenase complex (PDC) of *Escherichia coli*.[70] This well-characterized multienzyme complex, with a molecular weight of about 4.8 million, has been isolated in highly purified form and analyzed by Lester Reed and associates.[67,68]

PDC catalyzes a multistage oxidative decarboxylation of pyruvate,[67,71] and is composed of three enzymes. Each molecule of the PDC complex contains about 16 molecules of pyruvate decarboxylase (molecular weight 183,000), 8 molecules of dihydrolipoic dehydrogenase (molecular weight 112,000), and about 64 molecules of lipoic reductase-transacetylase (LRT). Reed and his associates have also succeeded in reconstituting the complex from the isolated enzymes.[68] The concept of the organization of the PDC complex that emerged from these classical biochemical studies is that of an organized assembly of enzymes in which each of the constituent enzymes is specifically located to permit efficient implementation of a consecutive reaction sequence.

Our correlative electron microscope studies[69,70] have confirmed and extended this picture through direct visualization of the exquisite molecular architecture of the PDC complex and its components. The negatively stained PDC complex appears in electron micrographs (Figure 5A) as polyhedric particles of about 300 Å to 400 Å in diameter surrounding this central tetrad (Figure 5B).

Electron microscopy of the isolated LRT aggregate revealed a typical tetrad structure that closely resembles the central tetrad of the native PDC complex and appears to determine its over-all structure. According to the model derived from the biochemical and electron-microscopic data, lipoic reductase transacetylase (LRT) is composed of 64 identical subunits situated at the eight vertices of a cube, while the molecules of pyruvate decarboxylase and dihydrolipoic dehydrogenase are respectively aligned on the twelve edges and in the six faces of the cube.[70,71]

The macromolecular organization of the α-ketoglutarate dehydrogenase complex (KGDC) isolated from *E. coli* appears to be similar to that of the PDC.[71,72] As shown by electron microscopy (Figures 5C and 5D), the smaller polyhedrical particles, with diameters of about 280 Å, feature a tetrad core surrounded by peripheral subunits, which are not clearly defined as in the PDC complexes. Recent studies by Reed and his associates[71] indicate that the macromolecular organization of the bacterial and mammalian PDC and KGDC multienzyme complexes are fundamentally similar, and may be governed by the principles of self-assembly that determine the functional organization of protein shells of regular viruses with icosahedral symmetry.[73]

ORGANIZATION OF L-GLUTAMATE DEHYDROGENASE L-glutamate dehydrogenase (GDH), which has a molecular weight of one million, consists of four major subunits. This molecule is of interest, as it is known to be reversibly dissociated by certain steroids and such hormones as thyroxin.[74] Disaggregation into much smaller units of about 40,000 molecular weight occurs at high and low *p*H values or on treatment with various agents.

Earlier electron microscope studies by Hall[75] revealed particles about 145 Å wide and 80 Å high, which were obtained from dilute L-glutamate dehydrogenase solution. Valentine[76] observed spherical particles of 125 Å, 90 Å, and 75 Å diameter in negatively stained GDH preparations. Although the objects varied in size, depending on the concentration of the protein, these electron micrographs were consistent with the concept of the undisso-

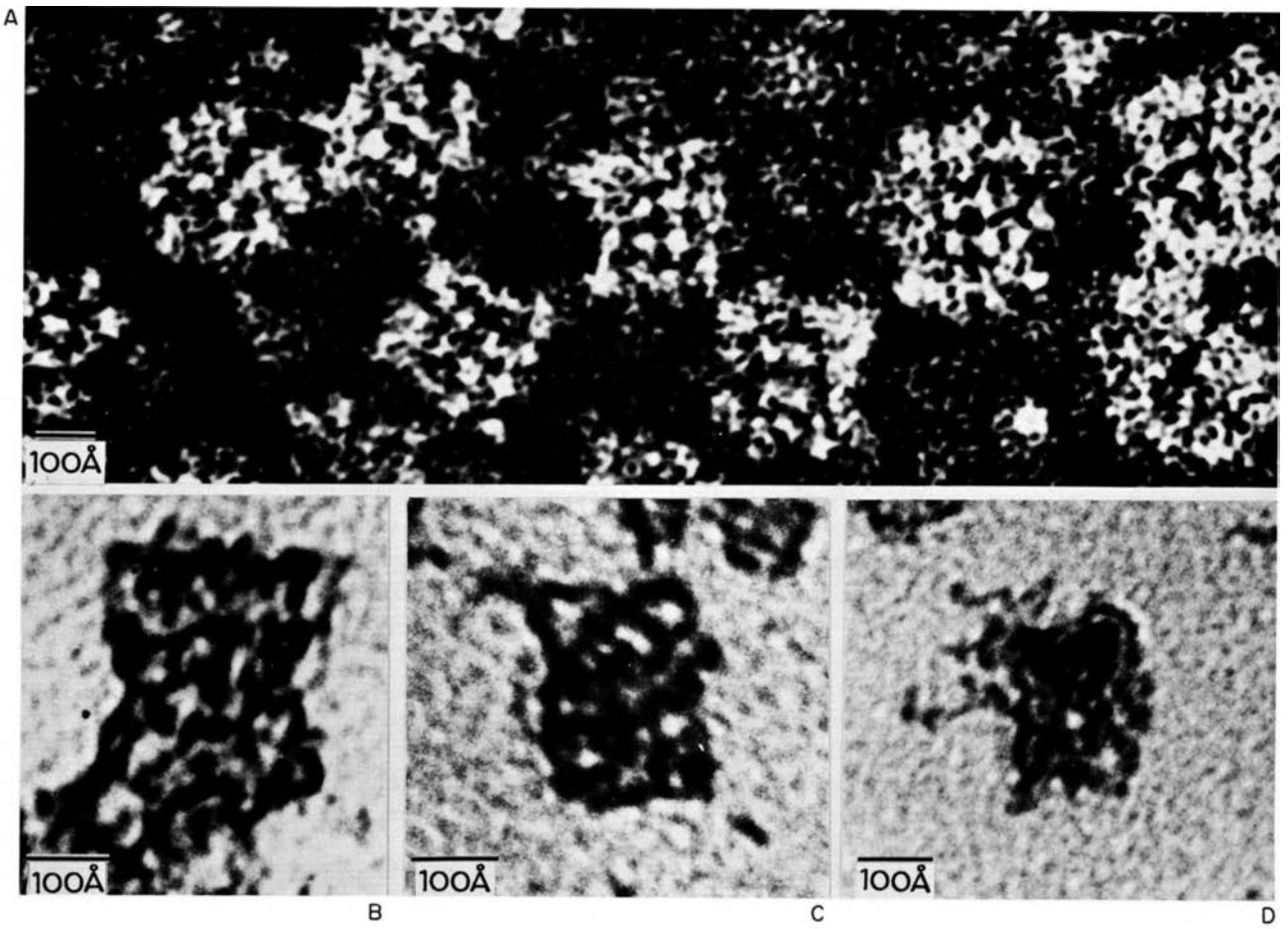


FIGURE 5 (A) *E. coli* pyruvate dehydrogenase complex (PDC) prepared by Dr. L. J. Reed, et al., showing characteristic central tetrad and associated subunits of this multienzyme complex negatively stained with phosphotungstate, and (B) positively stained with uranyl acetate. × 870,000. C,D: *E. coli* α-ketoglutarate dehydrogenase complex (KGDC), isolated in Dr. Reed's laboratory, displaying similar structural features of the positively stained particles in different orientations. × 1,100,000.

ciated molecule as a tetramer. Subsequent observations by Horne and Greville[77] indicate that the molecules tend to dissociate into typical tetrahedral subunits. However, attempts to observe the undissociated molecules have been hampered by technical difficulties.

Our recent investigations of bovine L-glutamate dehydrogenase crystals,[78] using microdroplet cross-spraying techniques and microbeam illumination for high resolution electron microscopy, have made it possible to observe the ordered aggregation and fine structure of the relatively well-preserved molecules in the thin microcrystals. As shown in Figures 6 A,B,C,D, individual spherical or polyhedral particles of diameter 130 Å to 150 Å frequently can be observed, either free (Figure 6 C,D) or closely packed in ordered aggregates (Figure 6B) of the thin crystalline strands. The individual polyhedral particles of 130 Å to 150 Å diameter may correspond to the undissociated individual molecules, while the numerous subunits of triangular shape could correspond partly to the GDH tetrahedral subunits described by Horne and Greville.[77] However, on the basis of available data, the organization of the undissociated molecules appears to be more complex than the suggested simple aggregate of four subunits. Further studies are being pursued in attempts to elucidate the detailed subunit structure of these enzyme molecules. Investigation of the postulated changes in enzyme activity that may be produced by alterations of their subunit conformation may also be of significance in relation to fundamental controlling mechanism in cells. Moreover, this enzyme is also of interest as a model system to illustrate the different stages of molecular and supramolecular organization in the observed paracrystalline arrays, which

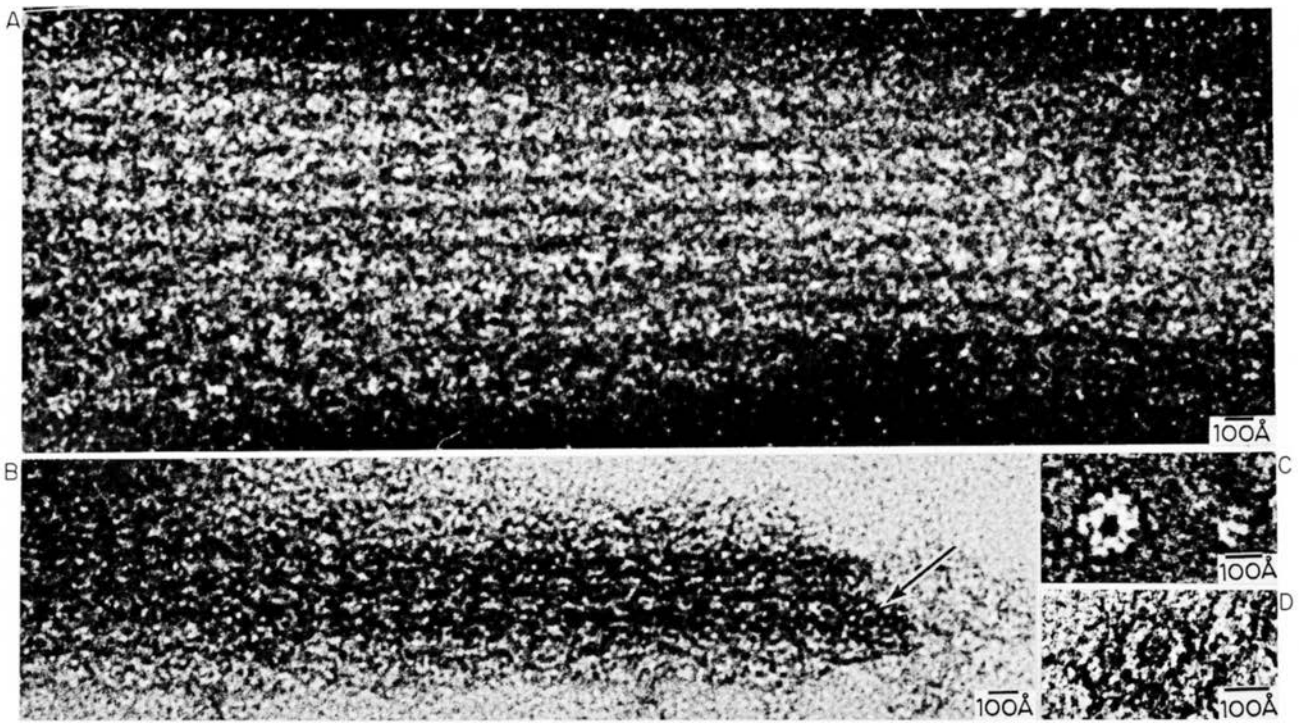


FIGURE 6 Bovine L-glutamate dehydrogenase (GDH) (Boehringer and Soehne, Mannheim). A: Microcrystals, negatively stained with uranyl formate by microdroplet cross-spraying technique, showing typical periodic lattice formed through ordered aggregation of the constituent molecules. × 400,000. B: In thinner, ribbon-like strands the regular substructure of individual molecules (arrow) can be discerned. × 400,000. C,D: As a result of the improved preservation and enhanced resolution achieved in these preparations, undissociated individual molecules (*ca.* 1 million molecular weight) are frequently detected, appearing as spherical or polyhedral particles of diameter 130 to 150 Å in different orientations. Subunits of triangular shape and size are also found. × 600,000. In addition to its specific value, investigation of this enzyme is also of interest as a model system to illustrate the different states of molecular and supramolecular association bearing on the general organization of enzyme complexes in membranes.

are relevant to the general problem of the organization of enzyme complexes in membranes.

FRACTION-I PROTEIN Another example of the characterization of the substructure of a protein enzyme can be achieved by combined electron microscopic and biochemical studies. Fraction-I protein, a major soluble protein fraction located predominantly in the chloroplast and comprising at least 50 per cent of the soluble proteins, has marked carboxydismutase activity. Recent studies in collaboration with Haselkorn, Kieras, and van Bruggen[79] show that Fraction-I protein of Chinese cabbage leaves consists of uniform cubical particles (Figure 7A) with an edge of about 120 Å.

In high-resolution electron micrographs of negatively stained preparations (Figure 7B), the cubical particles exhibit a characteristic subunit structure consistent with a model having 24 subunits; this agrees with the present physical and chemical data. In the proposed model, the subunits may undergo considerable conformational changes, and do not appear to be related by the principle of quasi-equivalence, which governs the structural organization of many of the regular viruses. That the substructure of a protein enzyme of only 120 Å can now be directly observed in electron micrographs is of particular relevance to investigation of the enzyme complexes of similar size, which are presumably associated with nerve membranes and derivatives.

Subunit patterns of neuronal membranes

STRUCTURAL SUBUNIT PATTERNS OF SYNAPTIC MEMBRANES Investigation of the molecular organization of the synapse is of particular significance in view of the specialized molecular switching componentry assumed to mediate information storage, transfer, and retrieval at these junctional regions.

Unfortunately, neuronal membranes have not yet

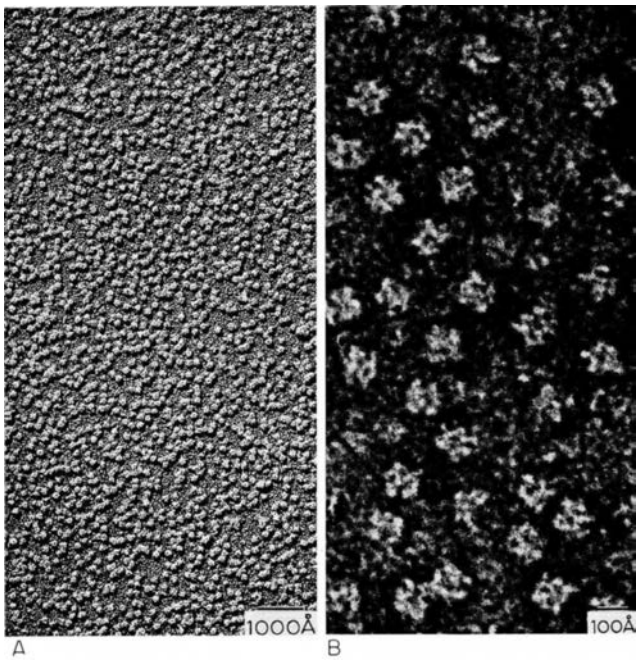


FIGURE 7 A: Shadow-cast particles of Fraction I protein isolated from Chinese cabbage leaves by F. J. Kieras and R. Haselkorn. This major soluble protein fraction, located predominantly in the chloroplasts, has carboxydismutase activity. × 60,000. B: As seen in negatively stained preparations, the individual particle appears to be a cube, about 120 Å along each edge, containing 24 subunits. × 400,000. This is one of the first demonstrations that the substructure of a protein enzyme can be directly resolved in the size range of the enzyme complexes associated with membrane systems.

yielded to correlated biochemical and electron microscopic studies of the type successfully applied to mitochondrial and chloroplast membranes. Difficulties in reliably isolating and preparing these labile structures without introducing serious artifacts impose serious limitations.

Typical subunit structural patterns in certain kinds of synaptic membranes have now been revealed by Robertson.[47] His comprehensive electron microscopic studies demonstrate a subunit pattern in the synaptic membranes of club endings of Mauthner cells in goldfish. Each of the alternating synaptic disks shows an internal beading with a repeat period of 90 Å to 95 Å in vertical sections. Transverse densities or lines spaced at 90 Å are seen in oblique sections, while frontal views of the membranes reveal a honeycomb pattern of lines and dots, approximating a hexagonal array. This network features dense granules in the center of each subunit facet and fine rodlet borders less than 20 Å in diameter. These patterns resemble the structure of Buckminster Fuller's geodesic domes, and it is not impossible that such an efficient design may generally be embodied in the organization of cell membranes and regular virus shells.[47,73]

Similar synaptic subunit structures have been seen in the Mueller cell of goldfish medulla.[47,80] Confirming and extending our earlier results,[6,21] Robertson has demonstrated the existence of a regular subunit structure in the lamellae of retinal rod outer segments and in erythrocyte membranes.[80] This subunit pattern, which can be more readily seen when two membranes are closely apposed, is therefore regarded as a structural pattern of general significance.[6,15,80]

Robertson assumes as a working hypothesis that this pattern represents a structural protein on the outer surface of the membrane.[80] Certain transverse densities give the impression of a transverse globular substructure of the membranes, and are regarded with reservations, as they may prove to be artifacts.[80] However, only further work with improved preparation techniques and appropriate X-ray diffraction analysis can establish or preclude the existence of such a regular subunit organization in native, undegraded neuronal membranes.

Organization of Synaptic Membrane Complexes

Several interesting ultrastructural features have been revealed in synaptic membrane complexes of different types.[80,81] Gray[81] describes synaptic membrane complexes in ventral horn cells of mammalian spinal cord that exhibit 500 Å dense particles in regular hexagonal array with a 1000 Å period. Certain preparation techniques reveal presynaptic fibrils and delicate, hook-like fibrillar extensions from the postsynaptic cytoplasm making contact in the synaptic cleft. In pyramidal cell synapses a patched differentiation of the synaptolemma is observed with two types of interlemmal elements that repeat at periods of about 235 Å.[80] An unusual, subsynaptic formation has been described by Taxi[80,81] in autonomic ganglia of certain amphibia. This complex is characterized by subsynaptic dense bands and granular regions that form a triangle with the subsynaptic membrane as its base.

Identification and isolation of the specific macromolecular componentry subserving the complex processes at these junctional regions appears to be a most worthwhile goal, requiring the full repertory of ultrastructural, chemical, and physiological investigations.

Fine Structure of Isolated Neuronal Membranes

The finest nerve fibers and neuronal extensions are fortunately of truly paucimolecular dimensions and, therefore, are ideally suited for direct examination by electron microscopy. Confirming and extending our earlier work,[11,30,82] we found that certain submicroscopic nerve fibers in frog spinal cord were usually attached to the

thicker nerve fibers. These fibers are thin enough to display their entire structure in a single electron micrograph. As shown in Figure 8A, these fibers appear as thin ribbons with diameters ranging between 0.1μ and 1μ and reaching lengths up to 50μ–100μ.

Essentially, each fiber, as displayed in a whole-mount preparation, consists of a thin sheath formed by a single, tubular membrane that contains long filaments 100 Å to 200 Å in diameter. In our original shadowed and osmium-fixed preparations, we observed no definite indications of fine, regular structure in these membranes. However, with improved techniques, new ultrastructural detail (Figure 8B) was revealed in these well-preserved, ultrathin nerve membrane specimens. They exhibit a characteristic surface structure featuring, in certain regions, hexagonal arrays of closely packed polygonal elements 50 Å to 90 Å in diameter. Various types of dense granular components are found attached to the membranes, particularly in junctional regions, as shown in Figure 8B, 9A, and 9B.

These techniques can undoubtedly be refined considerably and it should eventually be possible to examine these submicroscopic fibers in vacuum-tight microchambers under conditions approaching the native hydrated state.

The role of water in membranes

Water is a major constituent of nerve membranes, representing at least 35 per cent to 40 per cent, even in condensed, multilayered, fluid-crystalline systems like myelin.[2] The water layers at the aqueous interfaces of the fundamental repeating unit in myelin are about 12 Å to 15 Å thick. Their thickness appears to be determined largely by the electrical charge density at the interfaces, and by the ionic strength.[2,3]

Depending on the different environments, we may envisage various types of water structures, including water within the membrane structure itself, water in direct contact with the ordered macromolecular membrane surfaces, and water layers between closely paired systems of unit membranes.[91] Interconnected water channels within the myelin layers and related lamellar systems play an important role as possible pathways of diffusion of ions and certain molecules between the axon and extracellular fluids.[3]

On the basis of results derived from a broad interdisciplinary approach,[91,98] we must begin to consider water as a structured matrix—"the matrix of life," as was first pointed out by Szent-Györgyi.[89] Substantial experimental evidence is now available in support of a mixture model to account for the unique properties of water.[90] Thus, from neutron-scattering data[97] and other studies of water, we are now able to envisage a sample of liquid water as being made up of "flickering clusters" of about 50 to 100 molecules with an average lifetime of 10^{-11} seconds, which is long enough for a structure to have distinguishable existence.

The concept of organized water as an integral structural component of biological membranes must be given serious consideration.[2,6,88] We must assume that ordered water will become specifically integrated in the highly organized, three-dimensional, macromolecular structures of living systems. The abundance of postulated bulky species of water offers numerous possibilities, ranging from the crystalline hydrate structures to other frameworks of approximately tetrahedral, fourfold coordination.

Based on our earlier correlated studies of water, in which we used nondestructive methods such as nuclear magnetic resonance[2,4,34] and X-ray diffraction techniques,[4,14] we developed the concept of a three-dimensional, hydrated, lipoprotein system that provides a general structural framework for specialized macromolecular repeating units periodically arranged in the membrane layers.[4-6,15] Although the main functional differences between membranes would depend upon the types of transducing units present and the complementary stereospecific configuration of the underlying substrate, this hydrated lipoprotein matrix could introduce a common unifying feature. The water, particularly in close association with the ordered lipoprotein membrane systems, must likewise be highly ordered, resembling "ice-like" hydration sheaths or crystalline hydrate lattices.

The open structure of water favors the formation of these crystalline hydrates,[94] which are clathrate compounds with a hydrogen-bonded, ice-like framework defining cavities able to enclose molecules of noble gases, liquids, ions, protein side chains, etc., of appropriate size (4 Å to 6.9 Å). The hydrogen-bonded lattice of these microcrystalline hydrates, which are about 12 Å to 24 Å, is stabilized by van der Waals interaction, in contrast to ordinary ice. The hydrate crystals may therefore remain stable even in the range of normal body temperatures under special conditions. As suggested earlier,[5,6,87,88] these polyhedral hydrate structures with numerous built-in cavities could effectively enclose ions, or form closely fitting "molecular replicas" around protein side chains, thus adapting to the specific macromolecular configurations by three-dimensional interpenetration. Crystalline hydrate structures of this general type, or equivalent organized water structures permeating the ordered, lipoprotein-subunit, macromolecular assembly system, would provide an interconnected H-bonded substrate for fast protonic charge-transport mechanisms.[100]

Localized reversible phase changes in such ordered

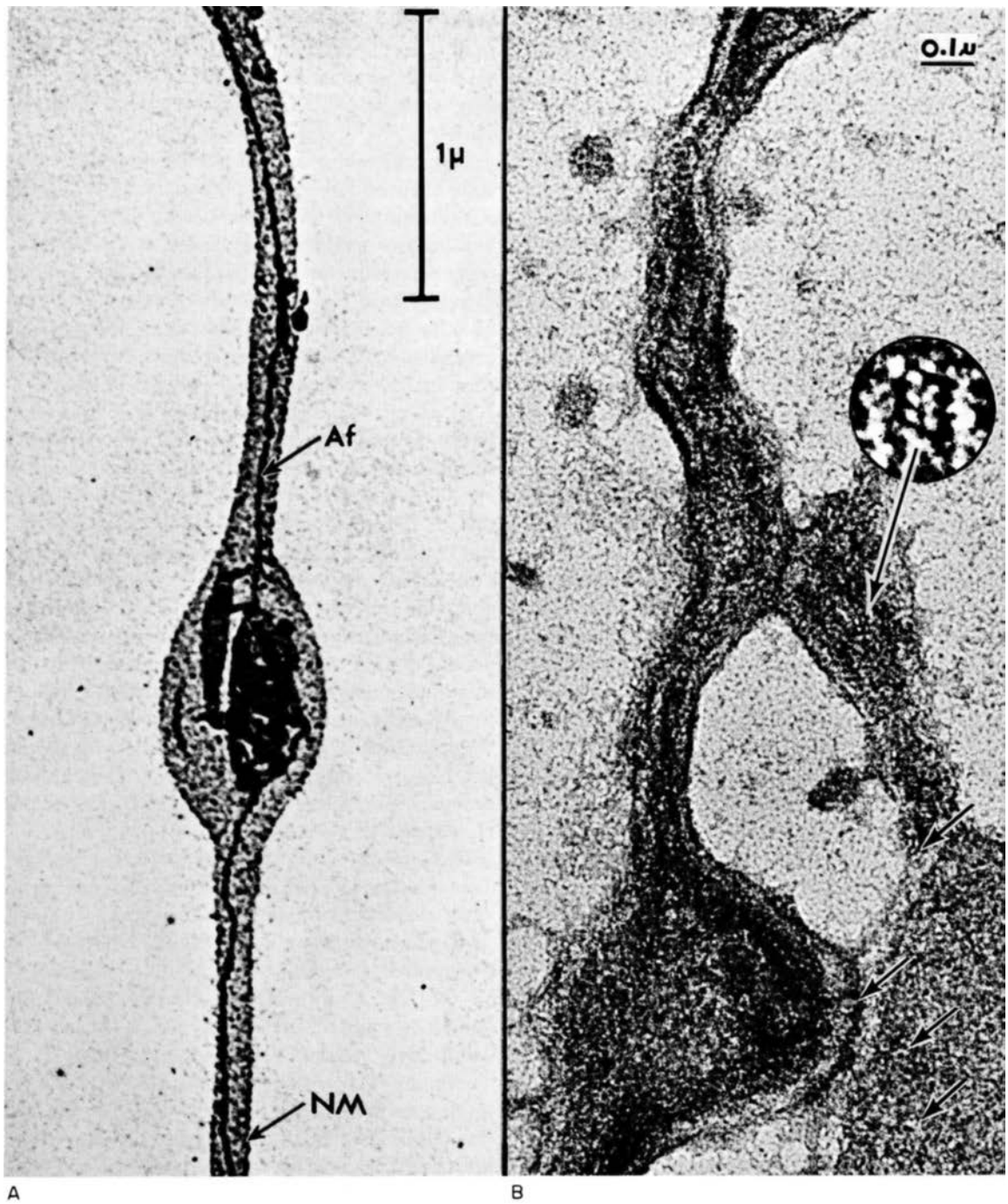


FIGURE 8 A: Fine submicroscopic nerve fiber from the lateral funiculus of the frog spinal cord. The fiber shaft has a width of 1000 Å and consists of a flattened, ultrathin tubular membrane (NM) enclosing an individual axon filament (Af) 200 Å in diameter. × 43,000. B: Submicroscopic nerve fiber ending and associated membrane components isolated from fresh frog spinal cord, stained with uranyl formate and examined by low-temperature electron microscopy. These well-preserved ultrathin nerve membrane preparations exhibit a characteristic surface structure, featuring in certain regions hexagonal arrays of closely packed polygonal elements, 60 to 90 Å in diameter (insert). Various types of larger particulate components (arrows) are found attached to the membranes, particularly in junctional regions. × 180,000. Insert: × 400,000.

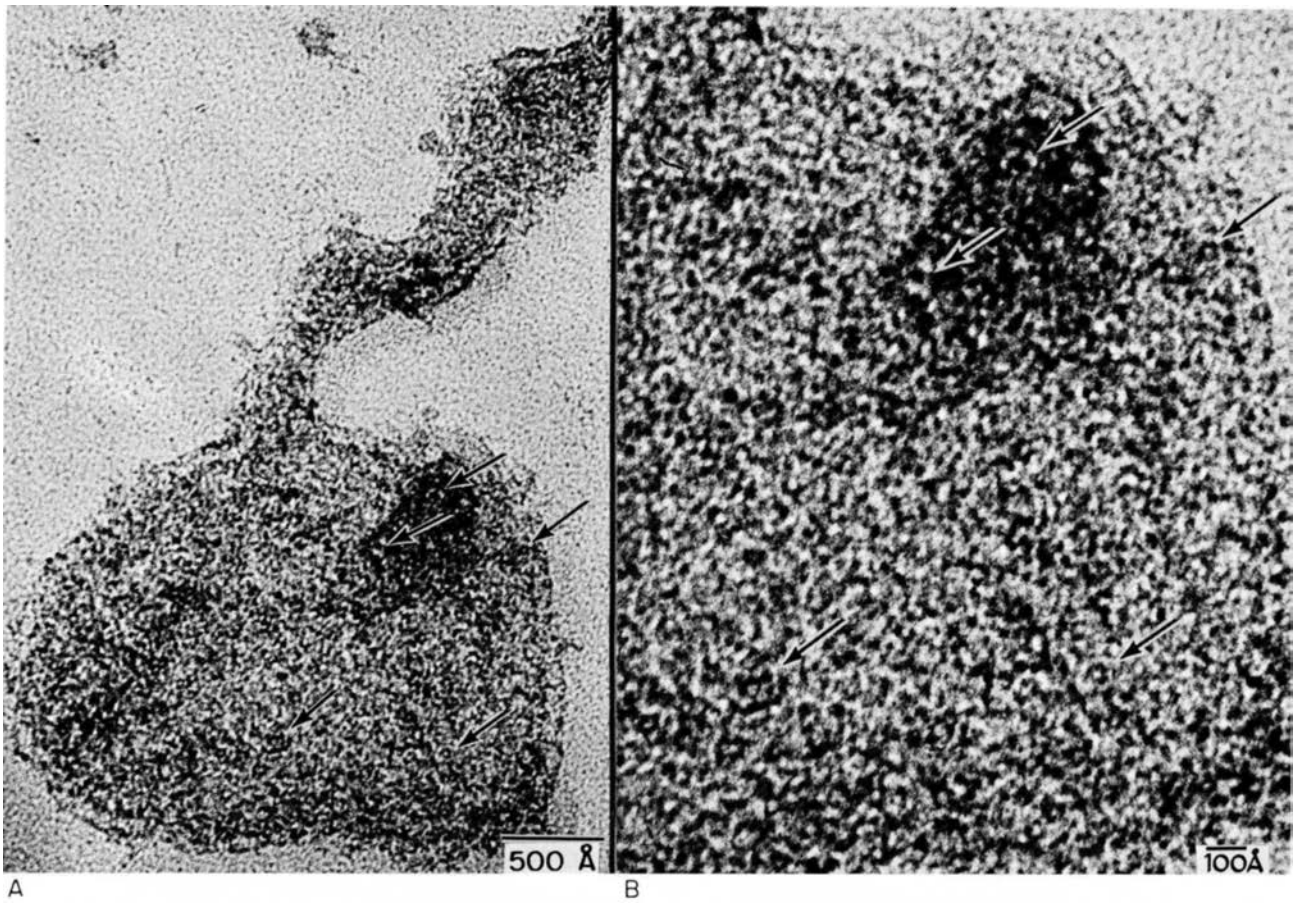


FIGURE 9 A: Submicroscopic nerve fiber ending isolated from fresh frog spinal cord using improved preparation techniques, which permit examination of whole mounts of ultrathin nerve membranes, without introducing the fixation, extraction, and embedding artifacts inherent in standard thin-sectioning methods. Under these favorable conditions an underlying subunit structure of the membrane surfaces can be resolved, with associated electron-dense components (arrows). × 270,000. B: As shown in the enlarged segment of this membrane, numerous polygonal components with electron-dense or annular cores (arrows), 100 to 200 Å in diameter, contribute to the mosaic-like surface pattern. × 530,000. Further methodological improvements should eventually make it possible to observe directly the molecular organization of individual nerve membranes in their native hydrated state.

water structures could also provide the basis for a variety of conformational changes in protein and lipoprotein layers, including reversible transitions from coherent bimolecular lipid layers to the subunit micellar arrays. Reversible phase transitions of this kind, spreading through the membrane matrix, may contribute significantly to the propagation of local perturbations. Also, selective permeability may be envisaged in terms of molecular "pores" lined with ordered water. In general, this concept of ordered water lattices forming an integral structural component of membranes provides a powerful conceptual basis for understanding many fundamental mechanisms of membrane function,[91,98] as pointed out by Hechter.

The precise molecular nature of the ordered water in membranes is, however, still unknown. Nevertheless, nuclear magnetic resonance techniques have been successfully applied in studies on the hydration structure of fibrous macromolecules.[99] Despite the uncertainties involved, there is recent evidence supporting the structural role of water in membrane systems. For example, Person and Zipper have shown that treatment of mitochondrial suspensions with a dry zeolite, which clathrates the water, produces the same type of membrane disruption as is usually achieved with detergents. This novel form of disruption can, therefore, be attributed to the avidity of the zeolite preparation for water, and its capacity to remove certain divalent cations selectively.

Extending our earlier work,[88,92,93] we have tried to ob-

tain direct experimental verification of organized water structures of the crystalline hydrate type through controlled local formation of microcrystalline, noble gas hydrates in selected lipid, lipoprotein complexes, and cell membranes.[98] As shown in Figure 10B, characteristic electron-dense microcrystalline hydrates, about 10 Å to 20 Å in diameter, are localized mainly in the hydrophilic regions of lipid micelles following application of argon and xenon under controlled high pressure and temperature in special specimen chambers. The noble gas atoms hereby occupy polyhedral cavities in a hydrogen-bonded framework of water molecules, contributing to stabilize and make visible electron optically any pre-existing organized water structures of the hydrate type in lipid or lipoprotein model systems.

These techniques for direct visualization of noble gas hydrates could eventually provide experimental data bearing on the existence of the hydrate microcrystals reversibly forming in cell membranes. This approach is relevant to the molecular theory of general anesthesia postulated by Pauling.[95] According to this theory, the formation of clathrate-like structures by anesthetic agents, including noble gases, in the aqueous portions of nervous tissues could cause anesthesia by modifying the electrical activity at junctional regions through entrapment of ions and electrically charged side chains of protein molecules. The formation of these hydrates would increase the structured water in the membrane systems, thus presumably making the water at synapses less available for facilitating conduction.

One might even go beyond this theory of Pauling and —in common with the earlier hypotheses of anesthesia advanced by Claude Bernard[96]—postulate reversible phase changes or "coagulations" in the synaptic membranes. It might not be unreasonable to assume that these reversible transformations in nerve membranes are not only associated with anesthesia, but perhaps also with certain rhythmic changes, such as sleep.

Reversible modifications of the molecular organization involving primarily the hydrated lipoprotein framework

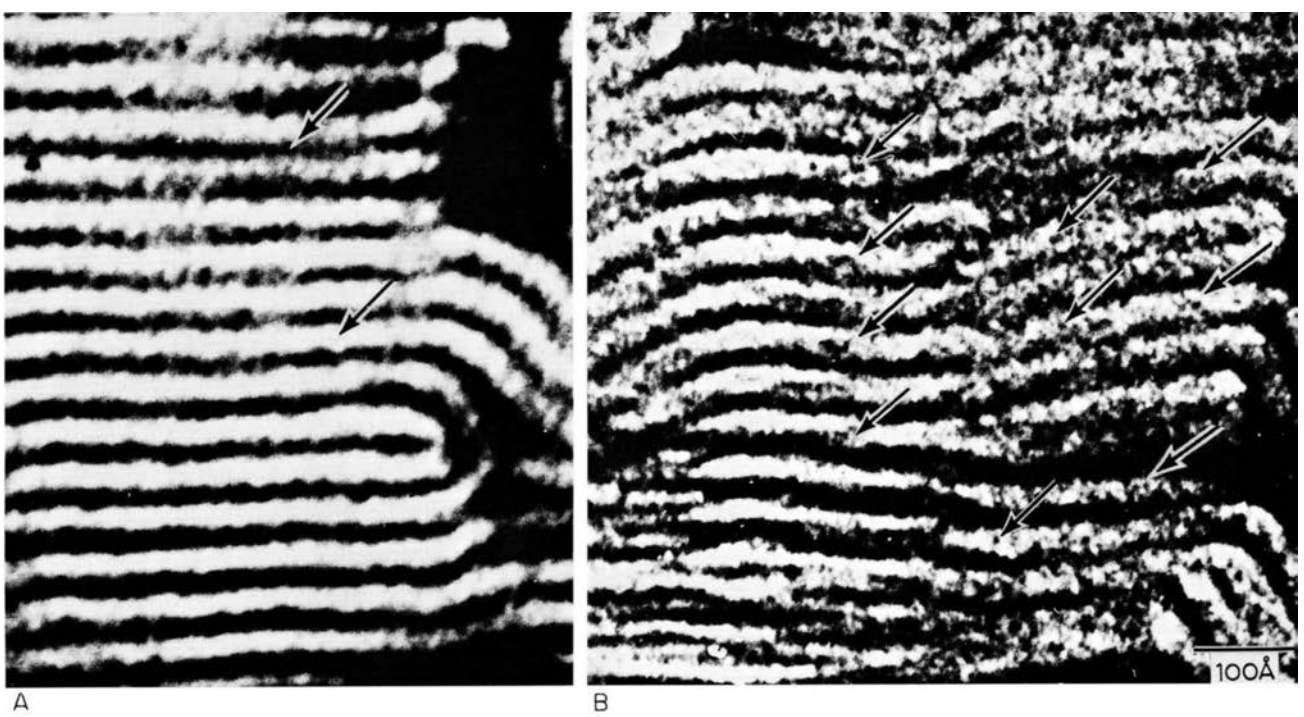


FIGURE 10 Low-temperature electron micrographs. A: Lecithin micelles embedded in thin, buffered phosphotungstate film, showing periodic dense lines separated by light bands corresponding to the hydrophilic and hydrophobic regions of adjacent bimolecular leaflets. B: Lecithin micelles similarly embedded in thin phosphotungstate film, and exposed to argon gas under pressure, showing dense particulate aggregates (arrows) along the dense and intermediate layers, which may represent the resulting argon hydrate microcrystals in the size range of 10–20 Å. × 1,330,000. Model experiments of this kind could provide direct experimental evidence for the existence of hydrate microcrystals forming reversibly in nerve membranes, as first postulated by L. Pauling.

of nerve membranes must, therefore, ultimately take into account the structure and cooperative ordering of water.

The biosynthesis of membranes

Membrane biosynthesis is of fundamental importance for all biological studies concerned with embryogenesis, morphogenesis, and differentiation. The available evidence indicates that characteristic mechanisms are involved in the biosynthesis of the protein and lipid membrane components, and also suggests that membranes may furnish the template for their "self-duplication."[109,110,122]

Investigation of nerve membrane biosynthesis may prove to be of particular value in elucidating the synthesis and assembly of the repeating subunits and their organization within the plane of the membrane layers. The nature of the association of cell membranes with the protein synthetic machinery and nucleic acids is therefore being studied by electron microscopy and biochemical techniques to serve as a basis for a better understanding of membrane biosynthesis.

MITOCHONDRIAL DNA, RNA AND PROTEIN BIOSYNTHESIS SYSTEM IN MEMBRANE REPLICATION The discovery of characteristic nucleic acids in mitochondria[101-103] and the demonstration of a protein biosynthesis system have recently confirmed and extended earlier work[102] to provide significant support for the semi-independence or autonomy of these organelles. DNA of mitochondria from various mammalian cell types[103-104] differ from nuclear DNA in guanine plus cytosine content, and in their renaturation properties. In contrast to nuclear DNA, which is linear, the molecules of mitochondrial DNA exist in circular form, their mean contour lengths measuring 5.24μ–5.45μ. Two to six ring molecules of molecular weight 10×10^6 may be present in a single mitochondrion. Attachment of membrane pieces to DNA, and the observed association of mitochondrial DNA with cristae may possibly represent sites at which replication is initiated.[104] Recent evidence also indicates that mitochondria contain mechanisms for incorporation of nucleotides into this DNA.[121]

In addition to DNA, ribosome-like particles,[102] RNA, and RNA polymerase-like systems[66,102] have been found in mitochondria from different sources.

This new information furnishes the basis for a working hypothesis implying that mitochondria possess their own genetic information and the necessary mechanism to express this information in an autonomous synthesis of proteins.[66] A 5.5μ-long DNA molecule is able to code the information for the synthesis of several hundred proteins. As pointed out by André,[66] part of this information may function as structural genes, controlling the duplication of mitochondria and the synthesis of the biochemical equipment. Another part could function as regulatory genes, controlling differentiation and response to the external medium. Further studies will help to establish to what extent these genes are hypothetical, and to what extent they are subordinate to information coming from the nucleus, or may be reacting independently to the external medium by mutation and adaptation.[66]

These hypothetical considerations may prove to be of operational significance in view of the characteristic association of mitochondria with synaptic endings.[80,81] In monkey spinal cord motor horn cells Bodian has described very large endings containing unusually densely packed mitochondria. The subsynaptic cytoplasm in many of these large endings is characterized by an accumulation of Nissl substance which consists of regular layers of endoplasmic reticulum cisternae with large numbers of ribosomes in between. It has been speculated that these Nissl bodies may be associated with protein synthesis in relation to specific endings.[80] However, in view of the dense accumulation of mitochondria possessing their own genetic information and the mechanisms to express it in an autonomous synthesis of protein, the interesting possibility of a more direct participation of mitochondria in regulatory aspects of these biosynthetic processes should be taken into consideration. Subunits of protein molecules would be synthesized locally and either be attached as specific macromolecular assemblies corresponding to coding and information storage in molecular switching componentry at the junctional regions, or they might pass through the ER membrane and the cysternal cavity into the extracellular space.[80] Similar considerations might also apply to the intimate interrelationship of mitochondria and associated granular components with photoreceptors of the insect retinula as shown in Figure 11.

ROLE OF RNA POLYMERASE IN TRANSFER OF GENETIC INFORMATION The enzyme RNA polymerase plays a key role in the transfer of genetic information through its participation in the differential RNA transcription upon DNA templates.[106,107] Confirming the results of earlier workers,[105] we have studied the physical properties of DNA-dependent RNA polymerase known to be relatively free of nucleic acid and capable of asymmetric transcription.

As shown in Figure 12 B, the RNA polymerase molecules from *E. coli*, prepared by the Chamberlin and Berg procedure, and having a sedimentation coefficient of 25S, appear to consist of six subunits arranged in a hexagon with a cross section of 120 to 130 Å in negatively stained


500 Å
Ut
Ut
T
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preparations. If the large hexagonal structures are the RNA polymerase molecules, as proposed by Fuchs, Zillig, et al., the basic structure is either a hexagonal disk or two of these stacked together. A few smaller structures are also observed; these might be degradation or dissociation products, some of which closely resemble the subunits in the hexagon. As shown by J. P. Richardson,[107] the ionic conditions strongly affect the form of the RNA polymerase. The low salt conditions favorable for observing the 21S form are the optimal conditions for enzymatic activity, while the high salt conditions that produce the 13S form completely inhibit RNA polymerase activity.

In general, these observations emphasize the critical effect of ionic conditions on all important systems participating in the process of biosynthesis. Bearing this in mind, one is tempted to speculate on the possible effect of ionic currents, which might have been modulated by bioelectric potentials at key junctional membrane regions, thus presumably affecting the processes that regulate information transfer and storage in nerve membranes and their derivatives.

In order to obtain more information on the manner in which RNA polymerase initiates transcription of RNA upon DNA templates, we are carrying out correlated electron microscopic studies on the binding of RNA polymerase to different types of DNA. Following the work of H. S. Slayter and C. E. Hall,[106] and J. P. Richardson,[107] it is assumed that certain sites must exist along the DNA molecules that specifically bind RNA polymerase, allowing transcription to begin at well-defined regions. Preparations of RNA polymerase were mixed in with different types of DNA, including ϕ X 174 DNA, bacterial, and salmon DNA, in varying concentrations under carefully controlled conditions.

As shown in a representative example (Figure 12A), the complexed enzyme generally appears to center itself upon the DNA axis, which is in agreement with the results reported by Slayter and Hall. Distribution of the RNA polymerase molecules along the DNA fibers varied according to the different preparative conditions. However, the disposition of enzyme particles is similar to the distribution reported by Slayter and Hall, with average separations ranging from about 1200 Å to about 2000 Å. These preliminary results are in agreement with values reported by other workers. However, further studies with improved preparation techniques are required before a reliable evaluation can be carried out. Particular interest attaches to correlated studies on the binding of RNA polymerase to circular forms of DNA (e.g.,from ϕ X 174) which are being carried out in collaboration with Dr. Samuel Weiss and his associates.

Cooperative features of nerve membranes

Biological membranes are the largest and most complex atomic aggregates known, spanning the widest range of dimensions as basic integrating components of all living systems. Elucidation of the molecular organization of cell membranes represents, therefore, not only one of the major fundamental problems of biomedical research, but it has also emerged as the next major barrier, cutting across molecular biology and neurobiology, which must be overcome to clear the way for further progress.

Membranes exhibit a unique degree of structural asymmetry, comprising a coherent lipoprotein film that is usually only a few molecules thick, but still capable of extraordinary lateral extension.

Nerve membranes exhibit these characteristics to a unique degree, as major components of the nervous system, which is generally regarded as the most sophisticated and complex biological system known. Thus, the total length of the peripheral network of the human nervous system is estimated at about 600,000 miles. All fibers of the human body's nerve fabric, which is essentially lined with multiply-folded membranes, would extend three times the distance from the earth to the moon. Having unraveled their exquisitely delicate, yet highly ordered texture, we cannot help wondering about the nature of these "paucimolecular" "living" films, singularly sensitive to "paucienergetic stimuli,"[108] which are so compactly infolded into the submicroscopic recesses of the relatively limited space occupied by the individual nervous system, that we could encompass a whole planet with the combined array of extended membranes.

In many respects, the entire complex membrane repository comprising the nervous system may be re-

FIGURE 11 Thin section through retinulae from a tropical Skipper butterfly, demonstrating the highly regular organization of the differentiated membrane-bound components in these photoreceptors. The matched rhabdomere pairs (R-arrows) are surrounded by rows of mitochondria (Mi), grouped together in radial columns by a network of fine tubular channels (Ut) arising from the tracheal compartments (T). The walls of the tubular rhabdomere compartments contain arrays of dense particles (insert), which may correspond to the ordered arrangement of the dichroic photo-pigment molecules, providing the basis for the polarization-analyzing property of the insect eye. × 12,390. Insert, × 354,000.

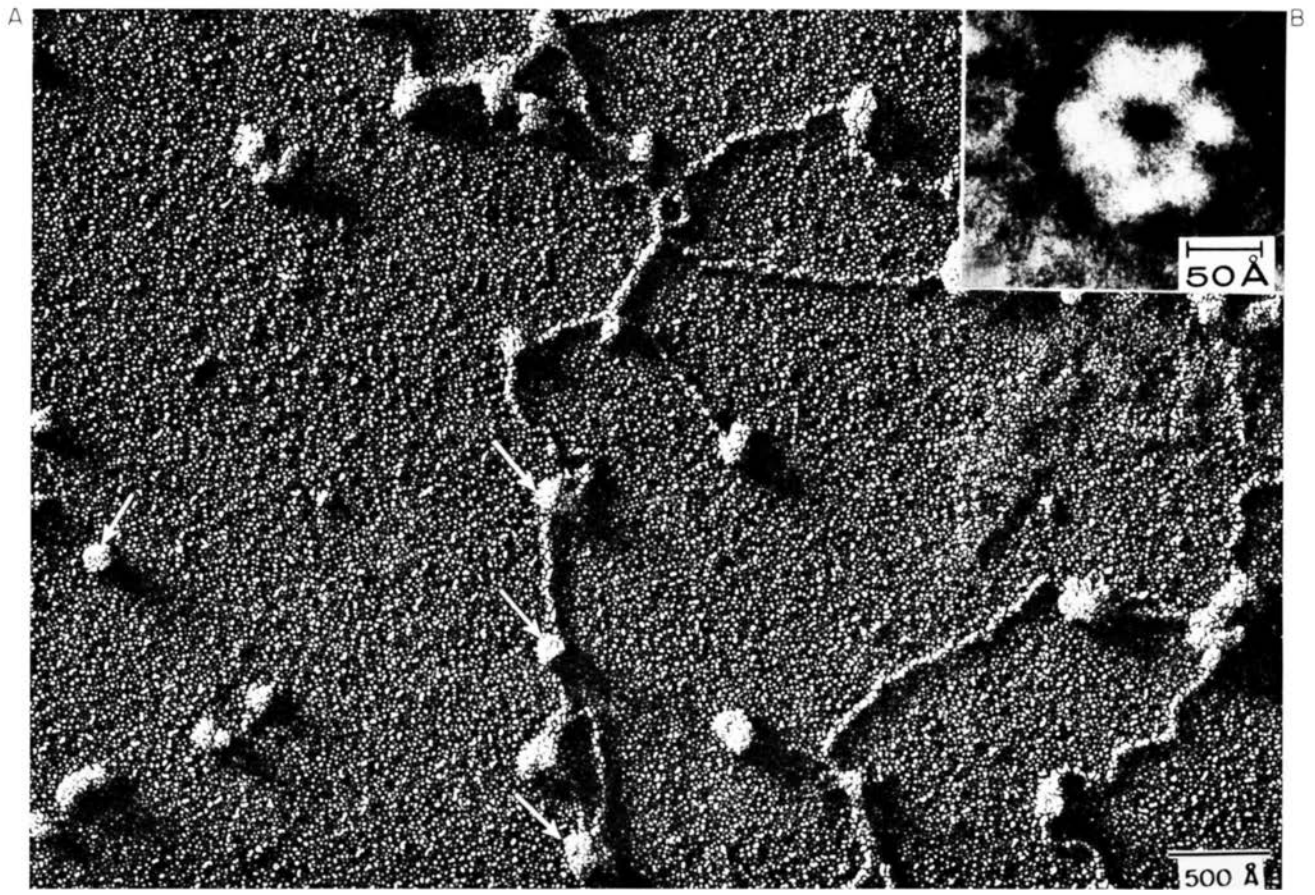

FIGURE 12 A: Characteristic attachment of RNA polymerase molecules (arrows) to DNA strands as seen in shadowed preparations. × 284,000. B: DNA-dependent RNA polymerase molecules from *E. coli*, prepared by the Chamberlin and Berg procedure, appear in negatively stained preparations to consist of six subunits arranged in a hexagon with a cross section of 120–130 Å. × 2,000,000. These correlated electron microscopic and biochemical studies are expected to yield further information on RNA polymerase and its participation in the differential RNA transcription upon DNA templates, which is of fundamental importance for the regulation of protein synthesis and function in nerve cell membranes and their derivatives.

garded as representing an essentially interconnected, continuous and "closed membrane system" where all junctional domains of the separate cell membrane entities establish functional contact and are interrelated to form an integrated, coherent whole of extraordinary dimensions. In this sense, if a diamond crystal may be considered as one giant "macromolecule" so it is justified to conceive of these CNS membrane aggregates as one giant "megamembrane" or "megamolecule." Such a coherent system would be uniquely suited to effect transfer, storage, and retrieval of information at the various hierarchies of organization.

Although this model is strongly idealized, it would, nevertheless, embody the basic features to serve as a suitable substrate for highly cooperative phenomena embracing the entire complex molecular aggregates, which are not unlike those involved with manifestation of quantum phenomena at the macroscopic scale, as, for example, in superconductivity. Although admittedly speculative and only intended to delineate salient features of a model, this approach may nevertheless prove to be a convenient working hypothesis which could provide the operational key to a deeper understanding of the molecular and supramolecular organization features of the CNS.

Macromolecular repeating units and domains in membranes

There is another feature of nerve membranes, which although derived from the results of ultrastructure re-

search, nevertheless attempts to correlate them with the over-all behavior of the system. Thus, membranes may also be regarded as arrays of organized domains or subunits, including macromolecular assemblies. Katchalsky, et al.,[123] conceive of such cooperative systems as exhibiting "memory-hysteresis behavior." It is pointed out that there exists also the possibility of a physical record in biopolymers based on conformational changes in single macromolecules or in cellular macromolecular structures such as membranes. Typical examples of physical memory devices are the magnetic tapes of tape recorders. Katchalsky conceives of the possibility that conformational transitions in macromolecules might also exhibit hysteresis phenomena. Also, the existence of various distributions of functional side-groups along the macromolecules makes possible the existence of different domains with a wide range of transition points.[123] Metastable macromolecular states of high permanence are linked with two-dimensional hysteresis phenomena based on domains in biological films.

According to Katchalsky, et al.,[123] if we assume that the smallest number of nucleotides is about 8–10 in a domain, the energy involved in a phase transition of a nucleotide is higher than 2 RT, which is a plausible figure in case memory recording in domain structures is considered. This figure is sufficiently high to make a signal changing the domain structure distinguishable from the thermal noise, but yet small enough to be of use in the low-energy transformations of living organisms. In this connection, it is pertinent to point out that membranes as two-dimensional ordered arrays of macromolecular assemblies, acting like information storage devices, would ideally fulfill the dual criteria of "low energy switching," and optimum size of domains as metastable forms of high permanence.

Cooperativity of membranes and their subunit structure

Review of the data on membrane organization as revealed by correlated ultrastructural and biochemical studies discloses certain general features. Coherent paucimolecular layers of indefinite lateral extension appear to consist of a periodic, hydrated lipoprotein substrate which is integrated with specific macromolecular repeating subunits organized in asymmetric, "paracrystalline," arrays within the plane of the layers. There is a fundamental underlying concept that membranes are made up by the stereospecific association of repeating macromolecular subunits, with specific conformations dependent on their association with the membrane substrate.

Recently, J.-P. Changeux and his colleagues[132] have extended their theory of allosteric transitions in enzyme-substrate reactions[125] to membranes composed of identical units, applying some of the principles and approaches proposed for regulatory enzymes. They discuss the cooperative properties of membranes on the basis of their highly ordered structure. Based primarily on data derived from correlated ultrastructural and biochemical studies of membranes, carried out during the past years by Fernández-Morán, Green, Perdue, et al.,[6,15,17] they consider the following significant aspects of membrane organization: (a) membranes are made up by the association of repeating macromolecular lipoprotein units; (b) the conformation of these units (protomers) differs when they are organized into a membrane structure or dispersed in solution; (c) many biological or artificial lipoprotein membranes respond in vivo, as well as in vitro, to the binding of specific ligands by some modification of their properties which reflects rearrangement of the membrane organization and presumably of the repeating unit's conformation.

From this they deduce a theoretical model of a biological membrane as an ordered collection of repeating globular lipoprotein units, or "protomers" organized into a two-dimensional, crystalline lattice. The protomer constitutes the "primitive cell" of the lattice and does not necessarily possess in itself any particular symmetry properties. Several conformational states are reversibly accessible to the protomer. The conformation of the protomer depends upon its association with neighboring protomers, and is thus submitted to a lattice constraint similar to the quaternary constraint involved in the organization of the quaternary structure of oligomeric proteins.

This interesting theory has been primarily developed to account for the cooperative phenomena accompanying the binding of ligands to a membrane. This model predicts the two classes of responses exhibited by biological membranes: a "graded" response or an "all-or-none" response. Basic to their theory is the assumption that "conformational changes of the protomer not only preexist the ligand binding but are not fundamentally different whether the ligand is bound or not." These authors emphasize that a number of important biological phenomena seems to be related to the highly cooperative structure of cell membranes.

Multiplex systems and possible ultrastructure correlates in nerve membranes

There appears to be a deep analogy between von Neumann's multiplexing concept[124] (which essentially in-

troduces redundancy so that the reliability of the whole system is greater than the reliability of its parts) and the hierarchically organized redundancy of structural substrates in the CNS at the macromolecular and molecular levels now revealed by electron microscopy. The microcomponentry required by the multiplexing technique may partly correspond to the immense number (in the order of *ca.* 10^{16} to 10^{19}) of specific macromolecular assemblies and related subunits periodically arranged on the nerve membranes and derivatives in close association with axon filaments, ribosomes, and other key components.

MAJOR PROBLEMS IN THE STUDY OF NERVE MEMBRANE ULTRASTRUCTURE With the improved methodology now available it is tempting to systematically extend the fine structure studies to all regions of the nervous system in vertebrate and invertebrate specimens.[126] However, one should bear in mind that this purely descriptive approach could easily consume the best efforts of entire generations of investigators. Thus, even if perfect techniques were currently available, a complete morphological analysis of three-dimensional relationships based on electron microscopy of serial sections would involve such a prohibitively large number of images to map only a few cubic centimeters of nerve tissues that total man hours would figure in the order of centuries.

Instead, it appears more profitable to concentrate on certain key problems of nerve ultrastructure and function:

(a) Correlated biochemical, electron microscopic and biophysical characterization of the major protein, lipoprotein, glycoprotein components, and the macromolecular and multienzyme assemblies associated with nerve membranes in a variety of specimens, including human. Attention would center on well-characterized enzymatic and protein components with specific physiological properties.[127]

(b) Systematic studies on the organization of subunit patterns within the plane of membrane layers, preferably examined in the native hydrated state under conditions of reduced radiation damage and of minimum perturbation, by using improved instrumentation and preparation techniques for high resolution, low-temperature electron microscopy correlated with polarized light and x-ray diffraction studies.[2,6,33,92,93,98,113,114,116]

(c) Correlation of ultrastructural studies with a comprehensive "macromolecular and molecular anatomy program" aimed at achieving reliable dissection and quantitative separation of the entire spectrum of discrete molecular species using Anderson's zonal centrifuge techniques[133] and related methods.

(d) Correlative studies of specific, dynamic changes on selected nerve specimens in vivo[129] and in vitro[130] (tissue and organ cultures) using light and electron microscopy.

It would be of special interest to carry out a correlated ultrastructural and biochemical analysis of the molecular organization of selected key sites of the central nervous system by performing a series of "submicroscopic or macromolecular biopsies." Such a submicroscopic biopsy, for example of key synaptic sites or cerebral nuclei, can be carried out "in vivo" using precision stereotactic techniques under conditions of minimum perturbation without inflicting more than "subliminal" damage, which is perhaps hardly greater than the natural rate of decay of nerve cells taking place in the adult brain. These submicroscopic CNS biopsies, carried out with specially developed ultramicrocapillaries, can be combined with subsequent tissue culture explants and related in vitro techniques. Hydén[129] has already carried out significant pioneering work in microbiopsies of living tissues. Further development of these techniques holds great potential for electron microscopy.

Ultrastructural studies could also be combined with appropriate non-destructive techniques, including nuclear magnetic resonance,[34] electron spin resonance, and methods for investigation of fast reactions.[100] Neutron diffraction and scattering studies[97] suitably modified for examination of nervous tissues should also yield valuable information.

Suggested methodological approaches in correlated studies of nerve membranes

Recent improvements in instrumentation and preparation techniques for electron microscopy appear to be especially suited for correlative investigations of nerve membrane ultrastructure at the molecular and pauciatomic levels[116]:

(a) High-resolution phase contrast electron microscopy with improved point cathode sources, low temperature stages and specially stabilized power supplies, as well as new types of phase contrast apertures has enabled us to visualize directly for the first time (Figure 13B) the hexagonal cells of single crystal graphite containing only 10 carbon atoms. Demonstration of this capability of point-to-point resolution in the range of interatomic distances now makes it appear feasible to record detail of molecular and pauciatomic dimensions in biological specimens. Extension of this work and the enhanced capability of interpreting phase contrast images should eventually enable us to achieve direct readout of molecular and submolecular structures, particularly in nerve membranes. Parallel advances in low-temperature electron microscopy at liquid helium temperatures using specially developed superconducting lenses,[113,114,116a] image intensifiers and improved recording techniques should

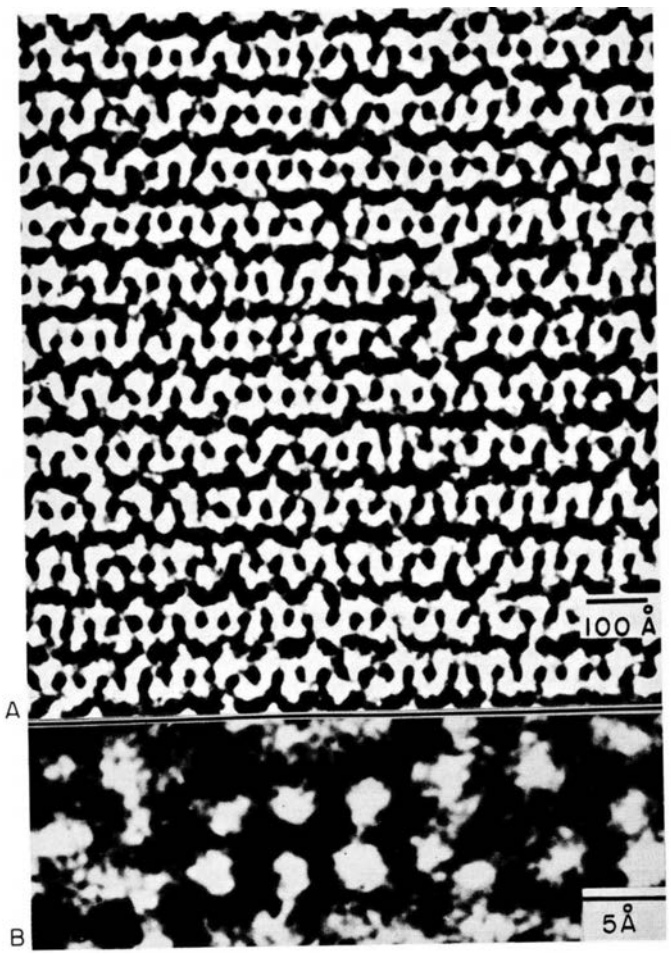

FIGURE 13 High-resolution electron micrographs showing A: The complex arrangement of the structural subunits of about 8–20 Å within the periodic lattice of catalase crystals stained with uranyl formate. × 800,000. B: Phase contrast image of the hexagonal array of carbon atoms in ultrathin layer of single-crystal graphite. × 22,000,000. This level of resolution of pauciatomic stuctural patterns, which can now be achieved under certain conditions with improved instrumentation and preparation techniques, should prove to be of key value in correlated studies for direct visualization of molecular organization of nerve membranes and associated enzyme systems.

eventually permit us to see directly the three-dimensional structure of key regions of synaptic junctions and of other nerve membrane areas.

(b) By using vacuum-tight microchambers[92-98] with ultrathin windows and related techniques, it is possible to observe membranes under conditions approaching their native hydrated state. Examination of living hydrated specimens can now be carried out thanks to recent advances in high voltage electron microscopy.[115] Larger microchambers for examination of intact, viable biological specimens have been successfully applied owing to the far greater penetration now possible with advanced high voltage electron microscopes. Moreover, by using pulsed T-F emission or stroboscopic microbeam illumination, one can, in principle, obtain a unique combination of high spatial and high temporal resolution for the study of fast reactions in nerve membranes.

(c) Scanning electron microscopy can likewise be profitably used in the study of nerve membrane specimens examined under favorable conditions. As shown by the recent remarkable work of R. F. W. Pease, et al.,[131] electron micrographs of living specimens of various developmental stages of the insect *Tribolium confusum* have been obtained with a scanning electron microscope at magnifications of about X1000. The requisite conditions of moderate vacuum and the relatively minor effects of irradiation by the imaging electron beam, which have been shown to be compatible with survival of certain specimens, may be applied in suitably designed experimental chambers to study selected nerve membranes and nerve cell specimens. Scanning electron microscopy can also be extended to the application of special bioelectric and biomagnetic probes for the study of rapid and ultrarapid phenomena in nerve membrane systems.

(d) We can also foresee the unique potential of electron microscopy "applied in reverse" for achieving electron optical demagnification of letters, diagrams, printed circuits, etc., in such a way that, in the optimal case, the contents of a several million volume library can be condensed on an area of a single page or its equivalent.[117,118]

(e) The same electron optical techniques that revealed the exquisite molecular componentry of nerve cells may someday serve to duplicate true homologous ultraminiaturized, "molecular computer and information storage systems." Although the packing density embodied in the human brain is of the order of 10^9 components per cubic inch, impressive advances made during the past years in manufacturing integrated microelectronic systems have already resulted in practical packing densities of about 10^5 elements per cubic inch, which is susceptible of further improvement. Likewise, functional printed circuits of micron or submicron dimensions which could fit on a red blood cell, for example, and transmit important functional data recorded in vivo at the cellular and subcellular levels are within the scope of present technology. One can conceive of integrated ultramicroelectronic circuits which begin to approach the dimensions of macromolecular assemblies, and could eventually be incorporated into key junctional sites of living nerve membranes without undue perturbation. Such submicroscopic prosthetic sensors, with their envelopes composed of biosynthetically, produced macromolecular "protein coats" to form integral components of the nervous system, could well subserve certain unique functions. For example, since they can be produced in large quantities and implanted in sufficient numbers throughout the central nervous system, they can provide a direct operational link at the crucial macromolecular level between the central nervous system and man-made information processing systems, such as computers of commensurate complexity.

There is increasing evidence that biological membrane systems may have many properties in common with semiconductors.[2,119,120] It may therefore be of interest to reconsider as a working hypothesis suggestive of new experimental approaches our earlier speculations on certain specific crystalline properties such as piezoelectric effects, semiconductor properties or equivalent phenomena which may be associated with the fluid-crystalline nature of myelin and related lamellar systems.[2,30] In connection with correlated studies on basic mechanisms of electroencephalography, I had previously discussed the hypothetical possibility that myelin and other paracrystalline nerve membrane systems might function as generators or specific amplifiers of coherent electromagnetic radiation, mainly in the infrared or UHF range between 1 and about 10 microns wavelength.[134] Similar hypothetical mechanisms involving far infrared quanta and coherent phenomena in the transfer of biological information on the macro- and supramolecular level are now being considered by J. Polonsky[135] in the scope of molecular exciton theory and quantum electronics.

Conclusions

Obviously, our current knowledge about nerve membrane ultrastructure and function is but a presage of many as yet unknown and unexpected properties of these basic constituents of the nervous system, awaiting further investigation.

Ultrastructure research should eventually enable us to learn how all of the prodigious activity of nerve cells and their appendages may be unfolding invisibly at the molecular and atomic levels in a strange, spatial-temporal framework, possessing an order that one may someday aspire to comprehend.

The old adage may thus prove to be invested with a deeper meaning when the simile is invoked that the neurons apparently have so little to show for all their incessant submicroscopic labors, because, like the mills of the gods, they grind exceeding small—between membrane interfaces at the molecular level.

The Role of Inorganic Ions in the Nerve Impulse

ROBERT E. TAYLOR

THIS CHAPTER begins the consideration of the nerve as a cell and as an element of a nervous network. Figure 1 lists most of the relevant phenomena that occur in nerve cells, and most of them have been or will be considered by others in this volume.

The known basic elements that enable a nerve to perform its role are indicated on the flow chart shown in Figure 2. In particular we shall discuss here the way in which ion permeabilities control the electrical potential across the nerve membrane and how the membrane potentials control the ion permeabilities. This loop, along with the geometry, is responsible for the propagated action potential. Others will be concerned with the phenomenology of the synapse, and it is sufficient to remark here that most nerve cells have a secretory function and in particular liberate substances in response to a reduction of the membrane potential at specialized endings. On the postsynaptic side, specialized regions are sensitive to these substances and respond by changes in ion permeability.

The boxes in Figure 2 that contain question marks represent processes about which little is known, and these are areas of great current interest. There is much to indicate that in each case some conformational change in a protein–lipid complex is involved, but we do not know the details.

Electrotonic spread

One very important item is not included in Figure 2. This is the matter of electrotonic spread. The basic phenomena are illustrated in Figure 3; it spans a period from 1834, when Peltier observed a voltage in the opposite sense following the application of currents to a muscle, until 1879, when Hermann produced the proper explanation. It was important historically, because measurements of this kind produced very strong evidence that the fibers of nerve and muscle were separated into an inside and outside by a surface phase with resistance and capacitance. Consider Hermann's model. It is a metal wire in a tube filled with salt solution. The side arms are for applying currents and recording potentials in a way that can avoid difficulties caused by electrode polarization and products of electrolysis. If the switch is closed, a current flows through the salt solution and some of it travels through the wire. What conditions are necessary to measure a potential at the other electrode connected to the voltmeter, both during the time the current is applied and for some time after the switch is opened? Simply that the metal–solution interface resistance for a given length of wire must be large enough, compared to the longitudinal resistance of the same length of wire; and to get the time delays, an electrical capacitance must be present at this interface.

The spread of current around some local variation is crucial to the operation of neurons. A detailed analysis of current spread in nerve membranes is seldom a simple matter, but we may appreciate the principles involved by considering an approximation, introduced by Weber for a cylinder. If the resistance of the membrane (r_m), or the metal–solution interface in Hermann's model, is high enough, we may neglect the radial component of potential drop in the internal and external phases and consider the longitudinal resistances inside (r_1) and outside (r_2) as geometrically linear elements. Then we may readily determine that if there is a disturbance V(0) of the potential across the membrane at some point, it will spread, in a region of no applied current, to produce a variation

Nerve Cells

GROWTH	MAINTENANCE	FUNCTION
DIFFERENTIATION	Metabolism	Reception
(Long Term Memory?)	Transmembrane-Transport	Transmission
REPAIR	Macromolecular-Synthesis	Secretion
	Intracellular-Transport	Integration at Cellular Level
	[Cooperation with Glial Cells?]	[Cooperation with Glial Cells?]

FIGURE 1 Areas of interest in the study of nerve cells.

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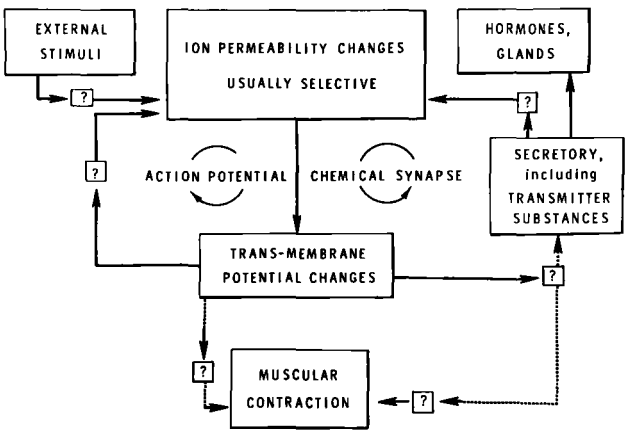

FIGURE 2 Schematic flow chart for most of the relevant phenomena peculiar to nerve.

in the potential at a distance (x) away in the amount of

$$V(x) = V(0) \exp(-x/\lambda),$$

where $\lambda^2 = r_m/(r_1 + r_2)$. We call λ the space constant of electrotonic spread.

The principles are the same for any geometry. The spread of potential ahead of an action potential is necessary for the transmission and the ultimate depolarization of the nerve endings for the release of transmitter substance. The transmitter substance released at a nerve ending may produce an increase or a decrease of the membrane potential in the postsynaptic region; or it may produce a change in the resistance of the membrane with no change in the potential. A resistance change can produce

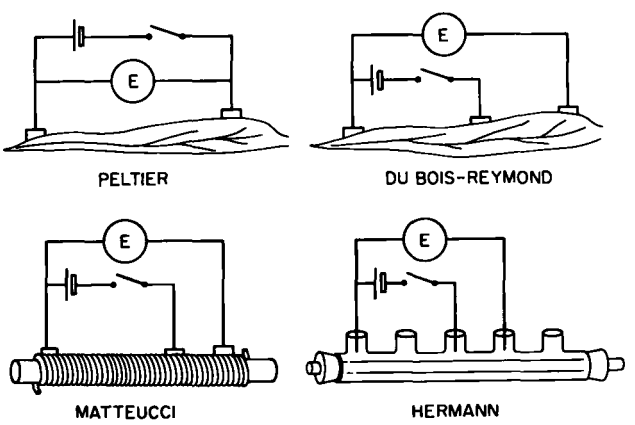

FIGURE 3 Early experiments leading to concept of electrotonic spread and the model of Hermann which explains it. This is a metal wire in a saline filled tube. The side arms contain electrodes connected to a battery and voltmeter. (From Taylor, Note 16)

effects because it modifies the extent of the electrotonic spread from other regions. Thus, a collection of endings that are not too far apart may have a highly complex net effect. Ultimately, these effects either result in sufficient depolarization at the axon hillock and a propagated action potential, or the distances may be so short that the electrotonic spread may itself invade the nerve ending, which, in turn, results in release of transmitter. We know that this occurs in certain nerve cells without axons.

The functional significance of purely electrical connections between neurons is not clear, but we do know that if two cells are in close contact, such as at a tight junction with low resistance, activity can spread from one cell to the other. Whether this is a one- or a two-way junction depends on the passive electrical characteristics of the junctional region. If the contact is not close and the resistance of the medium between the cells is particularly high, the activity in one cell could, and does,[1] result in hyperpolarization and inhibition at that region.

We must not be fooled into thinking that a cell is a cylinder or a sphere just because it seems to behave electrically like one or the other over a certain frequency range. Many cells have deep invaginations in the membrane, and the interior of parts of the endoplasmic reticulum may be electrically outside the cell, as is the case in the frog skeletal muscle fiber. For many purposes we may treat these cells as if they had a smooth surface, but we must not confuse the model with the cell.

Membrane capacitance

The pioneering work of Höber and of Fricke[2] on the impedance of the membrane of erythrocytes determined that this membrane had a capacitance of about one microfarad per square centimeter, while the membrane conductance was too small to be measured by their methods. The generally accepted idea that much of the membrane was composed of lipid, because lipid-soluble substances readily penetrated many cells, caused Fricke[2,3] to estimate that the thickness would be about 30 Ångstroms. Most measurements of cell membrane capacitances are somewhat higher than those found for artificial bilipid layers,[4,5] and our present estimates for natural membrane thickness are greater than 30 Ångstroms. Further, the capacitance of the artificial membranes does not depend on frequency, while that for the natural membranes does.

Based on work with W. Knox Chandler, I have estimated[6] the low-frequency capacitance of the squid giant axon membrane to be about 0.8 microfarads per square centimeter and the high to be about 0.6. This means that if the greater part of the membrane structure is based on a bilipid layer, a certain percentage of the area must con-

sist of a material with a higher and frequency dependent dielectric constant. An attractive and obvious possibility is that this material might be a penetrating protein bound to the lipid.

Membrane impedance

A most important series of observations were made by Cole[7] and Curtis on the phenomenology of the electrical behavior of the neuronal membrane. Impedance measurements on the single-celled plant *Nitella* showed that during the propagation of electrical activity, or during depolarization produced by high external potassium or by applied currents, a reduction of the parallel electrical resistance with little or no change in the capacitance caused impedance changes (Figure 4). The dotted lines show the expected alteration in the impedance if the underlying change was restricted to a decrease in the parallel resistance.

Later, Cole and Curtis and Cole and Baker extended these kinds of observations to the squid axon, and obtained similar results. One of their most dramatic examples is shown in Figure 5. Here the action potential of the squid axon is superimposed on the (20-kilocyle per second) alternating-current signal caused by imbalance of a Wheatstone bridge. The increasing size of this signal represents a decrease in the resistance of the membrane. It is not possible to conclude from impedance measurements alone that the membrane resistance change is the result of a change in the ion permeability of the membrane, but this conclusion is not necessary in order to use the equivalent circuit shown in Figure 6. Figure 6 is merely a pictorial way of expressing the experimental results. Combining the impedance measurements with action-potential shape and velocity enables one to deduce that during activity, changes take place in both the equivalent Thevenin, or open-circuit, potential and in the conductance.

Let us consider for a moment what these quantities mean physically. Charge may pass across the membrane in either of two ways—by charge transfer or by charge separation. In charge transfer there is no accumulation; the charges that move are made up by the external circuit, and the system remains electrically neutral. In charge separation there is a net loss of charge from the solution on one side of the membrane and a gain on the other. This difference, divided by the potential across the membrane, is the membrane capacitance. We note in passing that for a capacitance of 1.0 microfarads per square centimeter and a potential shift of 100 millivolts the charge is about 1.0 picomoles per square centimeter (10^{-12} moles per square centimeter). If no capacitative current is flowing, or if it is subtracted, the potential at which no remaining current flows is the Thevenin potential. The usual textbook treatment of the Thevenin potential and the equivalent internal resistance would consider only linear passive systems in which the equivalent resistance and potential are not changing with time, current, or potential. (May I emphasize that the Thevenin potential is in no sense an equilibrium potential!)

To elaborate from this point, we must consider other kinds of experimental results in which ion movement is observed directly by analysis of total content or inferred from the results of observations in which isotopes are

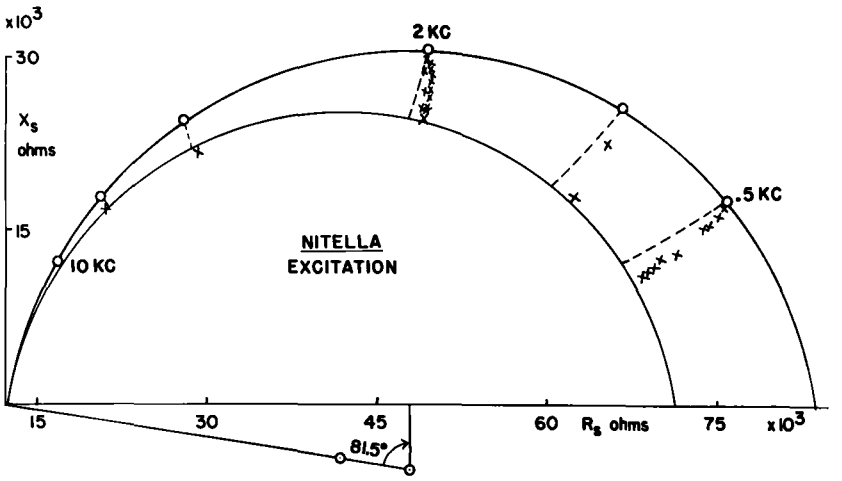

FIGURE 4 Impedance loci for *Nitella* at rest (open circles) and changes during activity (crosses). Frequencies are in kilocycles and X_s is the negative of the reactance.

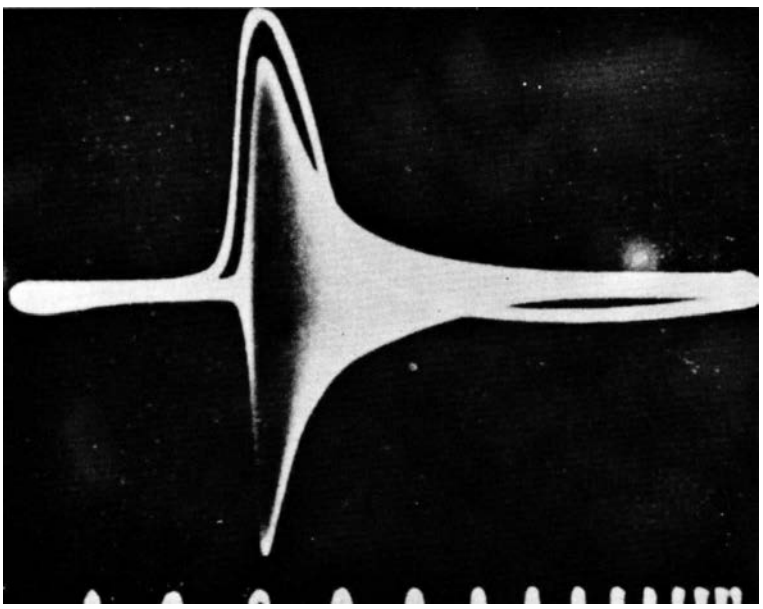

FIGURE 5 Impedance changes during action potential in squid giant axon. Intervals between marks on bottom are one millisecond. (From Cole and Curtis, Note 17)

used. It was known long ago that potassium ions were released from frog muscle fibers during activity, and Fenn[8] reported in 1936 that sodium ions entered the muscle fiber. The total concentration of sodium did not increase with time, and the electrochemical gradient for sodium is strongly inward, so the necessity for an active process for sodium extrusion followed immediately. The results of many experiments to date[9] give no indication that anything is happening except that ions flow down their electrochemical gradients through some sort of channel in which the permeability coefficients may be altered by potential changes, as in the axon, or by neurohumoral transmitter substances, as in the synapse, and are later pumped to restore the initial conditions.

All indications so far are that the individual ion species flow independently, i.e., there have been no results to indicate any cross coupling (except for active transport) This does not mean that the presence of one ion cannot affect the permeability for another ion; of this there are many examples. On occasion, failure to distinguish these two different phenomena has led to considerable confusion.

If the flows are independent, we may consider the current for each individual ion (I_i) as a separate system and write

$$I_i = G_i(V - V_i),$$

for the i'th ion where V_i is taken as the potential at which there is no current carried by the i'th ion, V is the membrane potential at time (t) and G_i is a conductance defined by this relation. Write $I_c = CdV/dt$ for the capacitative current; write I_p for the sum of the pump currents and write the total current as the sum of these parallel components as

$$I = CdV/dt + \Sigma_i G_i(V - V_i) + I_p.$$

This was the approach of Hodgkin, Huxley, and Katz,[9,10,11] and the Hodgkin–Huxley equations are the results of experimental determinations of the G_i and V_i plus their empirical description. The difficult part is the separation

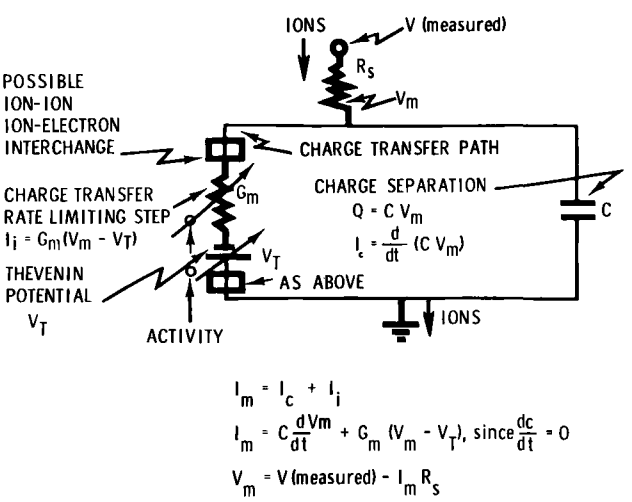

FIGURE 6 Diagram of element of nerve cell membrane indicating the experimental results of impedance and potential studies.

of the total current into the individual components. Once this is done, any V_i may be taken for any ion (i) and the corresponding G_i determined. It is clear, then, that the association of the V_i with the Nerst potential [i.e., $(RT/F) \ln (C_o/C_i)$ where R is the gas constant, T the absolute temperature, F the faraday, and C_o, C_i the external and internal ion activities respectively] as was done by Hodgkin and Huxley, is not an essential part of the empirical description. The only assumption is that the membrane capacitance remains constant and that the ion flows do not interact in any way not contained in the above equation. There is, however, a potential at which there is no flow of a given ion; this is, indeed, the Nernst potential, and if one does not want to use different V_i's for different V's, it is mathematically convenient to use the Nernst potential. Otherwise, the G_i would contain a singularity at $V = V_i$.

This may sound elementary, and indeed it is, but we shall see that the total picture is not quite so simple, because the channels—for example, those that normally carry sodium—are not infinitely selective.

For the resting potential at no total current flow and $dV/dt = 0$ we have simply

$$V_r = (\Sigma_i G_i V_i)/\Sigma_i G_i.$$

Thus, the potential at the postsynaptic membrane, say, is most heavily weighted in favor of that V_i for which the G_i is the largest. We shall not discuss pumps here, but in many cases (not all) it appears that the net pump current (I_p) is small.

The above way of treating the system may be convenient and it is certainly valid, but clearly it is not unique. Frankenhaeuser and Huxley[10] have fit data obtained on the Node of Ranvier by using a different set of equations appropriate for, but not limited to, the solution of the diffusion equation assuming constant field.

The ionic picture

I shall not describe the development of techniques introduced by Marmont and by Cole[7] for voltage clamping in the squid axon membrane or the remarkable success of Hodgkin and Huxley in developing their equations. For homework, one may consult the recent books by Hodgkin[9] and Katz.[11] The important point is that the conductances (G_i) are potential dependent and time dependent, and the potential is dependent on the G_i as given above. When the details are considered, this loop, as shown in Figure 2, is quite sufficient to describe the events during an action potential. A few examples will suffice for our purposes.

Figure 7 shows the result of applying a sudden depolarizing step of potential to the squid axon membrane. The middle curve is the total ionic current against time; the upper and lower are the potassium and sodium components, respectively. The currents during an action potential, as shown in Figure 8, are computer solutions of the Hodgkin–Huxley equations. Figure 9 diagrams the state of affairs along an axon carrying a propagated action potential. The potential-induced permeability changes allow

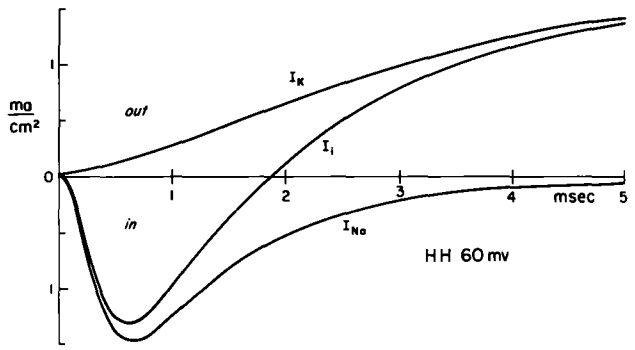

FIGURE 7 Time course of sodium (lower) potassium (upper) and net current during a depolarizing potential step according to the Hodgkin-Huxley equations.

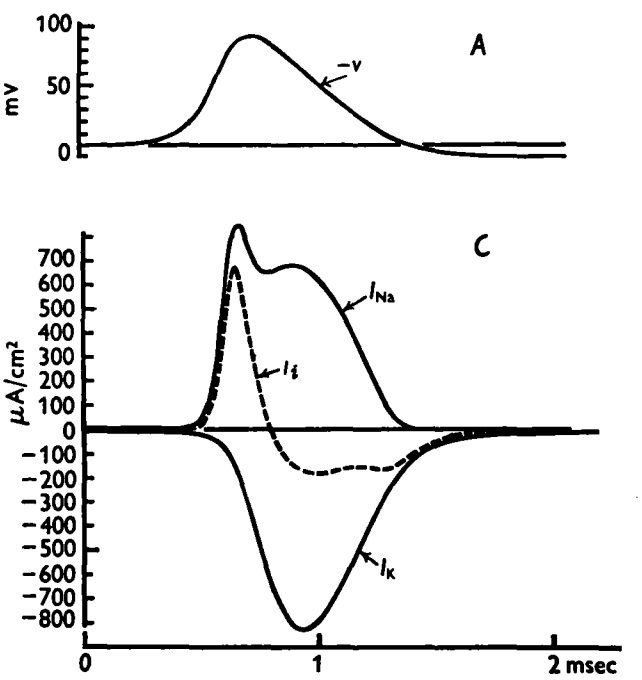

FIGURE 8 Current flow during action potential and the separation into sodium and potassium components according to the Hodgkin-Huxley equations.

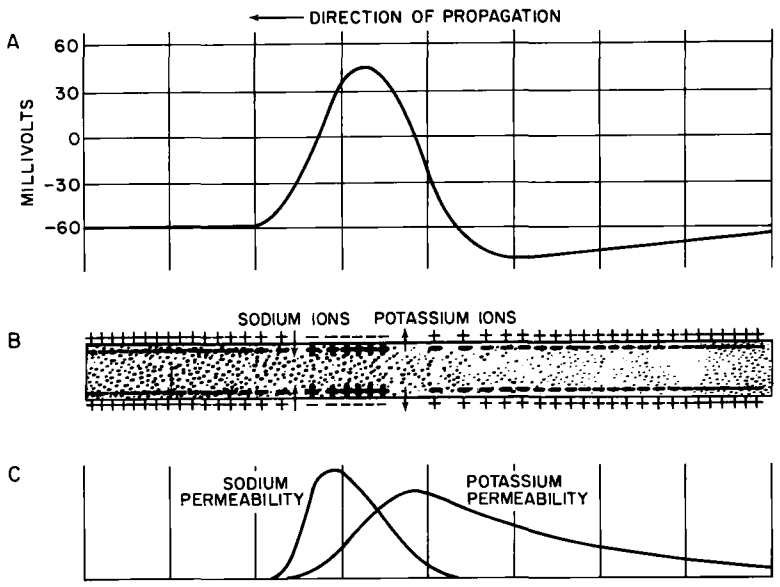

FIGURE 9 Diagram of conductance changes (lower) and current flows (middle) during propagated action potential. (From Keynes, Note 18)

sodium and potassium to flow down their electrochemical gradients; this in turn, produces current flow ahead of the active region, and the process is continuously repeated. A similar sequence of events occurs in jumps from node to node in the myelinated fiber.

Figure 10 gives an example of the effect of a local anesthetic agent on the currents through the membrane of the squid giant axon following a sudden change from the original resting potential to minus 10 millivolts (minus means inside negative). The initial inward transient peak is reduced and the later outward steady current is reduced and somewhat slowed. Other nondepolarizing blocking agents have been found to affect the early fast currents in a more selective manner. These phenomena are sufficient to explain the way in which these agents modify the ability of the axon to propagate activity. Probably most drugs that affect the central nervous system do not act in this way, but influence the course of events at the synapse, either by interfering with the release of transmitter substance, or other hormones, or by changing the reactivity of the postsynaptic receptor sites (see Kety and Kopin chapters, this volume).

Perfusion and selectivity

Since the early work of Baker, Hodgkin, and Shaw and that of Tasaki and Spyropoulos, a number of groups have successfully replaced the internal contents of the squid giant axon with known solutions. The experimental picture is changed considerably when it is possible to control the internal medium, and this is illustrated by considering the selective properties of the fast and slow channels and the effect of calcium removal. Clear-cut experimental results in these cases have not been possible without perfusion.

Chandler and Meves[12] showed that when the squid axon is perfused with a solution containing potassium but no sodium ions, following a depolarizing step to potentials greater than about 60 millivolts (inside positive), one still sees a transient outward bump of current. There is no sodium inside to carry this current. They showed in a quite conclusive manner that the current is carried by potassium ions but has the kinetics of the fast channel through which sodium ordinarily goes. They determined the ratios of the permeabilities for various cations; these

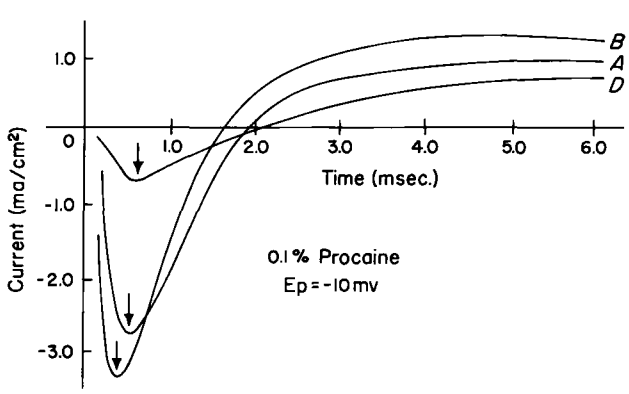

FIGURE 10 Total current during depolarizing step across squid axon membrane and the effect of procaine. B: before, D: during, and A: after application.

Relative selectivity of the sodium channel

Li	Na	K	Rb	Cs	Choline
1.1	1	1/12	1/40	1/60	< 1/73

FIGURE 11 Ratios of permeability coefficients for ions passing through the fast channels of the squid axon membrane that normally carry sodium ions.

are shown in Figure 11. The sodium-to-choline ratio was greater than 73. The work of Lüttgau and others established some time ago that the ammonium ion appears able to substitute for both sodium and potassium. Binstock has recently determined that the ammonium ion goes through the fast channels about one-third as well as sodium and through the slow channels about one-third as well as potassium. It has also been shown (Chandler and Meves, personal communication) that tetrodotoxin acts on the fast channel, not on the sodium ion itself. The same is true for tetraethyl ammonium chloride, which blocks the slow channels (Binstock, personal communication) for ammonium ions in the same way as for potassium ions. It will probably be found that the blocking action on outward potassium currents by internal cesium will have these same properties.

It is well known that cells generally do not tolerate the absence of calcium for long periods, and eventually become leaky to substances that are not lipid soluble. Frankenhaeuser and Hodgkin[13] stated that in the absence of calcium the squid axon membrane became rather linear. It is difficult, however, to eliminate the calcium by soaking nerves in external, calcium-free media, and internal perfusion is needed. The type of perfusion system combined with voltage clamping used by Rojas and Ehrenstein is shown in Figure 12. When perfused with potassium fluoride solutions and with no external calcium, the membrane loses all of the time- and voltage-dependent properties responsible for the usual electrical activity. The membrane resistance (in the intermediate time before the membrane becomes generally leaky) is about the same as the resistance when the normal slow channels for potassium are turned on, but we have found that the total number of slow channels has been decreased and replaced by channels with a conductance for potassium that is only about five times greater than that for sodium.

Artificial membranes

I will conclude with two examples of recent work that may eventually lead to an understanding of the basic molecular mechanisms involved. Using the techniques of Rudin and Mueller, del Castillo and collaborators[14] have found that if an enzyme or an antibody is put on one side of this artificial membrane and the substrate or antigen is added, a brief transient increase in conductance results. Whether the underlying mechanisms are similar to those responsible for the action of a transmitter at a synapse remains to be determined. Because nicotine has the same action on a choline esterase-treated membrane as does acetylcholine, but is not split, and because of a variety of other drug actions, these workers (*Ann. N.Y. Acad. Sci.*, in press) have concluded that the initial attachment results in the permeability changes, and that these changes are a function of the rate of attachment and not of the total number attached.

In this work with antibody–antigen or enzyme–substrate interactions on the membrane, both components must be placed on the same side. Another kind of experiment of the Rudin–Mueller type has been done by Bean and coworkers at Philco.[15] They use membranes made of purified phospholipid, tocopherol, and choles-

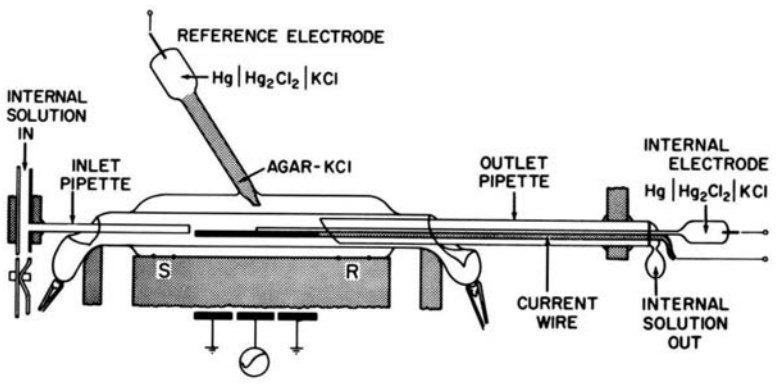

FIGURE 12 Arrangement for simultaneous perfusion and voltage clamping of the squid giant axon. (From Rojas and Ehrenstein, Note 19)

terol, and add what they call a membrane-activating factor, which results in a large decrease in the membrane resistance. Addition of chymotrypsin removes the effect of the activator. However, in this case it is not necessary to put the enzyme on the same side of the membrane as the activating factor (Figure 13). One of these substances has at least "poked a finger" through the membrane, and we may not be able to say which side of the membrane a protein is on; it may be on both sides at the same time.

Summary and concluding remarks

The flow chart shown in Figure 2, with the phenomena of electrotonic spread, sums up all the features (of which I am aware) involved in making it possible for a neuron to take its place in a nervous network. In no case is the molecular mechanism understood, but most of the phenomenology is clear, at least in broad outline. It is tempting to speculate that a process—much like an enzyme–substrate interaction in or on a lipid film—occurs both at the postsynaptic region and in the axonal membrane, and that this process determines the ion permeability and is somehow modulated by the applied electric field. While an axon perfused with potassium fluoride does not pump sodium ions actively (Rojas, personal communication), it will continue to produce action potentials for a very long time. For this reason it seems necessary to conclude that any energy needed to drive the reactions involved must come from the ion gradients. The one ion gradient that appears to be indispensable is that of calcium, but further speculation is probably not profitable without a great deal of further experimentation.

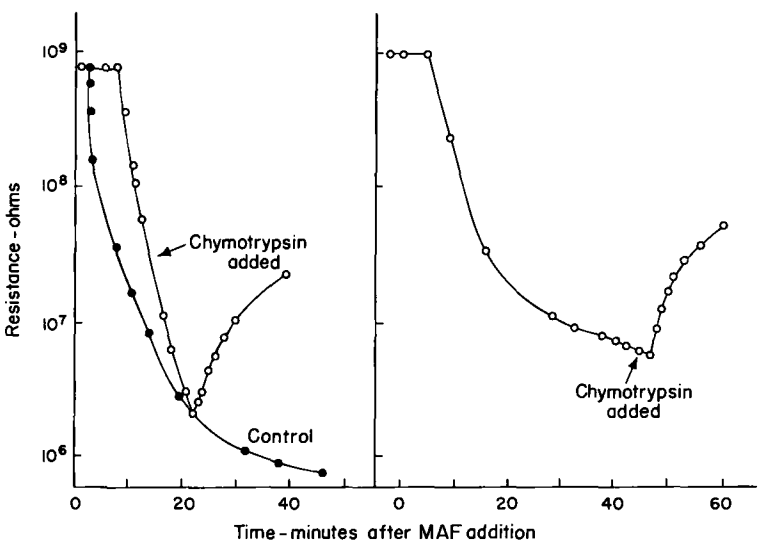

FIGURE 13 Effect of addition of activating factor to one side of artificial lipid membrane and the removal of this effect by chymotrypsin on the same side (left) or opposite side (right). (From Bean, Note 15)

The Molecular Mechanism of Active Transport

RONALD WHITTAM

ACTIVE TRANSPORT concerns the mechanism by which the structural organization and activity of enzyme molecules bring about a movement of ions. We do not yet know the details, but the mechanism can now be thought of in terms of the behavior of enzymes in the membrane.

Until about 30 years ago it was generally thought that ions cross the membranes of living cells only in a few special circumstances. Movements of salts across secretory or absorbing epithelia were well known, as was the chloride shift in red cells. Evidence was lacking, however, for the transport of sodium and potassium across red cells, and muscle and nerve fibers, which are not in the form of epithelia or tubules. Before World War II, new experimental techniques were developed that principally utilized radioisotopes and flame photometry; these led to radical changes in concepts of the permeability of cells to cations. Many workers showed that ions, like other body constituents, are in a state of dynamic equilibrium. Alkali metal ions in all the cells of the body exchange with other ions of the same element outside the cells. This kind of exchange is striking, because of the concentration gradients that are maintained across cell membranes. Sodium is always in a lower concentration inside the cell than outside, and cells are richer in potassium than in plasma.

Role of cell membrane

What, then, is the mechanism of these ion exchanges in cells that maintain concentration gradients? There are, in principle, only two alternatives. First, there could be a selective binding of ions or, more precisely, a change in ionic activity in cells to make the electrochemical potential the same inside and outside. Ions could still exchange, as they do between a salt solution and an ion-exchange resin, but the membrane would not play a direct part in regulating the cell's ionic composition. This view is advocated from time to time and might account for the internal potassium concentration; it could not explain the low sodium content. The concept has been not unequivocally verified in experiments. Second, the cell membrane could actually regulate the entry and exit of ions in a way that depends on chemical reactions and not just on membrane permeability. This means that ions move *with* the gradient of electrochemical potential in one direction and *against* it in the other direction. Sodium moves passively inwards and potassium passively outwards. These passive, "downhill" movements are balanced by an active transport of ions in the opposite direction—a process in which energy is expended. Active transport is from a solution of low electrochemical potential to one of high.

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THERMODYNAMIC ASPECTS Another way of considering active transport is as an irreversible process requiring energy from metabolism. As such, it can be analyzed by the thermodynamics of the steady state. In the thermodynamic sense, the system as a whole is open and in a steady state. Here the process is one of energy transduction—chemical energy is transformed into osmotic work by a chemiosmotic coupling reaction. This feature of active transport is particularly interesting because, in living matter energy transformation is a salient characteristic as typical as reproduction. The transformation is complex, for the enzymes are arranged as supramolecular assemblies that form a functional unit. The attachment of enzymes to membranes introduces a geometrical, or vectorial, feature into the mechanism of action, and the attachment cannot be explained solely by the classical methods of enzymology. The thermodynamics of irreversible processes is a guide to experimentation in this context, because the Curie principle states that a chemical reaction coupled to a vectorial flow of matter must itself show spatial asymmetry. Sophisticated criteria of active transport, based on steady-state thermodynamics, are helping to provide a valuable theoretical background to the process.[1]

Active transport is physiologically important for all cells, excitable and nonexcitable alike. First, the passive ionic changes that occur in excitable cells during the action potential are reversed. Second, the sodium pump is needed in cells in general to regulate cell volume; this process allows the cells to exist in plasma or interstitial fluid. Internal macromolecules exert an osmotic pressure that would attract water inward, were it not for the sodium pump, which maintains a low internal sodium concentration. The uneven distribution of sodium ions is essential for the survival of cells, for without it cells would swell and eventually burst. Third, potassium is needed as an activator of

some intracellular enzymes, and in its absence, or at low concentrations, important metabolic reactions can be impeded.

THE LINKED TRANSPORT OF SODIUM AND POTASSIUM In the 1950's there were two outstanding questions about ion movements. The first related to the kinetic nature of the transport of sodium and potassium, and in particular to possible linkage of the ion movements. That is, are there separate transport systems for sodium and potassium or, alternatively, are ion movements coupled, so that sodium is transported outward only when potassium is present in the external medium that is ready to be transported inwards? There are a few exceptions but, in general, it was found that there is a tight coupling of sodium and potassium movements in normal cells. Further, the joint transport of sodium and potassium is inhibited by cardiac glycosides in very low concentrations; their site of action appears to be the external surface of the membrane.[2]

The second question concerned the nature of the energy supply for active transport. One view was that oxido-reduction reactions of the respiratory chain were involved: another was that energy was required to maintain possible intracellular structures that might be needed for lowering ionic-activity coefficients. Most results, however, support the concept that energy from metabolism is supplied as adenosine triphosphate (ATP), which is then split by the sodium pump.[3] Thus, in red cells and nerve axons bereft of a supply of energy from metabolism, sodium can still be actively transported when ATP or one of its precursers, such as phospho-enolpyruvate, is present. We can, therefore, safely accept that there is a coupled transport of sodium and potassium in which ATP is utilized, and can now consider the nature of the reactions taking place.

Hydrolysis of ATP by cell membranes

If ATP is split by the active-transport system, does the rate of hydrolysis depend on whether sodium and potassium are present alone or together? Does potassium outside the cell affect the hydrolysis of ATP inside the cell? Early attempts to answer these questions with intact red cells were beset with analytical difficulties. The breakthrough came when Skou described the enzymatic breakdown of ATP that was stimulated synergistically by sodium and potassium.[4] A homogenate of crab nerve was fractionated, and the microsomal fraction, thought to contain the membranes, hydrolyzed ATP to adenosine diphosphate (ADP). Magnesium was needed for the hydrolysis, but, in addition, sodium and potassium together vastly increased the rate of breakdown. This was a novel finding, because enzymes that are activated by potassium are inhibited by sodium. Like other key discoveries, this one was foreshadowed in the literature. Utter[5] had described ATP hydrolysis in a brain preparation that was stimulated by sodium, but the new, important result was that the activity of the enzyme adenosine triphosphatase was stimulated by the transported ions. The requirement for magnesium has led to the generally accepted view that the enzyme is magnesium activated. However, magnesium actually is part of the substrate, which is MgATP.[6] Figure 1 shows how sodium activation needs potassium. Another feature of these ions is that at high potassium concentrations, potassium overcomes sodium activation.

INHIBITION BY OUABAIN An important finding was that strophanthin inhibited that part of the activity which needs both sodium and potassium. There was no inhibition of the activity that required only magnesium. The inhibition by very low concentrations of the cardiac glycoside ouabain can be overcome by raising the potassium concentration, and there appears to be a competitive relationship between potassium and ouabain, both as regards transport and adenosine triphosphatase activity.[7] The action of ouabain on intact red cells is always quantitatively comparably for the adenosine triphosphatase and the transport, so it is justified to regard ouabain as inhibiting that part of the enzymatic activity that is related to transport.[8] This point has not been established so precisely for any other cell or tissue, but can be reasonably assumed to ap-

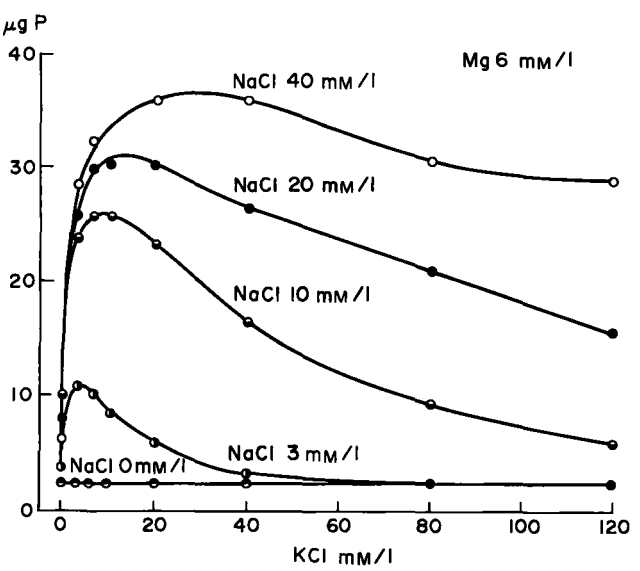

FIGURE 1 Enzyme activity in relation to the concentration of K^+ in the presence of Mg^{++}, 6 mM/1, and different concentrations of Na^+. Abscissa: potassium concentration in mM/1; ordinate, μg P removed from ATP in 30 minutes. (From Skou, Note 4)

ply generally. These inhibitory effects of glycosides are probably not connected with their inotropic action. In fact, in very low concentrations comparable to those of therapeutic doses, it seems that stimulation of the transport adenosine triphosphatase might occur.[9] It is pertinent that stimulation by glycosides, as well as inhibition, requires both sodium and potassium.

SIGNIFICANCE OF THE ACTIVITY OF ADENOSINE TRIPHOSPHATASE These are the main findings with fragmented membranes. Many other workers have extended the discoveries of Skou in studies of a wide range of cells and tissues.[10] Every cell that has a coupled transport of sodium and potassium has an adenosine triphosphatase that is activated by these two ions. It is widely accepted that enzymatic splitting of ATP is intimately connected with the working of the sodium pump. There is a particularly high activity of transport adenosine triphosphatase in membrane preparations from excitable tissues such as electric organ, brain cortex, and peripheral nerve, as well as in such secretory tissues as the kidney, the salivary gland, and the salt gland of marine birds. Again, there is a good correlation between enzyme activity, transport rates, and level of internal potassium in red cells from different breeds of sheep.[11] Sheep with high- or low-potassium red cells have comparable differences in activity of transport adenosine triphosphatase. I cannot cover the many studies that show interesting points about the hydrolysis. Instead, I shall concentrate on two aspects of enzyme activity; first, the role of the transport adenosine triphosphatase in cell metabolism and, second, its mechanism of action so far as active transport itself is concerned.

Active transport as a pacemaker of metabolism

Active transport utilizes ATP, and in any consideration of cellular energetics questions arise relating to the role of active transport in the energy turnover of cells. It is an old notion in physiology that cells are economical in adjusting their metabolic rates to meet energy demands for physiological processes. The general concept is that cells have a basal rate associated with resting conditions and, particularly in secretory epithelia that are subject to hormonal stimulation, a variable rate depending on the work done. Nervous stimulation also elicits an increased respiratory rate owing to the increased demands for energy for the reversal of the passive ionic changes that occur during nervous conduction. For conduction to continue, the passive ionic changes that occur during the action potential must be rapidly reversed. Active transport as a physiological process is more rapid in nervous tissue than in other tissues, and would therefore be expected to be a major component of energy utilization. Rapidity of sodium transport in neurons and axons is, of course, essential if the cells are to maintain a high conduction velocity. Thus, the rate of sodium efflux in cat motor neurons is about three times as high as the rate in rat diaphragm muscle, and at least a hundred times greater than sodium efflux in human erythrocytes.[12] It is obvious that the high rate of ATP utilization involved in active transport in nerve cells must have repercussions on the metabolic reactions that generate ATP.

Interdependence of transport adenosine triphosphatase activity and respiration

CONTROL OF METABOLISM IN RED BLOOD CELLS Red blood cells have provided a clear instance of control of metabolism by the transport enzyme.[8] In these cells the rate of lactate production is an indication of ATP synthesis, and the cells adjust their lactate production according to the rate of splitting of ATP in active transport. Thus, lactate production was changed when active transport had occurred at different rates. The metabolic rate was changed by varying the concentrations of external potassium and of internal sodium—variations that do not directly affect glycolytic enzymes in the ranges studied. The changes in ionic concentrations elicit changes in transport rates with attendant effects on the rate of ATP utilization. Just as ATP is produced by glycolysis, so the rate of glycolysis was regulated by the products of ATP splitting by active transport. There is a basal rate of metabolism that is independent of active transport but, in addition, there is a variable rate controlled by active transport. The transport-controlled part can vary from 25 per cent to 75 per cent of the basal rate. It is clear that even in human red cells active transport is a significant pacemaker of metabolism.

RESPIRING TISSUES ATP is supplied from metabolism solely by oxidative phosphorylation in mitochondria and by glycolysis. Because the functioning of nerves, and especially the cerebral cortex, is critically dependent on a supply of oxygen, the major source of ATP must be from oxidative phosphorylation. Much work on the relationship between metabolism and active transport has amply demonstrated that metabolic inhibition decreases the rates of active ion movements by lowering the rate of ATP production.[3] The question arises whether cellular respiration might, in turn, be controlled by the active-transport process. In what way is ATP production coupled to ATP utilization? Is there a positive feedback system, such that an increase in ATP splitting by active transport elicits an increase in oxygen consumption? Equally important, how

much of the total ATP produced during intermediary metabolism is devoted to active transport?

These questions cannot be tackled by work with such direct inhibitors of respiration as cyanide and azide, inasmuch as all energy-yielding processes are then blocked. An approach is afforded, however, by using procedures that modify the rate of active transport but do not involve assault upon the ATP-producing reactions. Thus, cessation of active transport by ouabain or by removal of sodium or potassium would affect the rate of oxygen consumption only insofar as the transport adenosine triphosphatase activity was inhibited, as this enzymatic activity is one that partly regulates the level of ADP upon which the rate of oxidative phosphorylation depends.

Tissue slices of brain and kidney cortex and liver provide good evidence that the rate of oxygen consumption is controlled by the rate of transport.[13] Inhibition of active transport by ouabain caused a decrease in oxygen consumption that was proportional to the degree of inhibition of active transport. In the absence of sodium ions ouabain showed no effects, presumably because there was then no active transport. There was also a parallelism between the oxygen consumption and transport rate when the rate of ion movement was regulated by changes in sodium concentration. Results with mammalian brain slices and renal cortex suggest that some 40 per cent of the energy derived from normal oxygen consumption is utilized by active transport.

In order to prove that the results with tissue slices were, in fact, the result of changes in transport adenosine triphosphatase activity, it was necessary to compare directly the activity with the rate of oxygen consumption. This is impossible in tissue slices, but it can be done with homogenates. The characteristics of respiration and enzymatic activity show that graded inhibition of activity caused a comparable inhibition of oxygen consumption. Figure 2 shows the parallelism of oxygen consumption and adenosine triphosphatase activity of rabbit brain homogenate after ouabain was added.[14] The same kind of parallelism has been shown in homogenates of kidney cortex.[15]

In analyzing the effect of active transport on metabolism, one must consider cellular activities at different levels of organization. First, to measure transport an intact cell is necessary. Metabolism (respiration or glycolysis) in intact cells and tissue slices has been shown to depend on the coupled active transport of sodium and potassium. Second, measurement of the enzyme activity and oxygen consumption in homogenates has shown that these two processes are interdependent. The two alkali metals have a cooperative effect on oxygen consumption; this can be attributed to their effect on the transport enzyme that is

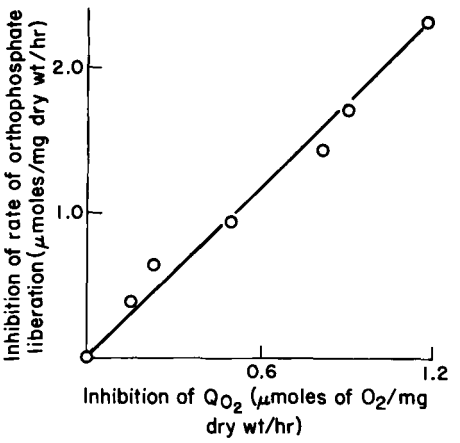

FIGURE 2 Parallelism of the graded inhibition of respiration and adenosine triphosphatase activity in brain-cortex homogenates, by different concentrations of ouabain. Each point is the mean of four determinations made in two separate experiments. (From Whittam and Blond, Note 14)

present in fragmented membranes in the homogenate. An important related point is that the enzyme activity of pure mitochondria was unaffected by the two ions and by ouabain. If the effect of the transport enzyme activity on respiration is caused by an effect on the concentrations of adenine nucleotides, there should be a relationship between the oxygen consumption and the ADP concentration. Figure 3 shows that in kidney cortex homogenate there was a correlation between ADP concentration and oxygen uptake.[16] Both were constant during incubation from thirty to fifty minutes, and the system was thus in a steady state in which ATP hydrolysis to ADP was balanced by ATP synthesis through oxidative phosphorylation. The level of ADP, determined by the adenosine triphosphatase activity, regulated the rate of oxygen consumption.

The observations can readily be explained according to the concepts for respiratory control discussed by Lehninger.[17] Figure 4 summarizes the mechanism involved. The scheme does not involve new mechanisms at any one level of cellular organization. It does, however, show the way in which active transport plays a crucial role in cellular metabolism. The energy supply from mitochondria is in the form of high-energy intermediates that are related to active transport. There are also high-energy intermediates in the cell membrane as well as in mitochondria, but as these compounds are present in small amounts it seems unlikely, a priori, that such compounds would diffuse between cell membrane and mitochondria in the same way as ATP. Earlier work has suggested that electrical activity in a nerve stimulates respiration, and

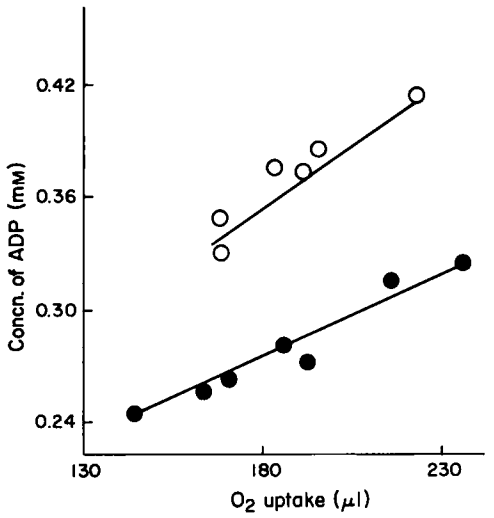

FIGURE 3 Dependence of O_2 uptake on ADP concentration controlled by Na^+-plus-K^+-activated adenosine triphosphatase. ○, with variations in K^+ concn. from 50 to 150 mM and in Na^+ concn. from 0 to 100 mM; ●, graded inhibition by submaximal concentrations of ouabain, at 100 mM-K^+ and 50 mM-Na^+. (From Blond and Whittam, Note 16)

these effects can be attributed to production of ADP following increased activity of the sodium pump.

Mechanism of action transport adenosine triphosphatase

One question now being investigated is whether there are two separate ATP-splitting enzymes in cell membranes, only one of which is activated by sodium and potassium. Alternatively, activation by alkali metals in unbroken normal cells may really apply to all the enzymatic activity.

Rendi and Uhr,[18] in their work with kidney tissue, found evidence for two different enzymatic systems. Thus, calcium and barium ions replaced magnesium for the "basic" adenosine triphosphatase, but not for the transport form. Other nucleotides were hydrolyzed by the basic form, but not by the transport enzyme. Skou and Hilberg,[19] on the other hand, worked with a membrane preparation from ox brain, and concluded that the adenosine triphosphatase activity with magnesium, and with magnesium, sodium, and potassium, is caused by the same enzyme. It appears from their experiments that the amount of sodium- and potassium-sensitive enzyme activity is increased when reagents that affect the structural configuration of proteins are added. Thus, treatment with deoxycholate, aging in a tris buffer or in a solution of urea, causes an increase of the component of enzymatic activity that requires sodium and potassium. The disappearance of adenosine triphosphatase activity with magnesium alone led to an increase in that component of the activity that required, in addition, sodium and potassium. It is attractive to suppose that these various reagents affect the structural configuration of a protein in the enzyme system. The presence of ATP seemed to hinder the changes in structural configuration that sometimes led to a decrease in activity of the transport enzyme.

It is somewhat difficult to relate this conclusion to the results that have been obtained with intact red cells. Here the total activity appears to consist of two components—one depending on the rate of active transport, and the other unaffected by the process. Of course, it is possible that the adenosine triphosphatase activity in erythrocytes has lost some of the high transport enzyme activity that would be necessary for the viability of more active cells. A further way of reconciling the results is to suppose that

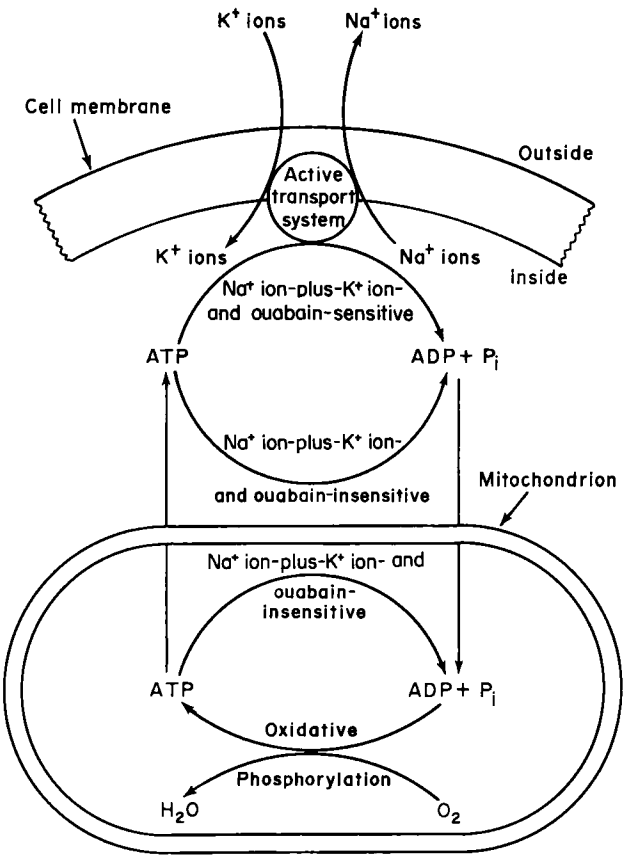

FIGURE 4 Coupling between active transport, respiration, and adenosine triphosphatase activity in rabbit kidney cortex. (From Blond and Whittam, Note 15)

the rate of transport in red cells—and hence the rate of transport enzyme activity—might be increased by the agents that have led to the increases in activity of the enzyme in fragmented systems.

There remains the question of whether an adenosine triphosphatase represents a single enzyme or a complex of two enzymes. There have been no clear experiments to distinguish between a straightforward hydrolysis of ATP, or a transphorylation of the terminal phosphate of ATP to intermediates by the action of a phosphokinase enzyme, followed by dephosphorylation caused by the action of a phosphatase. This is particularly important when dealing with the nature of the intermediate that has been found in the enzyme reaction.

LOCATION OF THE ENZYME IN CELLS Work with brain cortex has clearly established that the sodium–potassium-activated enzyme is found in cell membranes and not in the cell cytoplasm or intracellular particulate matter. Morphological work by Cummins and Hydén showed that the transport enzyme is located in the surface membrane of neurons.[20] Glia are relatively weak in the enzyme. Hydén isolated the neuronal membrane and demonstrated that it contained high sodium-activated adenosine triphosphatase. Microsomal preparations from homogenates can safely be assumed to contain the cell membranes. There is a high activity in this fraction compared with others.[14] Nerve endings or synaptic vesicles (synaptosomes) are particularly rich in the enzyme.

The intermediate of the transport reaction

Tissues vary widely in their rates of transport. As a result, cells convenient for transport studies, such as red cells or single nerve and muscle fibers, may not be right for studying enzymatic behavior from the detailed chemical aspect. In contrast, brain and kidney have drawbacks as systems for transport studied, but yield excellent material for enzyme studies. I must therefore select results from these two groups of cells in clarifying the mechanistic questions that arise.

There is a chemical reaction associated with a flow of matter. The ions can be regarded as activating factors in the reaction, as the reaction does not occur unless they are present. Although, as described above, it is not yet clear whether one or two enzymes are involved, I will refer simply to the enzyme system. There must be an enzyme–substrate complex and the question arises whether an actual intermediate is formed. The microsomal fraction from brain or electric organ has been incubated with gamma-labeled, radioactive ATP, and the fate of the tracer determined. Table I shows a result with electric organ.[21] The particles became labeled in a way that must have involved activity of the transport enzyme. The extent of labeling depended on the sodium and potassium present. Magnesium alone, or magnesium plus potassium gave about the same amount of labeling, but sodium almost doubled the counts. Furthermore, potassium opposed the action of sodium, so that when the two ions were present the labeling was somewhat decreased. Other similar studies with kidney preparations gave comparable results. Post et al[22] showed that ouabain interfered with the effect of potassium, and we showed that the antibiotic oligomycin also inhibited the potassium step of the reaction.[23] Oligomycin inhibits energy transformation in oxidative phosphorylation and also inhibits the active transport of sodium and potassium in red cells, in which ATP is provided solely by glycolysis. The inhibition of two such diverse processes by oligomycin may have to do with a common high-energy phosphate intermediate. In each of the tissues investigated, the labeled part of the particles was not lipid but protein.

It had been previously suspected that the turnover of a phosphoprotein—formed by a protein phosphokinase and hydrolyzed by a phosphatase—might be associated with the recovery of sodium and potassium concentration gradients in cerebral cortex slices after a period of electrical stimulation.[24] On the other hand,Hokins, et al., studying the salt gland of sea birds, thought that phosphatidic acid might be the intermediate; this, too, would depend on the action of a phosphokinase and a phosphatase. More recently, however, they have rejected the possibility that this compound or even phosphorylserine participate in the transport enzyme from the guinea pig brain.[25] Phosphatidic acid and other phospholipids have

TABLE I

Effect of Cations on Phosphorylation of Electric Organ Particles

Additions	Bound radioactivity cpm (25 -0)
None	92
Mg^{++}, 0.003 M	145
Mg^{++}, 0.003 M + Na^{+}, 0.06 M	345
Mg^{++}, 0.003 M + K^{+}, 0.025 M	114
Mg^{++}, 0.003 M + Na^{+}, 0.06 M + K^{+}, 0.025 M	264
EDTA, 0.002 M	94

The incubation volume of 50 μl contained enzyme, the indicated concentration of cations as chlorides, 0.02 M Tris phosphate *p*H 7.5, and 13 mμ moles ATP^{32}, 6×10^5 cpm. (From Albers, Fahn, and Koval, Note 21)

also been excluded as the intermediate in the transport enzyme activity of electric organ.[26]

If there is an intermediate, its amount or rate of turnover should be related to the rate of the over-all ATP splitting. An important result in this context is furnished by Post, et al.,[22] who plotted the amount of ^{32}P intermediate against the sodium-enzyme activity of a microsomal preparation of kidney (Figure 5). There was direct proportionality, suggesting that the amount of the labeled phosphate intermediate is a measure of the transport enzyme activity. The over-all reaction, therefore, seems to involve an intermediate that is markedly affected by sodium, potassium, and ouabain. The properties of the intermediate can be summarized:

1) Sodium is required for its formation.
2) Potassium is required for its dephosphorylation.
3) Ouabain inhibits both sodium- and potassium-activated steps, but inhibits the potassium step at lower concentrations than it does the sodium step.
4) Oligomycin inhibits dephosphorylation of the intermediate.

The intermediate is evidently a chemical entity intimately connected with transport adenosine triphosphatase activity. It is more closely concerned than is ATP itself with active transport, and represents the chemical compound of the chemiosmotic coupling reaction. As with intermediates in other processes, it is detected only in abnormal circumstances, in this case in the absence of potassium. The present evidence does not allow one to answer the criticism that the intermediate is really an artifact. In the normal membrane the transport enzyme works without a detectable intermediate. It certainly follows that the intermediate would not accumulate in the functioning sodium pump; only when active transport is abolished by deprivation of potassium or by addition of ouabain would it become detectable.

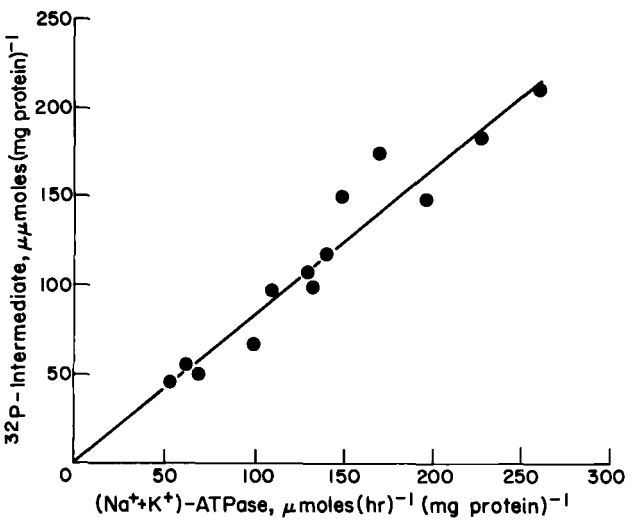

FIGURE 5 Comparison of the Na^+-dependent ^{32}P-intermediate of various membrane preparations from guinea pig kidney with their ($Na^+ + K^+$)-adenosine triphosphatase activity. (From Post, Sen, and Rosenthal, Note 22)

THE ACYL PHOSPHATE BOND The latest development has been to decipher the nature of the chemical bond linking phosphate to protein. Work with brain and kidney again yielded similar results, showing essentially that an acyl phosphate is formed. Thus, an acyl phosphatase caused liberation of P_i from the intermediate.[27] Further, in a brain preparation hydroxylamine caused breakdown (Table II), and there was greater lability towards alkali than acid, as was expected with an acyl phosphate.[25] Further evidence for the acyl nature of the phosphate in the intermediate is the finding that acetyl and carbamyl phosphates are hydrolyzed by the transport enzyme in a way that required potassium. Other phosphate esters, such as glucose-6-phosphate, were not hydrolyzed in the same way.[27] Inhibitors of ATP hydrolysis, like ouabain, also inhibited acetyl phosphate hydrolysis, and concentrations for half the maximal inhibition were the same as for ATP hydrolysis. There was competition between ATP and acetyl phosphate, in both the presence and absence of sodium, and it is not obvious why ATP should inhibit when no sodium was present, inasmuch as no intermediate would have been formed. At first sight, competition would be expected to occur between acetyl phosphate and the intermediate, not between acetyl phosphate and ATP.

The phosphate in the intermediate need not be attached to an amino acid, although it could be linked to aspartate or glutamate in a peptide chain, or to an α-carboxyl group of a terminal amino acid. Another possibility is a link with sialic acid, which is in cell membranes; phosphate could conceivably be attached to an acyl group in this acid or in neuraminic acid. This possibility is particularly attractive, for the negative charge on the outer surface of red cells is caused by sialic acid.

A point that has not yet been tackled is whether the intermediate is the enzyme itself or a separate entity. Can the intermediate be freed from enzyme? Can ATP be remade from the intermediate on adding ADP? It will be important to clarify the step of the reaction that requires potassium. Nevertheless, so far as mechanism is concerned, the important point is that a chemical intermediate has been demonstrated. It is probably high energy in nature, and can be taken to be the chemical compound that is immediately involved in the chemiosmotic coupling. The result of the fission of ATP by the transport enzyme is

TABLE II

Release of Inorganic Phosphate from the Labeled Protein by Hydroxylamine or Alkali

Fraction	Radioactive Phosphate in Fraction ($\mu\mu$moles/mg protein)			
	$Na^+ + K^+$	Na^+	Δ	Per cent
Total protein	100	644	544	(100)
Inorganic phosphate released by hydroxylamine	66	462	396	73
Inorganic phosphate released at *p*H 10.7	29	411	382	70
Inorganic phosphate released by 0.64 N NaCl	11	18	7	1.3

(From Note 25)

summarized in Figure 6. A direct relationship between the protein–phosphate intermediate and actual ion movements across membranes has yet to be demonstrated.

Functional significance of transport adenosine triphosphatase

ADENOSINE TRIPHOSPHATASE AS A FUNCTIONAL UNIT Classical purification techniques and, indeed, studies with fragmented membranes are evidently far removed from enzyme function in the living cell. It is natural, however, to attempt to purify the enzyme, and much effort has been directed to this end. There are various ways of preparing micellar units of adenosine triphosphatase activity, but so far a pure protein with characteristics of the enzyme has not been obtained. Indeed, the evidence suggests that the typical synergistic activation by sodium and potassium, and inhibition by ouabain, is a property of a functional unit of the membrane rather than just of a component. Thus, Ahmed and Judah[28] obtained a lipoprotein that had a rich transport enzyme activity. Again, Tanaka and Strickland[29] found that a protein preparation free from lipid was without such activity, yet the activity was elicited when lecithin was added. Preparations without lipid certainly appear to have lost the response to sodium and potassium.

The physiological significance of the enzyme system relates to its molecular mechanism. Fragmented membranes used in chemical studies are, of course, bathed uniformally by the medium in which they are suspended. Transport cannot be studied because there is no separation of the two fluid compartments that contain the ions. Perhaps the most striking aspect of the enzymatic reaction is its spatial asymmetry.[30] A fluid rich in sodium and poor in potassium bathes the external face of the membrane, while inside there is much more potassium than sodium.

Intermediate Reactions of Active Transport

$$\text{MgATP} + \text{PROTEIN} \xrightarrow{\text{Activated by } Na^+} \text{PROTEIN-PHOSPHATE} + \text{ADP} + \text{Mg}$$

$$\text{PROTEIN-PHOSPHATE} \xrightarrow[\text{inhibited by ouabain and oligomycin}]{\text{Activated by } K^+} \text{PROTEIN} + P_i$$

$$\text{ATP} \longrightarrow \text{ADP} + P_i$$

FIGURE 6 Result of fission of ATP by the transport enzyme.

Therefore it is essential to investigate enzyme activity in whole membranes under conditions in which concomitant measurements can be made of ion fluxes. What, for example, are the sites of action of sodium and potassium? This question can only be tackled with intact membranes. Further, is the hydrolysis of ATP stoichiometric in regard to the number of ions moved? Is enough energy available from ATP hydrolysis for the osmotic work that is done? Is the rate of the enzymatic reaction determined by the electrochemical potential gradients of ions, or just by the concentration of ions on the side from which they are pumped? There is the important question of regulation, inasmuch as cells vary in their concentrations of sodium and potassium in the steady state. Thus, some red cells have high potassium and others low potassium. On all of these questions most of the information derives from work with red cells, because active transport and enzyme activity can be measured simultaneously.

ACTIVATION BY INTERNAL SODIUM AND EXTERNAL POTASSIUM Ghosts containing MgATP can be prepared, and the internal and external cation content can be varied independently. The site of action of the cations can then be determined, and the results show that sodium activates from inside and potassium from outside. Figure 7 shows a typical experiment. In a medium containing 10 millimolar potassium, the adenosine triphosphatase activity depended on the internal sodium concentration; as the concentration was raised, so was the enzyme activity. In the absence of external potassium, this effect was not evident, indicating that internal sodium and external potassium are needed together in order to activate the transport enzyme. These results show that the transport enzyme is spatially oriented like the ion pump. The enzyme system responsible for the supply of energy for active transport shows the same spatial orientation and asymmetry in activation by ions as does the sodium pump. Cations activate the enzyme on the side of the membrane from which they are transported. An important point is that the enzyme activity resulting from internal sodium and external potassium is the part inhibited by ouabain.

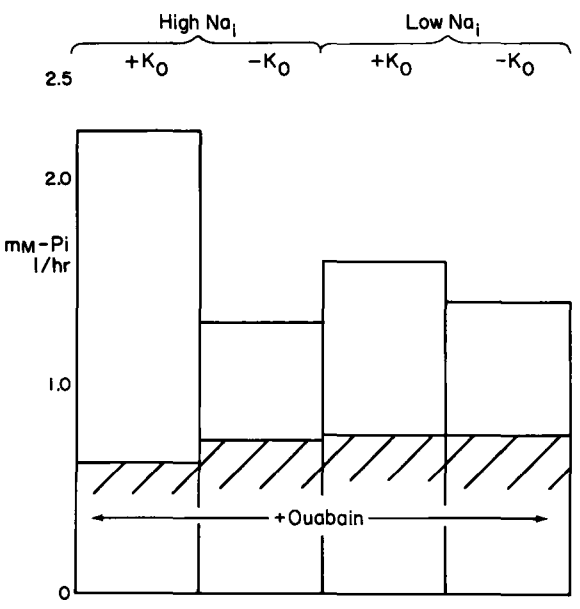

FIGURE 7 In potassium medium, adenosine triphosphatase activity increased as internal sodium concentration was raised. Without external potassium, effect was not seen. Results indicate internal sodium and external potassium are needed to activate transport enzyme. (From Whittam, Note 30)

COMPETITION BETWEEN IONS When the external sodium concentration is high relative to that of potassium, sodium hinders activation by external potassium.[31] Interference by sodium ions of potassium activation occurs at the external surface of the membrane. Sodium ions can apparently displace potassium ions from their site of activation when the potassium concentration is low. There was a greater stimulation of enzymatic activity by low potassium concentrations in various sodium-free media than in those that contained physiological concentrations of sodium. A similar situation has been found in sodium activation at the inner surface of the membrane. This illustrates further the spatial asymmetry of the membrane adenosine triphosphatase, which shows a dual response to sodium and potassium ions depending on their location. Active ion movements show the same dual response to sodium ions, because external sodium ions retard the influx of potassium.

In intact red cells, which produce lactate from glucose, transport enzyme activity can still be conveniently measured as the sum of the phosphate and lactate production that is sensitive to external potassium and to ouabain. When red cells are in a steady state there is no production of phosphate, and lactate is the only end product of metabolism. Lactate production that depends on external potassium can be taken as a measure of transport enzyme activity, because each molecule of lactate produced requires the esterification of one molecule of P_i when glyceraldehyde-3-phosphate is converted to 1,3-diphosphoglycerate. Table III shows that omission of potassium from the medium, or the addition of ouabain in the presence of potassium, caused a fall in metabolic activity. Furthermore, ouabain had no effect on a potassium-free medium. These results indicate that the effects arose through changes in the activity of the transport enzyme.[32]

TABLE III

Effect of External K and Ouabain on P_i and Lactate Production in Red Blood Cells

	Medium	μmole/ml. cells/hr. Fresh cells	Week-old cells
P_i	+K	0.62	1.62
	−K	0.46	1.24
	+K+ouabain	0.43	1.19
Lactate	+K	1.9	1.25
	−K	1.4	1.0
	+K+ouabain	1.5	1.0

Quantitative correlation of enzyme activity and rates of active transport

Transport rates can be varied in three ways without directly affecting the supply of ATP from metabolism (1) by altering external potassium concentration; (2) by altering internal sodium concentration; and (3) by partial inhibition with ouabain.

VARIATION WITH EXTERNAL POTASSIUM The enzyme activity (indicated by the phosphate and lactate production sensitive to ouabain) showed the same dependence on external potassium as does the potassium influx. There was a quantitative similarity in effects of external potassium on active transport and on the transport enzyme. It was possible to determine the relationship between active potassium influx and the transport enzyme by making measurements in the same experiment. The potassium influx sensitive to ouabain at different potassium concentrations (1 to 20 mM) was compared with transport adenosine triphosphatase activity that was also sensitive to ouabain. A change in active potassium transport was accompanied by a proportional change in an enzymatic activity (Figure 8). The ratio of potassium to ATP was constant over a range (1 to 20 mM of K), with a mean value of 2.2±0.1 (six observations).

VARIATION WITH INTERNAL SODIUM Internal sodium concentration was also a determinant of enzymatic activity and active transport rates (Table IV). Raising the internal sodium concentration from 12 to 100 moles per milliliter caused a doubling in the rate of potassium influx. The potassium/ATP ratio, however, was constant and independent of the internal sodium concentration with a mean value of 2.1±0.01 (four observations).

The internal sodium concentration regulated the rates of net transport of sodium and potassium (Figure 9). The net potassium uptake was always less than net sodium extrusion, but both ion movements increased in a similar way with increase of internal sodium concentration. The difference between the ion movements in the presence and absence of ouabain was again taken to indicate the rate of net active transport. When the differences for sodium and potassium were compared with the accompanying rates of adenosine triphosphatase activity in eight observations, the mean value of the sodium/ATP ratio was 3.2±0.2; the potassium/ATP ratio was 2.4±0.3. In each case there is the same relationship between the number of ions moved per molecule of ATP hydrolyzed.

An important conclusion emerging from these results is that the ratio of ions transported per molecule of ATP hydrolyzed is independent of the concentration gradient over the range studied, and is dependent only on the concentration of the ion on the side from which it is transported. This conclusion may point to the application of thermodynamics to active transport.

OTHER MOVING IONS There is a disparity in the number of monovalent cations that are transported in opposite directions across the membrane. More sodium ions move outward than potassium inward. A similar discrepancy is found in the passive, rapid ionic changes that cause the action potential, and in that situation chloride and calcium seem to be involved. In the red cell, it is likely that chloride

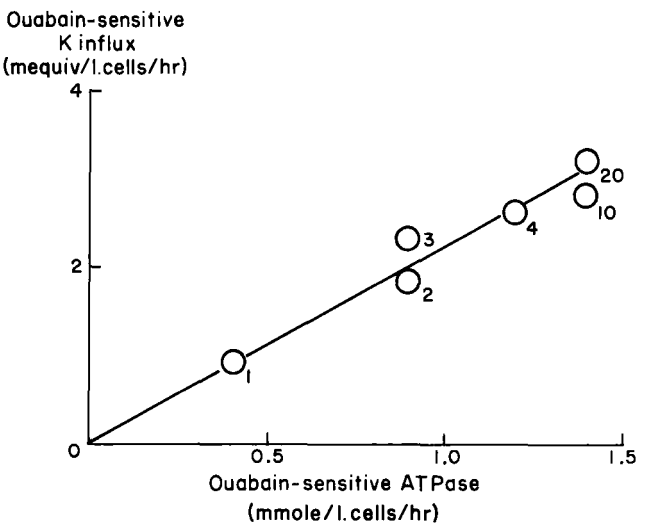

FIGURE 8 Linear relationship between the ouabain-sensitive components of K influx and ATPase activity. The measurements were made in the same experiment, and the numbers near the circles indicate the various K concentrations (1–20 mM) that were used.

TABLE IV

Values of the K^+/ATP ratio in erythrocytes of varied Na^+ content

Erythocytes were loaded with different concentrations of Na^+ by the "lactose" procedure, and suspended in a medium containing K^+ (10 mM) with or without ouabain (40 μM). P_i and lacate production and K^+ influx were determined after incubation for 1 hr. at 37°. Column (a) is the sum of ouabain-sensitive P_i and lactate production.

Internal concn. of Na^+ (Mequiv./ml. of cells)	Quabain-sensitive ATPase activity (μmoles/ml. of cells/hr.) (a)	Quabain-sensitive K^+ influx (Mequiv./ml. of cells/hr.) (b)	K^+/ATP (b) : (a)
12	0.8	1.8	2.3
18	1.6	2.8	1.8
31	2.0	4.3	2.1
100	2.0	4.2	2.1

(From Whittam and Ager, Note 8)

also moves in the same direction as sodium when a net transport occurs, so that two sodium ions exchange for two potassium ions, and one sodium ion is transported outwards in company with one chloride ion. However, a somewhat different situation may apply in the steady state, in which the same number of sodium (or potassium) ions move inward as outward. The discrepancy may be a clue to a specific mechanism, inasmuch as H^+ ions are liberated, according to *p*H, when ATP is hydrolized. It is possible that directionality involving H^+ ions may play a part in active transport of alkali metals, as appears to happen in mitochondria. Mitchell[33] envisaged that the mitochondrial membrane contains a parallel array of transporting units situated asymmetrically in the membrane in such a way that the microscopic ion movements caused by individual units result in a macroscopic flow.

STOICHIOMETRY AND MECHANISM The stoichiometry between sodium and potassium transport and ATP hydrolysis is of paramount importance in explaining mechanism. There are, on the average, two negative charges on each phosphate ion that is liberated during adenosine triphosphatase activity. This can be related to the finding that about two potassium ions are taken up per molecule of ATP hydrolyzed. The number of sodium ions extruded is somewhat greater, but the excess ions might be balanced, to maintain electrical neutrality, by protons or chloride ions.

Hydrolysis of ATP and ion pumping therefore go together as related features of an enzymatic reaction. To speak of ATP driving the pump may be meaningless because the rate of active transport and the rate of the enzyme activity regulate each other. The two processes are, in fact, inseparable; what affects one affects the other to a similar degree.

Mechanism of the transport adenosine triphosphatase

The crux of the problem is to explain how the activity of a phosphatase located in a membrane causes movements of activating cations. From the standpoint of molecular mechanism, we can ignore that ions move against electrochemical potential gradients. Thus, unpublished experiments have shown that internal sodium activates the transport enzyme to the same extent, whether or not sodium is present in the medium. A related point is that the rate of sodium extrusion—at least in cat spinal motor neurons—is independent of the resting potential.[12]

Like other enzymes, the transport adenosine triphosphatase has a stoichiometry, and perhaps the most useful way to think about the mechanism is in terms of the mech-

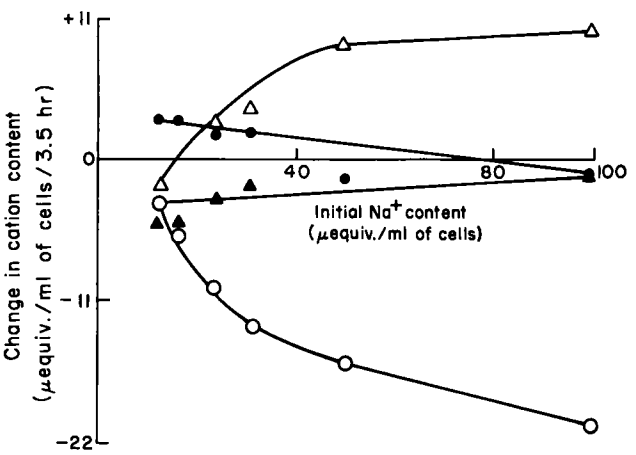

FIGURE 9 Dependence of net Na^+ and K^+ movements on internal Na^+ concentration. Erythrocytes were loaded with different concentrations of Na^+ by the "lactose" procedure, washed four times with a medium containing K^+ (10 mM), and suspended at about 15% hematocrit in the same medium, modified to include ouabain (50 μM) where appropriate. Samples for estimation of cell Na^+ and K^+ were taken initially or after incubation for 3.5 hr. at 37°, to allow calculation of the rates of Na^+ extrusion (○, control; ●, + ouabain) and K^+ uptake (△, control; ▲, + ouabain). Three further experiments gave similar results. (From Whittam and Ager, Note 8)

anism of action of enzymes in general. The stoichiometry that has been established for Na/ATP and K/ATP in red cells has a direct bearing on mechanism. The usefulness of this information may be illustrated by reference to the proposals that sodium and potassium are carried across the membrane in association with phosphatidic acid or phosphoprotein. The binding and release of the ions would depend on the sequential action of a phosphokinase and phosphatase. A difficulty is that the enzymes postulated as participating in ATP hydrolysis do not respond to alkali metals in a way that would be predictable from the asymmetrical dependence of the adenosine triphosphatase activity on alkali metals.

It is necessary, in dealing with the mechanism, to extrapolate from the results with fragmented membranes to transport studies with intact cells. Thus, we spent two years with red cells trying to detect a phosphate intermediate that would resemble the kidney or brain intermediate. No sign of such a compound was found, probably because there is so little pump activity in red cells compared with that in the brain and kidney. There is no correlation between adenosine triphosphatase activity and the amount of tracer phosphate incorporated into red cells, ghosts, or fragmented membranes. Yet, only in red cells, and in no other cell or tissue, has ATP proved to be directly associated with the pump. Let us accept that red cells provide a guide to cells in general and attempt to think of possible mechanisms.

It is worth summarizing the facts to be explained (Figure 10). ATP is hydrolyzed in such a way that the products of hydrolysis stay inside the cell. The splitting only occurs when sodium is on the inside and potassium on the outside, and the ions are moved as part of the vectorial reaction. Sodium is required to permit the substrate MgATP

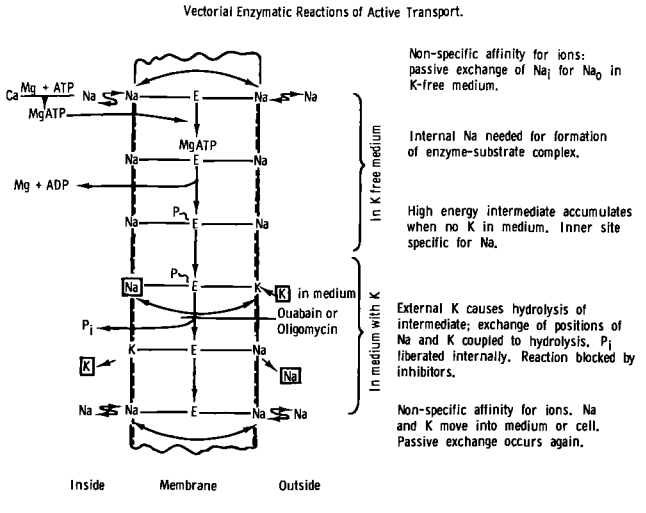

FIGURE 10 Vectorial enzymatic reactions of active transport.

to make a suitable attachment to the enzyme. In these circumstances, the phosphate intermediate is formed, and then is broken down by external potassium. The reaction catalyzed by potassium is really the chemiosmotic coupling reaction responsible for energy transduction. The enzymatic basis of transport may be clarified by answers to some simple questions that are clearly susceptible to experimental test. (Until it has been established whether one or two enzymes are involved, mechanism at the molecular level cannot be stated precisely.)

THE TRANSPORT ENZYME AS A FUNCTIONAL UNIT OF PROTEIN AND PHOSPHOLIPID Clearly a new dimension has been introduced into enzymology—directionality. Little is known about how the properties of enzymes change when they are in solution or bound to membranes. A clarification of mechanism may well come from the study of enzymes bound to well-defined artificial membranes, in which some vectorial features may be manifest. A striking example of what might happen is the discovery by Green[34] that the enzyme β-hydroxybutyrate dehydrogenase, which is located in mitochondria, is inactive when it is purified to be free from lipid (Kennedy chapter, this volume). If lecithin is added, enzymatic activity begins. Structure must be important, and the phospholipid evidently interacts with protein to give the catalytic unit. The transport enzyme is similar, in that lipid and protein are required for its activity. Lipids might affect the configuration of the protein in such a way that sensitivity to sodium and potassium is produced. Again, lipids might affect the attachment of the substrate, MgATP, to the active center. A conformational change in protein structure would seem to be probable when MgATP becomes attached to the enzyme, and sodium might be required to produce the appropriate induced fit of enzyme and substrate. Moreover, as Racker[35] has shown, the activity of purified adenosine triphosphatase from mitochondria differs in important respects from its activity when it is in its natural mitochondrial matrix.

These examples show the importance of structural factors, even in single enzymes in a homogeneous solution, and illustrated how vital these may be when dealing with vectorial effects of membrane-bound enzymes. The present evidence does not allow us to determine if the pump has two sites—one for sodium and one for potassium—or one site that is in a different position at different times, facing the inside when it has a high affinity for sodium and facing the outside when it has a high affinity for potassium. It has usually been assumed that there must be two sites for the two ions that are transported. On the other hand, the ion-specific sites may not be different chemical entities, but rather the same site in a different position.

MECHANISM BASED ON ALLOSTERIC EFFECT OF POTASSIUM AND CONFORMATIONAL CHANGES Before the concept of allosteric control was developed I suggested that the same site may be exposed alternately to the inside and outside of the cell, and this site might be the active center of the enzyme.[30] Recent developments, largely those of Monod, Changeux, and Jacob,[36] now make it possible to visualize an effect of external potassium on the hydrolysis of internal ATP without the need to postulate the movement in space or rotation of the enzymatic active site. It has become clear that enzymatic reactions can be regulated by agents that do not act directly on the active center—the so-called allosteric effects. An enzyme certainly has a catalytic active site but, in addition, there are other sites, spatially distant from the active center, where inhibitors and activators may act. Allosteric effectors increase or decrease the enzymatic activity through conformational changes in protein structure. Another aspect of allosteric control may lie in subunits of the enzyme that contribute in different ways to enzymatic activity. Physiological mechanisms such as active transport and oxygenation of hemoglobin are certainly amenable to analysis in terms of the concepts of allosteric control. The four heme subunits of hemoglobin undergo conformational changes in structure when one heme group is saturated with oxygen; the affinity for oxygen of the other heme groups is thus raised. This effect suggests the possibility of allosteric control of transport adenosine triphosphatase by ions (Figure 11). It appears reasonable to think that external potassium acts on a part of the enzyme system distant from the active center. Splitting of ATP occurs inside the cell. It can be enhanced if external potassium creates favorable conditions for the enzyme to lower the activation energy for hydrolysis through an allosteric effect on a subunit of the enzyme.

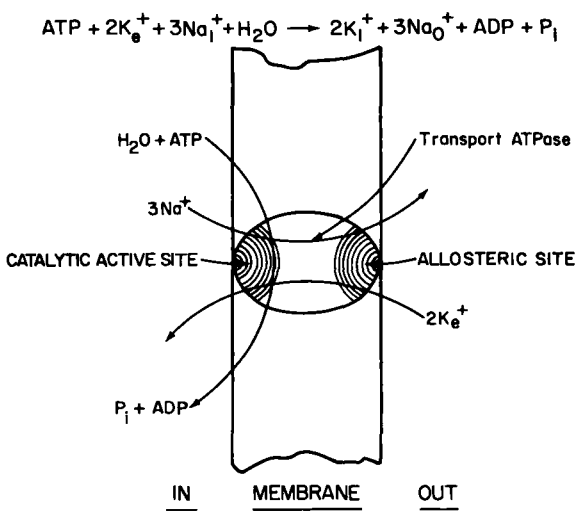

FIGURE 11 Net enzyme reaction of active transport.

It is necessary to postulate a conformational change when MgATP becomes bound to the enzymes; sodium is required for this step and for the subsequent phosphorylation. Conformational changes are known when an enzyme-substrate complex inhibitor is formed.[37] The enzyme system, including the phosphorylated intermediate, would be stabilized in this condition in the absence of external potassium. External potassium, however, may be assumed to induce an activation in such a way that hydrolysis takes place, and the two ions are ejected in opposite directions as a result of the further conformational change in protein structure that would inevitably accompany the reaction.

Summary and conclusion

Active transport might therefore be tentatively explained by the concept of allosteric control. Two conformational changes in structure must be postulated—one when sodium facilitates attachment of MgATP to the enzyme and the phosphate intermediate is formed, and another when hydrolysis occurs as a result of the effect of external potassium. The second change is accompanied by ion movements, and the enzyme resumes its initial structure. The sequence of reactions in active transport therefore appears to involve the creation of a high-energy intermediate associated with a protein conformation different from that found after its hydrolysis. The reaction is activated by ions that are located, after catalysis, on the opposite side of the membrane from their original location.

The idea that the transport of cations depends on configurational changes of macromolecules in the cell membrane is entirely compatible with the known facts about the system. First, macromolecular units could well be aligned asymmetrically in the membrane in such a way that the hydrolysis of intracellular ATP is controlled by the ionic composition of internal and external fluids. Second, it is reasonable to assume that the transport adenosine triphosphatase may discriminate between sodium and potassium, for other enzymes are known to respond differently to these two ions. Further clarification of the mechanism of active transport depends upon a better understanding of the behavior of enzymes in the restricted milieu of a membrane system, and of the means by which enzymes in this situation may bring about the transformation of energy.

Membrane Thermodynamics

A. KATCHALSKY

THE PHYSICO-CHEMICAL STUDY of membrane behavior goes back to the pioneering experiments of the French Abbé Nollet in 1774. In accord with the national tradition, Nollet investigated the selective permeability of membranes to alcohol from an aqueous solution, and observed the osmotic pressures developing across the membranes. This work was followed in the nineteenth century by Pfeffer and Traube, who prepared adequate semipermeable membranes and discovered the laws of osmotic pressure for dilute solution. It was, however, the father of physical chemistry, J. van't Hoff, who made use of membranes as an intellectual tool for the analysis of solution properties and developed a comprehensive theory of colligative phenomena. At the end of the nineteenth century, attention was drawn to electrolyte transport through membranes and to membrane potentials. This work culminated in the equations of Nernst and in the profound analysis of Planck, published in 1890.[1-4] A short while afterward, Donnan discovered the influence of colloidal solutions on ionic distribution across membranes and introduced the "Donnan potentials."[5-7]

From a theoretical point of view, the work of the masters of the nineteenth century was brought to a "dead end" by the monumental work of J. W. Gibbs.[8] The introduction of chemical and electrochemical potentials made the use of membrane models superfluous, because colligative properties as well as membrane equilibria could now be derived in a "trivial" or "self-evident" way from the equality of electrochemical potentials of permeable substances across phase boundaries.

A renewal of interest in membrane physical chemistry is discernable from the forties of this century when synthetic ion-exchange membranes were introduced and studied by L. Michaelis,[9] K. Sollner,[10] K. H. Meyer and Sievers[11] and by T. Teorell.[12,13] These "permselective" membranes consist of a polymeric matrix that carries numerous fixed charges and behaves as a dense polyelectrolyte gel. The fixed charges allow an easy transport of counterions and exclude (by Donnan exclusion[5]) to a large extent the entry and passage of coions. The pioneering theoretical work of Meyer and Sievers and of Teorell, which makes extensive use of the previous work of Nernst, Planck, and Donnan, laid the foundation for a kinetic theory of membrane transport and is still a powerful tool for the evaluation of the properties of these synthetic membranes, which are widely used for ion exchange and desalination.

Although the earlier physico-chemical studies of membrane behavior were encouraged by biological interest, the present-day approach to membrane biophysics differs appreciably from that of earlier generations. During the last twenty-five years, convincing evidence has been adduced that living membranes are not always the passive barriers for solute transport assumed by the physical chemists. Some transport has been shown to be "active" and based on an input of chemical metabolic energy that allows the accumulation or depletion of substances against the gradients of their chemical potential. The membrane of the cytologist and physiologist is not merely a transition layer between phases of different composition, but an organ endowed with a regulatory capacity and capable of transporting substances according to the needs of the organism.

Active transport of electrolytes and nonelectrolytes is found in the lowest forms of organization, and seems to be one of the earliest acquisitions of biological evolution. There is little doubt that the osmoregulatory ability, underlying the transition of animals and plants from the primeval sea to sweet water and land, is based on the capacity to regulate salt and water content by the "pumps" in tissue and cell membranes. Some of the more dramatic expressions of active transport may be found in organisms that have succeeded to penetrate saline lakes and salty deserts. The famous studies of Schmidt-Nielsen[14] demonstrated that sea birds such as gulls, cormorants, and others may drink sea water and excrete the excess of salt through their "tear" glands. The same research showed that the crocodile also weeps salty tears and is not as hypocrytic as has been assumed by the men of letters.

Living on the border of the desert, I had an opportunity to observe the tremendous power of desalination based on active transport. Close to the shores of the Dead Sea, on soil covered by crystalline salt, the visitor may see some lonely trees that survive the conditions of scorching heat and extremely high osmotic pressures. The tamarisk, which is prevalent in these surroundings, is often seen glistening in the sun—a phenomenon caused by the numerous crystals of sodium chloride excreted by glands sit-

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uated at the end of the twigs. Moreover, it was found that the Dead Sea is not absolutely dead; it contains bacteria and algae that metabolize and multiply in a 28 per cent salt solution—half of which is magnesium chloride. Unpublished results of B. Z. and M. Ginzburg show that these bacteria may accumulate a thousandfold concentration of potassium ion in their cells against the external osmotic conditions.

The recognition that living membranes are dynamic and complex structures, endowed with chemical reactivity and capable of selective transport, makes their quantitative study with the methods of classical physical chemistry an ungratifying task. Kinetic descriptions of biological phenomena have been useful, but such analyses often require detailed information that is not readily obtainable. We shall present the formal thermodynamic treatment for nonequilibrium processes, which requires less information but which also provides answers of less practical significance. Thermodynamics is not a substitute for detailed and explicit information about the structure and function of biomembranes. It is, however, a helpful guide in complex situations in which any quantitative statements may prove to be of value for analysis of the situation. The aim of this paper is to outline the thermodynamic approach to membrane phenomena and to indicate how useful conclusions can be made on the basis of this formal treatment. In the next section, the nonequilibrium thermodynamic approach is described and applied to simple and coupled transport processes. The third section encompasses a thermodynamic interpretation of ionic flows across simple and complex permselective membranes and correlates the thermodynamic expressions with the effects observed in biological and synthetic membranes. The final section is devoted to a brief discussion of active transport based on kinetic models that fulfill thermodynamic requirements.

The nonequilibrium thermodynamic approach

Classical thermodynamics is concerned primarily with equilibrium processes while for nonequilibrium processes it makes only general statements about the direction of change. It should be made clear, however, that Clausius and Kelvin, the founders of thermodynamics, were aware of the necessity of developing an adequate treatment of irreversible phenomena and made some advances in their quantitative evaluation. Important contributions towards a new formalism were made during the first half of this century by Jaumann,[15] Nathanson,[16] Eckart,[17] Eastman[18,19] and Wagner,[20,21] Meixner[22-25] and in particular by Brønsted[26] and by Bridgeman.[27] The crucial step was taken by Lars Onsager, who laid the foundation of nonequilibrium thermodynamics in two papers published in 1931.[28,29] Onsager's work was extended by Casimir[30] and further developed into a full-fledged branch of thermodynamics by Prigogine[31] and de Groot[32] and their coworkers. The steady-state treatment was supplemented by a comprehensive theory of relaxation phenomena, worked out by Meixner.[33] As pointed out by Onsager, however, the nonequilibrium thermodynamic treatment is limited to slow processes close to equilibrium and is insufficient for the description of rapid chemical processes. Therefore, in the discussion of active transport at the end of this paper we shall use a kinetic treatment that reduces to the formalism of nonequilibrium thermodynamics when equilibrium is approached.

THE DISSIPATION FUNCTION The central function of nonequilibrium thermodynamics is the rate of "irreversible" production of entropy, d_iS/dt, or the dissipation function $\Phi = T\,(d_iS/dt)$, which gives the dissipation of free energy per unit time and which is the more convenient function for the treatment of isothermal processes. In the range of validity of the Gibbs equation ($TdS = dU + pdv - \sum\mu_i \cdot dn_i$, etc.) the dissipation function can be expressed as a sum of products of flows, J_i, and forces, X_i:

$$\Phi = \sum_i J_i X_i \qquad (1)$$

The flows may be scalar, such as the flow of chemical reaction, measured by the formation of substance per unit time and per unit volume (dn_i/dt); or they may be vectorial, such as the rate of diffusion, or the rate of transport of an electrical current. The vectorial flows are given by the product of the geometrical velocity $\vec{v}$ and the concentration or density of the transported quantity. Thus, $\vec{J}_i = c_i\vec{v}_i$ equals the directional transport of substance i per unit area A and unit time t

$$J_i = \frac{1}{A}\frac{dn_i}{dt}.$$

The forces conjugate to the flows are a more sophisticated concept: the force driving the flow of a chemical reaction is, according to de Donder, the affinity of the reaction, A, given by the sum of the chemical potentials of the reactants minus the sum of the chemical potentials of the products of the reaction; the force driving the flow of an electrical current is the electromotive force; the force driving a diffusional flow of ions is the gradient of the electrochemical potential, $\tilde{\mu}_i$, where

$$\tilde{\mu}_i = \mu_i^\circ + RT \ln a_i + z_iF\psi \qquad (2)$$

There μ_i° is the reference chemical potential; a_i, the activity of the i'th component; and if the substance is charged z_i, the valence of the i'th ion; F, the Faraday num-

ber (96,500 coulombs/equiv); and ψ, the local electrical potential. If diffusion takes place across a membrane, the driving force is found to be the difference between the electrochemical potentials of the i'th component in the phases in contact with the membrane, $\Delta\tilde{\mu}_i$.

The dissipation function is important primarily because it permits a thermodynamically correct assignment of flows and forces to a system undergoing an irreversible process. It will be observed that the structure of the dissipation function imposes the condition that the product of every flow with its conjugated force (i.e., J_iX_i) must have the dimension of entropy formation or, in the isothermal case, of free energy dissipated, per unit time and unit volume. Thus, among all possibilities of choosing flows and forces, only those pairs with the right dimensions are amenable to further thermodynamic treatment and obey the laws of nonequilibrium thermodynamics.

The dissipation function (1) has another and more profound significance: entropy production in an irreversible process is positive definite, so we may write

$$\Phi > 0 \tag{3}$$

for any dissipative change. Let us apply eq. (3) to the case of two flows and two forces

$$\Phi = J_1X_1 + J_2X_2 > 0 \tag{4}$$

On a priori grounds, we would expect each of the terms in eq. (4) to be positive, for it is plausible to assume that a positive force will drive a positive flow and a negative force will produce a negative flow, so that $J_iX_i > 0$. Equation (3) does not, however, require all terms in the dissipation to be positive, and allows for the occurrence of negative terms, as long as the sum total is larger than zero. Negative terms in eq. (4) mean that a flow may proceed in a direction opposite to that of its conjugate force if a *coupled* process provides a large enough dissipation. Such cases of "contra-gradient" movement based on coupling are well known in physical chemistry, biochemistry, and biophysics:

If the two terms in eq. (4) are diffusional flows and forces and one of the flows moves against its conjugate gradient of chemical potential, the phenomenon is denoted by the physical chemists as *incongruent* diffusion.[34] When the two processes are chemical and one of the reactions proceeds in a contra-gradient direction through coupling with another chemical reaction that provides the dissipation, we are dealing with typical biochemical coupling well known from metabolic studies.

Finally, if a diffusional process moves against its concentration gradient through coupling with a chemical metabolic process, the phenomenon may be described as *active transport.*

Indeed, the quantitative treatment of coupled processes is the main concern of the thermodynamics of irreversible processes, and this aspect of nonequilibrium thermodynamics makes it a valuable tool for biophysical study. Our next step will be to write the explicit relations between flows and forces, which will comprise both direct and coupled interdependences.

THE PHENOMENOLOGICAL EQUATIONS Over a century ago the flow of slow irreversible processes was found to be directly proportional to driving forces. Well-known examples are Fick's law of diffusion and Ohm's law for electrical flow. However, even in the early nineteenth century it was recognized that in many cases of coupled phenomena the flow also depends directly on nonconjugated forces. Thus, in thermoelectric coupling, an electromotive force may produce a flow of heat, or a gradient of temperature can evoke a flow of electricity. To represent both the direct and coupled dependences of flows on forces, Onsager generalized equations relating flows to forces, and introduced the phenomenological equations, which read as follows:

$$\begin{aligned} J_1 &= L_{11}X_1 + L_{12}X_2 + L_{13}X_3 + \cdots + L_{1n}X_n \\ J_2 &= L_{21}X_1 + L_{22}X_2 + L_{23}X_3 + \cdots + L_{2n}X_n \\ &\vdots \\ J_n &= L_{n1}X_1 + L_{n2}X_2 + L_{n3}X_3 + \cdots + L_{nn}X_n \end{aligned} \tag{5}$$

Here the L_{ii}'s represent the straight phenomenological coefficients, while the L_{ij}'s are the coupling coefficients. In a shortened notation, eqs. (5) may be written as

$$J_i = \sum_j L_{ij}X_j \tag{6}$$

The number of coefficients in a multiflow system is very large, and Onsager's discovery of a means to reduce appreciably the number of parameters required for an adequate description of the system is most helpful. His famous symmetry theorem, based on statistical mechanical reasoning, states that

$$L_{ij} = L_{ji}, \tag{7}$$

or that the matrix of the coefficients in eq. (5) is symmetrical.

The ratio of a flow to a force may be regarded as a generalized conductance, so that $L_{ii} = (J_i/X_i)_{X_j = 0}$ is the straight conductance of the i'th flow, while the L_{ij}'s are coupling conductances. Onsager's theorem thus states that the coupling conductance of the i'th flow with the k'th force is equal to the coupling conductance of the k'th flow with the i'th force or

$$(J_i/X_k)_{X_{j\neq k}=0} = L_{ik} = (J_k/X_i)_{X_{j\neq i}=0} = L_{ki} \tag{8}$$

This important theorem has been amply verified experimentally in various fields of transport, including diffusion, electrokinetics, thermoelectricity, and membrane permeability (cf. D. Miller[35]).

To demonstrate the usefulness of the phenomenological equations, let us consider the simultaneous stationary transport of two uncharged solutes, A and B, across a membrane. The equations are general, so their application is not limited to synthetic membranes but also may be used to advantage for the description of passive transport across cellular membranes. We may write for the dissipation function

$$\Phi = J_A \Delta\mu_A + J_B \Delta\mu_B \tag{9}$$

and for the flows:

$$\begin{aligned} J_A &= L_{AA}\Delta\mu_A + L_{AB}\Delta\mu_B \\ J_B &= L_{BA}\Delta\mu_A + L_{BB}\Delta\mu_B \end{aligned} \tag{10}$$

In the case of nonelectrolytes in dilute solution (with equal pressures on both sides of the membrane)

$$\Delta\mu_A = RT \ln (c_A^o/c_A^i) \text{ and } \Delta\mu_B = RT \ln (c_B^o/c_B^i) \tag{11}$$

where the superscripts o and i denote outside and inside the cell, respectively. Let us now assume that the inner and outer concentrations of A are equal, so that $\Delta\mu_A = 0$. The uninitiated reader would expect that in this case $J_A = 0$ since the driving force for A vanished. Equation (10) shows, however, that

$$(J_A)_{\Delta\mu_A = 0} = L_{AB}\Delta\mu_B \tag{12}$$

which indicates that if the coupling conductance L_{AB} is nonzero, a flow of A will exist, driven by the coupled force $\Delta\mu_B$. The ratio of the driven flow to the direct flow is given by

$$(J_A/J_B)_{\Delta\mu_A = 0} = L_{AB}/L_{BB} \tag{13}$$

Equations (12) and (13) are quantitative expressions for the coupling possibility discussed above. Now let us consider another experiment in which we can make $J_B = 0$. In this case, a distribution of concentrations will be established governed by the requirement

$$(\Delta\mu_B/\Delta\mu_A)_{J_B = 0} = -L_{BA}/L_{BB} \tag{14}$$

According to Onsager's theorem, however, $L_{BA} = L_{AB}$ so that eqs. (13) and (14) yield

$$(J_A/J_B)_{\Delta\mu_A = 0} = -(\Delta\mu_B/\Delta\mu_A)_{J_B = 0} \tag{15}$$

Equation (15) is one of the numerous relations predicted by nonequilibrium thermodynamics. It will be observed that it does not involve the direct use of the L_{ij}'s but the symmetry of the coefficients alone. This is rather important, for the L_{ij}'s are not constants but functions of the parameters of the state such as concentrations, temperature, etc. However, it should be borne in mind that the L_{ij} *cannot* be functions of the *gradients* of the parameters, as in the linear case they are not functions of the forces or flows. The functional form of the L's is generally unknown, so that it is often only the cross-relations of the coefficients which are used, as in deriving eq. (15).

To enlarge the scope of cross-relations it is useful to consider also the inverse phenomenological equations, in which the forces are given as linear functions of the flows:

$$\begin{aligned} X_1 &= R_{11}J_1 + R_{12}J_2 + \cdots + R_{1n}J_n \\ X_2 &= R_{21}J_1 + R_{22}J_2 + \cdots + R_{2n}J_n \\ &\vdots \\ X_n &= R_{n1}J_1 + R_{n2}J_2 + \cdots + R_{nn}J_n \end{aligned} \tag{16}$$

Here the R_{ij}'s are generalized resistances which also obey Onsager's theorem, or

$$R_{ij} = R_{ji} \tag{17}$$

The cross-relations derivable from (16) are of the type

$$(X_i/J_k)_{J_{j\neq k}=0} = R_{ik} = (X_k/J_i)_{J_{j\neq i}=0} = R_{ki} \tag{18}$$

The observant reader will recognize the difference between eqs. (18) and (8) by the indices kept at zero value. These are different sets of relations whose usefulness depends on the choice of experimental conditions. Generally the resistances are not the reciprocal of the corresponding conductances, and the transition from L_{ik} to R_{ik} requires more advanced calculation.

Passive transport across simple and complex membranes

THE TRANSPORT OF SALT AND ELECTRIC CURRENT THROUGH A SIMPLE, CHARGED MEMBRANE The L_{ij}'s are not constant, but are functions of the parameters of state, so the phenomenological equations given in the previous section may be of limited value in practical application. It is often advantageous to transform the dissipation function to obtain forces related to the flows by simpler coefficients, some of which are known to be constants in a wide experimental range. The following elementary example of such a transformation of coefficients is that for monovalent salt transport across a simple charged membrane.[36,37] Two "practical" equations for the flow of current and the flow of salt will be derived here and will also be used in the next section for the evaluation of the behavior of complex membranes.

Let us consider a homogeneous membrane through which a flow of monovalent cation, J_1, and monovalent anion, J_2, take place. It is assumed that the ionic transport is not accompanied by a flow of solvent. The driving

forces are the difference of the electrochemical potentials of the two ions across the membrane, $\Delta\tilde{\mu}_1$ and $\Delta\tilde{\mu}_2$. The dissipation function is correspondingly

$$\Phi = J_1\Delta\tilde{\mu}_1 + J_2\Delta\tilde{\mu}_2 \tag{19}$$

If the electrical potential across the membrane is measured with two electrodes reversible to the anion, such as Ag–AgCl electrodes, it can be shown thermodynamically that the reversible electrode potential, E, is related to the difference of the electrochemical potential of the anion, $\Delta\tilde{\mu}_2$, by the relation

$$\Delta\tilde{\mu}_2 = z_2FE = -FE \tag{20}$$

It may be further noted that the electric current density, I, flowing across the membrane is related to J_1 and J_2 through the obvious equation

$$I = (J_1 - J_2)F \tag{21}$$

The insertion of eqs. (20) and (21) into the dissipation function, eq. (19), gives

$$\Phi = J_1(\Delta\tilde{\mu}_1 + \Delta\tilde{\mu}_2) + I\cdot E$$

The flow of the cation, J_1, which does not interact with the electrodes, is obviously identical with the flow of neutral salt, J_s, across the membrane. Further, by electroneutrality the sum of the differences $\Delta\tilde{\mu}_1$ and $\Delta\tilde{\mu}_2$ is equal to the difference in the chemical potential of the neutral salt across the membrane, $\Delta\mu_s$, so that

$$\Phi = J_s\Delta\mu_s + I\cdot E \tag{22}$$

The transformed dissipation function is now expressed in terms of conventional flows and forces and may be used for the construction of phenomenological equations with more familiar parameters:

$$\begin{aligned} I &= L_{11}E + L_{12}\Delta\mu_s \\ J_s &= L_{21}E + L_{22}\Delta\mu_s \end{aligned} \tag{23}$$

The physical meanings of the coefficients L_{11} and L_{12} become evident from a consideration of current and salt flow when $\Delta\mu_s = 0$. In this case, $I = L_{11}E$, but here L_{11} is evidently the *conductance* κ of the membrane or

$$L_{11} = \kappa \tag{24}$$

It will be further observed that at $\Delta\mu_s = 0$, $(J_s/I)_{\Delta\mu_s=0} = L_{21}/L_{11}$. Since $J_s = J_1$ and J_1F/I is the fraction of the current carried by the cation, $J_1F/I = t_1$, where t_1 is the *transference number* of the cation in the membrane. Thus

$$L_{21} = t_1L_{11}/F = \kappa t_1/F \tag{25}$$

and as by Onsager's theorem $L_{21} = L_{12}$ we may rewrite the first of eqs. (23) as

$$I = \kappa E + (\kappa t_1/F)\Delta\mu_s \tag{26}$$

which is a phenomenological equation that is practical.

Equation (26) may be cast into more familiar form if we recall from eqs. (20) and (2) that

$$-FE = \Delta\tilde{\mu}_2 = \Delta\mu_2 - F\Delta\psi \tag{27}$$

where $\Delta\psi$ is the potential difference measured with two *calomel* electrodes and $\Delta\mu_2$ is the difference in chemical potential of the anion. Inserting eq. (27) into eq. (26) and noting that the sum of the transference numbers of the cation and the anion is unity, $t_1 + t_2 = 1$, we get

$$I = \kappa[\Delta\psi + \frac{1}{F}(t_1\Delta\mu_1 - t_2\Delta\mu_2)] \tag{28}$$

If the solutions on both sides of the membrane are ideal and each side contains equal concentrations of cation and anion (i.e., no colloidal particles are present)

$$\Delta\mu_1 = \Delta\mu_2 = RT\ln(c_s^i/c_s^o) \tag{29}$$

and

$$I = \kappa\left[\Delta\psi + \frac{RT}{F}(t_1 - t_2)\ln\frac{c_s^i}{c_s^o}\right] \tag{30}$$

where i and o denote the inside and outside compartments separated by the membrane.

At $I = 0$

$$(\Delta\psi)_{I=0} = -\frac{RT}{F}(t_1 - t_2)\ln\frac{c_s^i}{c_s^o} \tag{31}$$

which is the well-known form of the liquid-junction or membrane potential. If the membrane is practically impermeable to anions, $t_2 \simeq 0$ and $t_1 \simeq 1$,

$$(\Delta\psi)_{I=0} = -\frac{RT}{F}\ln\frac{c_s^i}{c_s^o} \tag{32}$$

which is one of the forms of the Nernst–Planck equation.

We derive next the relationship between the phenomenological equation for the flow of salt and permeability. We shall write from the first of eqs. (23) $E = (I/L_{11}) - (L_{12}/L_{11})\Delta\mu_s$, and introduce E into the second of eqs. (23):

$$J_s = \left(L_{22} - \frac{L_{12}^2}{L_{11}}\right)\Delta\mu_s + \frac{L_{12}}{L_{11}}I \tag{33}$$

Neutral salt permeability is generally measured at $I = 0$, so the second term of eq. (33) can be set equal to zero; and the permeability coefficient P is defined as $(J_s/\Delta c_s)_{I=0}$. Introducing an average concentration, $\bar{c}_s$, which relates $\Delta\mu_s$ to the osmotic pressure difference $\Delta\pi_s$ across the membrane:

$$\Delta\pi_s = 2RT\Delta c_s = \bar{c}_s\Delta\mu_s \tag{34}$$

the thermodynamic expression for salt flow becomes

$$(J_s)_{I=0} = \frac{1}{\bar{c}_s}\left(L_{22} - \frac{L_{12}^2}{L_{11}}\right)\Delta\pi_s = \frac{2RT}{\bar{c}_s}\left(L_{22} - \frac{L_{12}^2}{L_{11}}\right)\Delta c_s$$

The permeability coefficient P is therefore

$$\left(\frac{J_s}{\Delta c_s}\right)_{I=0} = P = \frac{2RT}{\bar{c}_s}\left(L_{22} - \frac{L_{12}^2}{L_{11}}\right) \qquad (35)$$

In nonequilibrium thermodynamics it is often more convenient to use a "mobility," ω, of the salt, which is the true mobility divided by the thickness of the membrane and is related to P by the relation

$$P = 2RT\omega \qquad (36)$$

With this coefficient the flow of salt given in eq. (33) is transformed to the equation:

$$J_s = \omega\Delta\pi_s + \frac{t_1}{F}I \qquad (37)$$

The fact that κ, t_1, and P are well known to workers in the field does not make these coefficients characteristic constants, although in many cases their dependence on the parameters of state is less pronounced than is that of the L_{ij}'s. Thus P, or ω, is fairly constant for transport across neutral membranes, but it depends strongly on salt concentration in electrolyte transport through charged membranes. The reader is therefore warned not to regard the equations of nonequilibrium thermodynamics as convenient expressions that will provide him with simple answers about membrane behavior. We generally need some plausible model for the interpretation of the coefficients before numerical comparison with experimental data may be attempted.

COMPLEX MEMBRANES: NONLINEAR BEHAVIOR AND RECTIFICATION The example of the previous paragraph was analyzed on the assumption that the membrane may be regarded as a homogeneous phase. There is serious doubt whether this assumption applies to the complex structures revealed by electron microscopy and corroborated functionally by biochemical and pharmacological tests. As pointed out by Dr. Fernandez-Moran (this volume), cellular membranes, including axonal membranes, are anisotropic systems composed of different layers. This morphological conclusion is verified by the following physiological observations:[38] Whereas the application of proteolytic enzymes to the outer surface of a squid giant axon leaves the excitation capacity unchanged, the introduction of trypsin or α-chymotrypsin into a perfused axon causes an irreversible loss of excitability. It was further found that the fish poison tetrodotoxin has different effects when applied to the internal and external surfaces of the axon. While nanomoles of tetrodotoxin applied externally will abolish action potentials, the internal application in perfusion liquid leaves the activation of the axonal membrane virtually unchanged. These and other observations not detailed here led to the recognition of the axonal membrane as a *multilayer* structure, consisting of an internal layer of protein and a more external layer of lipid, with an intramembrane structure which comprises some ionic constituents, presumably divalent ions and Ca^{++} in particular.[38,39]

The quantitative treatment of a series array of charged membrane elements requires additional consideration that exceeds that of a single element. Before going into the detailed physical analysis of the behavior, we make the general observation that if one element has different permeability characteristics from another, the steady flow of ions will cause either an accumulation or a depletion of salt in the transition region between the membrane layers. This change in the intramembrane milieu will result in a nonlinear dependence of flows on forces, as the parameters of state, such as ionic concentration in the membrane, themselves become functions of the flows. This nonlinearity is expressed in electrical terms as rectification or nonohmic dependence of current on potential. It is found that, corresponding to current rectification, there is a nonlinear dependence of membrane potential on the logarithm of concentration, or that eq. (31) is not obeyed. Such nonlinear behavior is a fundamental characteristic of nerve membranes and has been amply demonstrated in both intact and perfused axons.[40,41]

We shall evaluate steady flows across composite membranes consisting of a series of charged elements, α and β, that are endowed with different electrochemical properties (Figure 1). The membrane maintains contact with two aqueous solutions of the same monomonovalent salt at different concentrations. In addition to the osmotic driv-

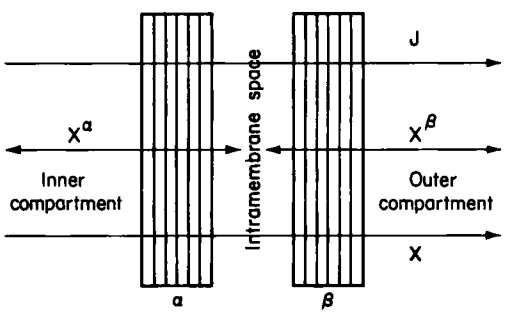


FIGURE 1 Scheme of a composite membrane composed of a series array of membrane elements α and β. The total force X acting across the composite membrane is additive in the forces acting on each element, $X = X^\alpha + X^\beta$. In stationary cases, the flows J are continuous across the system.

ing force, $\Delta\pi$, an electric potential, E, is imposed through two electrodes reversible to the anion.

The forces operating on the composite membrane may be considered as those acting on element α and on element β. In a steady state the total force is the sum of the partial forces, or $X = X^\alpha + X^\beta$ where X^α is the force between the inner solution and the hypothetical or real interelement space, and the force X^β acts between the intramembrane space and the outer solution. Thus:

$$\Delta\pi = \Delta\pi^\alpha + \Delta\pi^\beta;\ E = E^\alpha + E^\beta \qquad (38)$$

The condition of stationarity imposes the further requirement of continuity of flows across the membrane as a whole, or

$$J_s^\alpha = J_s^\beta = J_s \text{ and } I^\alpha = I^\beta = I \qquad (39)$$

Now each element is homogeneous and obeys eqs. (26) and (37), so that

$$J_s = \omega^\alpha \Delta\pi^\alpha + \frac{t_1^\alpha}{F} I = \omega^\beta \Delta\pi^\beta + \frac{t_1^\beta}{F} I \qquad (40)$$

and

$$I = \frac{1}{R^\alpha}\left(E^\alpha + \frac{t_1^\alpha}{F}\Delta\mu_s^\alpha\right) = \frac{1}{R^\beta}\left(E^\beta + \frac{t_1^\beta}{F}\Delta\mu_s^\beta\right) \qquad (41)$$

where $R^\alpha = 1/\kappa^\alpha$ and $R^\beta = 1/\kappa^\beta$ are the electrical resistances of the membrane elements.

Equation (40) lends an interesting insight into the mechanism of the rectification process. Let us assume that the space between membrane elements (intramembrane space) is large enough to assign an internal salt concentration, c_s^*. Then for ideal solution behavior we may write

$$\Delta\pi^\alpha = 2RT(c_s^i - c_s^*) \text{ and } \Delta\pi^\beta = 2RT(c_s^* - c_s^o) \qquad (42)$$

where i refers to the inside or to the solution adjacent to the α component of the membrane, and o to the outside solution in contact with the β component.

Inserting (42) into (40) we get for the internal concentration

$$c_s^* = \frac{\omega^\alpha c_s^i + \omega^\beta c_s^o}{\omega^\alpha + \omega^\beta} + \frac{t_1^\alpha - t_1^\beta}{2RT(\omega^\alpha + \omega^\beta)}\frac{I}{F} \qquad (43)$$

Equation (43) shows that under conditions of a steady flow of electricity, the intramembrane salt concentration must adjust itself to the value of I. If $(t_1^\alpha - t_1^\beta) > 0$, c_s^* will increase with increasing I, which means physically that the current will cause the accumulation of salt in the interelement space. On the other hand, for decreasing I, c_s^* will decrease and will ultimately reach the value of zero when

$$I_{min} = -\frac{2RTF\,(\omega^\alpha c_s^i + \omega^\beta c_s^o)}{t_1^\alpha - t_1^\beta} \qquad (44)$$

As negative concentrations are unknown, this is the lowest achievable value of current, whatever the potential E may be. The limit in current flow can be overcome only when E is low enough that current from the breakdown of the water molecules into ions becomes significant.

If the external and intramembrane solutions may all be assumed to obey the ideal equations for simple salt solutions, the differences of chemical potentials in eq. (41) become:

$$\Delta\mu_s^\alpha = \Delta\mu_1^\alpha + \Delta\mu_2^\alpha = 2RT \ln (c_s^i/c_s^*)\ ;$$
$$\Delta\mu_s^\beta = \Delta\mu_1^\beta + \Delta\mu_2^\beta = 2RT \ln (c_s^*/c_s^o)$$

The combination of this relation with eqs. (41) and (43) would give an explicit and rather bulky equation for the dependence of current on potential and on the concentration of salt on both sides of the membrane. We shall consider therefore only two special cases: (a) nonlinear behavior for the case of I = 0, for which the resting potential $\Delta\psi$ will be given as function of concentration, and (b) rectification for the case of equal concentrations on both sides of the membrane, in which the potential $\Delta\psi$ will be given as a function of the current density, I.

(a) When I = 0, the intramembrane concentration, c_s^*, given by eq. (43), reduces to a weighted average of the external salt concentrations, the weight functions being the permeability coefficients ω^α and ω^β:

$$(c_s^*)_{I=0} = \frac{\omega^\alpha c_s^i + \omega^\beta c_s^o}{\omega^\alpha + \omega^\beta}$$

This behavior at I = 0 plays an important role in determining the "resting potential" of a series array of membranes.

Similar to eq. (28) for a simple membrane, the resting potential for a double membrane is given by the following expression

$$(\Delta\psi)_{I=0} = -\frac{1}{F}\left(t_1^\alpha\Delta\mu_1^\alpha - t_2^\alpha\Delta\mu_1^\alpha + t_1^\beta\Delta\mu_1^\beta - t_2^\beta\Delta\mu_2^\beta\right) \qquad (45)$$

and hence, similar to eq. (31) for a simple membrane, under ideal solution conditions:

$$(\Delta\psi)_{I=0} = -\frac{RT}{F}\left[(t_1^\alpha - t_2^\alpha)\ln\frac{c_s^i}{c_s^*} + (t_1^\beta - t_2^\beta)\ln\frac{c_s^*}{c_s^o}\right] \qquad (46)$$

It will be observed that if the transport numbers across the two elements are the same, i.e. $t_1^\alpha = t_1^\beta = t_1$ and $t_2^\alpha = t_2^\beta = t_2$, eq. (46) reduces to eq. (31) for a simple membrane. In this case, c_s^* does not play any role in determining the magnitude of the resting potential. On the other hand, when the electrochemical properties of the membrane elements differ appreciably, the resting potential of a com-

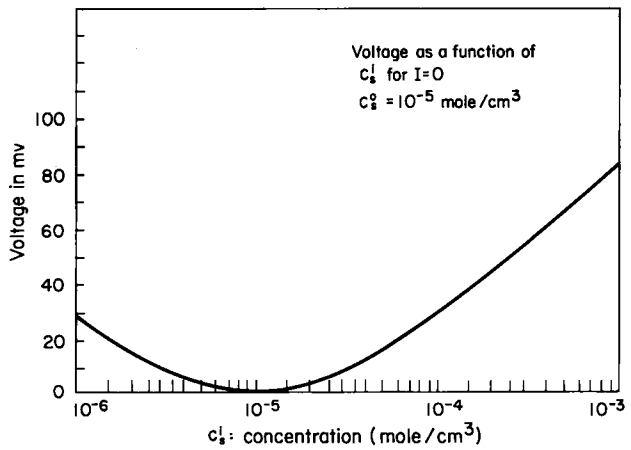


FIGURE 2 The resting potential, $\Delta\psi$, for a complex membrane composed of two highly permselective elements. The concentration on one side is kept constant ($c_s^o = 10^{-5}$ mole/cm^3) while the concentration c_s^i is varied. The flow of electrical current is zero (I = 0). (Calculated by J. Richardson, Note 42)

posite membrane assumes a form that bears no resemblance to the ordinary liquid-junction potential, eq. (31).

To get an idea about the dependence of $\Delta\psi$ on concentration in an extreme case let us assume that $\omega^\alpha = \omega^\beta$ so that $c_s^* = (c_s^i + c_s^o)/2$. Further, let membrane α be highly selective for cation, i.e. $t_1^\alpha \simeq 1$, $t_2^\alpha \simeq 0$, while membrane β be highly selective for anions with $t_1^\beta \simeq 0$ and $t_2^\beta \simeq 1$. In this case

$$(\Delta\psi)_{I=0} = -\frac{RT}{F} \ln \frac{4c_s^i \cdot c_s^o}{(c_s^i + c_s^o)^2} \qquad (47)$$

Figure 2 represents $(\Delta\psi)_{I=0}$ as a function of $\ln c_s^i$ at constant c_s^o for this interesting case. The figure is taken from the work of J. Richardson, now being prepared for publication.[42]

The remarkable feature of the curve is that there exists a range of concentration for which $\Delta\psi$ is nearly independent of concentration. The conditions under which this behavior is expected are derivable (differentiating eq. (46)) from the general expression

$$\left(\frac{\partial \Delta\psi}{\partial \ln c_s^i}\right)_{I=0;\, c_s^0} = -\frac{RT}{F} \frac{(t_1^\beta - t_2^\beta)\omega^\alpha c_s^i + (t_1^\alpha - t_2^\alpha)\omega^\beta c_s^o}{\omega^\alpha c_s^i + \omega^\beta c_s^o} \qquad (48)$$

When $(t_1^\beta - t_2^\beta)\omega^\alpha c_s^i + (t_1^\alpha - t_2^\alpha)\omega^\beta c_s^o = 0$, $\Delta\psi$ will not change the concentration. In the case considered in the previous paragraph this happens for equal concentrations on both sides of the complex membrane.

As shown in Figure 3 from the work of Baker, Hodgkin, and Shaw[43] and in Figure 4 from Tasaki and Takenaka,[44] the resting potential of the perfused squid giant axon does not follow the Nernst equation in its variation with the internal potassium concentration. Hodgkin[45] suggested that this may be the result of a potential-dependent change in the permeability of the axonal membrane to potassium. Indeed, the series model is endowed with a concentration-dependent change in permeability due to a selective accumulation of salt in the intramembrane space. Moreover, the nonlinear shape of the curves for the resting potentials indicates that the membrane is composite, and by a suitable choice of permeability parameters it may be possible to correlate the behavior with the known asymmetric structure.

(b) The final aspect of series membranes to be considered

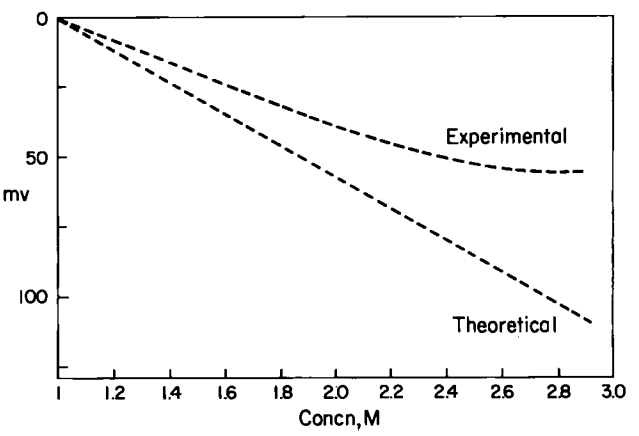


FIGURE 3 The effect of the internal potassium concentration on the resting potential of a perfused squid axon. External solution, sea water; internal solution, sodium chloride + potassium chloride solutions, isotonic with sea water. (After Baker, Hodgkin, and Shaw, Note 43)

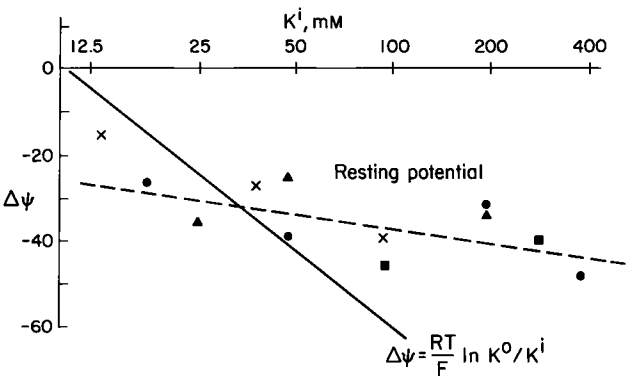


FIGURE 4 Effect upon the resting potential of diluting the K-perfusing fluid with isotonic sucrose solution. The ratio of Na^+/K^+ concentration was fixed at 1/10. Abscissa represents the K^+ concentration in the perfusing solution. 22° C. (From Tasaki and Takenaka, Note 44)

is that of electrical rectification at $c_s^i = c_s^o = c_s$. The value of c_s^* for this case is from eq. (43)

$$c_s^* = c_s + \frac{t_1^\alpha - t_1^\beta}{2RT(\omega^\alpha + \omega^\beta)} \frac{I}{F}$$

The corresponding value of the difference in chemical potential

$$\Delta\mu_s^\alpha = -\Delta\mu_s^\beta = -2RT \ln c_s^*/c_s$$
$$= 2RT \ln \left[1 + \frac{(t_1^\alpha - t_1^\beta)}{2RTc_s(\omega^\alpha + \omega^\beta)} \frac{I}{F}\right],$$

upon insertion into eq. (41) for the steady-state current gives

$$I(R^\alpha + R^\beta) = E + \frac{2RT}{F}(t_1^\alpha - t_1^\beta) \times \ln \left[1 + \frac{(t_1^\alpha - t_1^\beta)}{2RTc_s(\omega^\alpha + \omega^\beta)} \frac{I}{F}\right] \quad (49)$$

In the present case $c_s^o = c_s^i = c_s$, and according to eq. (27), $E = (\Delta\psi)_{\Delta\mu_s=0}$, so that

$$(\Delta\psi)_{\Delta\mu_s=0} = I(R^\alpha + R^\beta) + \frac{2RT}{F}(t_1^\alpha - t_1^\beta) \times \ln \left[1 + \frac{(t_1^\alpha - t_1^\beta)}{2RTc_s(\omega^\alpha + \omega^\beta)} \frac{I}{F}\right] \quad (50)$$

Let us consider again the extreme case of $t_1^\alpha \simeq 1$ and $t_1^\beta \simeq 0$

$$(\Delta\psi)_{\Delta\mu_s=0} = I(R^\alpha + R^\beta) + \frac{2RT}{F} \times \ln \left[1 + \frac{I}{2RTFc_s(\omega^\alpha + \omega^\beta)}\right] \quad (50a)$$

Equation (50a) is represented by curve I of Figure 5 taken from Richardson's work.[42] It is a rectification curve such as those observed with p-n junctions for semiconductors.

A closer inspection of eq. (50a) clarifies the reason for the rectification: for positive and large values of I, the contribution of the logarithmic term is smaller than that of the linear term, and the resistance approaches the Kirchoff term $R = R^\alpha + R^\beta$. On the other hand, for negative values of I when $I \to -2RTFc_s(\omega^\alpha + \omega^\beta)$, the term in the logarithmic brackets goes to zero, and the logarithm itself goes to minus infinity, indicating an infinite resistance at negative potentials. The effective resistance is given by

$$\left(\frac{\partial\Delta\psi}{\partial I}\right)_{\Delta\mu_s=0} = R^\alpha + R^\beta + \frac{2RT}{F[I + 2RTFc_s(\omega^\alpha + \omega^\beta)]} \quad (51)$$

which shows clearly the dependence of the resistance on the current.

The more general case of different concentrations on the two sides of the complex membrane is represented in curve II of Figure 5. It should be borne in mind that a wealth of rectification curves may be obtained for different values of ω, t, and the concentrations.

Rectification behavior is well known for axonal and muscle membranes. The steady state current obtained with voltage clamp experiments on squid giant axons has the same dependence on electric potential as that depicted in Figure 5. Cases of rectification in other excitable membranes may be found in the recent review of Grundfest.[46]

No detailed picture of the molecular structure of the axonal membrane is available, so any interpretation of the curves is perforce tentative. It may be stated, however, that both the rectification behavior and the nonlinear dependence of resting potential on concentration are consistent with the morphological finding of an asymmetric structure, and support the view that the causative factor behind the observations are ionic shifts in a complex membrane having a series array of elements.

NONSTEADY TRANSPORT IN COMPLEX MEMBRANES AND ITS RELATION TO ACTION POTENTIALS So far, we have treated only cases of stationary flow. In the realm of slow irreversible processes, steady states play the same role as do equilibria in static processes; their analysis is relatively simple and it is possible to reach numerous conclusions without specifying in great detail the properties of the system. The attractive character of

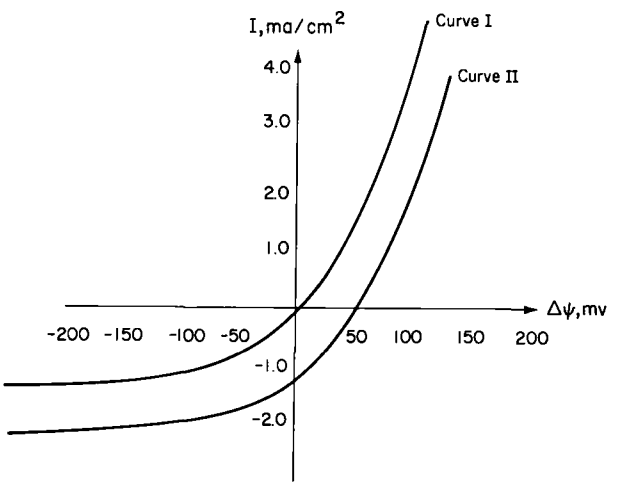


FIGURE 5 Rectification with a double membrane composed of cation- and anion-permselective membrane elements. Curve I for equal salt concentrations in outer and inner compartments. (Note that at $I = 0$ $\Delta\psi = 0$.) Curve II for unequal salt concentrations. (Calculated by J. Richardson, Note 42)

steady states made the theoretical biologists believe that mature organisms are in a stationary state, and maintained by the mechanism of homeostasis. Present-day theories tend to regard the organismic states as quasi-stationary with periodic oscillation around a steady average. But whatever may be our opinion about the organism as a whole, there are numerous biological processes that do not resemble stationary flows and must be treated as relaxation phenomena.

The theory of nonstationary behavior in complex membranes has not been worked out, although a rather extensive literature deals with such relaxation processes as the kinetics of diffusion in simple membranes. Some interesting observations exist on relaxation phenomena in complex membranes; these are still not worked out theoretically, but they deserve our attention nonetheless. For example, the experimental results described below resemble the "firing" of nerve membranes and give the correct sequences of action spikes. Although inanimate models for nerve firing have been known for many decades, the more recent observations are based on membrane systems that approximate the nerve membrane more closely in size. Dr. Shashoua kindly put at my disposal some of the results he has obtained with ultrafine complex membranes. The membranes were prepared by spreading a drop of polybase solution over a concentrated solution of polyacid. A surface reaction takes place, and the resulting thin membrane may be taken off, washed, and used for experimental testing. The membranes he prepared differ from the industrial in their thinness and in that their preparation makes it plausible to assume that the surface facing the polyacid solution is negatively charged while the polybase side is positive, according to the scheme in Figure 6.

When the membrane was inserted between two sodium chloride solutions of equal concentration and a constant potential applied, the current flow exhibited a remarkable behavior. Making the electrode on the polyacid side positive and the electrode on the polybase side negative, the current showed a series of spikes of the type observed with nerve membranes (Figure 7).

The resemblance is not only in the act of "firing," followed by regeneration of the original state during a "refractory" period; it also is in the order of magnitude of the times for a spike. It was found that the firing takes about 1 millisecond and the period of the sequence is several milliseconds.

On this basis, we may forward a hypothesis for the mechanism of the behavior of Shashoua's membranes: The application of potential of the right direction to a complex membrane will cause an accumulation of salt in the intramembrane space according to eq. (43). The local

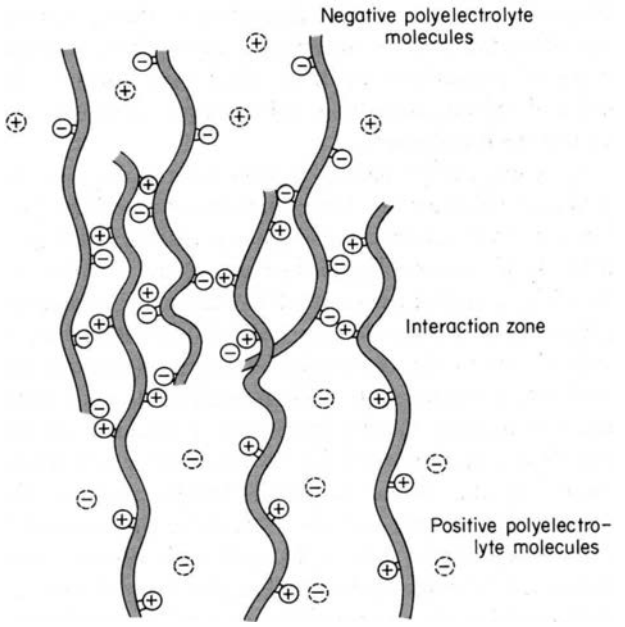


FIGURE 6 Schematic representation of double layer composed of two polyelectrolyte monolayers (after Shashoua).
O charges fixed on the macromolecules
◌ free mobile counterions

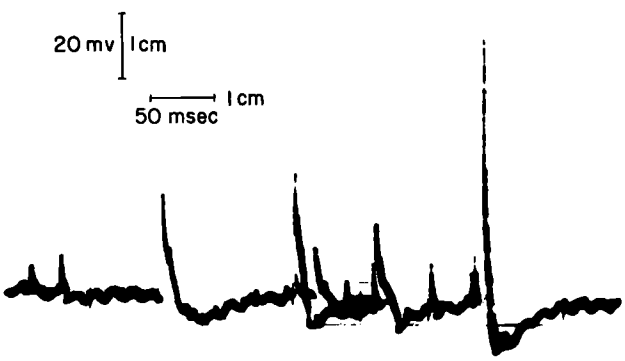


FIGURE 7 The "firing" of a polyelectrolyte bilayer with constant voltage applied across the membrane. Abscissa is time in milliseconds; ordinate, voltage in millivolts. (From Shashoua, private communication)

increase in concentration raises the conductance, and hence the sharp rise in the amount of current passing the membrane, until the peak of the spike is reached. The accumulation of salt has, however, an effect that counteracts it: It is known that polyelectrolyte molecules, which are stretched at low ionic strengths, contract or collapse at high ionic strength. Thus, the originally swollen membrane shrinks and becomes more permeable to salt with

the passage of current. We thus come to the conclusion that when the peak of the spike is approached, a breakdown of permselectivity takes place, water flushes the accumulated intramembrane salt, and the membrane returns to its initial state.

The danger of projecting an inanimate analogy on the excitation behavior of a living membrane is well known. It seems, however, that in the present case the analogy is not only phenomenological but of a more intimate nature. The complex structure of the nerve membrane requires intramembrane changes of salt concentration, as pointed out in the discussion of resting potentials and steady-state rectification. Salt accumulation may cause phase transitions in the lipoprotein complexes of the membrane, accompanied by an increased permeability resembling that found in polyelectrolyte systems. The phenomenon would become even more pronounced if the intramembrane salt exchanged with divalent ions such as Ca^{++}, which are believed to play a prominent role in maintaining the structural integrity of biomembranes. We shall have an opportunity to mention in the last part of this paper the large mechanical forces liberated by macromolecular phase transitions, and reconsider the effect of such phase transitions on transport across membranes.

A few words might be added on the reason for considering that changes in intramembrane ion concentration are the causative factor of stimulation phenomena. It is well known that an increase of the internal potential by about 20 millivolts suffices to release a firing of the nerve. The energy input in such a change per charged group is 2×10^{-2} electron volts. This is an amount much lower than that required to change any chemical structure or to open even weak bonds. We are driven to the conclusion that the stimulating potential is only a trigger mechanism that releases other forms of energy. A plausible explanation is that the potential causes an accumulation of ions that upon interacting with the macromolecular components of the membrane leads to phase transition and permeability changes.

We should not be misled by the term "phase transition" and identify it with a regular thermodynamic transition from a solid to a liquid, or from one solid to another solid phase. There is accumulating evidence that macromolecular systems are capable of existing in several metastable phases separated by sufficiently high energy barriers to withstand thermal impact. In view of some measurements of Tasaki[38] showing effects of salts perfused into the squid giant axon on its electrical properties, it seems that some nerve membrane constituents are metastable and amenable to ready transition upon interaction with ionic components.

CARRIER-MEDIATED PASSIVE TRANSPORT Our extensive discussions of complex membranes were dictated by the attempt to interpret nonlinear dependence of flows on forces observed in the covers of cells and tissues. In particular, the rectification of electrical current may serve to support the assumption that biological membranes are composed of two or more layers characterized by different electrical permeability properties. There is, however, another group of nonlinear transport phenomena which indicate that the membranes are complex in a sense different from that discussed above. It has frequently been observed in biological membranes that as the solute concentration is increased, the flux eventually ceases to increase and reaches a maximal limiting value. The existence of saturation phenomena is indicative of the existence of a finite number of sites participating in the transport. Moreover, it was found that the transport of substances that would be expected to be highly resistive to passage through the lipid layer of the membrane may be facilitated, presumably through combination with carrier molecules whose number is limited and amenable to saturation under experimental conditions.[47]

Following the formalism developed by Wilbrandt,[48,49] carrier transport may be depicted as shown in Figure 8. Here C^o denotes the carrier concentration on the side of the membrane adjacent to the outer solution, while CS^o is the outer concentration of the carrier loaded with nonelectrolyte solute S. The form CS^o moves across the membrane to the inner side, where it dissociates and transfers S into the cell. The stationary concentration of the carrier-

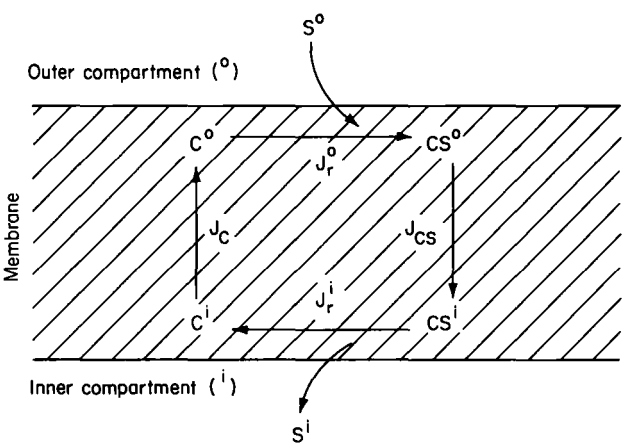


FIGURE 8 Schematic representation of carrier (C) mediated transport of the solute S. J_r^o is the rate of adsorption of S^o to the free carrier C^o in the outer compartment to give CS^o. J_r^i is the rate of solute desorption in the inner compartment. J_C is the flow of free carrier from the inner to the outer compartment; J_{CS} the flow of the solute-loaded carrier from the outer to the inner compartment.

solute complex in the inner side is $CS^i < CS^o$. The corresponding concentration of the free carrier is $C^i > C^o$. It is possible to describe this simple carrier system by four flows: the flow of the carrier–solute J_{CS} and the counterflow of the free carrier J_C; the "flow" of adsorption of S^o on C^o on the outer side J_r^o, and the flow of desorption from CS^i on the inner side J_r^i.

Let us now subject the model to a thermodynamic analysis. The rate of concentration change of the carrier forms is given by the following equations

$$\begin{aligned} dC^o/dt &= J_C - J_r^o\,; \quad dCS^o/dt = J_r^o - J_{CS}\,; \\ dCS^i/dt &= J_{CS} - J_r^i\,; \quad dC^i/dt = J_r^i - J_C \end{aligned} \tag{52}$$

Under conditions of stationary flow all the concentrations are constant, so that the above derivatives equal zero. Hence $J_C = J_r^o = J_{CS} = J_r^i$. But J_r^i is the rate of liberation of S into the cell and obviously equals the rate of the macroscopic steady flow of the solute across the entire membrane, J_S, hence

$$J_S = J_C = J_r^o = J_{CS} = J_r^i \tag{53}$$

The forces driving the flows are as follows:

Flow	Force
J_C	$\Delta\mu_C = \mu_C^i - \mu_C^o$
J_{CS}	$\Delta\mu_{CS} = \mu_{CS}^o - \mu_{CS}^i$
J_r^o	$A^o = \mu_S^o + \mu_C^o - \mu_{CS}^o$
J_r^i	$A^i = \mu_{CS}^i - \mu_C^i - \mu_S^i$

(54)

where the A's are the affinities of the respective reactions. The dissipation function may therefore be written according to the usual recipe:

$$\Phi = J_C\Delta\mu_C + J_{CS}\Delta\mu_{CS} + J_r^oA^o + J_r^iA^i \tag{55}$$

Equation (55) is the *microscopic* dissipation function for the detailed model and comprises all information used in its construction. Making use of the equality of the flows in eq. (53)

$$\Phi = J_S(\Delta\mu_C + \Delta\mu_{CS} + A^o + A^i)$$

But by addition of the forces in eqs. (54), we learn that $\Delta\mu_C + \Delta\mu_{CS} + A^o + A^i = \mu_S^o - \mu_S^i = \Delta\mu_S$ so that

$$\Phi = J_S\Delta\mu_S \tag{56}$$

Equation (56) is the *macroscopic* dissipation function, which could be written a priori, without taking recourse to any model. It states that macroscopically there exists only a single driving force, $\Delta\mu_S$, and a single external flow, J_S, and that their product gives the total dissipation, whatever the mechanism of transport. The reader should note that the identity of eqs. (55) and (56) reflects the general requirement that for any microscopic model, and any interpretation of experimental results, the microscopic dissipation functions must sum up to the macroscopic value of Φ, which would be written on the basis of observation alone, without involving any interpretation.

The formalism of linear nonequilibrium thermodynamics requires

$$J_S = L\Delta\mu_S \tag{57}$$

without providing any information about the saturation properties and transport facilitation. The model may then be used for the explicit evaluation of L, the value of which can never be given by thermodynamics alone. We shall proceed to the evaluation of L by kinetic methods, and the derivation may also serve as an example of the procedure generally used in the evaluation of the coefficients in the phenomenological equation of nonequilibrium thermodynamics.

Following the common practice, we assume that J_C and J_{CS} obey eq. (35) with the same permeability coefficient P

$$J_C = P(C^i - C^o) \quad \text{and} \quad J_{CS} = P(CS^o - CS^i) \tag{58}$$

But by eq. (53) $J_C = J_{CS}$ and hence,

$$C^o + CS^o = C^i + CS^i = C\,, \quad \text{a constant} \tag{59}$$

Now, although the intrinsic rates of adsorption and desorption are presumably quite fast, the net rate of the chemical reaction J_r is induced to equal the rate of carrier transport in the membrane by the condition of stationarity, eq. (53). The rate of any chemical reaction is proportional to the deviation of the concentrations from their equilibrium values, so we infer from the generally-assumed slow rate of carrier transport that these deviations are only slight. This assumption permits the substitution of the C's and CS's in eqs. (58) and (59) by their equilibrium values. If the equilibrium constant is taken to be equal on both sides of the membrane, we may write

$$\frac{C^o \cdot S^o}{CS^o} = K_m = \frac{C^i \cdot S^i}{CS^i} \tag{60}$$

and hence, using eq. (59),

$$CS^o = \frac{C \cdot S^o}{K_m + S^o} \quad \text{and} \quad CS^i = \frac{C \cdot S^i}{K_m + S^i} \tag{61}$$

Inserting (61) into the second of eqs. (58) and because in eq. (53) $J_S = J_{CS}$, we get:

$$\begin{aligned} J_S &= P \cdot C \left(\frac{S^o}{K_m + S^o} - \frac{S^i}{K_m + S^i} \right) \\ &= \frac{P \cdot C \cdot K_m (S^o - S^i)}{(K_m + S^o)(K_m + S^i)} \end{aligned} \tag{62}$$

Equation (62) exhibits the expected saturation properties, and if the product $P \cdot C$ is sufficiently large, it would ex-

plain a facilitation of transport through carrier mediation.

To use eq. (62) for a kinetic interpretation of the phenomenological coefficient L in eq. (57), let us rewrite eq. (57) with the aid of an average concentration $\bar{S}$, defined as in eq. (34)

$$J_S = L\Delta\mu_S = \frac{L}{\bar{S}}\Delta\pi_S = \frac{LRT}{\bar{S}}(S^o - S^i)$$

By comparison with eq. (62), L is readily identified with

$$L = \frac{P \cdot C \cdot K_m \cdot \bar{S}}{RT(K_m + S^o)(K_m + S^i)} \qquad (63)$$

Equation (63) deserves consideration from several points of view. It verifies the statement that the phenomenological coefficients are not constants but rather are complicated functions of the parameters of state—in the present case, the concentration S^o and S^i of the permeable solute on both sides of the membrane. Another important aspect is that while the macroscopic thermodynamic driving force for solute flow, $\Delta\mu_S$, does not and cannot contain any information about the facilitation or saturation of the flows, this information is implicit in the phenomenological coefficient, which comprises the frictional factor (contained in P) and the finiteness of the number of carrier sites (implied in C and in $K_m \cdot S/(K_m + S^o)(K_m + S^i)$). Thus, to gain greater insight into the thermodynamic equations and the ingredients of the L_{ij}'s in particular, it is also useful to treat the system from a kinetic point of view.

The discussion of complex membranes will serve us as an introduction to the last section of this paper, which is devoted to active transport based on the coupling of metabolic processes with diffusional flows.

Active transport across complex membranes

THE COUPLING OF DIFFUSIONAL FLOWS WITH CHEMICAL REACTION At the start of this discussion, we pointed out that the fundamental characteristic of active transport is the coupling of a diffusional process across living membranes with a chemical reaction. This coupling enables cells and tissues to drive flows against the direction of their conjugate forces and to establish stationary gradients of concentration across permeable membranes.

Nonequilibrium thermodynamics permits an a priori coupling between any flows and forces, whatever their nature. When certain symmetry conditions are imposed on the system, however, some of the coupling coefficients vanish, and the corresponding interdependences are abolished. At the beginning of this century, Pierre Curie stated that in an *isotropic* space no coupling is possible between scalar and vectorial flows.[50] Curie's principle was introduced into nonequilibrium thermodynamics by Prigogine (cf. Note 31); he observed that as a chemical flow is scalar, while transport across membranes is vectorial, no coupling between the two can take place in an isotropic space, such as a biochemist's test tube. Thus we come to the conclusion that to permit active transport the coupling space must be anisotropic, as is apparently the case with all biomembranes. In the previous section, we considered the rectification properties of complex membranes and adduced some evidence that the biological structures correspond operationally to a series array of membrane elements. Now we shall consider the complex membranes as a simple case of anisotropic arrangement permitting chemo-diffusional coupling for active transport.

To make the mechanism of the chemo-diffusional coupling more tangible let us consider a chemical reaction proceeding between two permselective membranes (α and β) of opposite polarity (cf. Note 51). The reaction is assumed to consume neutral reactants and produce J_r moles of monovalent cations and anions per unit time and per unit volume. If the distance between the membrane elements is h, $J_r h$ ion equivalents will be produced per unit time per unit area of the intramembrane space. To maintain a steady state, the intramembrane concentration of the cations (c_1^*) and anions (c_2^*) must be constant. Hence

$$dc_1^*/dt = 0 = J_1^\alpha - J_1^\beta + J_r h \qquad (64)$$

$$dc_2^*/dt = 0 = J_2^\alpha - J_2^\beta + J_r h \qquad (64a)$$

Subtracting eq. (64a) from eq. (64) and translating into electrical terms, we get the evident requirement $I^\alpha = (J_1^\alpha - J_2^\alpha)F = (J_1^\beta - J_2^\beta)F = I^\beta$; namely, in a steady state, the flow of electrical current (I) should be the same across both membrane elements despite the production of charged species by the chemical reaction.

As pointed out before, $J_1 = J_s$ when the electrode is reversible to the anion, so eq. (64) can be written as

$$J_s^\alpha - J_s^\beta = -J_r h \qquad (65)$$

The flows of salt across each membrane element obey eq. (37), independent of whether a reaction takes place in the intramembrane space. This makes eq. (65) equivalent to

$$\left(\omega^\alpha \Delta\pi_s^\alpha + \frac{t_1^\alpha}{F} I\right) - \left(\omega^\beta \Delta\pi_s^\beta + \frac{t_1^\beta}{F} I\right) = -J_r h \qquad (66)$$

In the special case of equal concentrations, c_s, on both sides of the complex membrane,

$$\Delta\pi_s^\alpha = -\Delta\pi_s^\beta = 2RT(c_s - c_s^*)$$

which upon insertion into eq. (66) gives for the intramembrane concentration

$$c_s^* = c_s + \frac{(t_1^\alpha - t_1^\beta)}{2RTF(\omega^\alpha + \omega^\beta)} I + \frac{J_r h}{2RT(\omega^\alpha + \omega^\beta)} \qquad (67)$$

Equation (67) is a generalization of that obtained for a composite membrane without chemical reaction (eq. (43)). It indicates that the third term—the chemical term—and the second term—the electrical term—may either cooperate or operate in opposite directions in determining the magnitude of the intramembrane salt concentration. As shown before (eq. (41))

$$I(R^{\alpha} + R^{\beta}) = E + \frac{1}{F}(t_1^{\alpha}\Delta\mu_s^{\alpha} + t_1^{\beta}\Delta\mu_s^{\beta}) \qquad (68)$$

where

$$\Delta\mu_s^{\alpha} = -\Delta\mu_s^{\beta} = -2RT \ln (c_s^*/c_s)$$

Hence (similar to eq. (50) without chemical reaction), insertion of eq. (67) into eqs. (68) gives

$$E = (\Delta\psi)_{\Delta\mu_s=0} = I(R^{\alpha} + R^{\beta}) + \frac{2RT}{F}(t_1^{\alpha} - t_1^{\beta}) \times \ln\left[1 + \frac{(t_1^{\alpha} - t_1^{\beta})\,I}{2RTc_s(\omega^{\alpha} + \omega^{\beta})F} + \frac{J_r h}{2RTc_s(\omega^{\alpha} + \omega^{\beta})}\right] \qquad (69)$$

Equation (69) is endowed with several interesting properties: It will be observed that even at $I = 0$ and the concentration on both sides of the composite membrane equal, the potential will not vanish for a finite rate of reaction. Indeed

$$(\Delta\psi)_{\Delta\mu_s=0,I=0} = \frac{2RT}{F}(t_1^{\alpha} - t_1^{\beta}) \times \ln\left[1 + \frac{J_r h}{2RTc_s(\omega^{\alpha} + \omega^{\beta})}\right] \qquad (70)$$

At very slow rates of the chemical process, for which

$$\frac{J_r h}{2RTc_s(\omega^{\alpha} + \omega^{\beta})} << 1,$$

by expanding the logarithmic term in series, eq. (70) reduces to

$$F(\Delta\psi)_{\Delta\mu_s=0,I=0} = \frac{(t_1^{\alpha} - t_1^{\beta})h}{c_s(\omega^{\alpha} + \omega^{\beta})} J_r \qquad (71)$$

Although these results have been derived from a detailed example of coupled chemical and diffusional flows, they provide two general principles for the thermodynamic analysis of active transport. The purely formal equation for active transport derived by Kedem[52]

$$\Delta\tilde{\mu}_j = R_{jj}J_j + \sum_k R_{jk}J_k + R_{jr}J_r \qquad (72)$$

linearly relates the driving force, $\Delta\tilde{\mu}_j$, for the j'th component undergoing active transport to the conjugate flow, J_j, to other diffusional flows, J_k, and to the flow of chemical metabolic reaction, J_r, by phenomenological generalized-resistance coefficients. First, comparison of eq. (71) with eq. (72) provides a physical interpretation of the coupling coefficient R_{jr} (cf. Kedem et al.;[51] Blumenthal, Caplan, and Kedem, in preparation) relating chemical reaction with over-all force:

$$R_{jr} = \frac{(t_1^{\alpha} - t_1^{\beta})h}{c_s(\omega^{\alpha} + \omega^{\beta})}$$

Second, it is apparent from eq. (69) that only if the current flow, as well as the reaction rate, is quite small, does the force $\Delta\psi$ depend linearly on the flows I and J_r, thus defining the conditions under which the phenomenological equation (72) may be applied to complex membranes.

ACTIVE ION EXCHANGE: THERMODYNAMICS AND A KINETIC MODEL The accumulation of K^+ and expulsion of Na^+ in active cells is described by Whittam in the previous chapter. The present-day evidence on this exchange indicates that its mechanism closely resembles that described rather generally in previous sections. In the case of the erythrocyte, it is possible to make more definite statements about the driving chemical reaction and about the nature of the exchange processes.

The work of Post et al.,[53] Glynn,[54,55] Skou,[56,57] and others has shown that the energy-providing process is a reversible breakdown of ATP to ADP + P_i. It was shown that the adenosine triphosphatase activity requires the presence of both sodium and potassium; in the presence of Na^+, phosphorylation of membrane components takes place until all available sites have been phosphorylated. Potassium is required for the normal rate of breakdown of the phosphorylated intermediate and for the continuation of the ion exchange process. Whittam[58] has adduced evidence that the process is anisotropic and that the sites of reaction for Na^+ and K^+ are spatially separated. By introducing adenosine triphosphate and sodium and/or potassium directly into the red blood cell ghosts he could show that *external* sodium and ATP are not necessary for the adenosine triphosphatase activity, and only the intracellular presence of these components favors the liberation of energy. Similar experiments proved that potassium must be present in the extracellular medium to permit the conclusion of the process. As a first model, it has been assumed that there is a phosphorylating site on the inner side of the cell membrane, at which magnesium–adenosine triphosphate is broken down in the presence of sodium, and an active intermediate, presumably a phosphorylated carrier, is formed. The active carrier takes up sodium selectively and transports it to the outer side, where the carrier is deactivated, say by dephosphorylation, in the presence of potassium. In the dephosphorylation process, the adsorbed sodium is liberated into the external solution and

potassium is bound to the free carrier, to move with the carrier back into the cell.

Recent studies on the nature of the ATP–membrane reaction lend support to the view that the phosphorylated forms are the active molecules involved in sodium transport. Post et al.[59] and Hokin et al.[60] (cf. Note 61) have achieved the isolation of activated protein molecules in which the phosphorylation takes place on the carboxyl groups with the formation of acyl-phosphate groups. Although proof for the direct participation of COO~P groups in ion transport is still lacking, a phosphorylated intermediate is a serious candidate for the role of a protein carrier.

There is also no direct experimental evidence for the preferential interaction of unphosphorylated carrier with K^+ and of phosphorylated carrier with Na^+. The plausibility of a model for active transport involving these assumptions is nevertheless supported by several lines of research. Glynn[62] has obtained evidence from isotope exchange rates for the transport of sodium bound to a carrier that shuttles back and forth within the membrane. The selectivity of polyphosphate molecules for monovalent cations is enhanced in organic media, so that phosphorylated carrier molecules might act as selectors between sodium and potassium within a lipid-containing membrane.

The complicated process of active ion exchange may be summarized in the reaction scheme shown in Figure 9, which is readily amenable to thermodynamic treatment. It is worth noting that the "outer" layer of the model need not be regarded as the outermost layer facing the external solution. There may exist additional *negative* layers that favor the exchange of Na^+ and K^+ and would account for the experimental observation that P_i is not released into the external solution. The model should be regarded as an aid to calculation and not as a representation of any real, structural pattern in biological membranes.

A microscopic dissipation function is readily constructed from this model, which condenses to the macroscopic expression

$$\Phi = J_{ch}A + J_{Na}\Delta\mu_{Na} + J_K\Delta\mu_K \tag{73}$$

where J_{ch} is the rate of ATP breakdown and A is the affinity for the over-all process ATP $\rightarrow$ ADP + P_i, or

$$A = \mu_{ATP} - \mu_{ADP} - \mu_{P_i} \tag{74}$$

In case of ion exchange, $J_{Na} = -J_K$, so that the dissipation function becomes

$$\Phi = J_{ch}A + J_{Na}(\Delta\mu_{Na} - \Delta\mu_K) = J_{ch}A + J_{Na}\Delta\mu_{exch} \tag{75}$$

where

$$\Delta\mu_{exch} = \Delta\mu_{Na} - \Delta\mu_K \tag{76}$$

is the driving force for ion exchange. Corresponding to the macroscopic dissipation function, we may write the phenomenological equations

$$\begin{aligned} J_{ch} &= L_{11}A + L_{12}\Delta\mu_{exch} \\ J_{Na} &= L_{21}A + L_{22}\Delta\mu_{exch} \end{aligned} \tag{77}$$

Here the phenomenological coefficients L_{ij} encompass the complexity of the membrane structure and the external and intramembrane reactions. The analysis of these coefficients in terms of kinetic parameters is considered next.

Several kinetic models for active transport and ion exchange in biological membranes have been developed during the past few years. Their purpose is to provide a quantitative basis for the correlation of existing data on cell and tissue transport and to predict the dependence of the rate of transport on the parameters of state. The models are generally based on the requirements discussed in the previous paragraphs: an anisotropic distribution of the elements of metabolic processes across the membrane; the existence of saturation and facilitation connected with the presence of carrier molecules in the membrane; and chemo-diffusional coupling based on carrier interaction with suitable metabolites. We shall present here a kinetic model for active ion exchange based on the general treatment of Rosenberg and Wilbrandt.[63]

The kinetic interpretation of active transport may be carried out in a manner similar to that used previously in the description of carrier-facilitated passive transport. We shall again assume that a quasi-equilibrium state prevails on both surfaces so the concentration of all reaction components may be evaluated from equilibrium equations. It is further assumed that the flows are steady so that the following relations hold for the "chemical cycle" (a) and the "diffusional cycle" (b) defined by Figure 9:

$$J^i_{ch} = J_{CK} = J^o_{ch} \tag{78}$$

and

$$J^i_r = J_{CPNa} = J^o_r \tag{79}$$

Since J^i_{ch} is the phosphorylation reaction, and J^o_{ch} is the dephosphorylation reaction, their combined effect determines the rate of ATP breakdown and equals the over-all rate of chemical change, J_{ch}, as represented in eq. (73).

Figure 9 shows clearly that the rate of external liberation of sodium J_{Na} is equal to the outer rate of exchange, J^o_r, so that according to eq. (79)

$$J_{Na} = J_{CPNa} \tag{80}$$

The rates of carrier transport across the membrane are taken to follow the simple pattern given for facilitated transport:

$$J_{CK} = P_{CK}(CK^o - CK^i)\,; \quad J_{CPK} = P_{CPK}(CPK^o - CPK^i)$$

and

$$J_{CPNa} = P_{CPNa}(CPNa^i - CPNa^o) \tag{81}$$

where the P's are permeability coefficients. No carrier molecules leave the membrane, so it is clear that

$$J_{CPNa} = J_{CPK} + J_{CK} \tag{82}$$

and if we assume for the sake of simplicity that all the permeability coefficients are equal, $P_{CK} = P_{CPK} = P_{CPNa} = P$, we get from eqs. (81) and (82)

$$CK^o + CPK^o + CPNa^o = CK^i + CPK^i + CPNa^i = C \tag{83}$$

where C is a constant. We may write the quasi-equilibrium equations for the chemical and exchange processes (incorporating the concentration of water into the equilibrium constants K_T and K_P):

$$\frac{CPK^i \cdot ADP}{CK^i \cdot ATP} = K_T\,; \quad \frac{P_i \cdot CK^o}{CPK^o} = K_P \tag{84}$$

and

$$\frac{CPNa^i \cdot K^i}{CPK^i \cdot Na^i} = K_e = \frac{CPK^o \cdot Na^o}{CPNa^o \cdot K^o}$$

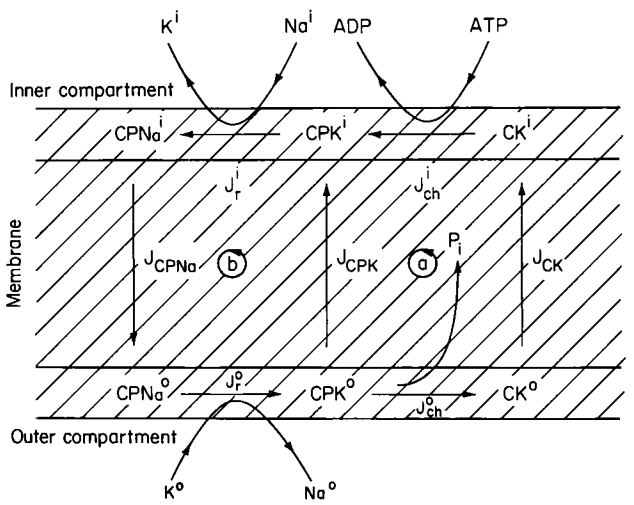


FIGURE 9 Schematic representation of carrier-mediated, active exchange of Na^+–K^+. Cycle (a) is the "chemical cycle," and includes J^i_{ch}, the rate of the chemical process that transforms the free carrier (CK^i) into a phosphorylated carrier (CPK^i) ($CK^i + ATP \rightarrow CPK^i + ADP$), and J^o_{ch}, the rate of dephosphorylation that regenerates the free carrier ($CPK^o \rightarrow CK^o + P_i$). Cycle (b) is the "diffusion cycle," which includes J^i_r, the rate of exchange of potassium by sodium on the inner side of the membrane ($CPK^i + Na^i \rightarrow CPNa^i + K^i$), and J^o_r, the rate of sodium liberation to the outer solution through exchange with external potassium ($CPNa^o + K^o \rightarrow CPK^o + Na^o$). J_{CK}, J_{CPK}, and J_{CPNa} represent the rates of flow of the different carrier forms across the membrane.

where the exchange constant K_e is assumed to have the same value on both surfaces of the membrane.

It is advantageous to introduce the following shorthand denotations:

$$r_T = K_T \frac{ATP}{ADP} = \frac{CPK^i}{CK^i} \quad \text{and} \quad r_P = \frac{P_i}{K_P} = \frac{CPK^o}{CK^o} \tag{85}$$

as well as

$$r_i = K_e \frac{K^i}{Na^i} = \frac{CPK^i}{CPNa^i}$$

$$\text{and} \qquad r_o = K_e \frac{K^o}{Na^o} = \frac{CPK^o}{CPNa^o}\,. \tag{86}$$

It is also found useful to denote

$$1 + \frac{1}{r_T} = \rho_T \qquad \text{and} \qquad 1 + \frac{1}{r_P} = \rho_P \tag{87}$$

Using eqs. (83), (85), and (87), we find from eq. (86) that

$$CPNa^i = \frac{C}{1 + r_i\rho_T} \qquad \text{and} \qquad CPNa^o = \frac{C}{1 + r_o\rho_P}$$

and hence the flow of sodium as given by eqs. (80) and (81) is

$$J_{Na} = PC\left[\frac{1}{1 + r_i\rho_T} - \frac{1}{1 + r_o\rho_P}\right] \tag{88}$$

Equation (88) has the expected saturation properties: when the inner sodium goes to high values

$$r_i = K_e \frac{K^i}{Na^i} \rightarrow 0$$

and hence the first term in the square brackets on the right side of eq. (88) reaches the maximal value of unity. Similarly, if the ATP concentration inside the cell is so high that phosphorylation is made complete,

$$\rho_T = 1 + \frac{1}{r_T} = 1 + \frac{ADP}{K_T \cdot ATP} \rightarrow 1$$

and hence the term

$$\frac{1}{1 + r_i\rho_T} \rightarrow \frac{1}{1 + r_i}$$

and becomes independent of the ATP concentration. Similar behavior is observed with the second term of eq. (88).

To evaluate the rate of chemical reaction we make use of the relation

$$J_{ch} = J_{CK} = P(CK^o - CK^i)\,,$$

which upon the evaluation of CK^o and CK^i gives

$$J_{ch} = PC\left[\frac{r_o/r_P}{1 + r_o\rho_P} - \frac{r_i/r_T}{1 + r_i\rho_T}\right] \tag{89}$$

Equation (89) also exhibits the expected saturation behavior and dependence on reactant concentrations. Despite the convenient form of eqs. (88) and (89), it is difficult to compare them with the thermodynamic expressions (77) and to evaluate the phenomenological coefficients. A useful transformation is to cast eqs. (88) and (89) into the following forms:

$$J_{Na} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)} \times \left[(1 + r_T)RT\left(1 - \frac{r_i}{r_o}\right) + RT\left(\frac{r_T}{r_P} - 1\right)\right]$$

$$J_{ch} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)} \times \left[RT\left(1 - \frac{r_i}{r_o}\right) + (1 + r_i)RT\left(\frac{r_T}{r_P} - 1\right)\right] \qquad (90)$$

It will be observed that eqs. (90) use the "kinetic forces,"

$$RT\left(1 - \frac{r_i}{r_o}\right) \quad \text{and} \quad RT\left(\frac{r_T}{r_P} - 1\right),$$

and that these forces are governed by a symmetric matrix of coefficients:

$$L_{11} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)}(1 + r_T)$$

$$L_{12} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)} = L_{21} \qquad (91)$$

$$L_{22} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)}(1 + r_i)$$

We will now show that the kinetic forces reduce to the corresponding thermodynamic expressions when the system is close to equilibrium. Let us first consider the ion exchange force $\Delta\mu_{exch}$, which upon substitution of the definitions of eq. (86) into eq. (76) becomes

$$\Delta\mu_{exch} = -RT \ln \frac{K^i/K^o}{Na^i/Na^o} = -RT \ln \frac{(K^i/Na^i)K_e}{(K^o/Na^o)K_e} = -RT \ln \frac{r_i}{r_o}$$

or

$$\frac{r_i}{r_o} = \exp\left(-\frac{\Delta\mu_{exch}}{RT}\right) \qquad (92)$$

Close to equilibrium, $\Delta\mu_{exch}$ approaches zero, so that the exponential in eq. (92) may be expanded in series and only the linear term retained:

$$\exp\left(-\frac{\Delta\mu_{exch}}{RT}\right) \simeq 1 - \frac{\Delta\mu_{exch}}{RT}$$

Hence

$$\Delta\mu_{exch} = RT\left(1 - \frac{r_i}{r_o}\right). \qquad (93)$$

In a similar manner, the intracellular affinity of the breakdown of ATP

$$A = \mu_{ATP} - \mu_{ADP} - \mu_{P_i} = RT \ln K_T K_P \frac{ATP}{ADP \cdot P_i} = RT \ln \frac{r_T}{r_P}$$

Again close to equilibrium A approaches zero and hence

$$A \simeq RT\left(\frac{r_T}{r_P} - 1\right) \qquad (94)$$

Thus, in the range close to equilibrium, the thermodynamic forces A and $\Delta\mu_{exch}$ equal the kinetic forces, and then eqs. (90) assume a form identical with those of eqs. (77), namely

$$J_{Na} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)}[(1 + r_T)\Delta\mu_{exch} + A]$$

$$J_{ch} = \frac{PC\, r_o/r_T}{RT(1 + r_o\rho_P)(1 + r_i\rho_T)}[\Delta\mu_{exch} + (1 + r_i)A] \qquad (95)$$

It is rather remarkable that the symmetry of the coefficients $L_{12} = L_{21}$ (91) holds in a wider range than does the symmetry of the thermodynamic coefficients, eq. (95), as a consequence of the approximations, eqs. (93) and (94). Comparison of these equations with experimental data may be found in a paper of Blumenthal, Ginzburg and Katchalsky (in press).

It is evident that the treatment outlined above is insufficient for the description of nonstationary states or of systems in which surface equilibria may not be assumed. A more powerful, recently developed approach is based on the lattice treatment of statistical mechanics. The attractive feature of this method is the decomposition of the transport process into elementary steps that may generally be described by monomolecular kinetics. In the work of Heckman[64] and of Hill and Kedem,[65] the membrane is represented as two parallel planes consisting of lattice points, between which a carrier shuttles back and forth. Hill[66] found that the calculational procedure is simplified appreciably if the lattice model is translated into cyclic diagrams equivalent to the diagrams for active ion exchange illustrated in Figure 9. Blumenthal, Ginzburg and Katchalsky (in press) have applied the lattice treatment to the analysis of active sodium–potassium exchange. As expected, the resultant equations reduce to those presented in this section for quasi-equilibrium states, but their range of applicability is wider, extending even to nonstationary cases far from equilibrium.

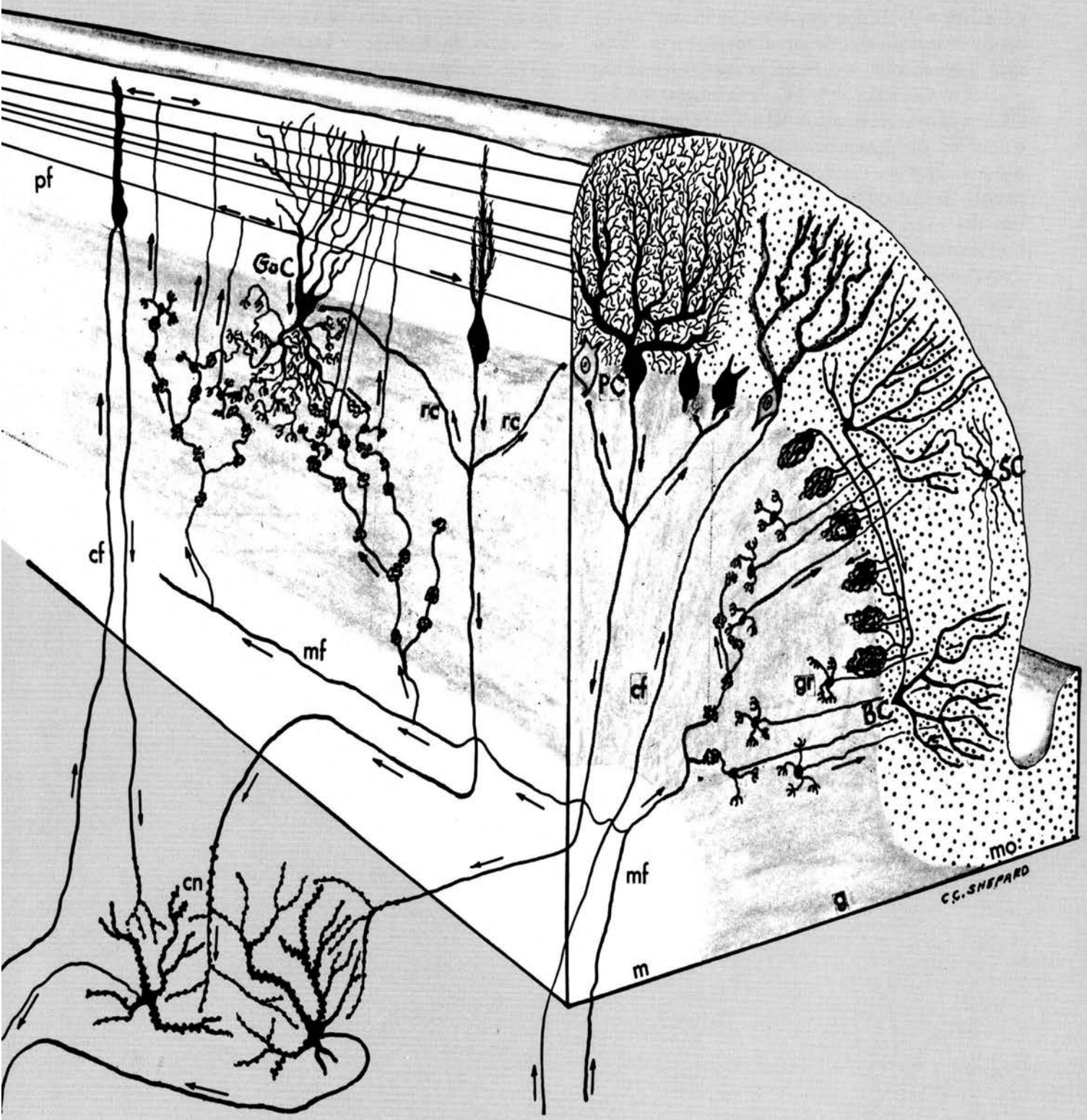
RELATION TO MECHANOCHEMISTRY Since we have ventured into a detailed model of active transport, it is difficult to stop before raising the question of the operational mode of the carrier in the membrane. The rheological analysis of erythrocyte membranes by Burton[67] and by ourselves[68] indicates that the membrane is a tough, rather rigid structure with viscoelastic parameters resembling those of swollen nylon. It is difficult to imagine that free diffusion of macromolecules could fulfill the task imposed on carriers. The passage of carrier through the complex and well-organized membranes of cells requires an appreciable mechanical effort, and it is inviting to speculate that this could be carried out by mechanochemical macromolecules. Contractile fibers and a collagen mechanochemical engine[69-71] show that macromolecular "phase transitions" governed by a chemical interaction may produce sufficient forces to transport low-molecular-weight substrates across membranes. It is therefore suggested as a working hypothesis that the free carrier molecules are contracted macromolecules that open up upon phosphorylation resulting from interaction with ATP. The phosphorylated protein molecules combine avidly with sodium and transport it across the barriers presented by the membrane. After dephosphorylation, the molecules combine selectively with potassium and contract back to their original shape.

It is possible to estimate the number of contraction–relaxation cycles performed by the carrier molecules: results obtained in various laboratories have led to the conclusion that the number of reaction sites involved in cation transport across the erythrocyte membrane is between 10^3 and 10^4. The known sodium flow at room temperature is $J_{Na} = 10^{-13}$ moles/cm²sec. Hence, the number of sodium ions extruded from a single red blood cell per second (assuming the area of a human erythrocyte to be $150\ \mu^2 = 150 \times 10^{-8}$ cm²) is

$$10^{-13} \times 150 \times 10^{8} \times 6.10^{23} = 10^5 \text{ ions/sec} \cdot \text{eyrthrocyte.}$$

This leads to the conclusion that the number of cycles per second per carrier molecule is between $10^5/10^3$ and $10^5/10^4$ or 10 to 100 cycles per second.

While it is intriguing to speculate about the cyclic performance of cellular components, the study of these important phenomena falls beyond the realm of nonequilibrium thermodynamics and its kinetic counterpart. Here, therefore, is an adequate time to finish the paper and leave the field open for further developments in the exciting realm in which steady processes pass into quasi-stationary periodic phenomena.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C.C. SHEPARD

NEURONAL PHYSIOLOGY

"*The essential requirement for nervously mediated phenomena is a nervous* system, *a system whose component parts are numerous but not chaotic, its essence the relations among components.*" BULLOCK, PAGE 347.
The importance of different cell types in a definite organization is emphasized in this semidiagrammatic drawing of the cerebellar cortex, PAGE 418.

INTRODUCTION

THEODORE HOLMES BULLOCK

Signals and Neuronal Coding

IN THE HIERARCHY of levels from molecules to the brain, we come now to that intermediate level that involves neurons and their signals and codes. Without waiting for answers to the questions posed at more basic levels, it is possible to inquire what is going on at the higher level and to achieve a certain understanding, at the same time uncovering principles of operation and phenomena to be explained at lower levels.

The constellation of natural wonders we are dealing with—nervously mediated behavior—is above all marked by its organized, adaptive, and coordinated, as well as complex, nature. We are not "simply" attempting to crack the problem of storage of memory but to move toward understanding of drives and instincts, habits and skills, recognition and decision.

The essential requirement for nervously mediated phenomena is a nervous *system*, a system whose component parts are numerous but not chaotic, its essence the relations among components. It must make sense. A nervous system is an organized constellation of cells (neurons) specialized for repeated conduction of an excited state from receptors or from other neurons to neurons or effectors, and for integrating converging signals, generating new signals, and commanding adaptive behavior. What are these neurons?

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A neuron is a cell specialized by its properties and connections to receive certain forms of signals, respond with special signals, conduct excitation, and make adaptive functional contacts with other neurons or with receptors or effectors. (See Bullock and Horridge, 1965, Glossary and Chapters 1–5 for further definitions and discussion of concepts.) The neuron doctrine is heuristically helpful even if soft in spots. It asserts that the nervous components of the nervous system are cells, called neurons, with their appendages or extensions called processes, and including axons and dendrites (for definitions see Bullock and Horridge, 1965, and Bodian, this volume). The doctrine was enunciated in the last century, with the discovery by Cajal of new evidence against the old idea that nerve fibers are components distinct from the cells and the later idea that even though connected with the cells they form a continuous reticulum, or meshwork. It emphasizes that the neuron is distinct, is usually only in contiguity with other cells (not continuity), and is the unit of the system. Each neuron is a developmental unit of maintenance, degeneration, and regeneration and, in a subtle way, of function. Neurons are units in much the same way as people are units.

As organization, order, and interrelation are paramount in the system aspect of neuroscience, that aspect is often thought of in terms of which neurons are specialized for which tasks, what pathways can be assigned, how neurons affect each other, and with what transfer functions. This approach deals with circuitry and connections, segregation of functions, and interrelations of subsystems. It is an article of faith among many physiologists that adequate knowledge at this level will explain much or all of behavior, but I put it this way to underline that we cannot yet test this article, and there may exist operational principles at higher levels understandable only in terms of large numbers of elements.

To introduce the problems dealt with in this third block of papers, I will pose two broad questions: (1) What are the kinds of neuronal events that could be doing the signaling between parts of the nervous system; in other words, what are the forms of meaningful energy or material flux for information processing? (2) What are the properties of neurons that could be integrative; in other words, what discriminable features of nerve cells might contribute to weighting inputs, encoding into the forms of signaling events, decoding and processing signals?

Forms of signals

Several kinds of neuronal events are known to be important as signals. Quite possibly there are others, or subdivisions of these, not yet recognized.

GLIAL–NEURONAL INTERRELATIONS The first category is a diffuse one, not yet well understood or assessed. The nonnervous cells specially characteristic of the nervous system are called neuroglia or glia cells, and in higher animals are extremely numerous, differentiated into types, and intimately related to neurons anatomically. Although many workers doubt that they play a neural or information-processing role, there is some evidence suggesting that glia participate with neurons in more than purely vegetative ways or merely as passive and unchanging paths for extraneuronal current flow. (We heard relatively little about this at the Intensive Study Program, but I include it for completeness.)

PASSIVE ELECTROTONIC POTENTIALS This term refers to the displacement of a transmembrane potential of a neuron as a result of current imposed from any source, without active response. We may speak of the electrotonic component of a response that has an active component superimposed on a passive one. The source can be other cells, other parts of the same cell, or artificial. The displacement is a function of the imposed electromotive force (emf), and of the standing ("passive") properties of resistance and capacity, and therefore is attenuated and delayed with distance from the source.

ACTIVE ELECTROGENIC POTENTIALS The term active means that the cell membrane changes its properties: virtually the only property to change in response to stimuli is resistance; that is to say, conductance to one or more species of ions. Such conductance change in the presence of the normal cell-membrane potential results in a gating of current that can represent a large power amplification. There are several distinct types of active potential change, logically grouped under three heads. Something about each of these appears in this book.

1) Exogenic types are responses to impinging stimuli from the environment or from other neurons. Sensory receptors respond to environmental stimuli with receptor potentials. Neurons receiving input from other neurons by presynaptic paths respond with synaptic potentials. Both classes can be thought of as transducing impinging events of specific kinds and responding with a graded potential and conductance change. There are mainly two subclasses. Certain inputs cause a depolarizing potential, called the excitatory postsynaptic potential, or EPSP, for its effect of increasing the excitatory level and likelihood of firing an impulse by the neuron. Other inputs cause a hyperpolarizing response, the inhibitory postsynaptic potential, or IPSP, for its suppressing effect. Parallel depolarizing and hyperpolarizing receptor potentials occur in sensory re-

ceptors in response to particular stimuli. There may be several distinct EPSPs and IPSPs in the same neuron, caused by different incoming presynaptic pathways converging on this neuron, which then integrates the various influences. Different junctions on the same neuron may give PSPs of different amplitude and form, as seen by an electrode in the soma. In some neurons the IPSP conductance change is more important, in others the potential change, in exerting the inhibitory effect.

2) Endogenic potentials are responses to antecedent activity in the same neuron. There are three types: local potentials, nerve impulses, and after-potentials. If a receptor potential or synaptic potential or one of the next following category succeeds in rising to a sufficient amplitude and in a favorable part of the neuron, it may induce a further active response in electrically excitable membranes. This can remain graded, and therefore local, or can reach a sharp threshold and trigger a nerve impulse. You will frequently see the term spike, a synonym for a nerve impulse, that is an all-or-none, depolarizing and then reverse polarizing ("overshooting"), brief (i.e., actively terminated or repolarizing) event that propagates without decrement because it draws current of more than threshold intensity through the nearby resting membrane, inducing it to fire in turn. Unlike the other forms of activity, the spike is always followed by a period of about its own duration (*circa* 1 millisecond) of complete refractoriness, thus assuring that spikes will occur in discrete numbers or trains. Following each spike, and graded with their number and frequency, we observe the third type of endogenic potentials—the after-potentials. These are after-depolarizations or after-hyperpolarizations or both in sequence, *pari passu* influencing excitability, and may last for tens of milliseconds up to many minutes.

3) Autogenic potentials are internally timed, therefore spontaneously occurring, events. Although it is dependent on the environment of the cell for steady-state conditions adequate to permit its expression, the moment of occurrence is determined by intrinsic mechanisms and is hence truly spontaneous. Among such potentials are pacemaker potentials of more or less regular recurrence that may be of relaxation oscillator character or of pendular, sinusoidal character. Another variety is the random miniature junction potentials, so-called from their quasi-Poisson sequence, small and quantal amplitude, and localization in immediately postjunctional membrane.

Note that we have quite a list of types of activity in the neuron. Only one of them is all-or-none and propagated. All the rest are graded, and decrement electrotonically to a small percentage of their original height within a distance that varies between microns and millimeters, according to diameter. These are very local signals. The spike is a specialized signal suitable for long-distance conduction and virtually confined to axons.

TRANSMITTERS BETWEEN NEURONS The neuron doctrine of the discreteness of the cellular elements, supported abundantly by modern electron microscopy, demands something we can call transmission between neurons. Because the agents of this process, the transmitters, must be released under the control of the antecedent nerve impulses in the same neuron and must determine the response of the postsynaptic receiving neuron, they are extremely important and have accordingly attracted a great deal of work. There is good evidence that some synapses transmit electrically and others chemically; some of each type are excitatory and others are inhibitory, and there are many subdivisions based on physiological properties, anatomical specifications, and pharmacological responses. We will read in three papers the indications that chemical transmitters are not everywhere the same but at least several in kind, although not really numerous. The electrical event arriving at tapering and branching axon endings probably falls from a spike to a graded event in many neurons and decrements to various degrees. The transfer function between this potential and the amount and rate of release of transmitter is known in one case, the squid synapse, and is strongly nonlinear. The possibility of feedback effects of the transmitter or of the postsynaptic membrane upon the presynaptic release is an example of a purely speculative complication that needs to be examined experimentally. The special class of chemically signaling neurons called neurosecreting cells are widespread among animals; their product is released at nerve endings, stored, and distributed by the bloodstream.

ELECTRICAL SIGNS IN MASSES OF NEURONS Macroelectrodes in many higher centers record gross potential shifts of slow to very slow time course. One class is so clearly elicited by applied stimulation (flashes, clicks, shocks, or the like) that it is called the evoked potential. Some very slow shifts are not obviously time-locked to discrete stimuli. Fluctuating potentials in the band, approximately 0.5 to 100 per second and ongoing under normal conditions, are especially a feature of vertebrates. These are the brain waves (electroencephalogram, or EEG). The question is still open: Are these only effects or are they also causes; can they be signals to other nerve cells? Do they bear some direct relation to the focus of cellular activity known and listed above? How do they vary with the state of the organism? There are three papers on these and related problems, and several later topics involve these potentials as indicators.

Here is a world of phenomena and questions between

the molecular level and the circuitry of the brain, dealing with the many forms of signals available for conveying information, for encoding and integrating it.

Attributes for integration

Even if we know the actual forms of events that are doing the signaling, it would not mean we know the way, or ways, in which different inputs that converge on an integrative cell are weighted or in which the sequences of spikes are read. What properties of neurons permit or contribute to the integration in time and space that determines the output as function of the input, often complicated by ongoing activity? As before, we are not sure we know all the mechanisms there are, but we know several, and I will quickly parade them as an introduction to the group of chapters that leans on neurons.

FORMS OF EXCITABILITY First we can recognize that a consequence of the several forms of response is several different forms of excitability. The classical threshold, which represents the spike excitability, is only one of these and one that is important only at limited loci in the neuron—the spike initiation locus of the moment, sometimes at branch points or near the axon terminals. But each of the other forms of activity has an excitability of its own, even though it is without so sharp a threshold. In addition, each is an independent property varying with local conditions in that part of the neuron. The local potential, for example, has an input–output curve that departs gradually from the 45° slope of passive, electrotonic response in a steeply accelerating manner below the spike threshold. The postsynaptic-potential amplitude depends on the amplitude of the presynaptic spike and the intervening chain of events involved in transmitter quantity and rate. In the case of the giant synapse of a squid, no postsynaptic potential was elicited below 80 per cent of a normal, full prespike, and between 80 per cent and 100 per cent the curve rises steeply. Each input can have a different curve, and they can change with time (see *Time Factors,* below).

FORMS OF INHIBITION There are several different types of inhibition. The longest known, and one so familiar it is sometimes contemptuously dismissed, is that which occurs in the wake of activity. After-potentials of hyperpolarizing sign can depress the neuron for quite a time. Even if the cell has not fired but has only had an excitatory postsynaptic potential, there may yet be a depression, which is a true inhibition. There are the inhibitory postsynaptic potentials. Sometimes they are mainly significant in subtracting potential from a near threshold excitatory event. In other places they are characteristically more influential as an impedance change, short-circuiting any excitatory potentials. Other forms of inhibition are slowly developing hyperpolarizations not time-locked to particular impulses but accumulating with many impulses and lasting a long time. Still another is caused by excessive depolarization.

Presynaptic inhibition is a variant based on arrangement. An inhibitory ending can terminate on the axonal terminals of an excitatory pathway and act to throttle transmission at the presynaptic level. Presynaptic enhancement of excitation is also believed to occur.

ANATOMICAL FACTORS Where the axon telodendria end on the neuron, how many knobs or end buttons there are, and how close together or far apart they may be are all decisively important for integrating different inputs. The presynaptic synapses just mentioned, climbing vine endings, clubs, nests, bottle brushes, and a host of other forms must have a role in determining output as a function of input. If we visualize all the graded forms of activity, the spontaneous synaptic and local potentials that precede a spike, and think of each of them as having a local focus, falling off in effectiveness electrotonically, summing, canceling, spreading to dendrite confluences, eventually acting at the spike trigger locus, we can appreciate the contribution to integration of length, taper, branching, and other geometric aspects of dendrites; and of shapes, distribution, and neighbors of axonal telodendria. We are not yet in a position to understand the physiological significance of cell arrangement in clusters, layers, glomeruli, and the like, which characterizes higher animals and higher centers.

TIME FACTORS There are several forms of enhancement and of depression of response as a function of previous activity. In some cases the change can be attributed to an excitability change without alteration of the responsiveness, whereas in other cases there is an altered responsiveness with or without a shift of excitability. If a stimulus gives a larger response as a consequence of antecedent activity, we infer an underlying process called facilitation, and there may be several consecutive phases of shorter-term and longer-term facilitation lasting up to many minutes or some hours, but usually for some tens of milliseconds. If a stimulus gives a smaller response because of antecedent activity we may speak of relative refractoriness or of antifacilitation. Particular synapses are characterized by being facilitating or antifacilitating even on the same neuron at the same time. Possibly these effects are mainly a result of changes in the presynaptic stages of transmission. Following a period of stimulation, for example a

burst of excitatory or inhibitory shocks, there may occur important after-effects—positive, negative, or both in sequence: the response may continue for a time after cessation of stimulation (positive after-effect) or a rebound may occur instead (negative after-effect).

Thus, the result of the same arriving input can be quite different depending on several kinds of history-dependent factors with differing rates of recovery.

ENCODING AND DECODING NERVE IMPULSES The chapters I have introduced in the preceding sections of this paper will demonstrate the neuron's formidable capacity for complex behavior. Permutations of the several properties and forms of activities give many degrees of freedom. But, apart from the electrical signs of masses of cells in brain waves, DC shifts, and evoked waves, we have dealt so far with elemental and unit aspects. We must go one step further at the neuronal level.

The vast flow of information from one part of the brain to another, from sense organs to brain and from brain to effectors, is contained in trains or sequences of nerve impulses, at least wherever the areas are at any considerable distance from each other and aside from certain kinds of messages carried by neurosecretions. These trains of spikes represent the coded information for our myriad discriminations, recognitions, and commands. To ask how the sequences are generated and what features of the sequences are read by the decoding cell, i.e., every postsynaptic cell, is equivalent to asking what might be the code or codes; what is the language of the nerves?

As far as we know, the integrative properties sketched above are adequate to provide both coding and decoding, that is, to determine the generation of a train of spikes as some function of input plus spontaneous activity plus noise, and to determine the postsynaptic response of the next cell as some function of this train. But knowing these properties does not tell us what code is used.

There are several a priori possibilities, given all-or-none events of variable number and spacing. Note that this is in no sense a digital communication link, but a pulse-coded analog system. The intervals between spikes are not quantized but are continuously variable. Because only number and spacing can change, it has long been assumed that there are only two ways messages can differ—in number and in frequency (spikes per unit time, arbitrarily chosen). Only recently has it begun to register that there are other ways, including the degree of variance of the intervals about the mean, the shape (symmetry, number of modes, etc.) of the distribution of intervals about the mean, the presence and sign or the absence of autocorrelation of successive intervals, the possibility of systematic temporal microstructure ("patterns") in impulse trains. Each of these has been found and is thereby a candidate code—although not ipso facto a code, because we must show that the postsynaptic cell reads or discriminates the feature in question, when other features are invariant.

Experiments of this kind are beginning—testing, for example, whether fine structure such as alternately long intervals and short intervals can elicit different postsynaptic responses from a train of evenly spaced spikes at the same average frequency—with positive results in several preparations. Moreover, from analysis of the changes in sensory trains on varying the natural stimulus we can see how these fibers encode information. Particularly favorable for this purpose has been the newly discovered modality of electroreception in fresh-water electric fish of different species. The finding of broad general interest is that there is not one code but several. The average frequency is doubtless the code in some fibers. In others a number code is employed—the number of spikes following each stimulus measures its intensity; the individual intervals do not systematically vary, and the average frequency is contaminated by the variable rate of recurrence of stimuli. A latency or phase code (these two can be distinguished in some cases) is found in several preparations in which regular events are measured in intensity by the time between some invariant signal of their happening and a delayed second signal (whose frequency is therefore constant). Some fibers appear to be like doorbells and simply carry a presence or absence signal. We will read about still other ways of coding in single lines, but in this volume will not get far into the consideration of how parallel lines represent information, converge, cross-correlate, and are read by higher levels.

It is evident, and several authors will illustrate, that in each of these areas of neuronal integration and signaling the subject is in a fluid state. We are still like naturalists hunting in the jungle, who, having found a strange creature, are trying to decide its place and significance in the natural scheme.

But when, in a given case of single input to single output, we have isolated the parameter of arriving impulse trains that the receiving cell looks at, we must still learn several features if we are to understand this simple communication system. One is the time over which the receiving cell integrates the train parameter. If we knew this, we would be able to evaluate the shape of the encoding or spike-generating curve: stimulus (input) being a dimension shown to be the one the system is concerned with, against spike response (coded message) measured along the parameter of the code. Another is the amount of noise introduced in the encoding process and its distribution and composition. This would permit an estimation of

the resolution at a given reliability, or vice versa. There is likely to be more than one dimension of a stimulus that will, under normal circumstances, be encoded; ambiguity is a common attribute of neurons and receptors. To understand even the simple one-line-in—one-line-out system we will have to look at the permutations of the relevant dimensions.

These are statements of the ideal. So far, actual data do not satisfy all these criteria, and the sample is a limited number of preparations under not fully comparable conditions. Still it seems important to summarize by saying that our information indicates a wide range of different input–output curves: linear, logarithmic and power functions of different steepness, nonlinear relations up to almost-square step functions, and curves with sudden inflections.

But it is really the special case and an unusual situation that can be represented fairly by a curve of output as function of input in the steady state. Much more general is the case in which output has a transient higher sensitivity, called a phasic response, and then adapts either completely or partially, leaving a steady or slowly declining weaker sensitivity, called a tonic response. Very few studies have quantitatively assessed the phasic component, especially by small sinusoidal stimuli over a wide frequency range.

Coming still closer to natural signaling in the nervous system we must recognize that even with the refinements of the preceding paragraphs we are well short of a typical elementary link in real nervous communication. A single line in is a rarity and the multiple lines arriving on a cell may all be of one modality, perhaps differing in a submodality like color or pitch or topographic receptive field, or of two or more modalities. In either event, the cell may be integrating spatially as well as temporally, cross-correlating the converging inputs and therefore changing the meaning of the signal in the output.

Here we start the transition between elementary neuronal processes and processes inherent in assemblages of neurons. Besides convergence and its consequences, there are the broad problems of principle and specific questions of fact in given places that depend on overlap functions, on lateral inhibition or, more generally, on interactions between parallel elements, on phase locking or coupling between oscillators of different frequencies, and still others.

In addition to all this we face the intriguing and fundamental question of the relation of nerve impulses to behavior, which is somewhat parallel to the old question of the relation of gene to character. We cannot discuss here the involvement of regions of the brain and its organizational aspects. But we can note that there is a wide spectrum of examples with respect to reliability: in some, the nerve impulse sequence in unit neurons is just as reliable as the overt action or response, whereas in others the latter is much more consistent than the former. Because averaging over many repetitions brings closer agreement, we are driven to invoke a population of nearly equivalent units whose average activity has a one-to-one relation with behavior.

This, in turn, raises the question: Who reads the activity of the population? Particularly when the behavior has a sharply alternative character, as in a fly standing or taking off, a bird giving its species-characteristic call, a person reading the price list—either–or behavior that is highly reproducible—the issue is real. How is the activity of an unreliable population of nerve cells read and integrated into a highly reproducible act? Answers to this important problem of principle are not forthcoming in this volume but it is not for lack of plausible possibilities (see Bullock, 1961). Rather, the possibilities have not yet been formulated precisely enough and experimental tests of them not performed except in seemingly special cases in arthropods and annelids.

Certain it is that as future experiments are directed to the great problem of the relation of nerve impulse to behavior, taking account of coding and signaling in naturally organized constellations of neurons, we will learn of new principles and emergent properties.

Synaptic and Ephaptic Transmission

HARRY GRUNDFEST

SPECIALIZATION for generating, conducting, and transmitting electrical messages is a principal function of nerve cells. Neurons manifest their epithelial origin by a functional resemblance to other excitable cells of epithelial descent—the receptor, gland, and neurosecretory cells.[1-4] In general, all these latter cells are responsive only to specific stimuli. With the exception of electroreceptors, they do not respond to electrical stimulation and do not generate spikes; i.e., their input component is electrically inexcitable and their output component is secretory in function.[5-9] These rather simple input–output relations are, however, complicated in neurons by the interposition of a middle component (Figure 1), which is conductile in function. To achieve this capability, the membrane comprising the middle component must be electrically excitable and must be capable of generating spikes.[7,10,11] Thus, the intracellular interfaces, between input and conductile components on the one hand and between the conductile and output components on the other, must develop special characteristics that shape the performance of neurons along markedly distinctive lines. The other excitable cells are to be regarded as principally secretory elements, and they are permissively electrogenic. Their activity may be accompanied by an electrical sign that may be depolarizing or hyperpolarizing (i.e., a shift to less or more inside negativity from the baseline of the inside-negative resting potential), or no electrical sign need be present.[1,2] Neurons, however, are obligatorily electrogenic, and their electrogenesis must submit to rather rigid requirements.[7,8,10,12] Furthermore, neurons function sequentially as well as in parallel, activity from one being transmitted to another. Neurons also transmit messages to effectors, including gland and neurosecretory cells, muscle fibers, and electroplaques. At each stage, intercellular transmissional interfaces occur; they may be excitatory or inhibitory in function, and their number may be multiplied, because both functional types may occur at a given stage.

The requirements that stem from the specialization of neurons for conductile activity are probably the most important elements in shaping the complexity of the intracellular interfaces. The conductile function demands the capacity to generate signals that can propagate rapidly along the cell and with a minimum of distortion of the information. In man, axons may be one meter or more in length, while in whales and other elongated vertebrates the conduction paths in the funiculi of the spinal cord presumably call for even longer axons. The requirement for speed thus ruled out the bulk transport of informational material like that which must take place in the secretory output of gland or receptor cells. It imposed, rather, the need for an electrical signaling system. However, the electrical conditions that are available to the biological machinery impose severe requirements upon the cable properties of the axon.[13,14] For example, a steady electrical signal applied at one end of the axon degenerates after propagation for only one millimeter to about the same extent as it would in some 1000 kilometers of long lines communications telephone or telegraph cables.[10]

The solution evolved by the neuron[15-19] and described in the preceding chapter[20] was essentially similar to that now used in communications engineering. The axon forms a cable with distributed amplifiers that utilize the local energy of ionic batteries. Thus, it is capable of generating an all-or-none response (spike) of rather constant shape. Once initiated, the spike can propagate itself along the conductile membrane component to the output of the farthest extension of the axon.

The ionic batteries that are normally available to the cell for spike electrogenesis are inside-positive, utilizing the difference between the steady-state electrochemical potential for sodium or for divalent cations (calcium and/or magnesium) and the inside-negative resting potential. The regenerative characteristic of the spike, which is essential for the conductile capacity, demands that the trigger for spike electrogenesis also be a positively directed (depolarizing) change from the resting potential. The conductile membrane component thus must be electrically excitable, responding to a threshold depolarization with a much larger positive-going electrogenesis.

These conditions, in turn, set requirements for the interface between the input and conductile components of the cell membrane. The input component must itself be electrogenic and must respond to its specific excitant(s) with depolarization, which, upon liminal or supraliminal input excitations, is capable of triggering the production

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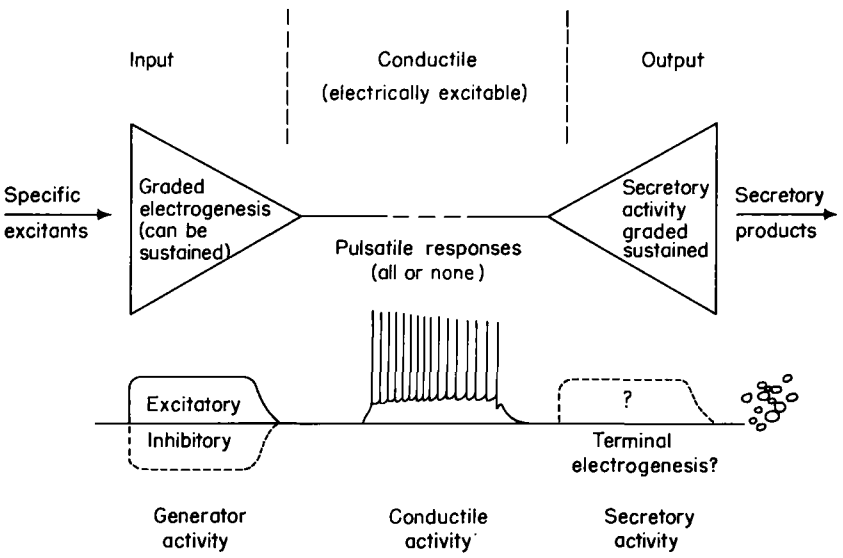

FIGURE 1 Diagrammatic representation of functional components and electrical responses of a receptor or correlational neuron. The electrically inexcitable input produces electrogenesis, which is graded in proportion to its specific stimulus and usually is sustained as long as the stimulus is applied. The possibility of hyperpolarizing electrogenesis, which may be produced by inhibitory synaptic membrane, is shown, but is not further considered. The depolarization at the input, operating upon the conductile electrically excitable component, can evoke spikes. The spikes are encoded by number and frequency in proportion to the generator depolarization. These pulsatile signals, propagated to the output, presumably command secretory activity there, roughly proportional to the information encoded in the message of the pulses and sustained as long as the message demands. The transmitter, which is believed to be released at the output, can initiate a synaptic transfer by operating upon the input of another cell. The possibility of a special output electrogenesis is indicated, but is not further considered. (From Grundfest, Note 6)

of one or more spikes. This electrogenesis of the input is accordingly termed the *generator potential* of sensory neurons or the *excitatory postsynaptic potential* (EPSP) of synaptic transmission. A second variety of input membrane may also be present, and probably is always found in neurons. It depresses the excitatory depolarizing electrogenesis, and thus has an inhibitory function, in the course of which it generates an *inhibitory postsynaptic potential* (IPSP).

In summary, these input components of the generalized neuron do not respond to electrical stimuli, but are excited by specific chemical, mechanical, photic, or thermal stimuli. Thus, there can be no electrical feedback, either positive or negative, and the responsiveness of the input membrane is essentially linear. The potential changes are fully graded and may last as long as the excitant is applied. The input component may be commingled with the conductile, electrically excitable portion of the membrane, or the two may be discretely localized. The topological relations of the components, the sensitivity of each component to its adequate stimulus, the amplitude of the input electrogenesis, the presence or absence—as well as the degree of effectiveness—of inhibitory inputs, all give scope to different permutations of interplay and give rise to many varieties of phenomenology. It is important to stress that all these manifestations may be observed only with tests on living cells. No morphological markers are currently available to distinguish the electrically excitable from the electrically inexcitable membrane components, let alone the different subvarieties of each type of component.[10,11,21]

Transfer of information from one neuron to a chain of others, or from neurons to effector cells, is another functional requirement of neuronal organizations. It introduces two additional interfaces. One is intracellular, between the conductile electrogenesis and the output membrane component. The other is intercellular, between the output of the neuron and the input of the next cell. The vast majority of the junctions form synapses in which the characteristics of the individual cells shown in Figure 1 are repeated at each synaptic stage (Figure 2). Because the input of the postcell is also electrically in-

excitable, transmission must presumably involve a chemical step, and this demand of the extracellular interface is met by the secretory capability of the output component of the presynaptic neuronal terminals. The intercellular interface usually is a pathway of high electrical resistance. For example, large changes in membrane potential, imposed by injecting current intracellularly into the presynaptic terminals of the squid giant axon synaptic complex, do not produce measurable electrical leakage to the postsynaptic axon nor does a large current injected into the latter leak across to the presynaptic axon in measurable amounts.[22] Much less frequently (and as yet unknown in the mammalian central nervous system) there also occur transmissional interfaces with relatively low electrical resistance. Depending upon the degree of the electrical coupling,[23,24] leakage currents from one component cell may affect the others. These electrical connections form electrotonic or *ephaptic* junctions.[9]

Both synapses and ephapses are usually accessible to electrophysiological tests as to the nature of the excitable membrane. These tests may provide direct or indirect evidence for electrical inexcitability,[6,9,42] and wherever this evidence is available it is legitimate to infer that transmission is chemical. On the other hand, pharmacological or chemical evidence regarding chemical transmission is poor at best, and is totally lacking for most synaptic systems.[24a,77] Thus, the term "chemical synapse" may be used legitimately only if there is evidence regarding an electrically inexcitable component, and the word "chemical" is superfluous.

Synaptic transmission

In general, the morphology of neurons exhibits a pattern based on the above functional relations.[25,26] As a rule, the dendrites and soma of neurons form the receptive region, while the conductile component is represented by the axon and the output components by the nerve terminals. However, that is by no means an invariant pattern. In vertebrate dorsal root fibers the receptive component is

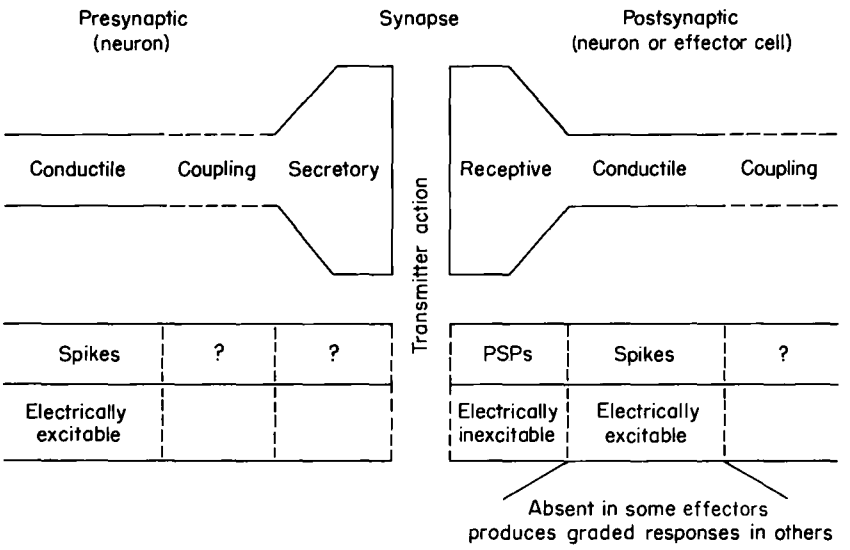

FIGURE 2 Structural and functional components of synaptic transmissional activity in electrogenic excitable cells. The upper portion shows the different structural elements diagrammatically. The lower part describes the physiological activity. The receptor portion of the presynaptic neuron is omitted. It may be a synaptic or ephaptic junction or the receptive membrane of a sensory neuron. The depolarizing potential generated in the receptor or synaptic membrane (Fig. 3), or induced in the postephaptic cell (Fig. 17), initiates spikes which propagate in the electrically excitable conductile membrane. At or near the presynaptic terminals, which are presumed to have secretory function, some unknown coupling mechanism translates the electrical signal into orders for the secretory activity. After release of the transmitter and its diffusion across the synaptic cleft, transmitter action on the electrically inexcitable subsynaptic membrane is probably extracellular. The PSPs generated by the synaptic membrane may be depolarizing (excitatory) or polarizing (inhibitory), and both types of activity may occur in the same cell. If the depolarizing PSPs are large enough, they can initiate conductile activity, provided the postsynaptic cells have an electrically excitable membrane component. (From Grundfest, Note 42)

far from the cell body, which lies in the ganglion close to the intraspinal output terminals. The receptive region, exemplified by the dendrite of the Pacinian corpuscle,[27,28] joins onto and acts upon a relatively long conductile pathway in the axonal component. While the cell body is essential for the survival of the other portions of the neurons, it may be bypassed functionally, information being delivered to the spinal cord by the spikes that are generated along the axon. The functional bypass of the cell body may be reflected in an electrophysiological manifestation. The soma membrane of amphibian spinal ganglion cells does not require sodium to generate a spike, but can utilize a large variety of cations.[29] Presumably the spike electrogenesis of these spinal ganglion neurons normally employs one of the several alternative ionic batteries that are available in the animal organism.[11,21]

In many invertebrates the neuron soma does not receive synaptic input and there is little or no dendritic surface. The synaptic junctions lie in the neuropil, which is formed of fine branches given off from the axon shortly beyond the site at which the latter arises from the soma. In some cases a distinct region of the cell, or even the whole cell, presents only an input electrogenic membrane that is electrically inexcitable. For example, the dendrite and soma of the eccentric cell of *Limulus*,[30] or the soma of cardiac ganglion cells in lobster[31] and crab,[32] have mainly or only electrically inexcitable membrane. In the crab the neurites of several of these cells are also electrically inexcitable, while those of the other cells generate spikes.[32] By and large, the dendrites of vertebrate neurons appear to lack conductile, electrically excitable membrane,[33-36] but in some regions of the central nervous system or under some conditions, spikes may be generated,[37,38] indicating that some electrically excitable membrane may be dispersed among portions that are electrically inexcitable. Similarly, electrically excitable components may be interspersed among the predominantly receptive regions of sensory neurons, as in the dendrite of the Pacinian corpuscle.[39,40] In axo-axonal synapses the postsynaptic component is close to or within the region of secretory membrane, while in dendro-dendritic synapses the presynaptic dendrite must have secretory as well as conductile components.

The intermingling and functional coexistence of differently excitable components is the general rule for the soma membrane of most neurons and for the membrane of many varieties of muscle fibers and electroplaques. The different permutations of components cannot be predicted from morphological data, but can frequently be detected by functional studies.[6,12,41,42] Pharmacological as well as electrophysiological tools are now available for such studies, and the latter have demonstrated that the different components maintain their individual properties despite close proximity and intermingling.

The output component of neurons is generally fairly well recognized morphologically by the presence of vesicles in its presynaptic terminals. In a few cases the vesicles have been shown to contain substances such as acetylcholine or the catecholamines, which are potent excitants of some postsynaptic membrane components (cf. chapters by Drs. Kopin and Kravitz[43,44]). However, the presence of such agents does not furnish direct proof that chemical transmission takes place. The strongest and most general evidence for the secretory step and for chemical transmission is the large body of evidence that the input of the next cell, whether it is a neuron or an effector, is also electrically inexcitable. That being the case, release of a chemical from the terminals is the only way we can conceive that information transfer takes place. The morphology of various synaptic complexes has been described in the chapters by Dr. Bodian[26] and Dr. Palay.[45] However, to what degree differences in the detailed morphology of the junctions play a functional role is unknown.

From the standpoint of membrane theory it is noteworthy that both excitatory and inhibitory (when the latter are present) postsynaptic input components of muscle fibers and electroplaques appear to have essentially the same properties as do these components in neurons. Furthermore, input membrane appears to have developed its specialized characteristics at an early evolutionary stage.[3,8] These facts are of considerable practical value, as it is often easier to study facets of synaptic transmission in various neuromuscular or electroplaque systems, or in neurons of invertebrates, rather than in neuronal arrays of the much more complex vertebrate central nervous system. Indeed, the pioneering studies using modern methods were first made by Katz and his colleagues on the frog endplate preparation.[46,47] These and the rapidly ensuing subsequent investigations on cat spinal neurons by Eccles and his colleagues,[48,49] of crustacean neuromuscular systems,[50] eel electroplaques,[51] and crayfish stretch receptors,[52-54] formed the basis for a general theory of synaptic electrogenesis,[6,9,42] which is described briefly in this chapter and which supports the major details of Sherrington's view[55] that, over and above the conductile activity of trains of impulses, specialized transmissional processes are responsible for the characteristics of central nervous activity.[56-58]

For example, an outstanding feature is the polarized character of synaptic transmission, as opposed to the capacity for bidirectional or unpolarized propagation of an impulse in an axon. Polarized transmission across the junctional interface is inherent in the functional differ-

entiation (Figure 2) of the secretory output of the presynaptic terminals and the electrically inexcitable chemosensitive input of the postsynaptic membrane. Other functional differentiations and polarizations may occur at each of the interfaces between the various intracellular components of each neuron and at each of the intercellular interfaces of each synaptic stage. In fact, each of the intracellular electrogenic components may itself involve a complex of different processes, and each presynaptic fiber may innervate many postsynaptic cells, each with different properties. Also, a single cell may receive inputs from many prejunctional neurons. The possibilities for *divergence* and *convergence* of intercellular connections give rise to n-body behavior among even a small number of neurons, as is exemplified by the complexity of signaling in the nine-celled cardiac ganglion of lobster (cf. Chapter 17 in Bullock and Horridge[59]). In the huge number of neurons of the mammalian central nervous system, the details of neuronal behavior assume the appearance of stochastic events, although the electrophysiological sampling of individual neurons clearly discloses that rigorous synaptic processes are really at play.

Some elementary characteristics of synaptic excitation are illustrated in Figure 3, which shows the EPSPs that were evoked in two lobster muscle fibers by neural stimulation. In these preparations a single axon innervates the excitatory synapses of all the muscle fibers, and another axon activates all the inhibitory synapses. One of the preparations had been treated with serotonin, an agent that does not affect the sensitivity of the postsynaptic membrane of the muscle fibers but does increase the amplitude of the EPSPs (although not of the IPSPs), presumably by a specific action on the secretory capacity of the terminals of the exciter axon. Two brief shocks were delivered to the axon in rapid succession. The first stimulus evoked an EPSP, obligatorily depolarizing in sign, as noted above. The degree of depolarization was insufficient to elicit electrically excitable electrogenesis in the muscle fiber. The second stimulus elicited a larger (*facilitated*) EPSP, which developed atop and summed with the depolarization remaining from the first EPSP. *Homosynaptic facilitation* is a common feature in synaptic activity,[9] although the degrees of increase in the secretory activity may vary considerably. In some cases, however, facilitation may be absent or the second response may be smaller than the first. In the latter case it is not always clear whether the diminution of the PSP results from a smaller secretory output of the presynaptic terminal or a diminished sensitivity of the postsynaptic membrane (*desensitization*).[60] *Summation* of PSPs is another feature that is common to synaptic electrogenesis and that stems from the characteristics of electrical inexcitability.[6]

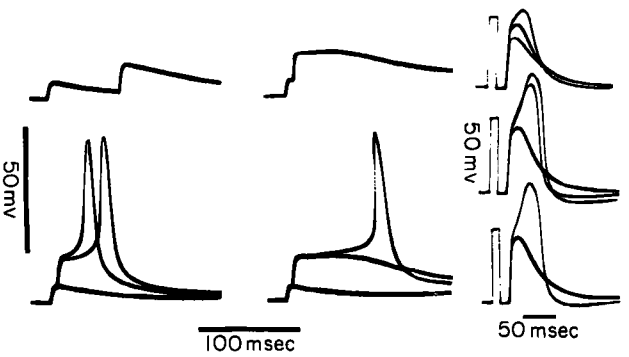

FIGURE 3 Effects of pharmacological agents on different electrogenic components. Two neuromuscular preparations of lobster. Intracellular recordings of responses of a muscle fiber to stimulation of the exciter axon. *Right:* The preparation had been soaked in a medium in which Cs had replaced the K. This procedure causes maximal or nearly maximal release of transmitter from the presynaptic terminals. During repetitive stimulation of the axon the large EPSPs were facilitated and produced large, spike-like graded responses. Note that the occurrence of the latter resulted in marked diminution of the terminal depolarization of the EPSP. *Left:* This preparation had been treated with serotonin, which also causes augmentation of transmitter release, but the EPSP increases only about two-fold (see Fig. 13). The muscle fiber also becomes capable of spike electrogenesis, which does not normally occur in these muscles. Facilitation and summation of two EPSPs elicited spikes. Note, however, that considerable depolarization caused by the EPSPs remained after the spikes had terminated. (From Grundfest and Reuben, Note 61)

The data of Figure 3 show that when the two stimuli to the axon were more closely spaced, the effects of summation became more pronounced and the depolarization was now adequate to elicit a small graded response of the electrically excitable component. Closer approximation of the two neural volleys caused a still greater degree of summation, and the excitatory effect of the synaptic electrogenesis was now clearly evidenced by the spikes it evoked. It is noteworthy, furthermore, that these muscle fibers do not normally produce spikes. The latter responses were caused by the treatment of the preparation with serotonin.[61]

The second preparation of Figure 3 had been exposed to a saline in which cesium chloride had replaced all the potassium chloride that is normally present in *Homarus* saline. The effect of this treatment is to increase markedly the amplitudes of the PSPs (both excitatory and inhibitory) that are evoked by single stimuli to the respective axons.[62,63] This effect often results in single EPSPs or IPSPs that are at, or close to, their theoretical maximal

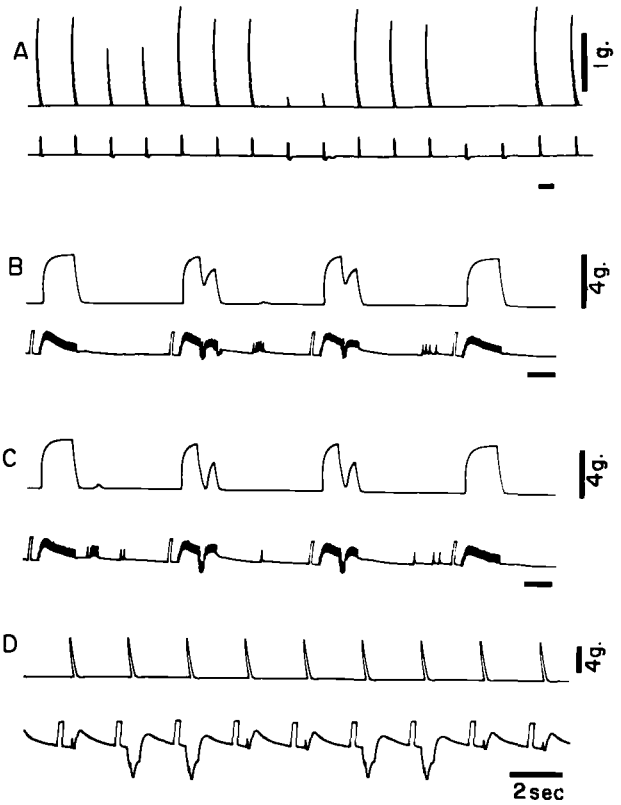

FIGURE 4 Examples of complete inhibition of "slow" mechanical responses in grasshopper preparations (A–C) and absence of effect on "fast" response (D). A: The slow exciter neuron was stimulated briefly at 12/sec., every 7 sec. The mechanical response (upper trace) was diminished to about half by stimulating the inhibitor neuron at 50/sec. Inhibition became more marked when the frequency was raised to 100/sec., and it was complete when the inhibitor neuron was stimulated at 200/sec. The lower trace is an intracellular recording from one of the muscle fibers. The resting potential was −70 mv and the IPSPs were small (10-mv calibrations in B–D, also apply to A). The EPSPs were diminished, however, during the IPSPs. B, C: Another preparation. The slow exciter axon was stimulated at 20/sec. for about 3 sec. in each sequence. During the two middle sequences the inhibitor neuron was stimulated briefly at 25/sec. (B) and 50/sec. (C). The tension evoked by the motor activity (upper trace) diminished during the inhibitory activity and in C it was abolished. The lower traces show intracellular recording from a muscle fiber which had a resting potential of −70 mv. Bursts of "spontaneous" EPSPs, which evoked small mechanical responses, are also seen. D: Another preparation. The upper trace shows the single twitch response of the muscle to stimuli delivered to the fast exciter axon. The lower trace is an intracellular recording from a muscle fiber which responded with EPSPs to both exciter axons and with an IPSP to stimulation of the inhibitor neuron. The hyperpolarizations in two sequences were produced by stimulating the inhibitor axon at 100/sec. The slow mechanical response (not shown) was abolished by the inhibitory stimulation, but as is seen in D the twitch response to the fast axon was not affected. (From Usherwood and Grundfest, Note 64)

amplitudes. The EPSP of lobster muscle fibers is usually about 5 millivolts in amplitude and it becomes about 10 millivolts after applying serotonin. In the experiment of Figure 3, however, the EPSPs after cesium treatment were about 40 millivolts. Facilitation by repetitive stimulation of the nerve increased the amplitude of the depolarization to about 50 millivolts, and this evoked a small local response in the electrically excitable component (upper record). Further repetitive stimulation increased the EPSP still more, so that it was now capable of eliciting large spikelike responses. However, in contrast to the effect of serotonin, cesium did not convert the normally graded responsiveness of the muscle fiber to spike electrogenesis. It is also evident that more than 50 millivolts of synaptic depolarization was required to elicit a maximal graded response, whereas spike electrogenesis in the serotonin-treated preparations was evoked when the synaptic depolarization had attained an amplitude of only about 20 millivolts. Another difference in the characteristics of the interplay of the two types of electrogenesis is also evident. The responses of the electrically excitable membrane of the cesium-treated preparation cut short the depolarization of the synaptic membrane, whereas the spikes elicited in the serotonin-treated preparation did little to "short circuit" the synaptic electrogenesis.

In 1906, Sherrington[55] stressed that inhibition is an activity of the nervous system fully equivalent to that of excitatory processes. The concensus thinking during almost the next 50 years denied this view,[52] but its validity has now been fully established. The interactions of excitatory and inhibitory drives on both the mechanical and the electrical responses of an insect muscle are shown in Figure 4.[64] Increasing the frequency of stimulation of the inhibitory axon caused more effective suppression of both responses (A). The effectiveness of the inhibition was diminished when the frequency of the excitatory synaptic bombardment of the muscle fiber was increased (B) and the respective excitatory and inhibitory effects could be pitted against each other (C). The amplitude of the excitatory synaptic depolarization fell during the course of repetitive activity (B, C), but when an inhibitory input was absent the mechanical response remained at a plateau level.

The decline of the EPSP might have been caused by depletion of the transmitter, impairment of the secretory activity, or desensitization of the postsynaptic membrane. However, a rest of about seven seconds was sufficient to restore the amplitude of the EPSP. Recovery was probably still more rapid, because fairly large brief bursts of "spontaneous" activity were observed within about one second after a train of stimuli, and this electrical activity also elicited small mechanical responses. In the preparation

shown in Figure 4 the metathoracic ganglion, which innervates the muscle, was intact, and such activity (*after discharge*) is a frequent characteristic of complex neuronal organizations.

Neither the mechanical responses nor the electrical activity of muscle fibers that are not innervated by the inhibitory axon were affected (Figure 4, upper trace of D) when stimulation of the latter nerve fiber caused large IPSPs in the muscle fibers that do receive inhibitory innervation (lower trace). It may be noted in passing that the excitatory and inhibitory synaptic components of arthropod muscle fibers are commingled within areas less than one micron apart and that there may be several electrically excitable components interspersed with the synaptic elements. While no morphological distinctions can be observed in the membrane even at the highest available resolutions of electron microscopy, electrophysiological and pharmacological tests can detect the activity of one or another element of the mélange.[12,41,42,65,66]

Living cells are in the have-not class as far as availability of electrochemical means is concerned.[10-12,21] For this reason it is not surprising that the ionic mechanisms of the different electrically excitable and electrically inexcitable types of electrogenesis are essentially similar (Figure 5).

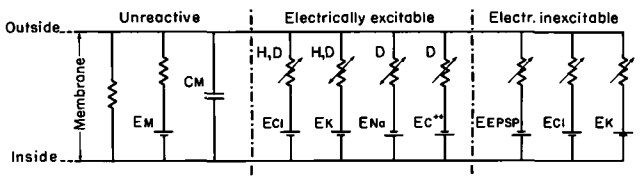

FIGURE 5 The different electrical components of the generalized electrogenic membrane. As a dielectric the membrane has a capacity C_M. Unreactive, nonselectively ion permeable membrane is shown as a simple resistance element. Electrogenically inert, selectively permeable sites are shown as a battery with fixed internal resistance and an emf equal to the resting potential E_M. The electrogenically reactive components are batteries that change their internal resistance in response to appropriate stimuli. The change may be to decreased resistance (*activation processes,* represented by arrows pointing up) or to increased resistance (*inactivation processes,* represented by arrows pointing down). The responses of electrically excitable membrane components may be evoked by depolarizing (D) or hyperpolarizing (H) stimuli. Electrically inexcitable components are activated only by specific stimuli, chemical, mechanical, photic or thermal. Batteries representing channels which are permselective for Na, divalent cations (C++) or for the depolarizing electrogenesis due to generator potentials and EPSPs are shown as inside positive. Batteries for K and Cl are shown as inside negative. Current paths that may be due to electrogenic pumps are omitted.

They involve activation processes that increase the mobility of specific ions across the cell membrane. Depolarization results when the electrochemical gradient for the ion involved is positive to the resting potential and, as already noted, sufficient depolarizing electrogenesis normally is elicited only when the activation process is for sodium or, in some instances, for calcium or magnesium. The change in potential is associated with an increase in the membrane conductance, but the essential difference between electrically excitable and electrically inexcitable electrogenesis is that the former has available to it possibilities for positive feedback in the coupling between the transducer action and the change in membrane potential which it elicits.[7]

The electromotive forces (EMFs) of the electrochemical batteries for potassium and chloride, although generally close to the resting potential, are not necessarily coincident with the latter, nor with each other (Figure 6). The differences, which may be either positive or negative to the resting potential, indicate that these two ions are not distributed in a Donnan ratio, and imply the existence of metabolically driven pumps for ions other than sodium. Activation processes for potassium or chloride also increase the membrane conductance. Thus, they tend to "clamp" or "poise" the membrane potential close to the appropriate electrochemical potential. However, because the latter is in the vicinity of the resting potential, increased conductance for potassium or chloride tends to exert negative feedback on those transducer actions of electrically excitable membranes which are useful for conductile activity. This negative feedback gives rise to the pulse shaping that is characteristic of spike electrogenesis in many cells,[12,16] but which may be absent from other cells.[11,21,67]

Inhibitory synapses also operate through an increased conductance for potassium and/or chloride, the mode of chloride activation apparently being far the more common. The inhibitory synaptic action, however, is not exerted through the change in membrane potential, but by the clamping action of the repolarizing electrogenesis. Thus, it is immaterial whether the IPSP is depolarizing or hyperpolarizing (Figure 7). In either case the clamping effect of the conductance increase diminished the magnitude of an accompanying EPSP. The effectiveness of the inhibitory action was mainly confined to the early period of the IPSP. This indicates that the period of conductance increase and electrogenic activity is far briefer than the change in membrane potential, and it leads to the inference that the duration of transmitter action must have been brief. Presumably the transmitter was released from the terminals of the inhibitory axon in a relatively brief span of time and was either destroyed or diffused away there-

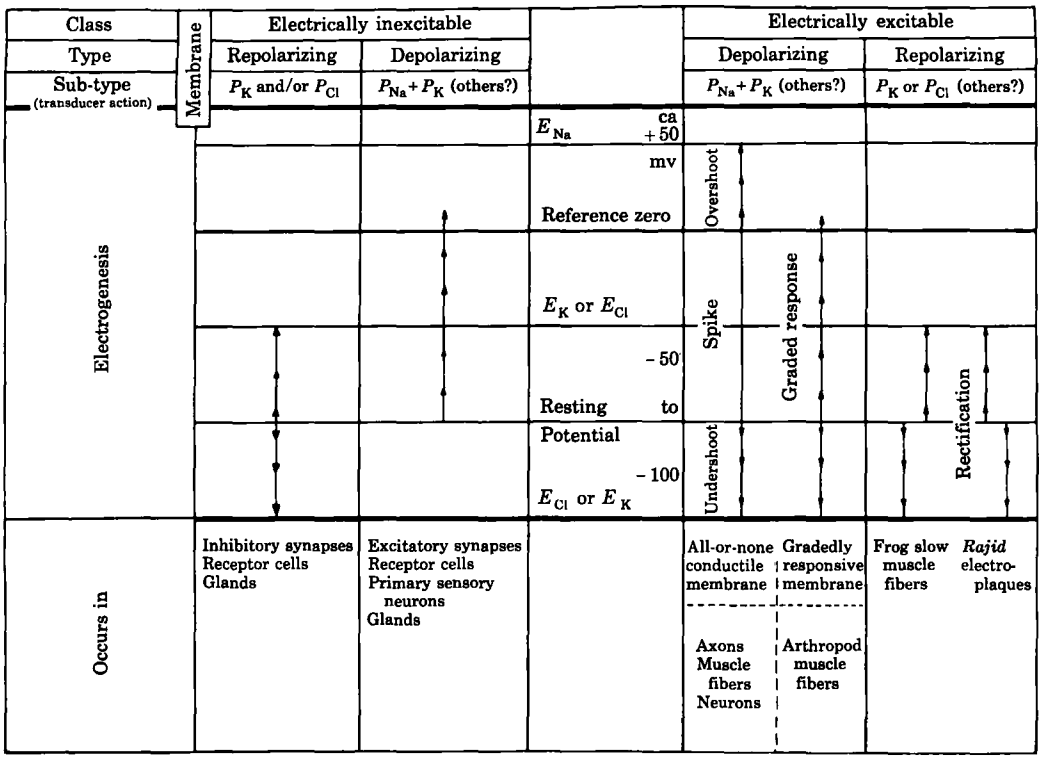

FIGURE 6 The ionic components of some varieties of electrogenesis resulting from activation processes for different ionic batteries. The resting potential is represented as between E_K and E_{Cl}, the electrode potentials for K and Cl, respectively. E_{Na}, the electrode potential for Na, is indicated as about 50 mv positive to the reference zero. The two classes of differently excitable membranes are each subdivided into electrogenic types and into subtypes characterized by the nature of their transducer actions which lead to increased permeability (P_K, P_{Cl}, P_{Na}) for different ion species. The types of cells or cell components in which the different activities occur are also shown. The membranes could be further subdivided by various pharmacological reactions. (From Grundfest, Note 12)

after. However, whether the change in membrane potential was negative or positive, it dissipated more slowly through the resistive–capacitative network of the electrical cable properties of the cell membrane.

Although the inhibitory electrogenesis is most effective upon the depolarizing electrogenesis of excitatory synaptic membrane, it may also exert effects directly on the electrically excitable component. If the inhibitory electrogenesis causes a hyperpolarization, this change in the membrane potential contributes to depressing the excitability of the electrically excitable membrane. Thus, an intense inhibitory synaptic bombardment of cortical neurons diminishes the excitability of the latter to electrical stimulation.[68] On the other hand, if the inhibitory membrane can be made to generate large depolarizations, the IPSPs become excitatory for the electrically excitable spike-generating component. The latter then responds with activation under the stimulus of adequate depolarization (Figure 8).

The patterns of chemical affinity of the postsynaptic membrane components provide some insight into the membrane's structure. A given agent may activate depolarizing electrogenesis in one cell and the repolarizing variety in another (Figure 9). Thus, at least two components of membrane microstructure are required. The ion-specific channels may be pictured as holes or as special "plugs" in a protein-covered lipid bilayer surface (Figure 10). The surround of this micromolecular electrode region is chemically specific and presumably causes "conformational" changes that open or close the ion-specific channels. The pharmacological specificity thus is to be ascribed to the boundary or "receptor region" of the complex, whereas the nature of the electrogenesis is ascribed to the ion-specific permeability of the channel. The number of such

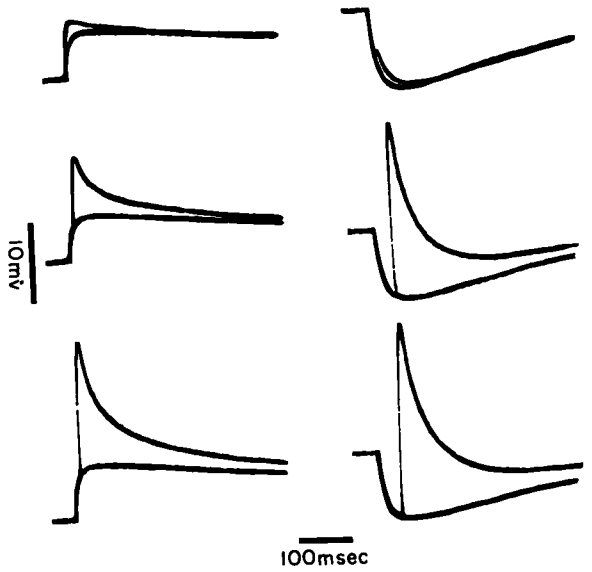

FIGURE 7 Inhibitory action of depolarizing and hyperpolarizing IPSPs of lobster neuromuscular synapses. The preparation had been soaked in Cs-Ringer's solution to augment both EPSPs and IPSPs. *Left:* Initially the resting potential was about −85 mv and the IPSPs were depolarizations of about 5 mv. Two sweeps are superimposed in each of the records, one showing the IPSP alone, the other with an EPSP elicited at progressively slightly later intervals in the three records. Note how rapidly the inhibitory effects of the IPSP on the EPSP declined. *Right:* An EPSP that was evoked without an accompanying IPSP produced a violent contraction, and the resting potential decreased to about −70 mv as a result of the damage. The IPSP now became hyperpolarizing by about 10 mv. It still diminished a concurrent EPSP and, as before, the inhibition was most marked when the two activities were nearly simultaneous. (From Grundfest, Note 42, after Reuben and Grundfest, unpublished data, 1960)

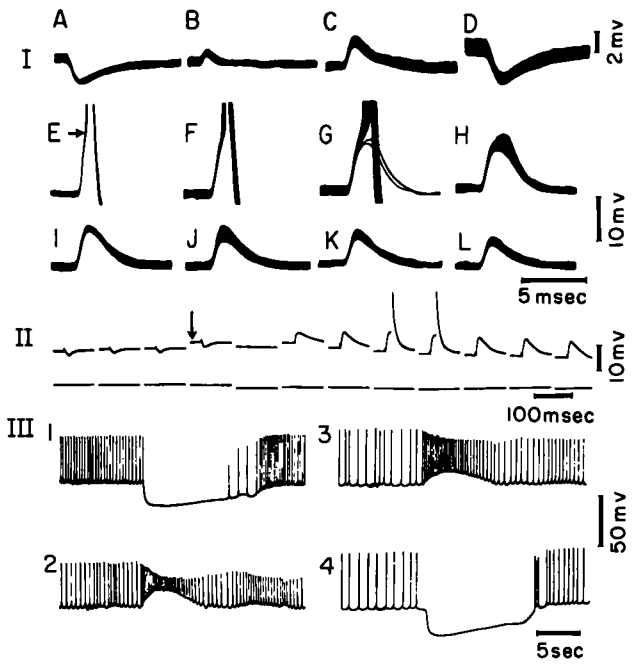

FIGURE 8 Excitatory effects of normally "inhibitory" membrane resulting from change in electrochemical conditions for chloride and consequent inversion of the electrogenesis from hyperpolarizing inhibitory action to depolarizing excitatory action.

I. Cat motoneuron. A: IPSP was hyperpolarizing before iontophoretic injection of chloride into the cell. B and C: Depolarization after the injection. D: The potential was again inverted to hyperpolarization during depolarization of the cell with an applied current. E to L: Another sequence of records after a large injection of chloride into the neuron. The resting potential changed relatively little, so that the large depolarizing IPSPs exceeded the critical firing level of the electrically excitable membrane components and elicited spikes (E to G). As the chloride leaked out of the cell, the depolarizing electrogenesis of the IPSPs became progressively smaller. (Modified from Eccles, Note 49)

II. Crayfish stretch receptor. The first three records show hyperpolarizing IPSPs. At the arrow, the normal saline medium was exchanged for a chloride-free (glutamate) saline. At the third record, the IPSPs became depolarizing and subsequently grew larger until they could evoke a spike of the receptor neuron. (Modified from Hagiwara, et al., Note 68a)

III. Neuron in abdominal ganglion of *Helix*. (1) The repetitive firing of the cell was stopped by a large hyperpolarization induced by application of acetylcholine. (2 and 3) The chloride-containing saline was replaced with a chloride-free medium. The "inhibitory" membrane now responded with depolarization to an application of acetylcholine, and the frequency of discharge was increased to such a degree that the amplitudes of the spikes diminished. (4) The electrogenesis again became hyperpolarizing and inhibitory after the chloride-saline was introduced. (Modified from Kerkut and Thomas, Note 68b)

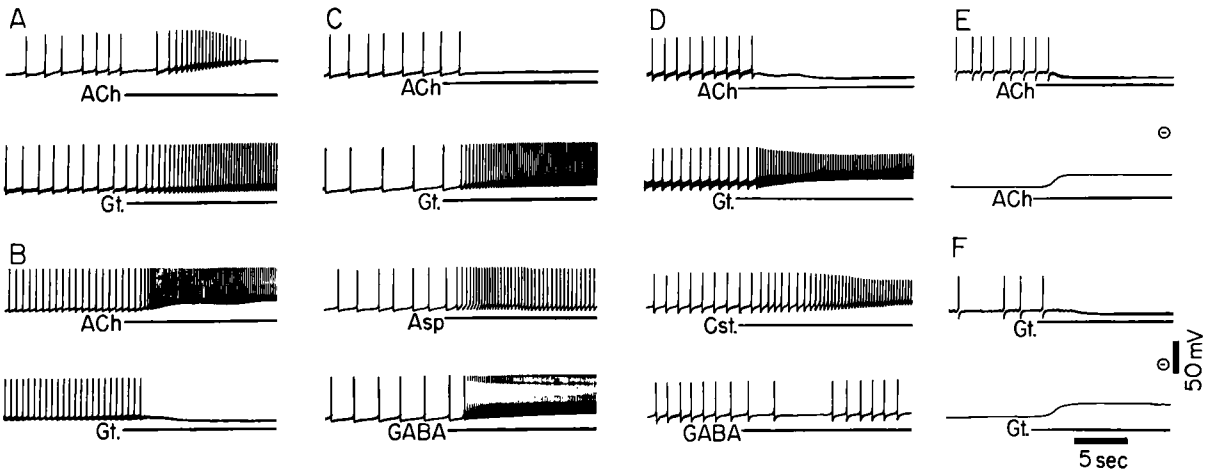

FIGURE 9 Some patterns of pharmacological reactivity in five neurons of *Cryptomphallus aspersus*. Intracellular recordings show spontaneous spike discharges. Bars indicate iontophoretic applications of drugs. *A:* Both acetylcholine and glutamate induced depolarization and accelerated activity. *B:* Only acetylcholine depolarized, while glutamate caused hyperpolarization and stoppage of spike discharges. *C:* Acetylcholine was the "inhibitory" agent in this cell, while various amino acids (glutamate, aspartate and GABA) were "excitatory." *D:* GABA as well as acetylcholine was inhibitory for this neuron, but glutamate and cysteine were excitatory. *E:* Both acetylcholine and glutamate caused hyperpolarization and stoppage of spike activity. The second and fourth records show the membrane potentials during hyperpolarization. The spontaneous activity ceased, and when the drugs were applied they evoked a response that was depolarizing from the new resting potential, demonstrating that the inhibition was due to an electrically inexcitable membrane component. (Modified from Gerschenfeld and Lasansky, Note 68c)

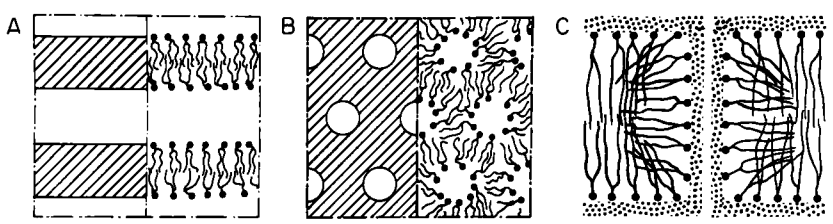

FIGURE 10 Hypothetical structure of cell membrane, based on X-ray data from lipid liquid crystal films. *A:* A double layer of the molecules is the stable lamellar form, with polar heads (black dots) facing outward into the aqueous medium and the nonpolar tails forming an inner zone. *B:* Surface view of hexagonal arrays that form aqueous channels (open circles) through the lamellar layer. *C:* Hypothetical channel through the cell membrane; the stippling indicates a proteinaceous complex that supposedly coats the walls of the channel formed by a hexagonal array as well as the lamellar surface. (From Grundfest, Note 11)

channels is quite small in the resting state. The resistivity of the membrane is about 10^9 ohm cm or about 10^7 times higher than that of saline of equal ionic strength.[10] Presumably there are only 10^{-7} as many mean free paths for ions through the cell membrane as there are in a layer of water of similar dimensions. Channels that are presumably of the same electrogenic variety also may differ in their microstructure. For example, some varieties of electrically inexcitable membranes that produce depolarizing electrogenesis do not accept lithium in place of sodium, whereas Li can replace Na in spike electrogenesis.[68d]

While the subject of transmitter agents is beyond the scope of this review, it is appropriate to note that axodendritic and axosomatic synapses of the mammalian brain

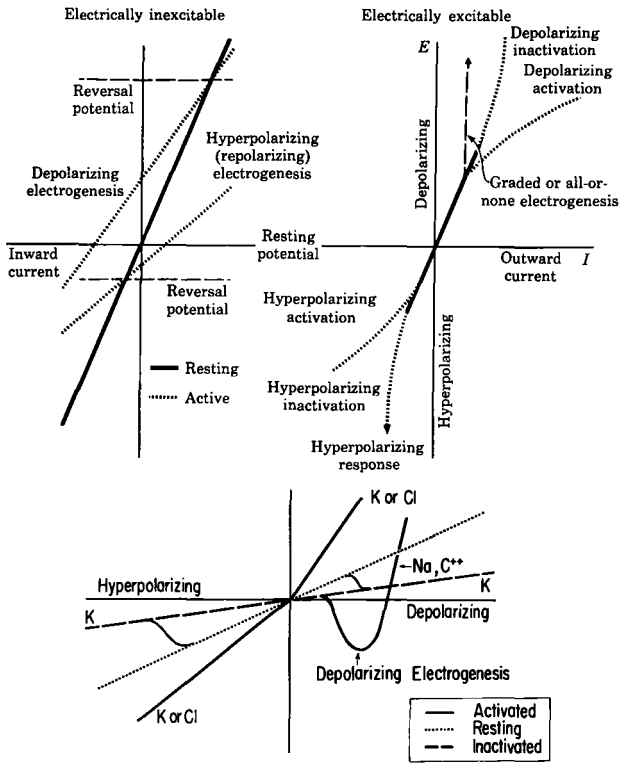

FIGURE 11 The I-E relations of electrogenic membranes. The origin of each graph is the resting potential. *Electrically inexcitable* membranes behave as ohmic resistances, E changing linearly with I. However, the slope of the relation changes during activation of the membrane by an appropriate stimulus. The broken lines represent active membrane of (depolarizing) EPSPs and of (hyperpolarizing) IPSPs respectively. The recorded amplitudes of the responses (given by the difference between the resting I–E line and that during activity) change with the change in membrane potential. Thus, a characteristic feature of electrically inexcitable electrogenesis is its change in sign when E exceeds the reversal potential specified by the intersection of the resistance lines for active and passive membrane.

Electrically excitable membranes exhibit nonlinear behavior, which is characterized by one or by several varieties of conductance changes. Most, but not all, develop graded or all-or-none depolarizing electrogenesis, which is accompanied by increased membrane conductance and is more or less regenerative. Decreased conductance (inactivation) may be evoked by depolarizing or hyperpolarizing currents and can also be regenerative. Increased conductance for K or Cl evoked by depolarizing stimuli, or for any ions by hyperpolarizing currents (inappropriately termed "rectification" processes) are usually not regenerative, but can become so when electrochemical conditions permit it.

A voltage clamp presentation (E on the abscissa, I on the ordinate) of the electrically excitable components, further identified as to ionic species, is shown in the lower diagram. Increased conductance for Na or C++ causes a shift of the characteristic toward inside-positivity. The link between the resting and activated states forms a negative slope characteristic, because the voltage and current change in opposite directions. The negative slope region is a "forbidden zone" and unless the membrane potential is clamped it undergoes a regenerative change to an all-or-none spike. Transitions from the resting state to the inactivated also go through a negative slope region. The change gives rise to regenerative depolarizing and hyperpolarizing inactivation responses. The various ionic processes which cause the nonlinear relations exhibit different degrees of time variance. Effects of pharmacological inactivation are not shown in the diagrams. (Modified from Grundfest, Note 71a)

exhibit different pharmacological properties.[69-71] It is also probable that in other cells different presynaptic pathways may have different pharmacological properties, even though they induce PSPs of the same electrogenic variety.

Only a general statement of evidence regarding the electrical inexcitability of synaptic membranes need be made at this time. One aspect of electrical inexcitability is the linear, or ohmic, relation between membrane potential and applied current (Figure 11). Electrically excitable membrane, however, exhibits a considerable variety of nonlinear relations, as the applied current may cause an increase or a decrease in the membrane conductance and for one or several ion species. The current-voltage (I-E) relation of electrically inexcitable membrane exhibits linearity over a range of ± 100 millivolts in some cells. The resistance does change when the membrane is excited by a specific stimulus and the change in the slope of the I-E line results in a crossing of the resting and active lines. Thus, it may be expected that the sign of the electrogenesis will be reversed by a current that sets the membrane potential of the resting membrane beyond the cross-over level (Figure 12). In the case of simple electrical and electrophysiological conditions, the reversal potential indicates the electrochemical potential of the synaptic battery, and the measurement can identify the nature of the ion or ions involved.[72]

Fatt and Katz[46] noted that when a directly evoked spike of a frog muscle entered the end plate region it did not evoke an end plate potential. If the synaptic membrane of the end plate does not respond to this electrical stimulus, the passage of the spike should be correlated with a passive electrical behavior of the membrane. The depolarization (and inside-positivity) caused by the spike electrogenesis should therefore cause an outflow of current

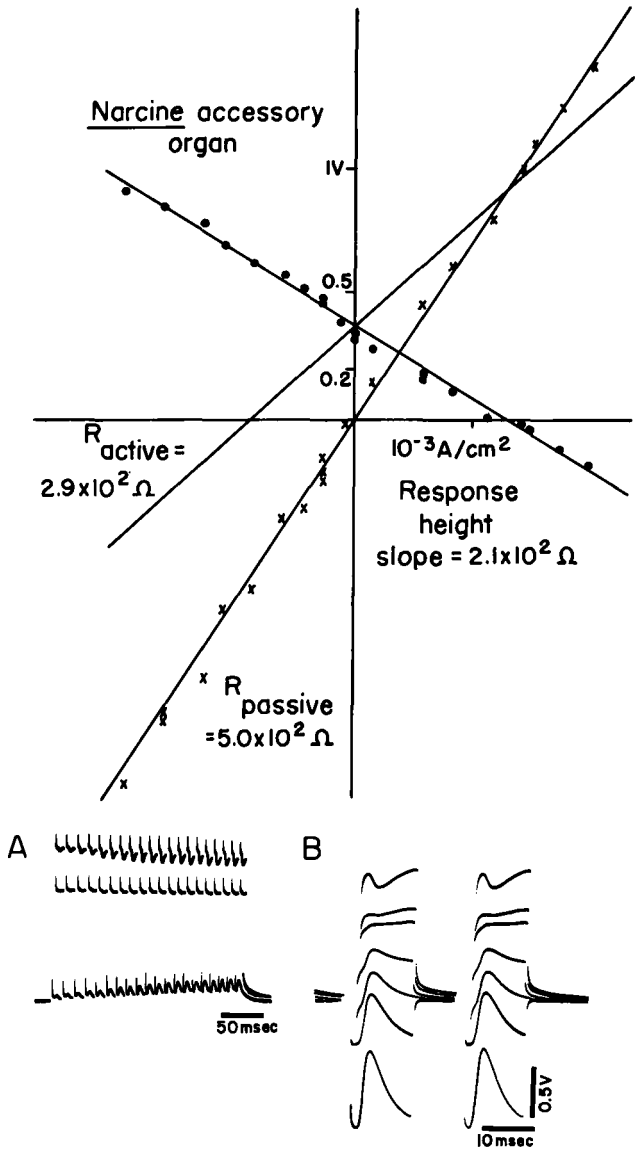

FIGURE 12 Effects of polarization on facilitated responses of the accessory electric organ of *Narcine brasiliensis*. (Below) A: Three records of responses to a train of stimuli at about 100/sec. The lowest record, taken before polarization of the electroplaques, shows the marked facilitation that is characteristic of this organ. A current was then applied outward through the innervated faces of the electroplaques. This diminished the amplitude of the responses, and the middle shows a stage at which the responses disappeared and left only the stimulus artifacts visible. When a stronger current was applied, the responses reappeared in reversed sign, a characteristic feature of electrically inexcitable activity. Facilitation was still present. B: Another experiment, recording at higher sweep speed. The last two responses of a train are seen, decreased in amplitude and inverted in sign by the depolarizing currents and augmented by the oppositely directed currents. Note that the latencies of the responses were not affected by large changes in potential. *Graph, top:* Same experiment as in B. Voltage-current relation of electroplaques before responses (crosses), and the amplitude of the maximally facilitated responses (circles) at different currents. Outward current through the innervated surfaces to the right of the origin. The difference between the two sets of values is the line marked "R_{active}." Note the diminution of membrane resistance during activity and absence of rectification during applied currents. (From Bennett and Grundfest, Note 86)

through the passive membrane, rather than the inward current that is associated with spike-generating activity. Highly circumscribed regions in which only such a passive efflux takes place were demonstrated in the frog endplate.[73] They are restricted to the sites at which "miniature" postsynaptic potentials are also observed and so are probably the sites of electrically inexcitable but synaptically reactive membrane. Werman's finding[73] has been questioned by Katz and Miledi.[74] However, Figure 11*h* of their paper also shows that the synaptic membrane does not generate an inward current when a spike traverses the endplate region.

The electrophysiology and pharmacology of the synaptic secretory activity are still imperfectly known. Some information is gained from deduction regarding the randomly occurring miniature postsynaptic potentials. These potentials appear to be caused by spontaneous release of small quantized amounts of transmitter agent, presumably from individual presynaptic vesicles.[75] In the crustacean neuromuscular preparations the release process occurs in both excitatory and inhibitory axons.[61] The randomness of the miniature PSPs with respect to two muscle fibers that are innervated by the same axon (Figure 13) and even with respect to two loci in the same fiber[65] indicates that the release of transmitter is highly localized. Thus, it must be independent of the spike mechanism, which would generalize the activity to the whole axon. Direct evidence that the secretory release is independent of spike electro-

genesis is also indicated by the persistence of the miniature PSP's after sodium activation in the axons had been blocked by tetrodotoxin or saxitoxin.[42,65,76] Furthermore, the persistence of the miniature PSP's also shows that the postsynaptic membrane is insensitive to these poisons.[65,77]

Recent data confirm the persistence of transmissional activity in nerve terminals after spike electrogenesis has been blocked by tetrodotoxin. Brief electrical stimuli applied to the axon in the immediate vicinity of the finer nerve terminals elicit postsynaptic potentials in muscle fibers of frogs[78] and crustaceans.[65] In the squid axon synaptic complex an EPSP can be evoked in the postjunctional giant fiber by applying a depolarizing current intracellularly to the terminals of the presynaptic giant axon (K. Kusano and S. Nakajima, unpublished, 1962; cf also Notes 78a, 78b, 78c).

The membrane correlates of the secretory activity are still obscure. It is generally assumed that the depolarization that is evoked by the spike is the direct trigger for excitation–secretion coupling.[22,79,80] As depolarizing potassium activation is unaffected when tetrodotoxin eliminates sodium activation and spike electrogenesis, it seems likely that the secretory activity of the nerve terminals may perhaps be correlated rather with the membrane changes that cause potassium activation.[65] This view would regard an increase in conductance, rather than the membrane depolarization, as the trigger or accompaniment of excitation–secretion coupling. An analogous difference of views exists with regard to excitation–contraction (e-c) coupling in muscle. The widely held view is that the coupling is effected by depolarization.[81] However, considerable evidence now available shows that e-c coupling is effected by a flow of current through the tubular system which invaginates into the muscle fibers.[21,82,83]

Direct evidence regarding the possible mechanism of excitation–secretion coupling is still sparse. What there is, however, tends to support the hypothesis of Ozeki et al.[65] When tetraethylammonium (TEA) ion is injected into the presynaptic axon of the squid synaptic complex in the region of the terminals, the *synaptic delay*, which is characteristic of synaptic transmission,[6] is increased.[78c] The onset of depolarizing potassium-activation is delayed by small amounts of TEA,[84] and the increased synaptic delay is thus consonant with the postulated role of the conductance change in excitation–secretion coupling. When a larger amount of TEA is injected the EPSP is blocked, as would be expected if potassium activation were eliminated.

The amount of transmitter that can be released by a single neural impulse appears to vary in different tissues. In the motor axon to frog skeletal muscle, a single impulse may release more than enough transmitter to excite a spike. The depolarizing PSPs of *Torpedo* and *Astroscopus* electroplaques are nearly maximal responses, and this is also the case for the electroplaques of the main organ of *Narcine brasiliensis*.[85] None of these systems show much, if any, facilitation. *Narcine*, however, also possesses an accessory electric organ,[86] which responds quite differently (Figure 12). A single impulse in the nerve elicits only a small PSP

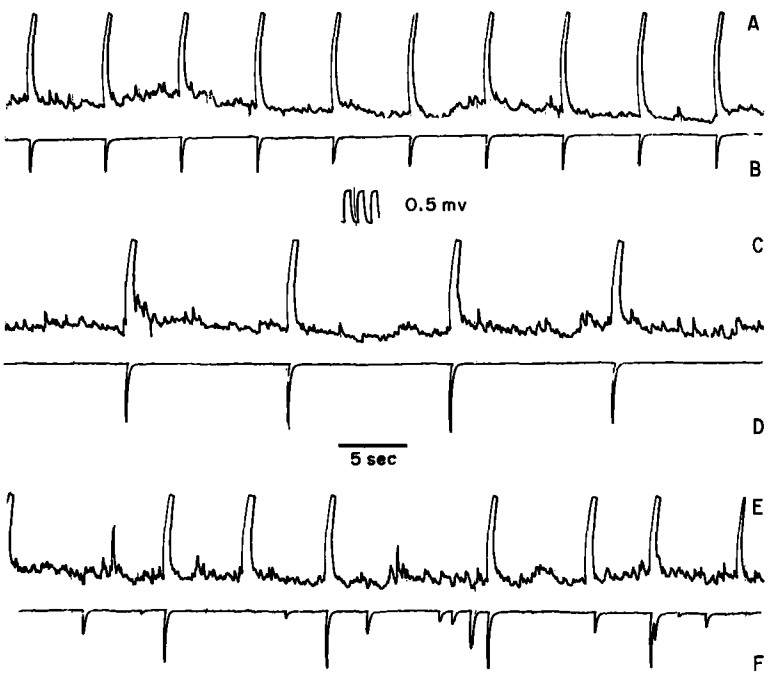

FIGURE 13 Independent spontaneous activity in two muscle fibers of a lobster neuromuscular preparation. Ink-writer records, upper trace in each set, at gain indicated in calibration; the lower trace recording from a second muscle fiber at 0.1 times this amplification. The EPSPs evoked by stimulating the axon appear simultaneously in both traces, with the potential inverted in the lower trace for convenience. A and B: before, *C–F*, after applying serotonin. The augmentation of the EPSPs is seen in the low gain traces (D, F). Spontaneous miniature potentials increased somewhat in *C*, but became very marked in E and F. Large potentials appeared in each muscle fiber without corresponding activity in the other. (From Grundfest and Reuben, Note 61)

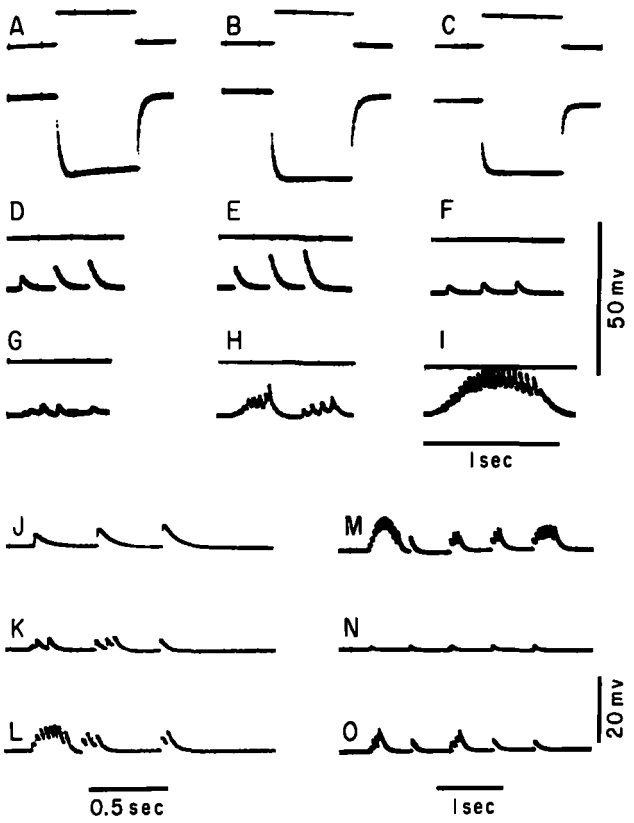

FIGURE 14 Pharmacological data on presynaptic terminals of lobster neuromuscular system.

A–C: Intracellularly recorded hyperpolarization (lower traces) induced in muscle fiber by intracellularly applied inward currents (constant strength as monitored on upper traces). A, in control saline medium. B, in medium containing picrotoxin (10^{-3} w/v). C, in medium containing 10^{-3} phenylethylamine (PEA). The drugs had little or no effect on membrane resistance.

D–F: Intracellular recordings of EPSPs of the fiber under the same conditions. The response increased somewhat in picrotoxin (E), perhaps because of removal of inhibitory synaptic activity; PEA markedly depressed the EPSPs. Note that facilitation was not affected by the drugs.

G–I: At different times after picrotoxin and PEA had been applied together. Repetitive activity was evoked, and broke up the pattern of the responses to the three orthodromic stimuli.

J–O: Another preparation. J shows EPSPs evoked under control conditions when three stimuli were applied to the exciter axon. K and L, repetitive activity early and later, after applying picrotoxin and PEA to the preparation. M, a later sequence (record at a slower sweep speed) in response to five stimuli to the axon. N, GABA (10^{-3} w/v) abolished the repetitive discharge. The responses became small because the inhibitory synapses were also activated at the same time by the GABA. O, reversal of the effect by adding more picrotoxin. (Modified from Grundfest and Reuben, Note 61)

but the responses are greatly facilitated during repetitive stimulation. The individual PSPs of the train may become nearly maximal.[86] Because both the main and accessory organ receive innervation from the vagus nerve,[87] the difference in secretory output must reside in some difference of the nerve terminals.[87a]

The secretory output may be varied by external agents (Figure 3). It is noteworthy that serotonin only augments the secretory activity of the terminals of the excitatory motor axon of crustaceans, while cesium increases the output of the inhibitory terminals as well. Phenylethylamine (PEA) is another agent that affects both excitatory and inhibitory axons, particularly in combination with picrotoxin or serotonin. When applied to either axon directly, PEA blocks spike electrogenesis and propagation. When applied to the terminals of the neuromuscular preparation it markedly diminishes the PSPs (Figure 14). However, after some time of PEA action, single stimuli applied to the axon cause an avalanche of activity in the muscle fiber. This is the result of repetitive activity that is initiated at the terminal portion of the axon and can be propagated back antidromically as a train of spikes; it also causes a burst of secretory activity at the terminals. Presumably, the train of spikes is initiated by a prolonged depolarization of the terminal portion of the axon following the invasion of a single *orthodromic* impulse. It is unknown, however, whether the prolonged depolarization arises in the secretory membrane of the nerve terminals or whether there is some intermediate region of the axon that reacts to PEA with a specific variety of electrogenesis. The effects of PEA can be counteracted by γ-aminobutyric acid (GABA), and the action of the latter drug can be eliminated again by picrotoxin. These two agents, which act very powerfully on the synaptic membrane of the muscle fibers, also can exert effects on the membrane of the presynaptic terminals. Both GABA and picrotoxin also affect the nonsynaptic membrane of the muscle fibers.[66,88]

A mechanism for control of the secretory activity appears to be present in some cases in the form of axo-axonal synapses on the presynaptic terminals. In general, this control appears to diminish the secretory output and is therefore termed *presynaptic inhibition.*[89] The mechanism of the effect is not yet clear. Excitatory controls of a similar type conceivably might also occur, and have been invoked to account for a *heterosynaptic facilitation,* which has been observed in molluscan neurons.[90]

A complex mechanism for controling the effectiveness of synaptic transmission is indicated by data on crab neuromuscular junctions (Figure 15). Many, but not all, neuromuscular preparations of the walking legs of crab respond to GABA quite differently from those of crayfish,[91] lobster,[92] or insect.[64] In all these latter forms, GABA activates

the inhibitory synaptic membrane and this may be shown by the decreased resistance of the muscle fiber. The activation by GABA and the inhibition of EPSPs are eliminated with picrotoxin, while the resistance returns to its original, higher value. In the crab preparation of Figure 15 the resistance was not affected by addition of GABA, but the excitatory PSP was markedly decreased.[42,93-95] The decrease was counteracted by adding picrotoxin, again without affecting the resistance of the muscle fiber.

Thus, it seems necessary to postulate that GABA has one or several of the following actions. (1) It lowers sensitivity of the membrane of the excitatory synapses to their normal transmitter; i.e., it behaves as does d-tubocurarine or other *synapse inactivator* drugs. (2) It blocks output of transmitter from the presynaptic terminals of the exciter axon. (3) It neutralizes the excitatory transmitter once the latter is released.

Neural activation of the inhibitory synapses of the crab muscle fibers causes only a small increase in conductance; this appears to be inadequate to account for the degree of their inhibitory effect on the EPSPs of the fibers.[94] Presumably, the additional inhibitory effects exerted by the inhibitory axon are similar to the effects exerted by GABA. Presynaptic effects of the inhibitory transmitter have also been observed in crayfish neuromuscular preparations.[96] These presynaptic actions are perhaps explicable[97] in the light of the effects of GABA shown in Figure 14 and of the general increase in chloride permeability which GABA can exert in crustacean muscles.[11,88]

Other complexities in the dynamics of nervous function arise from still unexplained "trophic" interactions between neurons or between a neuron and its effector terminus. Transneuronal degeneration, which often occurs when the innervation of a pool of neurons is removed, is an example of the neuron–neuron interaction. Examples of neuron–effector interaction are supplied by the profound changes that take place in the membrane properties of muscle fibers when they are denervated.[98-101] In mature, innervated, vertebrate skeletal muscle fibers, the sensitivity to acetylcholine is mainly confined to the endplate region. However, in fetal animals, or after the muscle fiber has been denervated, the membrane of the whole surface of the fiber develops chemosensitivity. The electrophysiological data show that this newly formed component is electrically inexcitable.[41,42] Thus, denervation either has converted patches of formerly electrically excitable membrane into the electrically inexcitable variety, or patches of membrane that previously had been *electrogenically inert* are now converted to the chemosensitive electrogenic type. The widely disseminated chemosensitivity shrinks again when the denervated muscle fiber is reinnervated, indicating a dynamic relation of the neuron–effector interaction. The axon and its connective tissue also exhibit an obligatory symbiosis. When an axon is interrupted and its distal part degenerates the myelin sheath of this portion is also destroyed. Conversely, when the myelin is affected by demyelinating processes the axons also lose their functional capacity. Even changes in ionic conditions may profoundly alter electrophysiological and pharmacological properties of the cell membrane.[101a]

Inasmuch as synaptic transmission involves many interfaces of components with different properties, the transmissional process inherently possesses many possibilities of

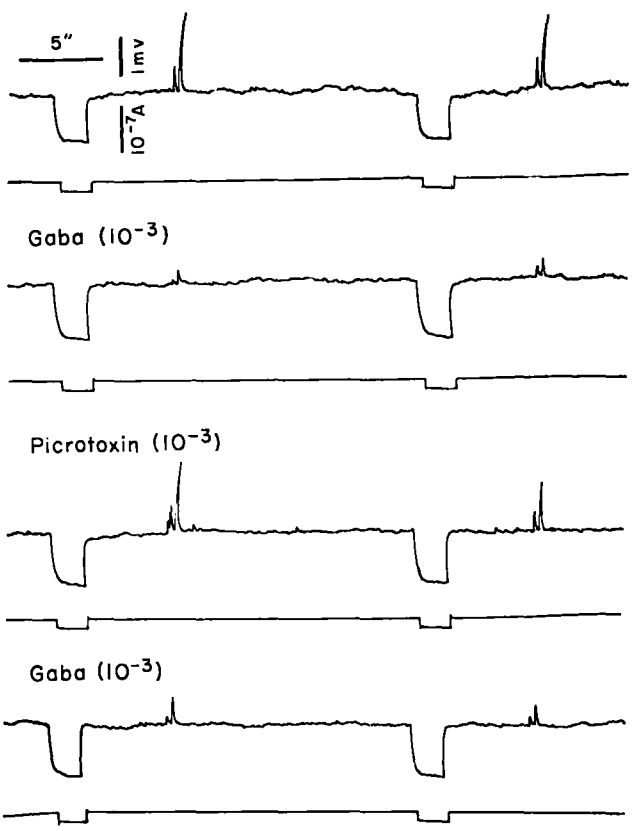

FIGURE 15 Effects of GABA and picrotoxin on crab neuromuscular synapses. Intracellular recordings, with ink-writer registration. An inward current, monitored on the lower of each pair of traces, was applied at the beginning and in the middle of the records. The amplitudes of the hyperpolarizations changed little, if at all, following application of GABA or picrotoxin, yet the EPSPs evoked by stimulating the exciter axons (upward deflections on voltage traces about 5 sec. after the end of the hyperpolarizations) diminished markedly with GABA and were restored by picrotoxin. Thus, GABA and picrotoxin were mutually antagonized. See further discussion in text. (From Reuben and Grundfest, unpublished data, 1961)

dynamic variation. This is particularly true for the intricately interconnected, interlocking sequences of synaptic relays that characterize the central nervous organizations of higher animal forms. Thus, functional as well as anatomical complexities may well grow factorially with the number and type of synaptic inputs to each neuron and again with the number and variety of interconnections.

Ephaptic junctions

The process of ephaptic, or electrotonic, transmission is of a rather different order. As already noted, the cellular partners of a synaptic junction are remarkably well insulated electrically one from another. However, the local circuit of the current that is generated during an impulse must have an extracellular component. Thus, the cell membrane and the sheaths surrounding a neuron cannot be perfect insulators. If some portion of this current enters an adjacent neuron it may affect the threshold of its electrically excitable membrane component. Such *field effects* have been observed by a number of workers[102-105] as an interaction caused by the activity of one axon on an adjacent inactive fiber (Figure 16). At a given point the effect on the recipient fiber is triphasic, because the apposed region of the donor fiber first acts as a source of current to the approaching active region, causing an inward current and hyperpolarization in the recipient axon. When the impulse arrives in the active fiber at the level of the site where the excitability of the inactive fiber is being tested, the current flowing through the latter becomes outward and depolarizing. As the impulse moves away, the recipient axon is again hyperpolarized. Thus, the electrical excitability at the tested region of the recipient fiber develops a sequence of depression, augmentation, and depression.

A much more intense flow of current may be expected if the relatively poorly insulated cells in a pool of neurons were to become active,[106,107] and considerable field effects, both excitatory and depressant, have been observed in the spinal cord.[108,109] However, the effects would be likely to be random, depending upon the orientation of the field current relative to individual neurons, and shifting as activity waxed and waned in the different cells of the neuron pool.[5] This randomness is quite unlike the precisely deterministic but richly textured effects of the structured synaptic interactions.

Massive electrical fields produced by contracting muscles can excite axons that supply the muscles,[110] but it is doubtful that this effect is of functional significance. A purely inhibitory effect, also of questionable significance, is exerted on the axon hillock of the Mauthner cell by the impulse coursing toward the spiral endings of the axons surrounding the hillock.[111] In general, spikes do not appear to invade presynaptic terminals.[22,79,85,112] Thus, the terminals would act as a source of inward current for the postjunctional cell, causing hyperpolarization and depression of excitability. While similar inhibitory effects might also be expected in other synaptic systems, they require an appropriate geometry of the junction to form a sufficiently strong field current.

The effectiveness of interactions by an electric field may be enhanced by experimental procedures that increase the

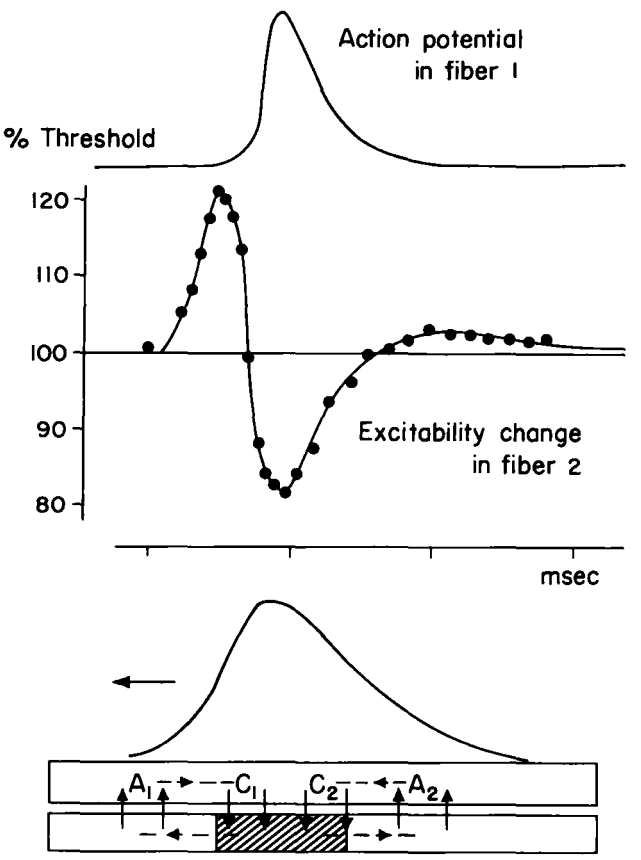

FIGURE 16 Changes in electrical threshold of one axon (fiber 2) caused by the action current that is associated with an impulse propagated in an adjacent axon (fiber 1). The diagram at bottom shows the mechanism of the change in excitability. The impulse is drawn as propagating from right to left. The active region of fiber 1, marked by diagonal lines, is the site of inward current. The current is outward ahead (A_1) and behind (A_2) the active region. These (anodal) current hyperpolarize fiber 2 upon entering it and raise its threshold. An asymmetry of the two anodal currents supposedly causes the difference in their effects on fiber 2. (Modified from Katz and Schmitt, Note 104 and Eccles, Note 105a)

excitability of the recipient cell or decrease the resistive pathway of the field current. This was first demonstrated in 1882 by Hering[113] and has since been confirmed by a number of workers.[105,114,115] Arvanitaki[115] coined the term "ephapse" for the "artificial junction" so formed. More recently this term and its equivalent "electrotonic junction" have been used to designate naturally occurring regions of relatively low resistance between two neighboring cells.[9,23,24] In the Mauthner cell system the electrical inhibitory effect is made particularly evident by the electrophysiological conditions. The inward current at the electrically excitable axon hillock region must be accompanied by an outward current in the cell body. However, this current flow does not cause a counterbalancing increase in excitability, as the soma of the goldfish Mauthner cell appears to be electrically inexcitable.

Ephaptic junctions carry electrical interactions to a higher level. They are based upon good electrical contact between cells, so the two cells behave as if they shared a common intracellular current pathway. In some cases, as, for example, the septate giant axons of earthworm[116] and crayfish,[23,117] the resistive paths between adjacent cells may be very low (Figure 17). This can be demonstrated and measured by the degree of electrical coupling when a current applied to one cell causes a considerable change in potential in the other cell. The degree of the electrical interaction may vary from the very tight coupling in the septate axons to looser coupling where the coupling resistance is relatively high.[23,24] In tightly coupled ephaptic junctions, a spike evoked in the prejunctional cell will excite a spike in the postjunctional unit. In the more loosely coupled systems, however, the postjunctional effects may be much weaker, depending upon the various degrees of attenuation of the electrical signal across the various magnitudes of electrical coupling.

A morphological correlate of the electrotonic coupling resistance has been elucidated recently, largely through the work of Bennett and his colleagues.[24,118-123] In a number of cases it has been shown that electrotonic transmission is associated (Figure 18) with the fusion of the cell membrane of the coupled cells. These fusion sites[124,125] are found commonly in epithelial tissues and have been termed "tight junctions."[126] In fusing, the unit membranes of the two apposed cells obliterate the intercellular space, whereas in synaptic junctions such a space (*synaptic cleft*) is always evident. A similar correlation has been made for the ephaptic junctions of chick ciliary ganglion[127,128] and of Mauthner cells with their presynaptic terminals.[129,130] A somewhat different type of tight junction has been observed between cells of epithelial tissues, which also exhibit good electrical contact.[131,132]

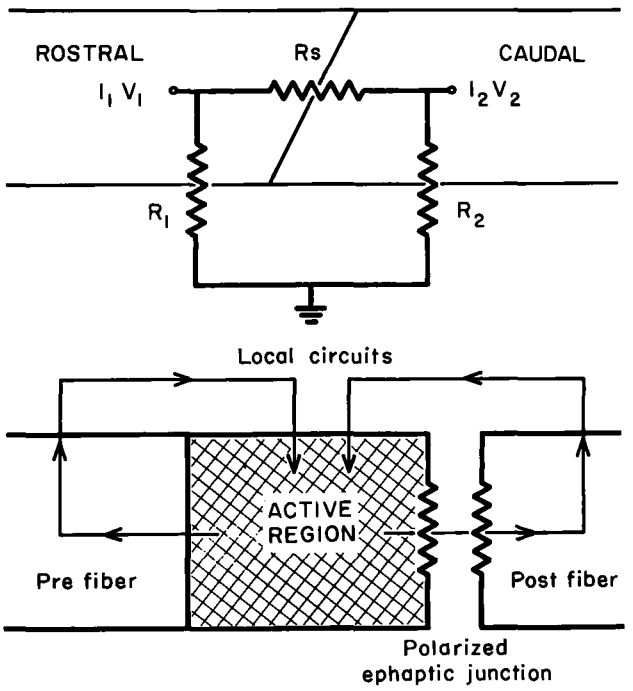

FIGURE 17 Top: Equivalent circuit of an unpolarized ephaptic junction in the septate axon of crayfish. The resistivity of the septum (R_s) is low relative to the membrane resistance of either segment (R_1, R_2). Thus, currents injected at I_1 or I_2 can flow across the septum. These relations are reciprocal. If V_1 is a spike generated within the rostral segment it can excite a similar spike in the caudal segment and vice versa. (Modified from Watanabe and Grundfest, Note 23)

Bottom: Transmission across a polarized ephaptic junction, cord to root giant fibers of the crayfish. A current from the depolarized active region of the prefiber can pass to the postfiber and the transjunctional local circuit excites this axon. Some form of rectification hinders the flow of current in the reverse direction. (Modified from Grundfest, Note 9)

The coupling resistance might be a passive element, with a linear I-E relation (Figure 11) indicative of electrogenically inert membrane, or there might be an electrically excitable component that would be denoted by a nonlinear I-E relation. The septal membranes of the lateral giant axons of crayfish exhibit a linear I-E relation.[23] This also appears to be the case in all but two known ephaptic junctions. Thus, electrical effects are transferred symmetrically in either direction across the passive coupling resistance. Consequently, ephaptic junctions are basically unpolarized, in contrast to synaptic junctions, in which transmission is always polarized by the inherent functional differentiation of the interfacial components.

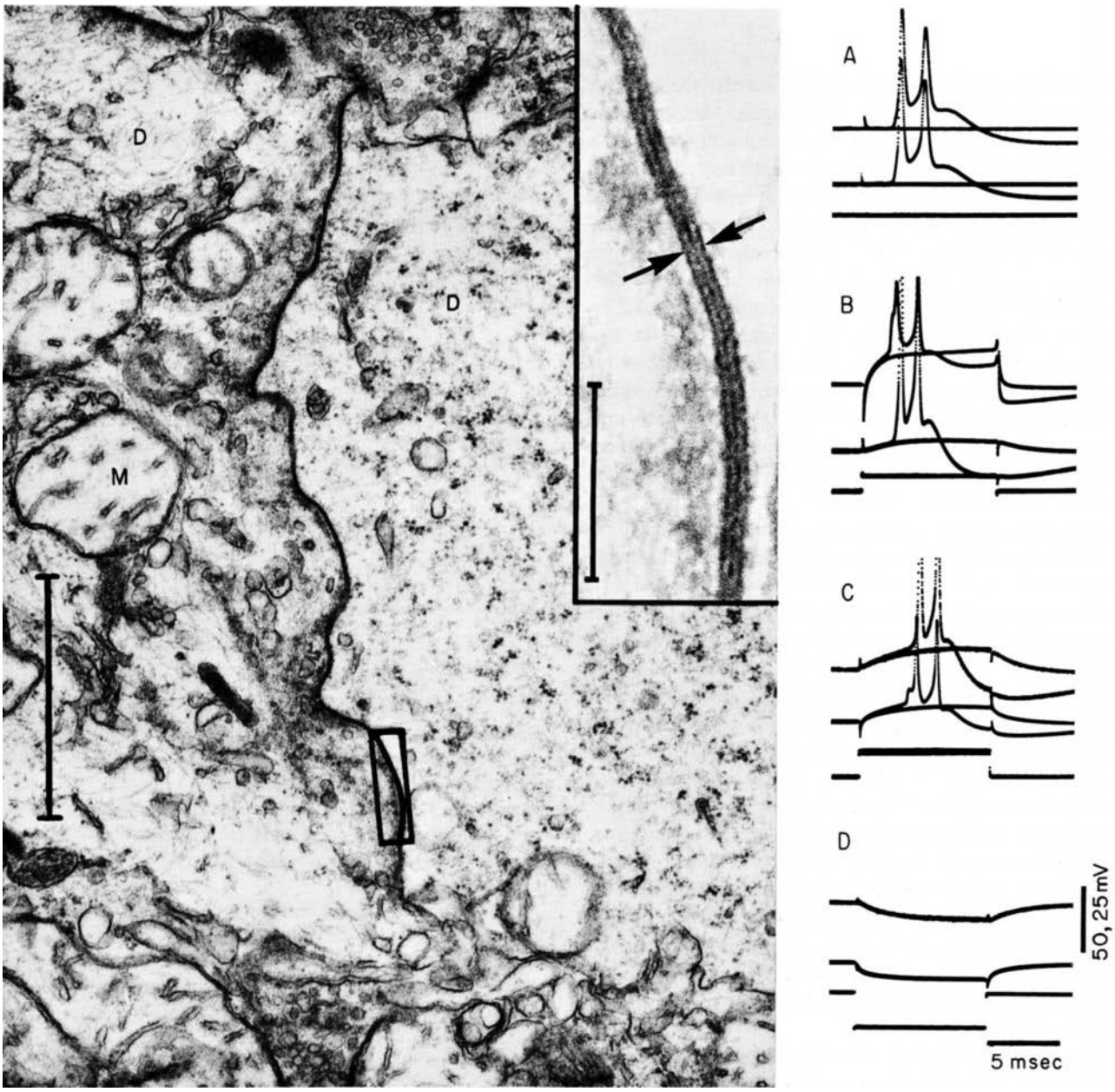

FIGURE 18 Ephaptic transmission and tight junction structure between electromotor neurons in spinal cord of Mormyrid electric fish. Electrophysiological evidence for tightly coupled ephaptic connections is shown (at right) by simultaneous intracellular recordings from two neurons. A: Stimulation of descending spinal tract evoked spikes nearly simultaneously in both cells. B, C, D: Current pulses were applied to either cell, the recording sensitivity for the other cell being doubled. The lowest trace shows the applied current. B, C: A subthreshold depolarization of the directly stimulated cell also evoked a smaller more slowly rising depolarization in the other cell. When a stronger stimulus evoked spikes the second cell also responded with spikes, but after a delay. D: An inward current was injected into the neuron of the middle trace. The cell of the upper trace was also hyperpolarized, but the change in membrane potential was smaller and developed more slowly, indicating that the spread of the potential was electrotonic.

Electron micrograph showing the juncture of two dendrites (D) of two electromotor neurons is at left. At the surface of contact the two apposed membranes are fused and the extracellular space is obliterated. Mitochondria (M) are seen in the dendrite on the left. In the upper and lower center the dendrites make synaptic contacts with nerve terminals that contain synaptic vesicles. Vertical line represents 1 μ. The rectangle outlines a portion that is shown enlarged in the inset (vertical line represents 0.1 μ). The fused membrane complex is about 140 A thick at the arrows and shows an intermediate dense line. (From Bennett, Pappas, Aljure, and Nakajima, Note 121)

One of the two known polarized ephaptic junctions is that transmitting from the cord giant axons to the root motor giant fiber in the crayfish.[133] The other is in the hatchet fish (*Gasteropelecus*) and couples giant axons from the spinal cord to the pool of motoneurons that activate the pectoral fin ("flying") muscle of the fish.[134] The polarized transmission is the result of a marked nonlinearity of the I-E relation; that is, the electrically excitable membrane responds with strong "rectification," so that the resistance to the ephaptic current is low in one direction of flow and relatively much higher in the other (Figure 17).

Rectification may be caused by at least four varieties of nonlinear I-E responses (Figure 11), and each type may involve one or several ion species. Which one, or what combination, of the possible membrane mechanisms for nonlinear behavior are involved in the two polarized ephaptic junctions are unknown. In both, the direction of transmission is functionally valid (orthodromic). However, probability considerations suggest that rectification might also operate in the "wrong" direction, so that the ephaptic transmission would be in the antidromic direction and nonfunctional.

Because electrotonic coupling through tight junctions is basically intracellular and must activate electrically excitable membrane, only spike electrogenesis in the prejunctional cell is likely to be large enough to deliver an appreciable current across the coupling resistance. Thus, ephaptic junctions are inherently excitatory. Bennett[24] has used this fact as a guide in searching out ephaptic junctions in various fishes. Both electrotonic coupling and tight junction structures have been observed in neuron pools in which it is advantageous that control of activity be strongly synchronized; e.g., in the discharge of pacemaker neurons, of electric organs, sound-producing muscles, and muscles controling escape reactions.

In epithelial tissues, tight junctions apparently function to seal off intercellular spaces to prevent the exchange of fluid across laminar sheaths.[126,132] The low resistance that obtains between epithelial cells, which are coupled by tight junctions, is of little moment in the functional relations of these tissues, but in the nervous system it leads to mass activity of populations of neurons.[118,122] To achieve selective activity of individual units, therefore, must have been an evolutionary advance in the nervous system, ephaptic junctions being retained (or redeveloped) where massively synchronized excitatory activity was essential. No ephaptic junctions have been observed thus far in the mammalian nervous system by either electrophysiological or morphological criteria. However, given the known capacity of the central nervous system to undergo dynamic changes, it would not be at all surprising if some pathological conditions might result in the formation of tight junctions. For example, the formation of such junctions at dendritic and/or somatic interfaces might lead to circus activity, such as can occur between the crayfish septate giant axons,[117] and might be one possible cause for epileptiform activity.[58]

Summary

Synaptic transmission from one neuron to another is a cooperative activity. It not only involves the presynaptic and postsynaptic cells as units, but also calls upon the different functional components of the cell membranes. The receptive component of each neuron responds to specific stimuli and in general is electrically inexcitable. The information received is converted into several varieties of electrogenesis. The electrical activity imparts the information to a conductile component that must be inherently electrically excitable, because it is required to generate all-or-none impulses that alone can propagate the message along the axon. The output component, residing at the presynaptic terminals, must possess secretory activity, which reproduces the information that is encoded in the spikes, but in a form that is capable of acting upon the synaptic input of the next cell. Therefore, each synaptic stage involves the participation of a minimum of four interfaces, three of which are intracellular, the other intercellular. Convergence of a number of inputs on a single postsynaptic cell, and the possibility that some inputs are excitatory while others are inhibitory, increases the number of interfaces correspondingly.

The ionic mechanisms of excitatory (depolarizing) and inhibitory (repolarizing) postsynaptic electrogenesis utilize the same electrochemical batteries as those that develop the electrically excitable electrogenesis. However, the properties of the two systems are differentiated by their respective excitabilities. The electrogenesis of the postsynaptic components, both excitatory and inhibitory, is essentially linear with respect to the specific stimuli, and it can exhibit both temporal and spatial summation. Conductile electrogenesis is subject to positive and/or negative feedback from the very changes in membrane potential. Thus, it is inherently nonlinear.

The stimulus specificity of the excitatory and inhibitory input components of the postsynaptic cell call for different varieties of secretory processes in the respective presynaptic elements. These give rise to different "excitatory" and "inhibitory" transmitter agents. Diversities of chemical structure of input membrane introduce a still wider range of transmitters. The logistics of synthesis, mobilization, and delivery of these different transmitters increase the complexity of synaptic transmissional processes, giving

rise to the variations of facilitation, "fatigue," and related phenomena. The kinetics of the action of transmitters upon the receptive membrane, and their rates of diffusion or enzymatic destruction add complexities at the intercellular interface.

Excitatory interactions can occur without specialized junctional structures, simply by electrotonic spread of the currents that are generated by active cells. The spread may be facilitated by the occurrence of relatively low-resistance electrical pathways in ephaptic junctions. Particularly in certain neuronal systems in fish, the low resistance is apparently achieved by fusion of the membranes of adjacent cells, giving rise to structures that are similar to the "tight junctions," which are common among other cells of epithelial origin. In ephaptic transmission the action current of the prejunctional cell flows into the post-cell, depolarizing the electrically excitable membrane of the latter. Thus, transmission is essentially excitatory and involves only the electrically excitable membrane components. Therefore it lacks the rich texture that the participation of different functional components imparts to synaptic transmission.

Comparative Physiology of Dendrites

DOMINICK P. PURPURA

THE EXTRAORDINARY ELABORATION of dendrites to provide an expanded surface area for synaptic contacts between an ever-increasing number and variety of multipolar neurons may be viewed as one of the major driving forces in the evolution of the vertebrate brain.[1] The extent to which the development of dendrites has occurred in the mammalian central nervous system is evident from estimates of dendrite-cell body surface area ratios that are in excess of 15:1 for large pyramidal neurons of the cerebral cortex or Purkinje cells of the cerebellum. In view of this, it is not surprising that in the past few decades numerous attempts have been made to define the functional properties of dendrites, their role in the regulation of neuronal excitability, and their contribution to the production of spontaneous and evoked electrical activities of the brain.[2]

Even the most cursory examination of the general morphological features of neurons in the mammalian brain (Figure 1) is sufficient to caution against sweeping generalizations concerning "dendritic properties." Differences in the structural complexity of dendrites have long been viewed by neurohistologists as evidence for diversity of function.[3,4] Although the application of microphysiological techniques to the study of neuronal activities in different parts of brain has permitted some confirmation of this notion, it must be allowed that these techniques can at best provide only inferential clues to the functions of dendrites. An ultrafine microelectrode inserted into the cell body of a neuron with extensive dendritic ramifications "sees" only a small proportion of the synaptic transactions taking place on the surface of a typical neuron in the mammalian brain. This "iceberg phenomenon" is all the more impressive when one substitutes the conventional picture of dendrites as revealed in the usual Golgi preparations with an electron-microscope view of dendritic elements (Figure 2).[5] The demonstration of fine dendritic terminal processes less than 0.3 μ in diameter, the vast majority of which are devoid of specialized synaptic elements, argues against too rigid a view of the function of dendrites as receptor surfaces for synaptic contacts to the exclusion of other functions related to exchange and transport of ions, metabolites, etc.[6] Unfortunately, there are few data to support this "article of faith" and further consideration of its validity is beyond the purview of the present essay. The concern here is with those physiological concepts that have influenced current notions of dendritic functions, examination of the salient experimental findings upon which these views have been founded, and a survey of more recent data concerning the diversity of functional properties of dendrites of different varieties of neurons in the mammalian brain.

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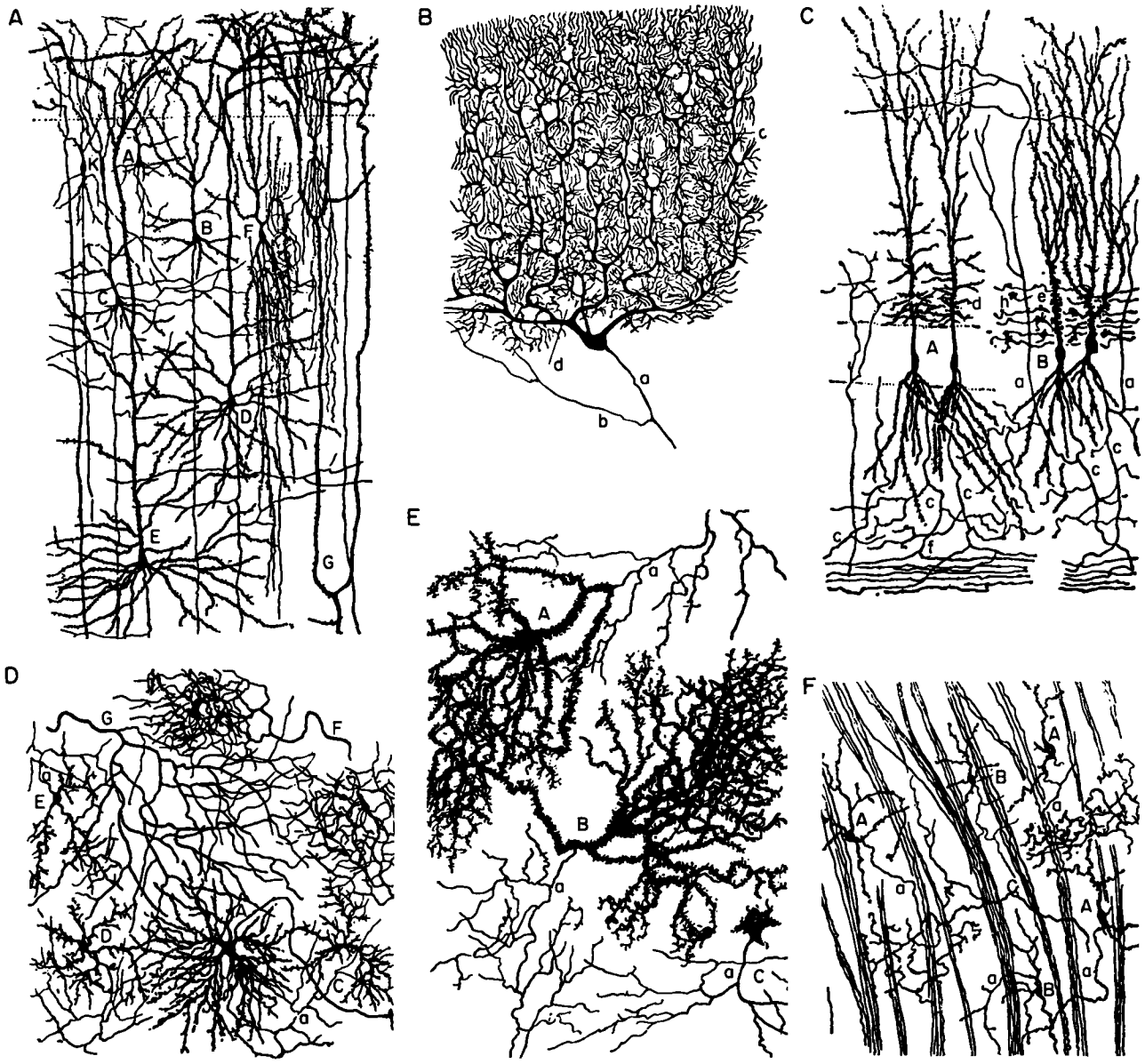

FIGURE 1 Examples of dendritic patterns of neurons which constitute the major cell types of elements in different structures of the mammalian brain. A: Outermost three layers of frontal cortex of a 1-month-old human infant. Small (A, B, C) and large (D and E) medium pyramidal neurons as well as the dendritic process of a large pyramidal cell (G) of the fourth layer are shown. Cells with double dendritic bouquets (F) and fusiform appearance (K) are also identified.

B: Purkinje cell of the cerebellum from adult human. Axon (a) and axon-collateral (b), capillary spaces (c), and spaces occupied by basket cells (d) are indicated.

C: Pyramidal cells of hippocampus from a one-month-old rabbit. A, small pyramidal cells of the superior region; B, large pyramidal cells of the inferior region. Large ascending collaterals (a), axons (c), and sites of contact of mossy fibers (h) are to be noted.

D: Frontal section of the thalamic somatic sensory nucleus of the cat a few days old. A, cell with a long axon; C, D, and E, cells with short axons; F, sensory fibers; G, axons of cortical origin terminating in the sensory nucleus.

E: Cells of the pons from a human infant, few days old. A and B, cells with axons arising from dendrites; C, cell with a bifurcated axon.

F: Sagittal section of the caudate nucleus from a newborn rat. A, cells with a long axon; B, cells with short axon; C, ascending afferent fibers. (From Ramon ý Cajal, Note 4)

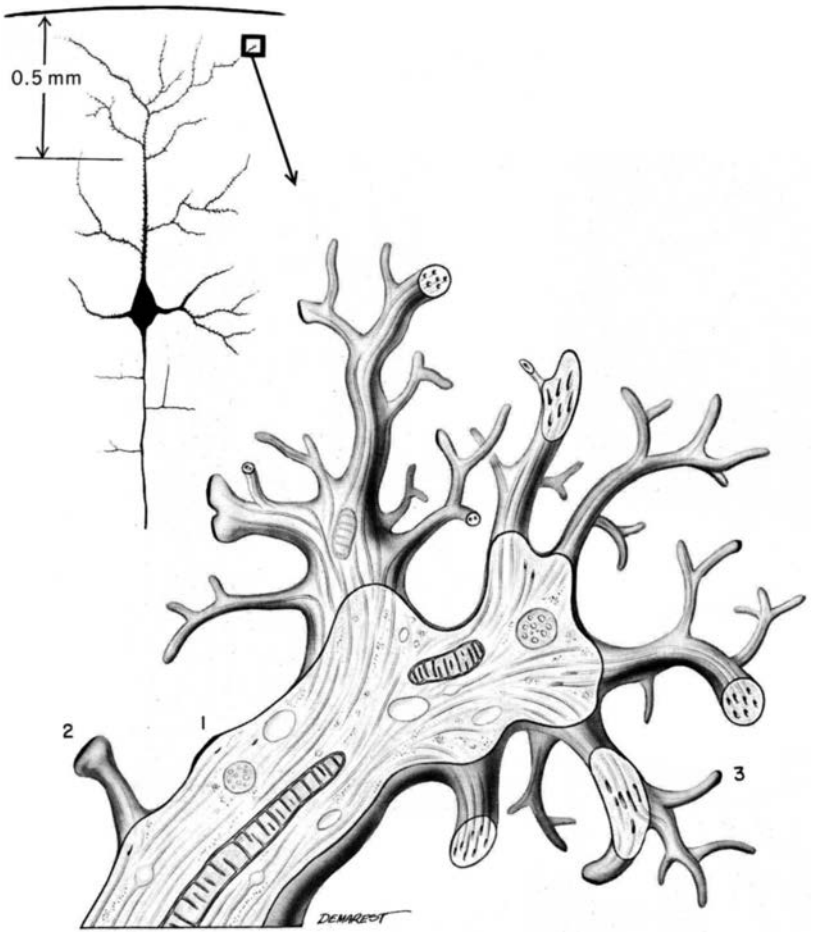

FIGURE 2 Representation of the general morphological characteristics of a small dendrite and its terminal processes in the superficial neuropil. The inset (upper left) shows a typical pyramidal neuron with its apical dendrite in the superficial neocortex (upper 0.5 mm). The central drawing depicts the small segment of the dendrite, within the square shown in the insert, as it might appear in a composite of electron micrographs. Postsynaptic membrane thickenings are shown on the dendritic trunk (1) on a spine (2) and on a terminal process (3). Intracytoplasmic profiles include mitochondria, elements of the endoplasmic reticulum, vacuoles, multivesicular bodies, and dendritic tubules with occasional areas of local dilatation. The dendritic tubules extend characteristically into the finest terminal processes. Profiles of these tubules in small processes are shown as they appear in sections of the neuropil. (From Pappas and Purpura, Note 5)

Early studies of "dendritic potentials" and the concept of graded response mechanisms

Developments in electrophysiology nearly four decades ago resulted in the rapid accumulation of a large body of information on the properties of axons, the first type of excitable tissue studied in considerable detail with modern instrumentation. It was but a few years after the initial characterizations of the spike potentials and after-potentials of peripheral nerves that "axonologists" turned to the problem of electrocortical potentials. Once inside the skull these early investigators encountered a variety of spontaneous and evoked potentials with characteristics that were fundamentally different from the usual spike potentials of axons or muscle fibers. The most reasonable approach to this problem, and operationally the most simple, was to record from the surface of the exposed cerebral cortex. An electrical stimulus could be applied directly to the cortex, or close to the recording electrode (thus replicating the typical "axonological" experiment), or a stimulus could be delivered to a peripheral receptor system and the responses of cortex recorded with different assemblies of electrodes.

The results of these types of experiments showed that, whether local stimulation of cortex[7] or stimulation of the optic nerve[8] was employed to evoke responses of cortex, the shortest duration potentials so elicited ranged from 10 to 15 milliseconds in duration, or many times longer than the usual spike potentials of axons. Now there was no reason to suppose that central nervous system axon spikes should differ in any way from peripheral axon spikes. Consequently, the idea that axon spikes might contribute to the production of potentials recorded from the surface of the brain was rejected early in the development of electrocortical physiology. With axons eliminated as sources for brain waves, logical necessity called for examination of cell bodies and/or dendrites as generators of this activity. The specific identification of the 10- to 15-millisecond local negative response of cortex to surface stimulation as a response of the "nerve cells with dendrites running laterally, e.g., the horizontal cells of Cajal, or the superficial branches of apical dendrites of pyramidal cells"[7] may be considered one of the major departures from a strictly axonological interpretation of brain wave activities. Other early concepts relating to the origin of brain waves were also based on the notion that "cells or specialized

parts of them rather than fibers must produce these large potentials."[8]

It is not possible here to consider the various lines of investigation that led to early concepts concerning the origin and nature of brain waves. These have been treated in detail elsewhere in an extensive review of the subject.[2] However, some discussion of this subject is unavoidable, inasmuch as current concepts of the origin and nature of brain waves derive from notions concerning the properties of dendrites.

Until the early 1940's, it is difficult to find any clear experimental data specifically relating to dendritic activities from the time of Adrian's study[7] of the "superficial negative response" of cortex. In one of the most historically significant studies reported during this period, O'Leary and Bishop[9] recorded the distribution of potentials in the avian optic lobes following optic nerve stimulation. Analysis of the potential changes observed during movement of a rather gross electrode through the dendritic plexuses of neurons in the optic lobes led O'Leary and Bishop to speculate that such responses might be "essentially nonconducted or stationary potentials" generated at synaptic sites on dendrites.[9] Somewhat earlier, Renshaw et al[10] had successfully used microelectrodes for the first time in the brain to record unit and slow wave activities. Their conclusions are also noteworthy: (1) waves and spikes are not interdependent; (2) monophasic waves of 20- to 100-millisecond duration recorded from the surface and depths of the hippocampus following afferent stimulation are presumably the result of the activity of vertically oriented pyramidal cells, polarity being dependent on differences in the location of synapses; and (3) "If the position of the active synapses determines the electrical field set up by the pyramidal cells, the existence of a process more or less localized to the region of an active synapse and characterized by an electrical sign is indicated."[10]

To achieve a respectable synthesis of the data leading to the later development of concepts of dendritic activities, it is necessary to note several discoveries that preceded the reports of O'Leary and Bishop[9] and Renshaw.[10] Relevant to this discussion are the findings of Hartline and Graham[11] in 1932. They observed a slow potential distinct from the spike discharges recorded from optic nerve of *Limulus*. Therman[12] noticed a similar slow potential in the optic nerve of *Loligo* and Bernhard[13] showed that the slow, electrotonically conducted potential, subsequently referred to as the "generator potential" by Granit,[14] could clearly be separated from spike potentials following cocainization of the optic ganglion of *Dytiscus*. Equally important in this respect were the almost simultaneous discoveries by Göpfert and Shaefer[15] and Eccles and O'Connor[16] of the endplate potential of skeletal muscle and the detailed description by Hodgkin[17] of the local response mechanism of axons. Following Katz's[18] studies of the mechanisms of excitation at muscle spindles, in which important differences were shown between the generator potential initiated at the terminal and the discharge of impulses in the afferent fiber, the stage was set for a more comprehensive treatment of the properties of dendrites of neurons in the brain.

Two divergent views of dendritic activities arose in the early 1950's. In re-examining the superficial negative response of cortex, Chang proposed that this response was due "to the passage of nerve impulses along the apical dendrites of the pyramidal cells."[19] Chang considered this response comparable to the soma-dendritic response of motoneurons studied with microelectrodes several years earlier by Lorente de Nó.[20] However, findings (Figure 3A) of distant responses of greater magnitude than those close to the stimulating electrode were not discussed by Chang, who assumed that apical dendrites might extend along the cortical surface for distances up to 2 mm (a figure now known to be incorrect). Following a series of studies on the site of origin of different components of the primary response of visual cortex, Bishop[21] and his associates formulated a major concept of dendritic properties,[22] which was based in part on data relating to graded responses in other excitable cells, e.g. eel electroplaques,[23] muscle spindles,[18] and the crustacean stretch receptor organ.[24,25] In their studies of the negative response of cortex, Clare and Bishop[22] showed that this response was graded and decremental in nature (Figure 3B), that it exhibited no refractoriness (in the axonological sense), and that it could be summated to give response durations typical of the configuration of most brain waves. In climaxing a long and extraordinarily fruitful preoccupation with the analysis of electrocortical potentials, Bishop[26] could state with considerable authority that "the chief physiological business of the nervous system is transacted in graded response elements." The manner in which graded electrotonically propagating responses of dendrites might influence the excitability of the neuron cell body was considered by Bishop[26] to be similar in many respects to the operation of sensory receptors. Strong support for this hypothesis was forthcoming from the detailed studies of Eyzaguirre and Kuffler,[24,25] who showed that the generator potential of the crayfish stretch receptor was initiated at those sites in dendritic terminals that were electrogenically different from membrane sites giving rise to conducted responses (Figure 3C).

Here it should be pointed out that while the graded response properties of dendrites were being elucidated by Bishop and his associates, other workers had taken a different approach to the interpretation of so-called "den-

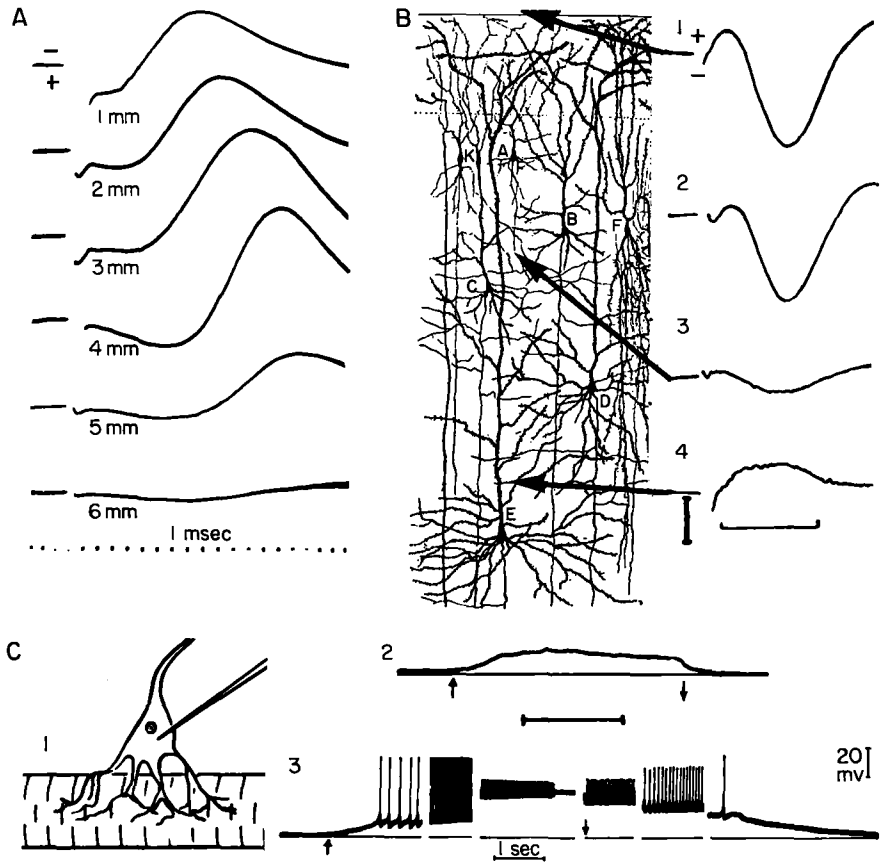

FIGURE 3 A: Series of surface-negative "dendritic potentials" recorded at different distances as indicated from the site of stimulation of the cortical surface in monkey. Note increased amplitude of responses at 3–4 mm from stimulating site. (From Chang, Note 19)

B: Right. Evidence of nonconduction downward in apical dendrites. Stimulus to suprasylvian white matter, leads from optic cortex. Record 1, surface to white; 2, surface to electrode 0.5 mm below surface; 3, 0.5 to 1.0 mm; 4, 1.0 mm to white. Most of the negativity appears in the upper 0.5 mm. Reversal of record in 4 indicates that the electrode at 1.0 mm is below the activated region. Calibration 0.2 mv; 10 msec. (From Clare and Bishop, Note 22)

Left. Replication of Figure 1A (taken from Ramon ý Cajal, Note 4). This is intended to provide a general picture of the approximate intracortical sites at which records of 1–4 might be recorded. Emphasis is placed on the extent of cortex subtended by apical dendrites of medium and large pyramidal neurons.

C: 1, schematic representation of a sensory nerve cell making contact with a receptor muscle strand. Capillary microelectrode in place. 2, membrane potential change during stretch in the absence of sensory discharge. Intracellular record from the cell body of a slowly adapting cell in the third abdominal segment of the crayfish. Stretch and relaxation marked by arrows. Resting potential of 65 mv reduced by 6 to 7 mv for the duration of stretch. Time calibration 1 sec. 3, overstretch of impaled slow receptor cell in the eighth thoracic segment. Continuous stretch progressing over 10 sec presented in small sections. Initially, frequency and firing level have increased, but spike peaks are little changed. In third record the impulse decreases to about 10 mv before complete block occurs at a depolarization level around 35 mv. Blocking stretch was maintained for 2 minutes. With slight relaxation (arrow) the membrane potential returns into the firing range and discharge frequency reduces during stages of relaxation. (From Eyzaguirre and Kuffler, Note 24)

dritic potentials" of cortex. Eccles[27] showed the resemblance of superficial negative responses of cortex to excitatory postsynaptic potentials of spinal motoneurons. This hypothesis was also championed by Purpura and Grundfest,[28] who extended the concept to include the existence of hyperpolarizing, inhibitory postsynaptic potentials in the overt evoked potentials of different cortical structures.[29,30] Much of the initial data bearing on the "postsynaptic potential" theory of cortical electrogenesis was reviewed several years before the extensive application of intracellular recording techniques to the study of neurons in the brain.[31-33] Since that time new analytical approaches and techniques have provided much information on the properties of dendrites and the relationships between dendritic synaptic activities to different varieties of brain waves. The remainder of this essay is devoted to a survey of these major findings that have guided the further development of current concepts of the functional properties of dendrites.

Ontogenesis of dendritic synaptic activities in mammalian cortex

Until recently there have been relatively few attempts to utilize the postnatal developmental characteristics of neurons in the mammalian brain to provide information on the nature and origin of evoked potentials such as the aforementioned superficial negative response or "dendritic potential"[19] of cortex. The usefulness of an ontogenetic approach is particularly evident in respect to the mechanisms and anatomical substrate of the negative components of several varieties of evoked activities.[34] Examination of Golgi–Cox preparations of kitten neocortex from birth to three to four weeks postnatally has amply confirmed the sequential changes in cortical pyramidal neurons and other elements indicated by Ramoń y Cajal.[4] Emphasis is on the fact that in the earliest postnatal period as well as one to two weeks antenatally in the fetal kitten, pyramidal neurons have well-developed apical dendrites which exhibit limited tangential spread in the molecular layer (Figure 4A). At this stage, large basilar dendrites are absent and axon-collaterals of pyramidal elements are poorly developed.[35] The extraordinary density of apical dendritic elements in the immediate neonatal period is clearly seen in the electron micrograph of Figure 4B which is from the neocortex of the newborn kitten.[36] Thus light (Golgi–Cox) and electron-microscope studies provide abundant evidence for a predominantly "neural' type of cortex in the early postnatal period prior to the elaboration of glial elements.

One of the chief characteristics of the synaptic organization of elements in the neocortex of newborn kittens is the preponderance of axodendritic synapses related to relatively large dendritic processes (Figure 4C). Axodendritic synapses related to dendritic spines are not present in the neonatal kitten neocortex, and axosomatic synapses are encountered only rarely[36] at this developmental stage. These axodendritic synapses in newborn animals are identical in all respects to the axodendritic synapses found on large dendritic trunks of adult cats[5] and other animals.[37,38]

Stimulation of the surface of the neocortex in newborn kittens elicits a graded surface negative response at sites as far away as 4 to 5 mm from the locus of stimulation[39] (Figure 4D). Although these "superficial negative responses" are considerably longer in duration than in adult animals, it can be shown that this is largely attributable to the summation of several negative components, each of 10- to 15-millisecond duration. This summation probably results from the packing density of dendritic elements in the newborn animal.[36] When care is taken to record as close as possible to the site of stimulation, the local negative response is found to be identical in all respects to that of adult animals (Figure 4E). Thus, at an ontogenetic stage characterized by the presence of a well-developed superficial neuropil consisting largely of axodendritic synapses, the local negative response may be recorded from sites well beyond the limits of tangential spread of apical dendrites in the molecular layer. From this it follows that the local negativity of neocortex as well as many of the negative components of evoked responses revealed in immature neocortex are postsynaptically evoked responses of dendrites.[34] It might be added that studies of the type described by Clare and Bishop[22] (Figure 3B) amply confirm the decremental nature of the superficial negativity in immature animals. The fact that similar responses with an increased latency are detectable several millimeters from the site of stimulation indicates that in all instances the "local" surface negativity is a compound postsynaptic potential of dendrites.[2,28]

In order to further characterize the nature of "dendritic potentials," it is instructive to compare the morphophysiological relations in neocortex with those of the cerebellum in immature animals. Stimulation of the folial surface in adult animals elicits a 10- to 15-millisecond negativity with features similar to the superficial negative waves of adult neocortex. This response of cerebellar cortex was originally described by Dow,[40] who thought it was caused by stimulation of parallel fibers, which then elicit synaptic activities in dendrites of cerebellar Purkinje cells. Ontogenetic studies of this response provide support for this hypothesis.[41,42]

The postnatal morphogenesis of elements in the cerebellum differs significantly from that of the neocortex in two respects. Unlike neocortical pyramidal neurons, Purkinje

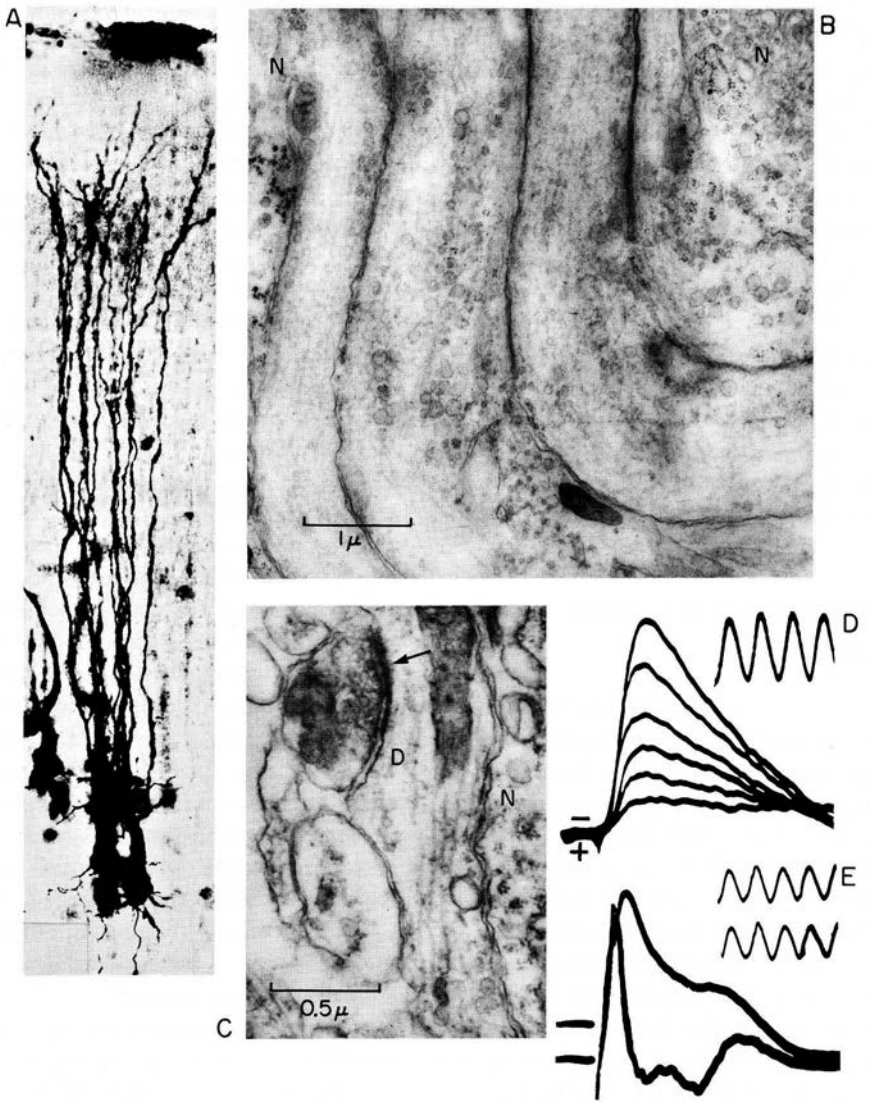

FIGURE 4 A: Golgi-Cox preparation of pyramidal neurons in neocortex of newborn kitten. Note well-developed apical dendrites and absence of basilar dendrites. Dendritic spines are not observed at this stage and tangential spread of apical dendrites in the molecular layer is less than 50μ. The apical dendrites are about 0.3 mm in length in this photomicrograph. (From Purpura, Carmichael and Housepian, Note 39)

B: Electron micrograph of elements in superficial regions of neocortex of fetal kitten. N, two neuron cell bodies with 4–6 dendrites packed between them. Note absence of glial processes between neural elements at this developmental stage and indistinct appearance of dendritic tubules. (From Voeller, Pappas and Purpura, Note 36)

C: Axodendritic synapse from the superficial neuropil of newborn kitten. D, dendrites. N, neuron cell body. Arrow indicates region of postsynaptic thickening. The presynaptic axon terminal exhibits clusters of synaptic vesicles and mitochondria. (From Purpura, Note 34)

D: Characteristics of long duration graded superficial cortical responses recorded 1.5 mm from stimulating electrodes on suprasylvian gyrus of a near-term kitten. Stimulus frequency 0.5/sec. Six superposed responses at different stimulus strengths. Cal. 100 cps; 0.1 mv.

E: Comparison of superficial cortical responses recorded at 1.5 mm with a large (0.5 mm) ball-tipped electrode (upper channel) and response recorded at the site of stimulation with a 0.1 mm wire electrode in a newborn kitten. Cal. 100 cps; 0.1 mv. (From Purpura, Carmichael and Housepian, Note 39)

cells have rudimentary dendrites that terminate below the 9 to 12 layers of densely packed external granule cells. External granule cells undergo marked proliferation, differentiation, and inward migration during the first six postnatal weeks. They leave behind an axon that bifurcates in the molecular layer to form the parallel fiber system.[4] During the progressive attenuation of the external granule layer the dendrites of Purkinje cells proliferate, in part in response to the simultaneous development of the parallel fiber system.[42] By the third to fourth postnatal week, Purkinje cell dendrites develop many secondary and tertiary branches with spiny branchlets. The latter represent the axodendritic synapses effected by parallel fibers.[43] The general appearance of Purkinje cell dendrites in the kitten during this postnatal period is shown in Figure 5A, B and C. Comparison of the different responses evoked by local folial stimulation at different postnatal developmental stages indicates that only after the third week does such stimulation evoke a typical surface negative response (Figure 5D). The response becomes identical to that of the adult animal, especially after the sixth postnatal week, when the elaborate dendritic plexuses of the Purkinje cell attain the pial surface and receive their full complement of parallel fiber axodendritic synapses.[41,42] Thus, failure to elicit a local surface negative response in the cerebellum following weak stimulation of the folium is referable to the absence of a synaptic substrate for the production of this response in the immediate neonatal period. The appearance of the superficial negativity occurs *pari passu* with the morphogenesis of axodendritic synaptic substrate. Two conclusions may be drawn from studies such as those summarized in Figures 4 and 5. The elaboration of dendritic synaptic organizations proceeds at different rates in different brain structures. In all instances examined thus far, alterations in evoked activities accompany the differential rate of development and elaboration of axodendritic and axosomatic synaptic pathways.

While ontogenetic studies of evoked potentials have emphasized the contribution of axodendritic synaptic activities to these responses, analysis of evoked potentials alone cannot provide sufficient information relating to the changing properites of dendrites during their "functional maturation." Suffice it to say that intracellular recordings from neurons of immature neocortex, as described below, indicate that although many elements of neocortex have acquired mature *morphological* characteristics by the end of the first postnatal month, dendrites may retain physiological properties that are rarely encountered in the mature brain.[44] Before proceeding to a discussion of these findings, it will be profitable to examine some of the data obtained from intracellular recording in the brain of adult animals to determine the extent to which these data provide additional clues to the understanding of the properties of dendrites.

Dendritic activities as summations of postsynaptic potentials (PSPs): the relationship of PSPs to spontaneous and evoked responses

The discussion thus far has been concerned primarily with the least complex evoked potential of cortex, the superficial negative response, and with some data that have permitted its identification as a postsynaptic potential of dendrites subtending the outermost layers of cortex. The question may be raised as to how synaptic activation of superficial dendritic elements is capable of influencing more remotely located parts of the neuron, i.e., cell body and initial axonal segment.[45-47] Some answers to this have been provided in studies of the relationship of the superficial negative response and neuronal discharges.

A typical finding[48] in extracellular studies is illustrated in Figure 6B. In this experiment, weak surface stimulation elicited a surface negative response not associated with cell discharge of a unit located in the cortical depths (Figure 6B 1). Only when the stimulus was increased to elicit positive spikes preceding the surface negative wave was there an associated cell discharge (Figure 6B 2). Essentially similar findings are observed in intracellular recordings,[49] in which surface stimulation evokes a depolarizing excitatory postsynaptic potential (EPSP) in deep-lying neurons. The EPSP may or may not initiate spike discharges in these elements, depending upon the strength of the surface stimulus and the magnitude of the EPSPs elicited (Figure 6C). Unfortunately, strong surface stimulation that evokes early spikelike components that precede the usual surface negativity[48] also sets into operation a variety of synaptic events other than those related to superficial apical dendrites (Figure 6D).[50] Not the least of these complications is that such strong cortical surface stimulation usually directly excites elements in the cortical depths, as well as axons and cells in superficial regions that make synaptic contacts with excitatory and inhibitory elements in neural organizations remote from the site of stimulation.[2,51-54] Perhaps the "ideal" experiment to test for the excitability properties of apical dendrites and the manner in which axodendritic EPSPs initiate spikes in cortical neurons should be one that attempts to reproduce the operational design employed in studies of the crustacean stretch receptor.[24,25,55,56] A generator potential, initiated in dendritic terminals[25,55,56] (Figure 3C), which electrotonically influences sites of impulse initiation in the axon close to the soma[24] (Figure 6A) provides a model of "dendritic function" without parallel in studies of dendritic activities in the mammalian central

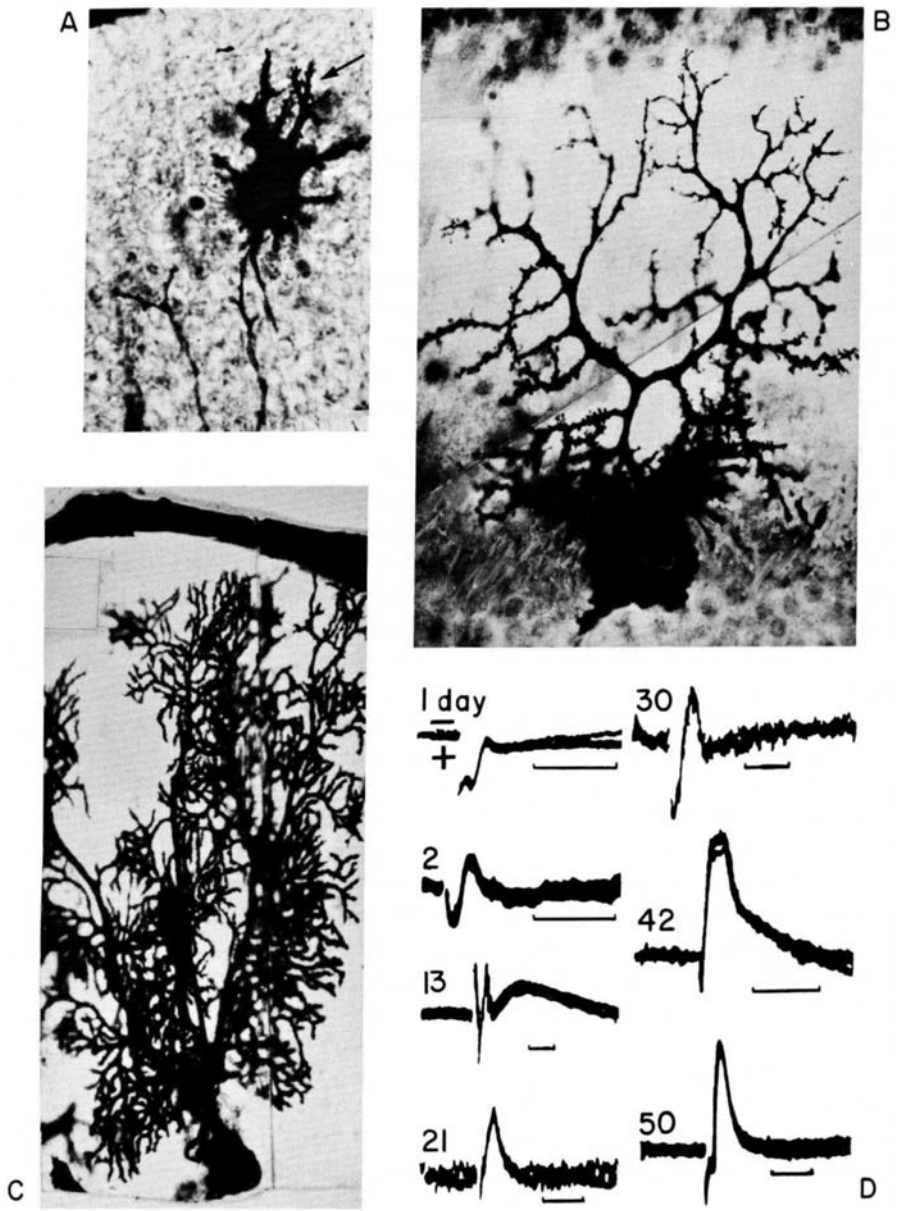

FIGURE 5 A–C. Microphotographs of Purkinje cells revealed in 200μ thick Golgi-Cox sections of cerebellum in kittens of various ages. A: Two-day-old kitten. Main stem dendrites are short, occasionally branched and have several protruberances. "Dendrite-like" ramifications are seen emerging from the cell body. B: Appearance of Purkinje cell in 8-day-old kitten. C: Characteristics of dendritic growth of Purkinje cell by the 6th postnatal week. In A and B, superficial portions of dendrites do not penetrate into lower border of external granular layer. D: Development of superficial cerebellar responses to local folial stimulation in kittens of various ages as shown. Recording electrode placed 1–3 mm from site of stimulation and parallel to the long axis of the folium. Spikelike responses seen in first week are succeeded by 'transitional' responses with minimal negativity and late surface-positivity. Superficial negative responses are clearly detectable by the end of the 3rd postnatal week. Evoked responses in 2nd month are similar to those recorded in adult animals. Time cals: 20 msec. (From Purpura, et al., Note 41)

nervous system. However, the problem in respect to cortex is not completely insoluble.

It is known that apical dendrites of large pyramidal neurons, such as those that send their axons in the corticospinal tract, extend well into the molecular layer, where they spread out under the pial surface. Intracellular recording from the cell body of these elements can be secured without difficulty, and a particular pyramidal cell can be clearly identified by its backfiring antidromic response to pyramidal tract stimulation in the medulla.[53,54] Furthermore, it is known that such elements participate in the synaptic events initiated by stimulation of afferent pathways arising in the ventrolateral (VL) thalamus.[57-60] Figure 7B shows the intracellularly recorded activities of such a pyramidal tract neuron in the motor cortex during VL stimulation.[61] By applying polarizing currents across the cortex it is possible to compare effects on evoked responses[62] with these intracellular activities. The results observed in this experiment are striking. Weak anodal polarization produces dramatic increases in the negativity of the evoked response and elimination of the positivity (Figure 7D) whereas weak cathodal polarization eliminates the negativity and augments the surface positivity (Figure 7F). These changes may not be accompanied by a detectable change in membrane potential or the timing and magnitude of synaptic events recorded at the level of the cell body. In contrast, increases in the strength of polarizing currents produce the following effects on large pyramidal neurons: surface anodal polarization depolarizes the cell body and initial axonal segment, thus leading to repetitive cell discharge (Figure 7), whereas surface cathodal polarization hyperpolarizes and suppresses cell discharge evoked by thalamic stimulation.[61] These overt effects of externally applied currents have been confirmed in intracellular studies of others.[63]

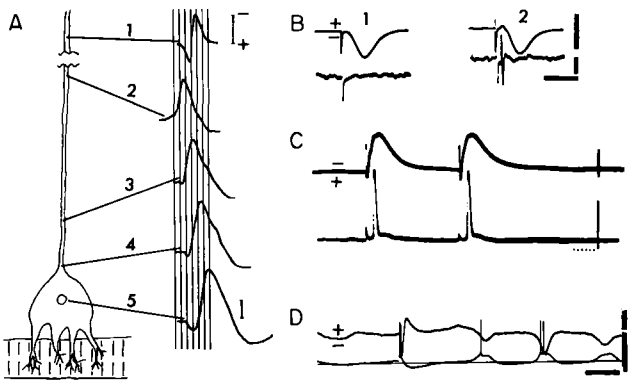

FIGURE 6 A: Tracings of orthodromic impulses set up in the lobster stretch receptor recorded with external leads at various indicated points on the nerve cell. One electrode was kept fixed on the cell at 5, while the other was moved to the other positions. Point 1 was about 1.3 mm from cell body-axon boundary; 2 was about 500μ distant. Time intervals are 0.1 msec. Amplitude calibrations 0.5 mv. (Modified from Edwards and Ottoson, Note 55)

B: Simultaneously evoked direct cortical responses and unit discharge. 1 and 2 are responses to weak and strong stimulation. Upper record is cortical response, lower one is microelectrode derivation. When the stimulus is too weak (1) to cause positive spikes to appear in the surface response, no response follows the shock in the microelectrode tracing. However with stronger stimulation (2) a positive spike appears in the surface record and a unit discharge having the same latency as the positive spike is seen in the microelectrode record. Amplitude calibrations: upper, 250 μv; lower, 1 mv. Time bar is 10 msec. (Modified from Stohr, Goldring and O'Leary, Note 48)

C: Synaptic excitation of a cortical neuron by direct cortical stimulation with threshold strength. Simultaneous recording from cortical surface and a cortical neuron. Note surface-negative waves and synaptic potentials preceding spike discharges. Vertical bars represent 1 mv for surface recording, 50 mv for intracellular recording. Time marks 1 msec. (From Li and Chou, Note 49)

D: Association between evoked intracellular spike and brief positive deflection initiating direct cortical response. Primary negative potential corresponds to membrane depolarization and direct cortical response rhythmic waves occur in association with a train of membrane depolarizations. Amplitude calibrations: Upper, 0.5 mv; lower 50 mv. Time bar, 50 msec. (Modified from Sugaya, Goldring and O'Leary, Note 50)

Four conclusions can be drawn from the data of Figure 7: (1) a considerable proportion of the current applied to the pial surface is distributed into a number of shunt pathways (i.e., along vascular elements, intercellular spaces, etc.); (2) dramatic changes in evoked potentials produced by weak polarizing currents occur as a consequence of alterations in PSPs initiated in dendrites remote from the site of generation of those EPSPs associated with cell discharge; (3) strong polarizing currents affect a significantly larger surface area of the soma-dendritic membrane. Surface anodal polarization excites the cell by virtue of the outward flow of current in the soma-initial axonal segment region; and (4) *Surface cathodal currents that effectively depolarize apical dendrites (and hyperpolarize cell body–initial axonal segment regions) do not elicit spikes in dendrites.* It must not be inferred from this that surface-applied polarizing currents produce no effects other than depolarization or hyperpolarization of vertically oriented pyramidal neurons. Actually, such currents have been shown to produce a number of alterations that suggest actions on presynaptic terminals and low-threshold effects on neurons with different orientations from those of the large pyramidal neurons.[61] However, the data provide support for the view that PSPs generated in dendrites make the major contributions to evoked potentials[2] and that dendrites of neocortical

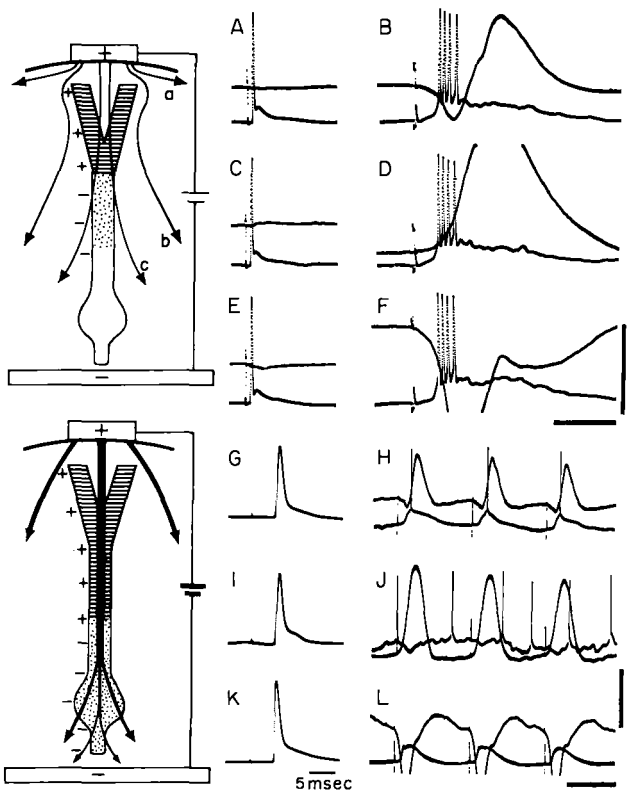

FIGURE 7 A–F: Dissociation of effects of weak surface polarizing currents (50μ A/mm^2) on evoked cortical responses and intracellular activities of a pyramidal tract neuron. Upper channel records are surface responses to stimulation of ventrolateral thalamus. A, antidromic spike with a prominent delayed depolarization; B, patterns of synaptic drive during stabilization phase of augmenting response; C and D, during weak surface anodal polarization; E and F, during surface cathodal polarization. Patterns of intracellularly recorded activities are uninfluenced during dramatic changes in surface evoked responses. Cals: 50 mv; 20 msec. Diagram, upper left, shows probable distribution of currents during weak anodal polarization: a, fraction of current flowing along surface, etc.; b, extraneuronal current flow; c, proportion of current inward at terminals of apical dendrites, outward across proximal dendritic regions. No effect of this current is observed at the soma level with weak intensities as indicated in D.

G–L: Effects of strong cortical surface polarization on a pyramidal tract neuron. G, I, K, antidromic responses; H, J, L, activities evoked by repetitive ventrolateral thalamic stimulation; G and H, controls; I and J, during strong surface anodal polarization (150 μ A/mm^2); K and L, during strong surface cathodal polarization. Note changes in delayed depolarizing potential and amplitude of antidromic spikes. Anodal polarization produces depolarization of soma regions and increase in cell discharge. Cathodal polarization of surface hyperpolarizes soma and prevents EPSPs from attaining firing level. Diagram, lower left: probable distribution of strong anodal currents. Increase in inward current through apical dendritic terminals of pyramidal neuron is associated with outward depolarizing currents through soma-initial axonal segment region. Calibrations at right 50 mv; 100 msec. (From Purpura and McMurtry, Note 61)

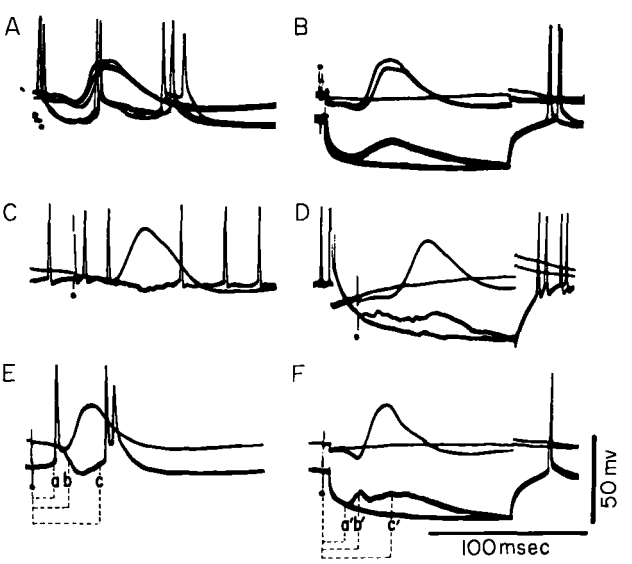

FIGURE 8 Different patterns of PSPs evoked in cortical neurons during recruiting responses elicited by 6/sec stimulation in thalamic midline nuclear complex (CM). A, C and E, examples of relationship of cortical surface-negative recruiting responses to intracellular activities of three neurons impaled during the same experiment; B, D, and F, corresponding superimposed traces of effects of injected hyperpolarizing currents alone and combined with CM stimulation. A, neuron responding with two phases of discharge during peak and near termination of surface-negativity exhibits a prolonged, slowly developing EPSP during membrane hyperpolarization (B); C, neuron in which peak of recruiting negativity is associated with membrane hyperpolarization exhibits an inverted (depolarizing) potential during inward current injection. Analysis of temporal relations of early EPSP (a, a^1), onset of repolarization potential (b, b^1), and late EPSP (c, c^1) is shown in E and F before and after induced membrane hyperpolarization, respectively. In B and F, thalamic stimulation preceded start of current injection by several milliseconds. In F note superimposed anode-break response following periods of current injection before and during thalamic stimulation. (From Purpura and Shofer, Note 64)

pyramidal neurons of adult animals do not give rise to conducted spikes in response to directly applied depolarizing currents. The results also indicate that surface anodal currents are effective in exciting cortical-pyramidal neurons because inward currents through apical dendrites are associated with outward (depolarizing) currents at impulse initiation sites (cell body–initial axonal segment region) remote from the site of application of the anodal current.

The concept that postsynaptic potentials constitute the only variety of response generated by dendrites of neocortical neurons and that such activities are the major source of spontaneous and evoked potentials[2] obviously oversimplifies an extraordinarily complex problem. It is evident that the local negative wave evoked by weak stimulation of the cortical surface represents the net depolarizing activity of combinations of excitatory and inhibitory PSPs generated at various loci in superficial dendritic elements. However, considering the origin of other varieties of evoked potentials, contributions of axosomatic and axodendritic PSPs in various proportions must be taken into account. It has already been shown in Figure 7 that the "augmenting response" elicited in motor cortex by ventrolateral thalamic stimulation is associated with both axosomatic and axodendritic PSPs on pyramidal neurons (as well as on other types of elements).[60] Intracellular recordings from neurons at different depths in the cortex during nonspecific, thalamocortical "recruiting responses" reveal that even those responses believed to consist of "pure" axodendritic EPSPs are actually composed of depolarizing and hyperpolarizing axosomatic and axodendritic EPSPs (Figure 8).[64]

Several efforts have been made in recent years to provide a more precise definition of the relative effects of axodendritic and axosomatic EPSPs on different types of neurons and to specify the organizational features of different inputs to these elements. The basis for these analyses derives largely from a series of studies on frog motoneurons reported by Brookhart and his associates.[65-67] These investigators compared the time course and duration of focal responses elicited by dorsal root and lateral column stimulation and examined the effects of membrane potential alterations on the EPSPs evoked by these two inputs (Figure 9A). They showed that monosynaptic axodendritic EPSPs elicited in frog motoneurons by dorsal root stimulation exhibit a slow rise time and are unaffected by membrane hyperpolarization. In contrast, EPSPs evoked by lateral column stimulation are generated on or near the soma, exhibit a more rapid rise time, and are increased by membrane hyperpolarization.[67] It was concluded from these studies that there was not only an excellent correlation between anatomical locations of activated synapses and properties of PSP's, but that axodendritic EPSPs produced their excitatory action on frog motoneurons exclusively by virtue of their electrotonic effects on impulse initiation sites in the initial segment region of the soma. Absence of spike generation in dendrites of frog spinal motoneurons was inferred from these data.[65,66]

The failure of membrane hyperpolarization to increase the amplitude of EPSPs has been observed in the monosynaptic EPSP of cat motoneurons,[68,69] the EPSPs evoked in neocortical neurons by stimulation of nonspecific thalamocortical projections,[70,71] the monosynaptic EPSPs elicited in red nucleus neurons by stimulation of corticorubral axons,[72] and the monosynaptic EPSP of ventrobasal neurons activated by lemniscal stimulation[73] (Figure 9). The distribution of synapses involved in monosynaptic EPSP of cat motoneurons[74,75] and the anatomical pathway involved in the nonspecific evoked responses of neocortex are problems that have not been sufficiently clarified. However, there is good histological evidence for a predominantly dendritic distribution of lemniscal afferents on thalamic ventrobasal neurons[76] and for corticofugal axons on neurons of the red nucleus.[72] Thus, the lack of effect of membrane hyperpolarization on EPSPs would appear to reflect the remote location of the activated synapses in dendrites.[67] Alternative explanations involving nonchemically mediated PSPs[69,77-80] or the development of membrane reactions to hyperpolarizing currents[33] that could alter the linear relations between membrane potential and EPSP amplitude are entirely feasible, but are not as yet supported by adequate data in the case of mammalian neurons.[81]

The preceding discussion of the effects of membrane hyperpolarization on PSPs emphasizes the manner in which intracellular stimulating and recording techniques are beginning to supply information concerning the synaptic organization of pathways to different types of neurons in the mammalian brain. These results suggest that the development of particular response patterns in central neurons has little to do with the anatomical configuration of dendrites of a neuron or with its so-called dendritic potentials. *Rather, the major determinant of response patterns relates to the nature and distribution of PSPs in a particular neuron.* This is most clearly illustrated by comparison of the different PSP patterns observed in neurons in three fundamentally different types of neuronal organizations during their participation in a common variety of activity. The sample records of Figure 10 show the PSP sequences observed in thalamic neurons[82,82a,83] (Figure 10A) caudate neurons[84] (Figure 10B), pyramidal neurons of motor cortex[60] (Figure 10C), and cortical interneurons[60] (Figure 10D) during evoked synchronization of neural activity in these structures produced by low-frequency stimulation of medial

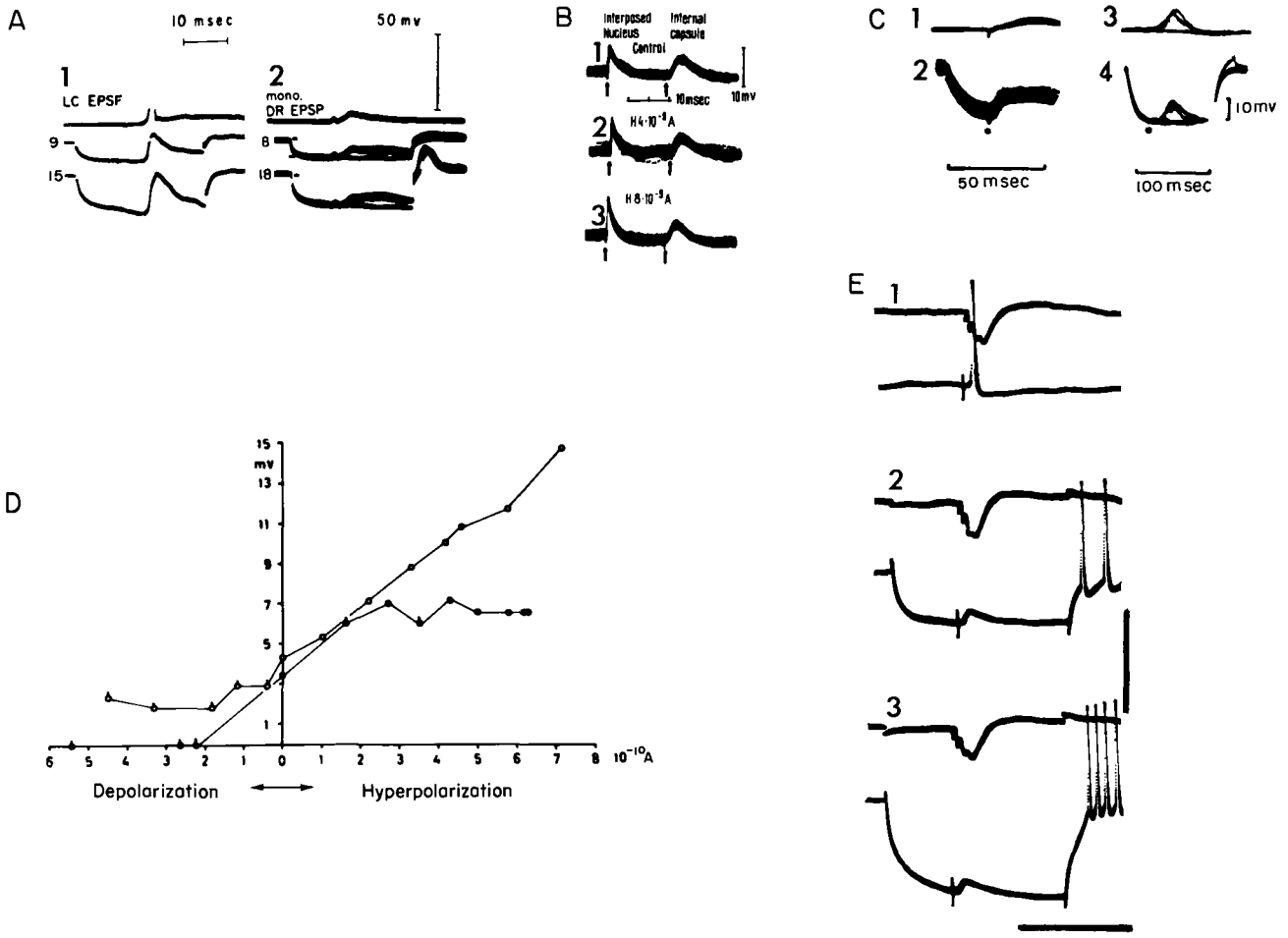

FIGURE 9 A: Comparison of effect of hyperpolarization on EPSPs in frog motoneurons. In each column top record illustrates the unconditioned response; lower records illustrate responses recorded during hyperpolarization induced by intracellular currents of the values shown in microamperes. 1, LC-EPSP: lateral column volleys initiated EPSP which underwent an increase in amplitude during hyperpolarization. 2, monosynaptic-DR-EPSP: in a different cell (resting membrane potential, 48 mv) the earliest portion of the complex EPSP was due to monosynaptic excitation. During hyperpolarization this component remained unchanged, whereas the later, polysynaptic EPSPs were augmented. Each record in 2 is made from four superposed responses. Values indicated $\times 10^{-9}$ A. (Modified from Kubota and Brookhart, Note 67)

B: Different effects of membrane hyperpolarization on EPSPs evoked in cell of the red nucleus by stimulation of n. interpositus and internal capsule. 1, control; 2, during passage of hyperpolarizing currents of 4×10^{-9}A; and 3, 8×10^{-9}A. Records taken by superposition of 20 faint traces. Note increase in EPSP evoked by interposed nucleus stimulation and lack of effect of membrane hyperpolarization on EPSPs evoked by internal capsule stimulation. (Modified from Tsukahara and Kosaka, Note 72)

C: Enhancement of "augmenting" EPSP (1 and 2) in a motor cortical cell during hyperpolarizing currents. 3 and 4, lack of effect of current injection on recruiting EPSP in the same unit. Each trace shows superposition of 20 EPSPs of a long lasting 6/sec stimulation in the ventrolateral thalamic nucleus (1 and 2), and in center median nucleus (3 and 4). Controls, 1 and 3; current injections in 2 and 4 were about 2×10^{-9} A. (Modified from: Lux, Note 71)

D: Plot of EPSP amplitude against current strength. Open circles: ventrolateral EPSPs, closed circles: center median EPSP. A short line on circle indicates that the firing level was reached by the respective EPSP. (Modified from Creutzfeldt and Lux, Note 70)

E: Lack of effects of induced membrane hyperpolarization of a neuron in the ventrobasal thalamic nucleus activated by medial lemniscus stimulation.

1, Control cortical surface (upper channel) and VB cell response.

2 and 3, Two different intensities of current injection. Membrane hyperpolarization to 20–30 mv above resting levels does not alter the amplitude of the EPSP. Cals. 50 mv; 50 msec. (From Maekawa and Purpura, Note 73)

thalamic regions. It will be recalled that the dendritic patterns of the cell types in these different structures are markedly different (cf. Figure 1). Nevertheless, the PSP patterns produced in these cells are remarkably similar, with the exception of the inhibitory PSPs and intense excitatory drives observed in thalamic neurons.[82,83] It is probable that no two cell types could be more remotely different in respect to their dendritic patterns than the large pyramidal neurons of neocortex (Figure 1A) and the neurons of the caudate nucleus (Figure 1F). Relatively large-amplitude EPSPs, as well as similar discharge patterns, are usually observed in both types of neurons during thalamocortical recruiting responses. *Thus the temporo-spatial characteristics of PSPs are the sole determinants of the potentials observed in extracellular recordings from different elements not of the size or geometrical orientation of their dendrites.*

Partial spike responses in dendrites

The concept that dendrites of vertebrate neurons function chiefly as integrators of synaptic activities that electrotonically influence impulse trigger sites in the region of the soma and initial axonal segment has been repeatedly substantiated in studies of a wide variety of cells (cf. references to intracellular studies in Note 85). Some exceptions to this have been observed in special situations, and it is instructive to consider these in respect to the problem of the potentiality for spike generation and propagation in dendrites.

It has already been noted that earlier extracellular studies of brain stem and spinal motoneurons had suggested that antidromically propagating responses invade some portion of the dendritic tree.[86,87] Recent intracellular record-

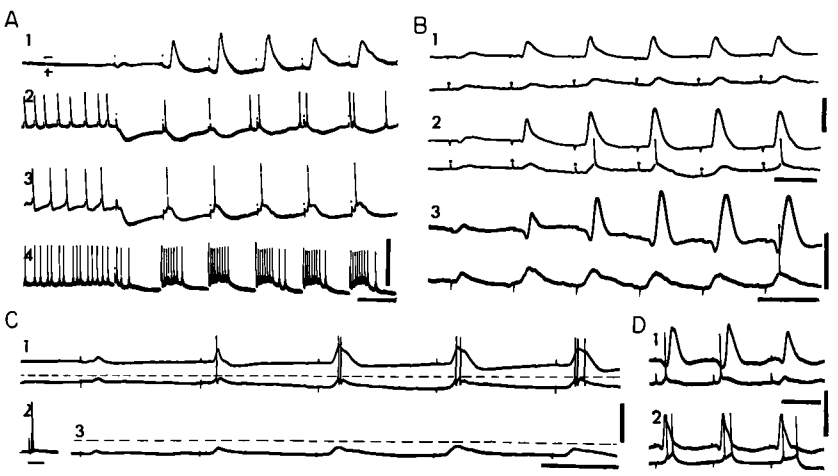

FIGURE 10 Patterns of evoked synaptic activities observed in intracellular recordings from different types of neurons during thalamocortical synchronization induced by low-frequency (7–10/sec) stimulation of medial nonspecific thalamic nuclei. Calibrations throughout, 50 mv for intracellular records; 100 msec.

A: 1, example of surface-negative cortical recruiting response. 2–4, EPSP-IPSP sequences recorded in thalamic neurons in different nuclei during recruiting responses. IPSPs, prominent in thalamic elements during this type of activity play a major role in synchronizing thalamic neuronal discharges. (From Purpura and Shofer, Note 83)

B: Upper channel records, recruiting responses from motor cortex; lower channel records, intracellular activities of caudate neurons. 1, threshold stimulation elicits prominent EPSPs in a caudate neuron. 2, increase in stimulus strength produces cell discharges. 3, cell discharges are rare in caudate neurons despite large amplitude EPSPs during recruiting responses. IPSPs are not common in contrast to findings in thalamic neurons. (From Purpura and Malliani, Note 84)

C: Characteristics of EPSPs elicited in a pyramidal neuron of motor cortex during recruiting response. 1, EPSP builds up with repetitive stimulation. 2, antidromically evoked response (cal, 10 msec). 3, slow increase in membrane potential reduces the effectiveness of EPSPs. Dashed line through "firing level" as indicated in 1.

D: PSP patterns and associated cell discharges in two cortical interneurons. These elements generally exhibit a larger number of spike discharges than large pyramidal cells during recruiting responses. (From Purpura, Shofer and Musgrave, Note 60)

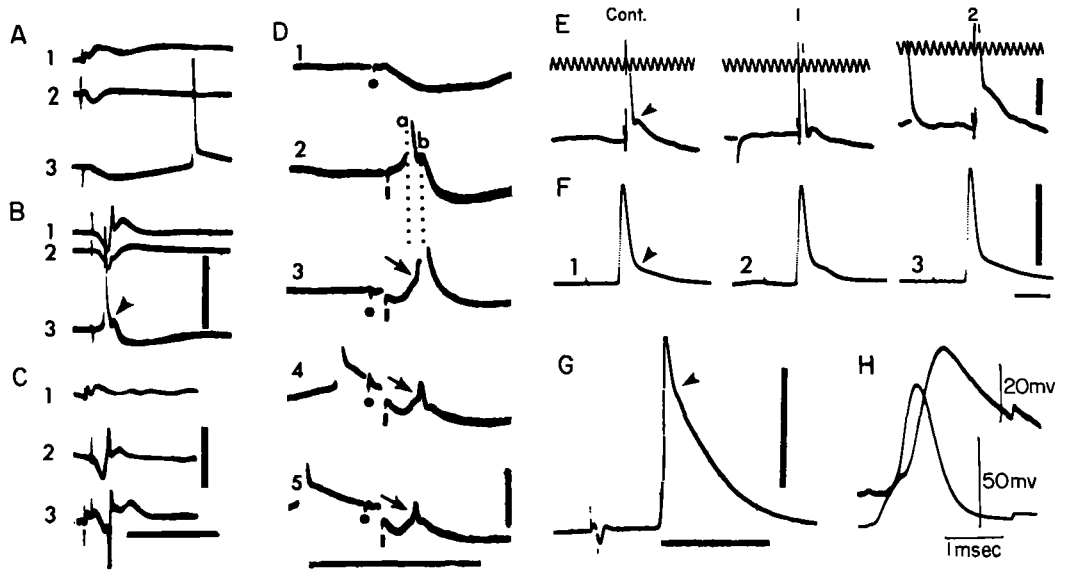

FIGURE 11 A–D, Alterations in mode of firing of a hippocampal pyramidal neuron. A: 1 and 2, surface and extracellular activities recorded from the cell body region of a hippocampal neuron. 3, intracellular response to stimulation of fimbria. Prolonged IPSP is followed by spike potential with a prominent depolarizing after-potential. B: Responses to subiculum stimulation as in A. Note (at arrow head) subiculum stimulation elicits a spike that is succeeded by a depolarizing notch and an IPSP. C: 1, unconditioned response as in A 1; 2, as in B 1; 3, change in polarity of extracellular recorded spike evoked by subiculum when the subiculum stimulus is preceded by fimbrial volley. This is indicative of a change in the mode of impulse propagation. D: Details of intracellular activities observed during events such as that in C 3. 1, conditioning fimbrial-evoked IPSP; 2, testing subiculum evoked response as in B 3; a refers to EPSP triggering cell discharges; b is the delayed depolarizing notch. 3, fimbrial evoked IPSP depresses the EPSP, but cell discharge is secured by a rapid depolarizing prepotential which is temporally related to delayed depolarizing notch of unconditioned response. 4 and 5, partial response is seen in isolation (at arrows) when spike discharge is blocked by IPSP and prior spontaneous discharges. Calibration B 3, 50 mv; C, 5 mv; 50 msec. (From Purpura, Note 99)

E: Antidromic spikes of spinal motoneurons to show the influence of membrane potential changes on delayed depolarization (at arrow head). Control 1, depolarizing current 1.47×10^{-9} A; 2, hyperpolarizing current 3.54×10^{-9}A. Time in msec. Amplitude cal., 10 mv. (Modified from Granit, Kernell and Smith, Note 88)

F: Antidromic responses of a pyramidal neuron of motor cortex. 1, control response with a delayed depolarization; 2, during anodal polarization of cortical surface; 3, during cathodal polarization. Note changes in delayed depolarization during soma depolarization (2) and hyperpolarization (3) by surface-applied polarizing currents. Cal., 50 mv; 5 msec. (From Purpura and McMurtry, Note 61)

G: Intracellular potentials recorded from a preganglionic neuron (T 3 and T 4 level) activated by a single antidromic stimulus. Hump on the falling phase of the spike (at arrow head) may be result of sustained changes in ionic conductance in the soma or the electrotonic recording of impulses which have propagated into the adjacent dendrites. Cal., 50 mv; 5 msec. (Modified from Fernandez de Molina, Kuno and Perl, Note 89)

H: Intracellular recordings from a single motoneuron with two parallel-type microelectrodes. The upper channel record is presumably from the region of proximal dendrites, whereas the lower record is from the cell body. The "dendritic potential" is delayed and has a prolonged falling phase. (From Terzuolo and Araki, Note 90)

ings from spinal motoneurons[88] have revealed that under appropriate conditions antidromically evoked spikes may exhibit a prominent delayed depolarization in the form of a notch, hump, or plateau (Figure 11E). Similar components of delayed depolarization have been observed following antidromic stimulation of pyramidal neurons of neocortex[61] (Figure 11F), and extraordinarily large and prolonged delayed depolarizations have been noted in sympathetic preganglionic spinal cord neurons[89] (Figure 11G). A dendritic origin of these delayed depolarizations has been inferred from studies of the relationship of extracellular and intracellular potentials, effects of membrane potential variations,[88] and in very rare instances by recording with two parallel microelectrodes from cell body and proximal portions of dendrites of spinal motoneurons[90] (Figure 11H). In the latter instances, spike potentials smaller in amplitude but longer in duration and with delayed peaks were observed from "dendritic" sites of impalement in contrast to soma sites.

The question of how much dendritic membrane is invaded by an antidromically propagating spike has not been satisfactorily answered in the case of spinal motoneurons or cells of the mammalian brain. In large, easily accessible elements, such as Mauthner cells, it is clear that antidromic invasion of dendrites does not occur.[91] Studies of the distribution of extracellular potentials of single spinal motoneurons have also been interpreted as evidence against extensive invasion of dendrites by antidromically propagating spike potentials.[92] However, modifications of these potential fields by orthodromic activities has suggested that synaptic depolarization of soma-dendritic membrane may facilitate antidromic invasion of the spike potential.[93] The problem of defining the contribution of passive and active membrane sites to extracellularly recorded responses of neurons with different dendritic patterns has raised many issues concerning "dendritic spike propagation" in some elements where propagation has been inferred from extracellular laminar studies.[94,95] Those hypotheses concerning spike conduction in dendrites that have been based exclusively on latency measurements of evoked responses are likely to require close re-examination in view of the possibility that such results may reflect passive electrotonic spread in dendrites.[92,96]

Probably a question of greater functional significance than that concerning the extent of dendritic membrane involved in antidromic spike propagation is whether, under appropriate conditions of synaptic activation, partial or conducted spikes might occur in dendrites. Data are now available that indicate this to be the case in some types of neurons.

The first observations on the possible dendritic origin of partial spikes recorded from the cell body were made in chromatolyzed spinal motoneurons.[97] In these elements monosynaptic EPSPs not infrequently initiate a series of small partial responses that are interposed between the EPSP and the spike potential (Figure 12A). Partial responses designated fast prepotentials (FPPs) have also been reported during spontaneous or subiculum-evoked responses in about 25 per cent of hippocampal neurons[98] (Figure 12B). It has been proposed in accordance with the suggestion of Eccles, et al,[97] that such FPPs arise in the dendritic tree.[98] To account for the small amplitude of FPPs and the fact that they are not blocked by hyperpolarizing currents sufficient to block soma spikes, it has also been necessary to postulate conduction block of the dendritic spike somewhere in the region between its point of origin and the cell body. Partial dendritic spikes or FPPs are considered evidence that "patches" of electrically excitable membrane do exist within the dendritic trees of these particular cells (hippocampal neurons) and that the patches act as booster zones for otherwise ineffectual distal dendritic synapses."[98]

The finding of partial responses or FPPs in chromatolyzed spinal motoneurons and hippocampal neurons has spurred an intensive inquiry into the factors influencing the appearance of partial spikes in dendrites. In contrast to the frequent occurrence of FPPs in hippocampal neurons, similar types of responses have been encountered only rarely in neocortical neurons of adult animals.[64] Of particular interest in the studies of neocortical neurons is the finding that when spikes are triggered by FPPs no inflection is detectable on the rising phase of the spike (Figure 12C-1), whereas when the spike potential is triggered by a spontaneous EPSP an inflection is clearly evident (Figure 12C-2). It appears from this that the FPP can electrotonically activate soma membrane before the initial axonal segment region, as in the case of chromatolyzed motoneurons.

Two additional situations may be cited in which FPPs have been observed; in intracellular recording from neurons of immature neocortex (Figures 12D and E) and following prolonged weak anodal polarization of neocortex of adult animals (Figure 12F).

During recordings from immature neocortical neurons slow increases in membrane polarization frequently result in the appearance of FPPs that trigger spike potentials at a firing level similar to that of slow EPSPs.[44] The appearance of FPPs in immature neurons is illustrated in Figure 12E. The spike potential in these records was triggered by an EPSP under "normal" conditions (Figure 12E-1-a). Injection of a weak hyperpolarizing current resulted in the appearance of a second, more rapid depolarizing prepotential that was superimposed on the EPSP (Figure 12E-2-b) as in studies of hippocampal neurons (Figure 12-B-3). Fail-

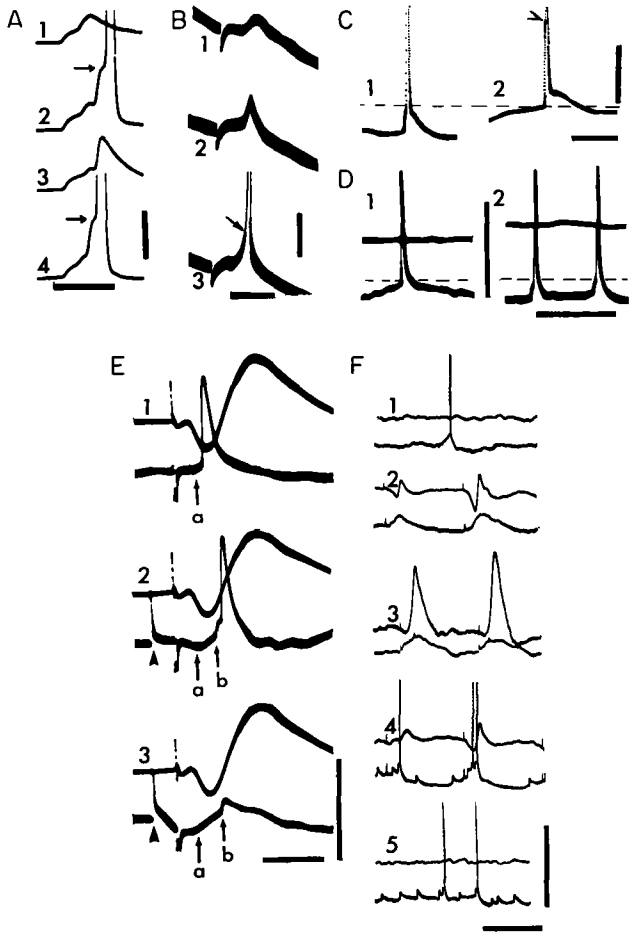

FIGURE 12 A: Records showing partial responses and the discharge of impulses from chromatolyzed motoneurons. 1 and 2, responses to show the wide variability of partial responses superposed on EPSPs. Partial responses and EPSPs evoke full spikes when the depolarization attains the critical level (about 13 mv) shown by the arrow in 2 and 4. Calibration 10 mv; 5 msec. (Modified from Eccles, Libet and Young, Note 97)

B: Orthodromic production of fast prepotential (FPP) in a hippocampal pyramidal neuron following single shock subiculum stimulation. Responses were recorded during passage of a strong (approx. 1×10^{-9}A) hyperpolarizing current which distorts baseline. 1, complex subliminal synaptic potential. 2, FPP in isolation on crest of the synaptic potential. 3, orthodromic spike with FPP indicated by diagonal arrow. Calibration 10 mv; 10 msec. (Modified from Spencer and Kandel, Note 98)

C: 1, spontaneous spike triggered by an FPP in a neocortical neuron of an adult animal. The spike potential was approximately 75 mv in amplitude. 2, spontaneous EPSP triggers a spike potential in the same cell without an FPP. Note that when the cell is fired by a spontaneous slow EPSP an inflection is clearly evident on the rising phase of the truncated spike but this inflection is absent when the spike is triggered by the FPP. Calibration 20 mv; 20 msec. (From Purpura and Shofer, Note 64)

D: Spontaneous discharges recorded in an immature neocortical neuron from a five-day-old kitten. 1, a spike potential is triggered by a slow depolarizing prepotential. 2, during spontaneous increase in membrane polarization spikes are triggered by fast depolarizing prepotentials (FPPs). Calibration 50 mv; 100 msec.

E: Appearance of partial responses during induced soma hyperpolarization in a neuron from a 24-day-old kitten. 1, ventrolateral thalamic stimulation elicits a 5 msec latency small EPSP (at arrow, a) and cell discharge. 2, during soma hyperpolarization (at arrowhead) the EPSP is augmented and a second component is revealed (b) which is succeeded by a spike potential equal in amplitude to that shown in 1. 3, the spike potential is blocked by the induced soma hyperpolarization and the EPSP and partial responses are seen in isolation. Compare (3) with A 2 and B 3. Calibration 50 mv; 20 msec. (D and E modified from Purpura, Shofer, and Scarff, Note 44)

F: Development of fast prepotentials in an unidentified neuron in the motor cortex of an adult cat after prolonged weak surface anodal polarization. 1, control spontaneous discharge. 2, cortical surface and intracellular activities during repetitive ventrolateral thalamic stimulation prior to polarization. 3, alteration produced during anodal polarization. 4, after-effects of anodal polarization. Note facilitatory action of polarization seen in the increased effectiveness of thalamic stimulation. Many FPPs are detectable, especially during spontaneous activity (5). Calibration 50 mv; 100 msec. (From Purpura and McMurtry, Note 61)

ure of spike generation revealed the partial response in isolation (cf. Figure 12E-3 and 12B-3).

In rare situations partial spikes have been induced in neocortical neurons during weak anodal polarization (Figure 12F). This suggests that in some elements hyperpolarization of dendritic terminals in superficial cortical regions and depolarization of proximal portions of dendrites (cf. Figure 7D, diagram, upper left) results in a focus of augmented excitability somewhere in the dendrites, with subsequent development of partial, electrotonically propagated responses. That surface-applied polarizing currents may, in exceptional cases, alter the excitability properties of dendrites should be borne in mind when considering the complex effects of extracellular polarizing currents as noted above and elsewhere in this volume.

The demonstrations of FPPs in "occult form"[98] have provided clues to the possible relationship of delayed depolarizations in some hippocampal neurons to partial responses of dendrites.[99] An experiment in which this hypothesis was tested is illustrated in Figure 11A-D. In this study fimbrial stimulation was employed as a conditioning stimulus to elicit an IPSP in the soma of a hippocampal

neuron (Figure 11-A-3). The testing stimulus to the subiculum elicited a spike that arose from an EPSP and was followed by a depolarizing notch that preceded an IPSP. Suitable timing of the intervals between conditioning and testing stimuli resulted in a change in the mode of firing caused by an FPP superimposed on the EPSP (Figure 11D-2 and -3). The appearance of the FPP was temporally related to the delayed depolarizing notch observed in the unconditioned response. These findings suggest that with synaptic activation of a large portion of the soma-dendritic membrane of hippocampal neurons, EPSPs ordinarily initiate discharges in the usual fashion.[100] When soma-EPSPs are depressed by IPSPs, activation of dendritic loci may permit cell discharge to be attained by the FPP. It is of interest that in the experiment illustrated in Figure 11 this change in the mode of spike initiation was associated with a dramatic alteration in the polarity of the extracellularly recorded spike (cf. Figure 11C-2 and -3). It would appear that cells such as hippocampal neurons and immature neocortical neurons, which exhibit a marked potentiality for partial spike generation in dendrites, may reveal this polarity change under conditions in which the "primary" impulse trigger sites are depressed by axosomatic IPSPs or other mechanisms. These factors may also lead to the development of dendritic spikes that propagate in an all-or-none fashion into the soma.

Spike generation and propagation in dendrites

Results that have been interpreted as evidence for the development of conducted responses in dendrites of immature neocortical neurons are shown in Figure 13.[44] In this

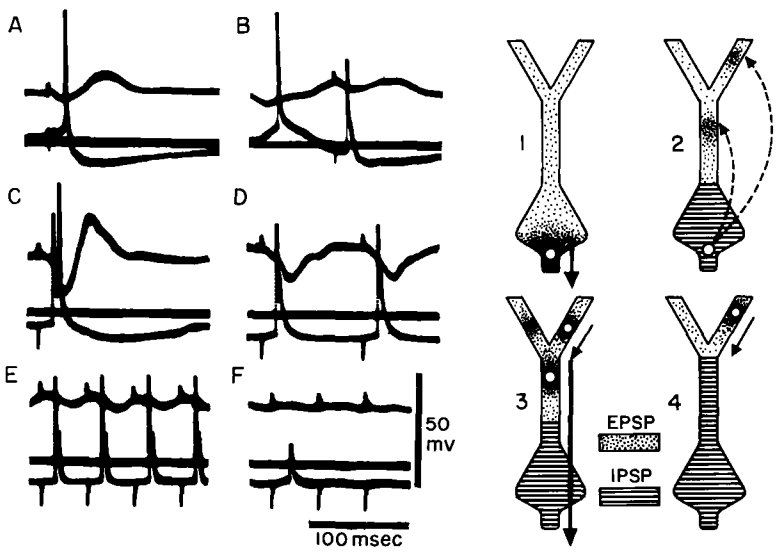

FIGURE 13 Impulse initiation and propagation in dendrites of a neuron from a 21-day-old kitten. Upper channel record, cortical surface evoked response to ventrolateral thalamic stimulation. Third beam of oscilloscope was placed at the "resting" membrane potential level established in the absence of stimulation. A: Single shock thalamic stimulus evokes a small EPSP which triggers a "normal" spike potential. The latter is succeeded by an IPSP. B: A spontaneous discharge initiated by a slow EPSP precedes the stimulus. When the stimulus occurs on the small succeeding IPSP, the evoked spike is smaller in amplitude and arises from a lower firing level. C: Early phase of 5/sec thalamic stimulation during which IPSP-summation leads to sustained membrane hyperpolarization. Spike potential is evoked with a shorter latency than in A. Spike arises directly from the baseline and exhibits a rapid rise time. Note second component on shoulder of first spike. D and E: Increase in stimulus frequency results in reduction of second component and finally in F, failure of all-or-none spikes and appearance of partial response whose latency is similar to the second component of spikes in E. Diagrams to the right show: 1, original conditions similar to those in A when EPSPs trigger impulse at normal spike initiation sites (white dot); 2 IPSPs produce transitory shift in impulse trigger site to dendrites; 3 repetitive stimulation as in C–E leads to depression of primary impulse trigger site by IPSPs and activation of dendritic loci by axodendritic EPSPs. Large arrow indicates all-or-none propagating spike. Small arrow represents partial spike. 4, conditions such as that in F at a stage when partial response arising in distal dendritic site is incapable of initiating conducted dendritic spike. (Modified from Purpura, Shofer and Scarff, Note 44)

experiment, stimulation of the ventrolateral thalamus in a three-week-old kitten resulted in the production of an EPSP in a somatosensory cortex neuron that triggered cell discharge in the usual fashion (Figure 13A). The spike potential was succeeded by an IPSP. In some instances, a spontaneous spike preceding the stimulus might be followed by an IPSP during which stimulation elicited a smaller spike that was initiated at a lower firing level (Figure 13B). It is important to emphasize that long-duration IPSPs in immature neocortical neurons exhibit a remarkable capacity for summation during repetitive stimulation.[44] During this IPSP-summation, spike potentials may be observed that arise directly from the baseline of membrane hyperpolarization. Such spikes exhibit rapid rise times (Figure 13C) and second components, or partial responses, which become more obvious with increasing stimulus frequency (Figure 13D-F). The diagrams of Figure 13 show in schematic form the possible sequence of events underlying the alterations in synaptically evoked spikes in Figure 13A-F. Initially EPSPs are generated in the soma-dendritic membrane, and these trigger cell discharge at the primary spike generating site in the soma-initial axonal segment region (Figure 13-1). During the IPSP there may be a tendency for secondary (or tertiary) sites of impulse initiation (Figure 13-2). With the buildup of IPSPs and depression of primary impulse trigger sites, foci in dendrites become active, because of increasing axodendritic excitatory synaptic drives. Spikes initiated in dendrites propagate into the soma in an all-or-none fashion. Consequently, they lack the usual depolarizing prepotentials (Figure 13-3). Finally, a stage may be reached in which are observed only partial responses that fail to propagate from dendrites into the soma.

These and other data indicate that there are two factors that condition the appearance of orthodromic spikes without prepotentials in immature neocortical neurons: (1) The suppression of primary impulse trigger sites; and (2) the sustained excitatory bombardment by well-developed axodendritic synaptic pathways that leads to impulse initiation in dendrites (cf. Figure 4).[34] These factors have been sought in other experimental designs, particularly those involving neurons that are activated by powerful excitatory axodendritic synaptic pathways and have a tendency to exhibit FPPs, e.g., hippocampal neurons of adult animals.[98]

Recent intracellular studies of hippocampal neurons have furnished additional evidence that dendrites of these neurons are potentially capable of generating and conducting spike potentials as a consequence of repetitive activation of axodendritic synaptic pathways and suppression of primary impulse initiation sites.[85] In order to demonstrate these spike potentials it is required in most instances that such stimulation induce a form of seizure activity in which marked depolarizing or hyperpolarizing shifts of soma membrane potential do not occur. At a stage in the buildup of this seizure activity, extraordinarily large extracellular field potentials (10 to 20 mv) representing the synchronized discharges of large numbers of hippocampal neurons are observed.[85] Such field spikes are seen in intracellular recordings as negative deflections associated with spikes that lack depolarizing prepotentials (Figure 14). Spikes triggered by EPSPs as well as orthodromic spikes that arise directly from the baseline are observed (Figure 14G) during recovery phases from such seizures.

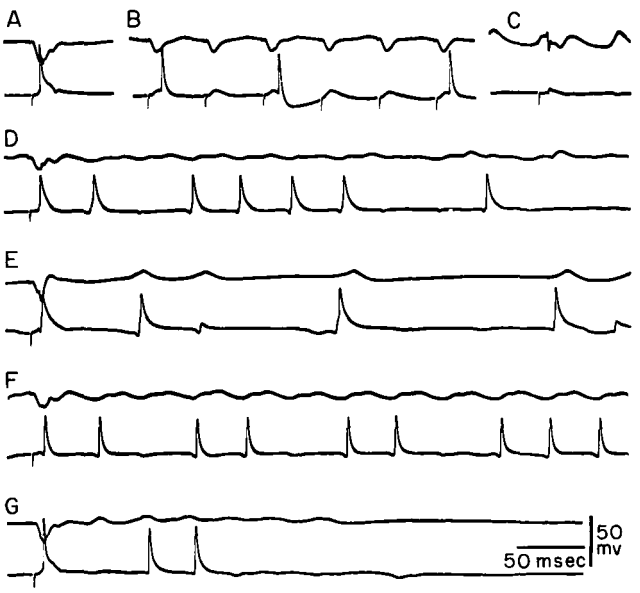

FIGURE 14 Changes in spike potentials of a hippocampal neuron during and after repetitive stimulation of the temporoammonic pathway in the subiculum. Upper channel records, recordings from exposed ventricular surface. A: Control subiculum evoked response. B: Repetitive subiculum stimulation. Note failure of many EPSPs to trigger cell discharges. C: Following cessation of repetitive stimulation and build-up of seizure activity. Subiculum shock fails to evoke a response. D–F: Various stages of seizure activity during which spontaneous spikes without depolarizing prepotentials are observed. Such spikes are associated with prior negativities which represent the intracellular reflection of summated synchronized discharges of nearby hippocampal neurons. Occasional failures of spikes reveal these deflections as well as partial responses in isolation. G: Early phase of recovery of subiculum evoked response. Spike is triggered by an EPSP. Two spontaneous discharges also shown arise directly from the baseline without depolarizing prepotentials. (From Purpura, et al., Note 85)

In several instances it has also been possible to demonstrate the transition from one mode of excitation of hippocampal neurons involving FPPs to another involving conducted responses in dendrites, particularly in elements exhibiting prominent and prolonged depolarizing afterpotentials (Figure 15).

The tendency for dendrites of hippocampal neurons to develop conducted spikes appears to be a satisfactory explanation of the markedly different effects observed in these elements during the action of applied polarizing current[101] as in experiments on neocortical neurons.[85] It is to be recalled that when "viewed" from the exposed ventricular surface, hippocampal pyramidal neurons are "upside down," i.e. with cell bodies close to the ependymal surface. This inverted orientation of hippocampal pyramidal neurons is responsible for the finding that cathodal polarization of the ventricular surface depolarizes basilar dendrites and cell bodies and hyperpolarizes apical dendrites, whereas anodal polarization of the ventricular surface produces opposite effects (Figure 16). These overt effects of transhippocampal polarizing currents are opposite to those observed following polarization of the neocortical surface (cf. Figure 6). The differences in orientation of hippocampal and neocortical neurons in the direction of applied currents accounts for the different effects these currents produce on soma membrane potential. Additionally, the capacity for applied depolarizing currents to evoke spikes in dendrites of hippocampal neurons appears to be a major factor in determining the spike potential changes observed during hippocampal polarization (Figure 16).

The foregoing survey indicates that a single model of "dendritic function" cannot satisfy the increasing volume of data obtained in studies of neurons in different organizations and under different conditions of activity. There is

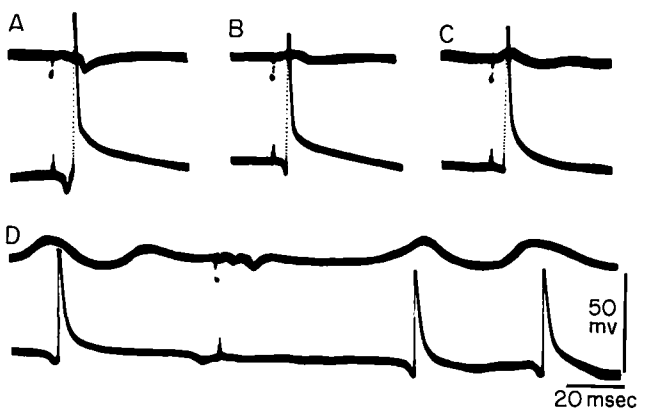

FIGURE 15 Spike potential changes in a hippocampal neuron during and after repetitive subiculum stimulation. A: Single shock subiculum stimulation elicits a field response which is seen as a negative deflection intracellularly. A rapid depolarizing prepotential is produced which triggers a spike discharge. The latter is succeeded by a prolonged depolarizing after-potential. B: During repetitive stimulation spikes, which are elicited on declining phases of depolarizing after-potential of a preceding discharge, lack the fast prepotential. C: With continued stimulation an orthodromic spike without a depolarizing prepotential is observed, indicating conduction into the soma from dendritic sites. D: "Spontaneous" discharges with negative deflections preceding spikes which lack depolarizing prepotentials. These discharges were initiated during seizure activity as noted in upper channel hippocampal surface records. (From Purpura, et al., Note 85)

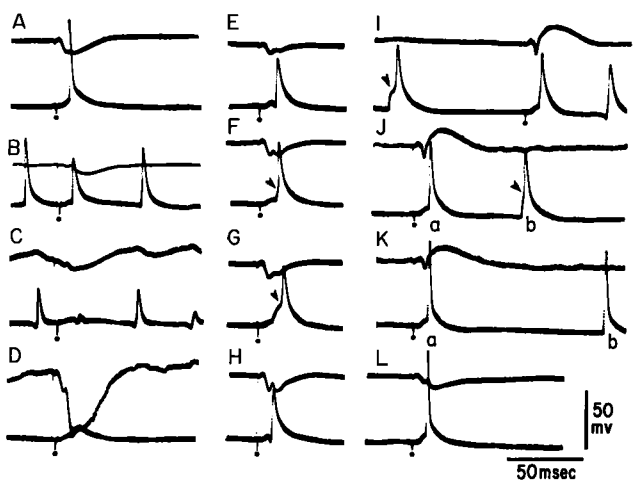

FIGURE 16 Effects of transhippocampal polarizing currents on intracellular potentials of a hippocampal pyramidal neuron. A: Control evoked response to subiculum stimulation. B: Changes produced during early cathodal polarization. Onset of spikes without depolarizing prepotentials are evident during induced seizure activity. C and D: Continued cathodal polarization leads to spike attenuation and the blockade of impulses. E–H: Progressive recovery of responses following cessation of cathodal polarization. Note transitional changes and spikes with a variety of prepotentials and inflections. Recovery is facilitated in I by the onset of anodal polarization. Anodal polarization is continued in J and K. A spontaneous spike exhibits a prominent inflection. Firing level differences are also evident in J and K between evoked (a) and spontaneous (b) spikes. The firing level difference is indicative of two different sites of impulse initiation of the orthodromic spikes. It is inferred that the orthodromic spikes without depolarizing prepotentials are propagated into the soma from remote dendritic sites of origin. L: Recovery after cessation of anodal polarization. (Modified from Purpura and Malliani, Note 101)

little doubt that in respect to their electrogenic properties, dendrites of the vast majority of neurons in the vertebrate central nervous systems do not behave like axons or, for that matter, like cell bodies. But whether such behavioral differences are largely referable to geometrical factors that determine cable properties, intrinsic membrane differences, or both, remains largely unclarified.

There are reasons for assuming that the functional properties of dendrites in the mammalian brain are a consequence of ontogenetic changes that have operated to "suppress" spike electrogenesis in dendrites. Findings of spike generation and conduction in dendrites of immature neurons grown in tissue culture[102] and of neurons in immature neocortex[44] suggest that in early periods of development, when neurons exhibit largely bipolar characteristics and few synaptic contacts, multiple sites for impulse initiation may exist. "Restriction" of spike initiation sites to soma-initial axonal segment regions is probably an acquired property in most mature neurons. But it is likely that under appropriate conditions activation of dendritic loci for impulse initiation may also occur in some mature neurons. On the other hand, there is nothing to indicate that dendrites of multipolar neurons of the vertebrate brain ever exhibit excitability properties such as those that characterize the dendrites of some bipolar sensory neurons of crustacea.[103]

Excitability modulation of low-threshold, impulse-initiation sites in soma-initial axonal segment regions has long been considered a major functional property of dendrites of neurons with elaborate dendritic trees.[2,6,32] It is known that the site of impulse initiation may progressively shift to more proximal regions of some multipolar sensory neurons in invertebrates during intense dendro-somatic depolarization induced by stretch activation.[56] In similar fashion, it is possible that under conditions of intense axodendritic synaptic activation, conducted spikes may arise in dendrites of some vertebrate neurons. In this context, it is of interest that in those experimental situations in which impulse initiation has been suspected in dendrites of mature mammalian neurons,[85,104,105] anatomical evidence has indicated the operation of relatively powerful axodendritic excitatory synaptic pathways in the production of dendritic spikes. Thus, the factors of strategic location of excitatory synapses[3] may be as important as geometrical and intrinsic membrane characteristics in determining "dendritic function."

The notion that dendrites constitute "94 per cent of the story"[106] of the brain is not without significance for the interpretation of many complex "higher nervous activities." That dendrites provide more than 94 per cent of the available surface for synaptic contacts between neurons satisfactorily accounts for the predominant role of axodendritic synaptic activities in the production of many electrocortical phenomena.[2]

The different properties of dendrites discussed here have been based on considerations of dendrites as "integrators" of excitatory and inhibitory synaptic activities initiated by conventional presynaptic elements (i.e., axons). Recent suggestions of electrical interactions between dendrites of frog spinal motoneurons[79] and dendro-dendritic synaptic interactions in the olfactory bulb[107] raise the question whether these singular modes of dendritic activation may, under special conditions, also be set into operation in other central structures.

No attempt has been made here to explore the possible existence of nonsynaptic, metabolically driven, "pacemakerlike" activities in dendrites of neurons in the vertebrate brain. Cyclic variations in membrane potential are detectable in many types of central neurons of vertebrates, particularly in structures involved in rhythmically recurring events. For the most part, such rhythmicities have been analyzed in terms of organizational features of synaptic activities.[60,82,83] However, it would be surprising indeed if alterations in intrinsic metabolic machinery and extraneuronal influences on extensive dendritic ramifications did not contribute to some of the periodic behavior of neurons involved in various integrative activities of the brain.

Summary

Data obtained in the past decade have provided considerable information on the properties of dendrites, their role in the regulation of neuronal excitability, and their contribution to the production of spontaneous and evoked electrical activities of brain. Current views concerning the functional properties of dendrites are traced in relation to the development of concepts of graded response activities from different varieties of excitable cells. Studies of the ontogenesis of superficial axodendritic synaptic pathways of cerebral and cerebellar cortex, as well as information obtained in intracellular studies of neurons in different organizations, indicate that much of the electrical activity of the brain represents summated excitatory and inhibitory postsynaptic potentials generated at various loci in dendrites.

The extent to which dendrites participate in spike electrogenesis is considered in the analysis of orthodromic and antidromic spike potentials of neurons located at several neuraxial sites, studies of the effects of transmembrane and extracellular polarizing currents, and investigations involving neurons that exhibit partial spikes or fast prepotentials. It is concluded that the vast majority of dendrites in the mammalian CNS do not ordinarily exhibit spike

electrogenesis. Partial or conducted spikes in dendrites are inferred from studies of chromatolyzed spinal motoneurons, hippocampal neurons, some mature neocortical neurons, immature neurons of neocortex, thalamic somatic sensory relay elements, and Purkinje cells, under appropriate conditions of activity. In most of these neurons, spike initiation and propagation in dendrites is viewed as a possible mechanism for enhancing the efficacy of synaptic pathways distributed predominantly in relation to dendrites.

The Problem of Sensing and the Neural Coding of Sensory Events

VERNON B. MOUNTCASTLE

IN EARLIER CHAPTERS, special attention has been given to the subject of excitation and conduction in nerve fibers. The transmission of excitation between cells—synaptic transmission—was discussed by Dr. Grundfest (this volume) in terms of the special properties of presynaptic axon terminals and of the postsynaptic areas of nerve or muscle cells upon which they impinge, with special emphasis upon the neurosecretory functions of the one and the chemo-receptive properties of the other. Such a general paradigm fits synaptic actions known in mammalian brains, but Dr. Grundfest has rightly called attention to the fact that it is not universal, for in some animals cellular interactions are effected by the flow of extrinsic ionic currents independently of any special transmitter action, and both he and the parsimony of nature suggest that we look carefully for such cellular interactions in brains with large numbers of cells. Dr. Purpura has paid particular attention to the properties of the dendrites of central neurons, and more generally to the important integrative functions of the local nonconducted responses of nerve cells, and elucidated their relation to the impulsive, conductive properties of axons.

Thus the stage is set for study of central nervous function at the level of groups of cells, of large populations, of neural systems; indeed, of whole brains and behaving organisms. In this progression I now take up the problem of sensing and the coding of sensory input in primary afferent axons.

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It is obvious, but perhaps worth emphasizing, that our brains know nothing of the real world directly; we face it through sensory receptors, our experience of it is mediate, once removed, slightly delayed in time, an abstraction determined by the functional properties of receptors. There is no reason to believe that these latter are high-fidelity recorders of every aspect of the world about us, but that certain of its aspects are emphasized; others, in a relative sense, are neglected. The total input picture after receptor transformation is composed of information of several different types and of varying precision and detail:

(1) The quality of a stimulus is signaled with some certainty. This is later referred to as the principle of labeled lines, a development obviously of great survival benefit for organisms posessing it.

(2) The input patterns signal with accuracy the spatial position of a point stimulus, and the spatial extent and form of more complex ones; and we are spatially oriented and spatially efficient animals.

(3) The transformed input signal allows a precise estimation of the intensity of a stimulus when it can be compared directly with another, but performance is poor when we are asked to categorize a series of stimuli distributed along an intensive continuum.

(4) There is an especially elegant measure of the spatial translation of stimuli, of the speed and direction of movement, as well as the serial order and temporal patterns of stimuli; and we perform well behaviorally in the temporal domain.

Certain aspects of sensory encoding relevant to these general statements will appear in that which follows.

The primary event in the sensory process—the way in which stimuli from the external world elicit trains of

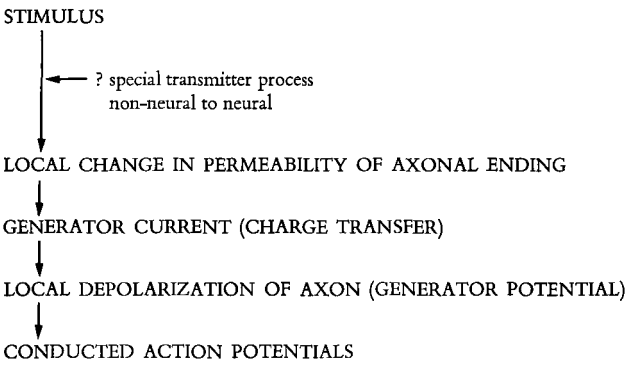


FIGURE 1 Schema outlining a probable chain of events in the excitation of peripheral nerve fibers by sensory stimuli.

nerve impulses in first order fibers—is an important subject in itself, and one which continues to receive the attention of many investigators. The general outline of events is shown in Figure 1. The generator process is not conducted, but invades adjacent regions of parent axons electrotonically; it can be summed in both space and time. When the depolarization produced by it in spike-generating membrane reaches threshold, conducted action potentials result. The sequence of events is remarkably similar to that in synaptic transmission. Indeed, one important recent finding is that in some cases the non-neural cells that surround peripheral nerve endings are related to them in a synapselike arrangement, and we must consider the proposition that the stimulus elicits from the non-neural cells the release of a transmitter agent, which in turn produces the permeability changes in the axonal membrane.

Definitions of terms used in sensory neurophysiology

It is important to make certain definitions. The terms sensory receptor, sensory ending, and sense-organ are used loosely and interchangeably by physiologists. More precisely, sensory endings or *receptors* are the peripheral endings of the first-order neural elements themselves. In some cases these end in close relation to specialized non-neural cells that play an important role in the sensory transducer process; for example, the hair cells of the cochlea, the specialized non-neural cells of the taste buds, certain organized endings within the skin, etc. These sensory endings possess *thresholds;* that is to say, there exist physically definable stimuli to which they do not respond. The strength of a stimulus just sufficiently strong to excite is a variable quantity, and is usually defined by some statistical estimator and considered in the context of decision theory.[1]

A *sensory unit* is a single primary afferent fiber, including all of its peripheral branches and central terminations. In a more extended sense, such a unit also includes all the non-neural transducer cells that are closely related to its ending. The definition of the *peripheral receptive field* of such a sensory unit is that area in space in which a stimulus of sufficient intensity and proper quality will evoke a discharge of impulses in the afferent axon. In a sensory surface such as the skin, the eye, or the ear, the peripheral branches of one sensory unit are intertwined with those of its neighbors to a considerable degree, and this overlap shifts gradually across the population as it moves across the sensory surface. This *principle of partially shifted overlap*[2] holds both for the peripheral endings of the first-order fibers and for the central distribution of their terminals upon the second-order neural population of the system. It has important implications for problems of coding: the code must be considered in terms of its distribution across a neural population, as well as over time in each element.

Confusion frequently exists as to the term *specificity* when applied to sensory receptors, sensory systems, and sensory experiences. A modern statement of the Mullerian law of specificity is to say that when active different sets of nerve fibers elicit different sensations by virtue of their central connections, and a given set therefore elicits an identical sensation no matter how activated. Such sets are differentially sensitive to stimulus quality. The importance of this selectivity for the problem of coding is that a given stimulus applied to a sensory sheet may activate only a certain fraction of the population and not others, emphasizing again the population problem in understanding sensory coding; and this case, the code for quality is the labeled line.

Receptors differ in the ways they respond to continuing stimuli. Some are called "quickly adapting" because they discharge only one or a small number of impulses at the onset of a steady stimulus, are silent during that application, and may discharge a second burst upon removal of the stimulus: these are motion detectors, and I shall later use one to illustrate coding in the temporal domain. In many sensory systems they make up a majority of the first-order afferents, and an increasing proportion of central neurons appear to be especially tuned to transients, the higher (i.e. the more central) the population considered. This fits with the exquisite capacity of humans to detect and evaluate transients, as against our comparatively much less precise analysis of steady states. Other receptors, termed "slowly adapting," respond to stimulus application with a high-frequency onset discharge whose peak

level and time course are functions of the rate of stimulus application. During a steady stimulus, moreover, they discharge at nearly regular rates, which are functions of stimulus intensity, and I shall later use one of these to illustrate coding for intensity. A general principle emerges; in many instances, the cascaded central chains of neurons composing a sensory system execute a differentiating operation upon the input to that system.

It is necessary now to define what neurophysiologists mean by the term I have just used—a *sensory system.* I do so at some risk, for considerable disagreement exists about it. Indeed, there can be no doubt that given the multiple interconnections of the large numbers of cells in a mammalian brain, the ongoing and powerful interplay between afferent and efferent systems, and the activity of those generalized thalamocortical systems that influence the excitability of the forebrain, an impulse in any afferent fiber has—in the limit—the capacity to influence the excitability of any central neuron anywhere. Yet order reigns: light flashes are not felt, nor pains heard. Such a functional segregation in subjective experience implies a corresponding anatomical one. It therefore seems reasonable to me to define as a sensory system all those interconnected central neuronal populations which receive their major input via one sensory portal and which, moreover, can on other grounds be shown to play an important role in the sensation considered. Thus, the visual cortex should certainly be classed as a part of the visual system, although it may receive input in addition to that from the eye. But the association areas of the posterior parietal and temporal lobes should not, for although of the greatest importance for the higher-order processing of visual input, they stand in the same relation to other inputs—auditory and somatic—as well. They occupy different positions in the hierarchy of neural populations.

The variety of neural codes

All the information transmitted between receptors and brain is carried by nerve impulses, which, being brief, discrete, all-or-none events, occupy only small durations of time compared to the intervals of time between them. They may thus be regarded as the individual items in the neural code. Let me first state the degrees of freedom available. There are undoubtedly other variants of those I list, and perhaps some fundamentals I have missed.

1) *The frequency code in single axons:* the proposition that a single axon signals changes in intensity of a stimulus by changes in frequency per unit time; the signal is thought to be independent, over a certain range, of the exact positioning of each impulse within that measuring time unit. You will see that the signaling of certain sensory attributes can be understood on the basis of this code.

2) *The frequency profile in a population:* the first proposition extended across numbers, usually very large numbers, of neural elements. In neural coordinates the frequency profile maps the contour, spatial position, and extent of a stationary stimulus. The transform is isomorphic. This leads to what I consider an important and conceptually most difficult problem. That is, how does a central neural mechanism read this profile? How does one measure and or calculate information transfer through such an *n*-dimensional matrix? How is that information coded in reduced numbers of elements for further processing, for action, for storage?

3) *The time derivatives of the frequency profile* of activity moving in space across a population of neurons, or stationary in neural space and changing the form of the profile with time. This is a likely candidate for the way in which information is provided for a central mechanism to read direction, velocity, and acceleration. All the unknowns pertinent to proposition 2 hold here, also.

4) What is known as the "*internal structure of the neural message*": the proposition that, given two trains of impulses in a single neural element of the same over-all frequency, messages of different informational content may be signaled during each measuring unit of time by the positional differences of the impulses in the two trains compared—a technique of pulse-interval modulation. What is meant is something much more subtle than obvious grouping and pausing, or the variance of the second of three impulses from a position close to the first to one close to the third. Pulse interval modulation is probably that aspect of neural coding that has been most intensively studied in recent years. How important a role it plays is uncertain, but later I shall give one example of sensory information transfer that can hardly be explained on any ground other than that the central nervous system can measure such intervals. There are several variants of this proposition, two of which follow.

5) *Coincidence-gating as a code* (or its reciprocal, produced by inhibition) expanded to include the axiom that a particular coincidence or locked sequential pattern in two or *n*-converging elements—or, in a more general sense, the temporal relations between pulses in adjacent lines—will transfer specific information by virtue of their temporal relation. This method of coding is apparently operative in some invertebrate systems. An elegant example has recently been described by Rose, et al.,[3] for some central auditory neurons in the cat, which are thought to serve the function of localizing sound in space, using differences in latency of activation/inhibition from the two ears—at the microsecond level—as signals of azimuth.

6) *Frequency modulation,* a rhythmic variation in inter-impulse intervals, a discharge pattern now shown to occur in several neural systems, for example, the carotid presso-receptors, visual, somesthetic. We have recently observed an example of coding by frequency modulation, with the additional and, to me, surprising finding that the central nervous system of man is apparently insensitive to it, in this particular case, short of an intensity that produces obvious grouping and pausing, which is a different code.

7) Coding by virtue of *labeled lines,* a principle mentioned earlier: the proposition that impulses in a certain set of afferent nerve fibers always evoke sensory experiences of the same quality, and are differentially tuned to the relevant form of stimulation.

The list grows too long, and new codes are discovered at a great pace. Consider, for example, Roeder's recent discovery of two different codes in the CNS of the moth, used by two different interneurons which are part of the system activated by the ultrasonic cries of predator bats, and which lead to evasive maneuvers by the moth.[4] The first discharges one impulse for each burst of impulses evoked in a first-order auditory fiber by each ultrasonic pulse emitted by the bat, and relayed to it via repeater cells. The cell discharges but a single impulse independently of the frequency or duration of the train impulses reaching it—it requires a period of silence before discharging again—and thus marks each pulse in the series of bat's cries. Another internuncial studied by Roeder is inactive in the absence of acoustic stimulation, but discharges a more or less regular train of impulses whenever a series of ultrasonic sound pulses of sufficient intensity reaches the ear. It thus marks the total duration of the entire series of bat cries. Now, both types of cell are activated by one and the same first-order input. It is as if, as Roeder puts it, the first-order message contains a number of cueing signals, each of which may open a different central door among the several upon which it impinges.

It seems pertinent to me to consider the problem of sensory coding in relation to sensation itself and, by comparing neural codes with sensory performance, to try to discover which codes are meaningful for particular aspects of sensation and to make quantitative comparisons between the two as they vary with varying stimulus parameters. That is, compare sensory encoding with psychophysical observations. It will certainly be more fruitful to study neural coding in terms of its functional meaning for the organism, rather than in isolation, for it is already clear that by experimental manipulation of a neural system one can elicit certain codes that have no particular significance for the brain receiving them. Before proceeding on this line, I believe it is useful to set down in rather simple terms some of the problems a brain faces in sensing.

What does a brain face in sensing?

The systematic development of these problems sets the demand upon the sensory codes used, and for the measurement of sensation itself. Sample problems are as follows.

1) The questions "*Has anything happened?*" or "*Is anything there?*" lead to the problem of detection: what aspect of an afferent signal is detectable against the ongoing activity of the nervous system? This problem is one of a statistical nature, now usually approached from the viewpoint of decision theory. What is threshold for a human observer can, for example, be manipulated by declaring rewards for correct answers, penalties for false positives, etc. The idea that there is a constantly recognizable nueral quantum should not, however, be dismissed without further study. Indeed, psycho-physical evidence for such a quantal jump has been obtained in experiments on hearing, with a severe reduction of background "noise."[5] And in the limit, neural activity always does increase by one more impulse in an already active element, or by the addition of one more neuron to an active population—quantal increases.

2) The question "*What is it?*" leads from the problem of detection to the identification of quality, which has occupied the attention of neurophysiologists for 75 years. The evidence for the specificity of the larger myelinated sensory fibers now seems completely convincing, and that for the smaller myelinated fiber groups grows steadily. Whether some of the C-fibers, which are sensory as well as afferent, are specific for modality while others are non-specific, or whether they occupy a continuous spectrum, is yet to be settled.

3) The question "*How much of it is there?*" leads from detection to the problems of quantification and scaling. We must discover along what dimension neural activity should be scaled, and which changes in stimulus intensity provide a significant change in signal for a central discriminating apparatus; are discriminable increments equal over the full discrimination scale?

4) The question of how much is there leads immediately to a related one: "*Is this stronger than that?*" The problem is of differential discrimination between different intensities.

5) Given that something of an identifiable quality and measurable intensity is present, an important—indeed, for behavior an essential—question is: "*Where is it?*" The answer is not to be found in particular codes in single elements, but must be approached in terms of the population of active elements. The isomorphic central replication of the spatial extent of receptor sheets reduces the problem from one of location in actual space to one in neural space, and points again to the central problem I

mentioned earlier: How is this distributed pattern of activity in a population read and more efficiently coded in the central nervous system?

6) Given that something of a given quality and intensity has happened in a known location, the last question introduces the dimension of time, to which I have already alluded: *Is the stimulus moving in space or changing its properties as functions of time?*

A general hypothesis

Before proceeding to some specific examples of sensory coding, I wish to put forward the general hypothesis that, as regards sensory functions, the brain operates in a linear manner. That is, when all the subsystems intervening between input and output are lumped together, a comparison of the input, defined as that in first-order fibers, with the output, defined as a behavioral response or subjective sensory experience, reveals no nonlinear transformation. Further, some of the component subsystems—in more physiological terms, some of the nuclear transfer regions —have already been shown to operate in a similar way. Perhaps it will not be necessary to postulate an additive reciprocity of nonlinear transformations between related subsystems to account for the over-all linearity observed. The implication is that whatever nonlinear aberration our image of the real world may suffer, it occurs in the initial stage at which impinging stimuli are transduced into nerve impulses. The central nervous system follows in linear fidelity whatever image of the world is reflected to it over primary afferent fibers.

This is a hypothesis of unexpected simplicity. I am led to it by comparing the results of recent studies and relevant psychophysical observations. If this general hypothesis is valid, sensory coding in first-order fibers becomes a subject of considerable importance.

Coding for stimulus intensity

The first example of coding I wish to illustrate is the classical frequency code for intensity. The afferent chosen is the slowly adapting myelinated fiber innervating the glabrous skin of the monkey's hand. I shall compare this with the human performance in the subjective estimation of the intensity of similar stimuli delivered to the hand, under exactly the same experimental conditions.[6] The raw data for the first are indicated in Figure 2, which illustrates trains of nerve impulses set up in a single fiber of the monkey's median nerve by brief indentations of the skin it innervated. Details aside, the number of impulses appears to be a function of stimulus intensity; that this is indeed the case is illustrated in Figure 3. For this graph

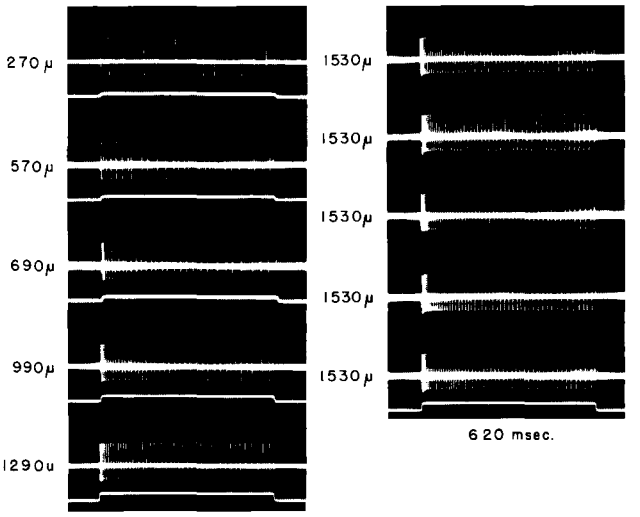


FIGURE 2 Oscillographic records of trains of nerve impulses evoked in a single fiber of the median nerve of a monkey. The axon innervated dermal ridges on the distal finger pad of the middle finger, and continued to respond steadily during application of a steady indentation of the skin of its receptive field. Records to the left obtained for stimuli of different depths of indentation. Those on the right obtained during serial presentation of a stimulus of one intensity, to show metronome-like repetition of response. Lower trace of each pair indicates intensity and time course of the stimulus. Small electrical artifacts at the onset and removal of the stimulus vary with the amplitude of the stimulus, and are not signs of discharges in other fibers.

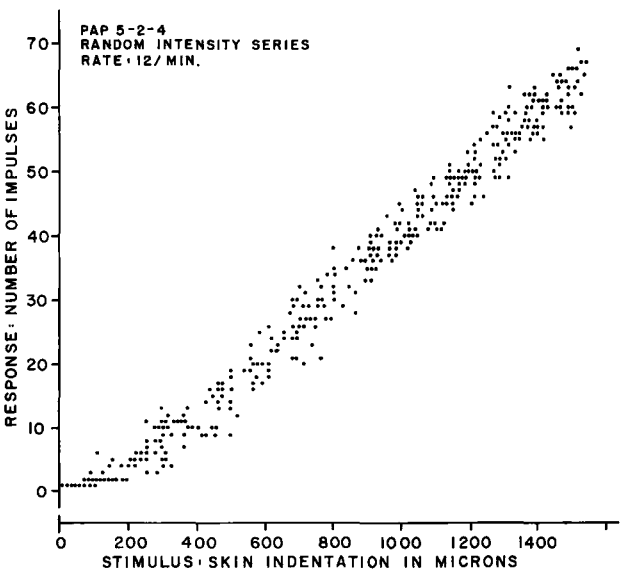


FIGURE 3 Results obtained in a random-intensity series study of a myelinated axon of the palmar branch of the median nerve of a monkey. The axon innervated a field extending across 5 dermal ridges on the thenar eminence. The stimulator probe tip, 2.0 mm in diameter (one-third spherical surface) approximately covered the receptive field. Intensities were delivered in random order and data collected and analyzed as described in Note 15. The measured value of the analog electrical signal of each stimulus is plotted on the ordinate. Identical S-R pairs appear as single dots. Stimulus duration 600 msec. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

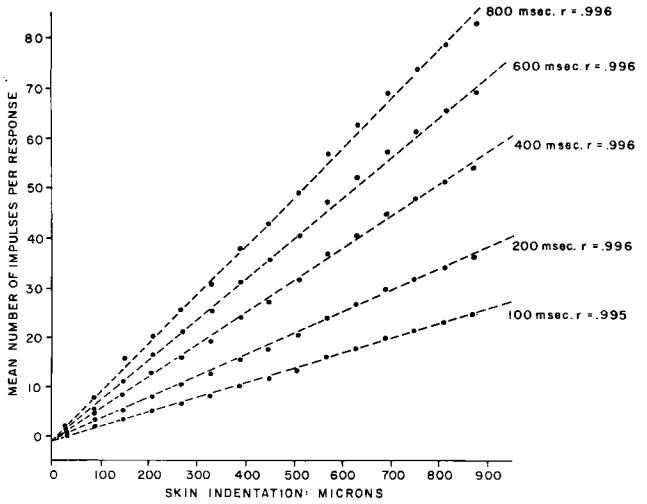


FIGURE 4 Results of a random-intensity series study of a myelinated axon of the median nerve of a monkey. The fiber innervated a receptive field extending across 4 dermal ridges of the distal pad of the fourth finger. The series consisted of 320 stimuli distributed among 16 stimulus categories. Stimulus values were converted to net skin indentation and plotted along the abscissa. Ordinate values are mean numbers of impulses evoked by each stimulus strength, counted in each successively summing period of time up to the 800 msec duration of the stimulus. Pearson product moment correlation coefficients for fit of each straight line calculated for all items, not for means of response populations. Stimulating tip 1.0 mm in diameter; one-third spherical surface. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

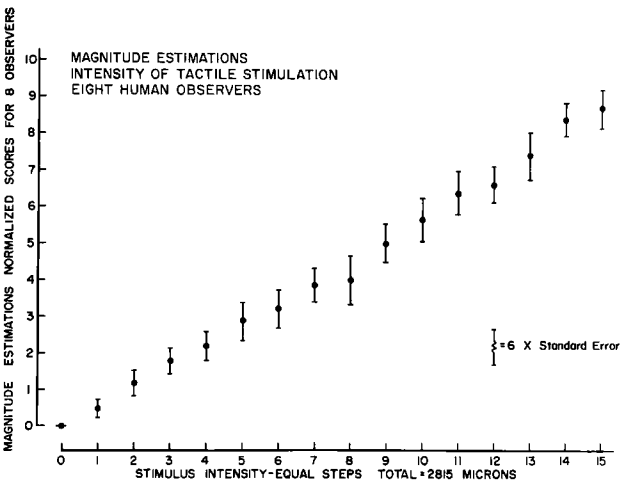
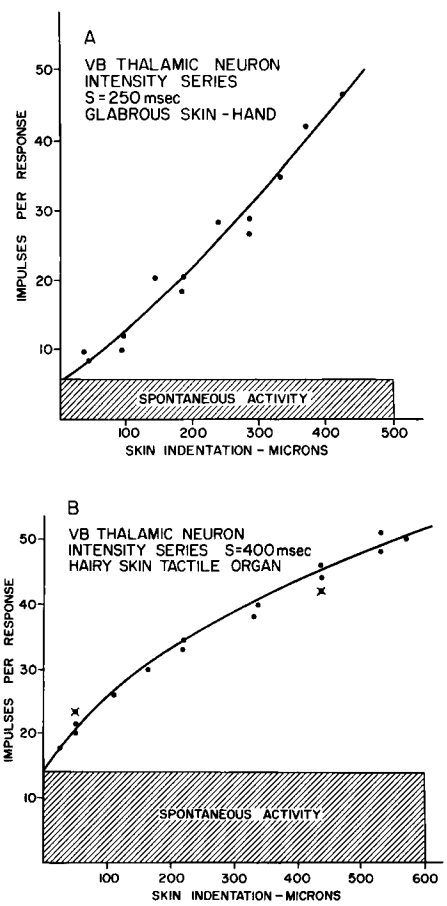


FIGURE 5 Results of subjective estimation of the intensity of mechanical stimuli by human observers. Stimuli delivered to distal pad of middle finger at rate of 12/min. Probe tip 2 mm in diameter, machined to a one-third spherical surface. Intensity continuum divided into zero and 15 equal steps; maximum—2815μ indentation. Observers usually rated along a scale of 10 to 20 steps; these have been normalized to a scale of ten for the ordinal values plotted here. Each observer received 5 each of the 16 stimuli, randomly ordered. Means and S.E.s are of all estimates of all observers, not means of means. Step 8 was given three times as an orienting stimulus before the series began, which may account for the slight displacement of the estimated value for eight from the otherwise nearly perfect linear relation (r's for individuals were all above 0.90; mean r = 0.93).

FIGURE 6 A: Results of an intensity series study similar to that illustrated in Fig. 4, but now carried out for a single neuron of the ventrobasal nuclear complex of an unanesthetized monkey. The impulse discharges of the neuron were observed by microelectrode recording. The cell was activated by mechanical stimulation of a field on the glabrous skin of the contralateral hand; it adapted slowly to steady stimuli. Mean rate of spontaneous activity indicated by lined area. The stimulus-response function showed a slightly curving foot, similar to that of Fig. 3, then remained nearly linear for more intense stimuli.

B: Results of a similar experiment on a thalamic neuron of an unanesthetized monkey, located in the ventrobasal thalamic complex, but this one related to a *single* tactile corpuscle of the hairy skin of the contralateral arm. The fitted line is a power function with an exponent of about 0.5. (From unpublished experiments of Dr. Gerhard Werner, Laboratory of Physiology, The Johns Hopkins University, 1965)



there is no averaging—the number of impulses is plotted as a function of the individually measured skin indentations. The stimulus-response relation is certainly linear over a wide range of values of the independent variable; it saturates with indentations beyond 1.5–2.0 mm, which tend to damage nerve endings. A second important feature of the data given in Figure 3 is that the error in the neural signal for stimuli of the same intensity appears to be independent of the intensity level or of the magnitude of response, a finding with an important theoretical bearing on what may be regarded as a discriminable neural increment. Figure 4 plots these linear functions for averaged responses, for sequentially summing time periods of inspection of the response.

The data presented in Figure 5, on the other hand, illustrate the human performance when asked to estimate the magnitude of similar stimuli. The linear relation is obvious. In sum, for the intensive attribute of mechanoreceptive sensibility the receptor transform is a linear one, and upon this input the brain operates in a linear fashion to produce one particular behavioral output—and a simple frequency analysis seems to satisfy the requirements for neural encoding, with some assumptions about the similarity of function for the hands and brains of monkeys and men.

Now you may inquire whether this linearity around the long loop from first-order input to behavioral output might not result from a summing of sequential transforms that are nonlinear, but reciprocal. The question cannot be answered completely, but some observations made on the ingoing side of the loop suggest that this is not so, and that intervening transforms are linear as well. The data illustrated in Figures 6-a and 7, for example, show that the relation between the response evoked in thalamic and cortical cells bears a nearly linear relation to the intensity of the mechanical stimulus, as does the first-order element.

I wish now to bring other evidence supporting this hypothesis that our relation to the world about us, and to the stimuli from it that impinge upon us, is set by the transforming properties of peripheral receptors, and that the brain operates upon those inputs in a linear manner. Consider now another mechanoreceptor projecting into the same neural system, but arising from the touch corpuscles of the hairy skin. In this case the transformation between the stimulus and first-order input is *not* linear, but negatively accelerating, and is best described as a power function with an exponent of about 0.5–0.6[7] (see Figure 8). Of great importance, Jones, when dealing with stimuli delivered to the hairy skin of man, found that the intensity function is not linear, but is also a negatively accelerating function, a power function with an exponent

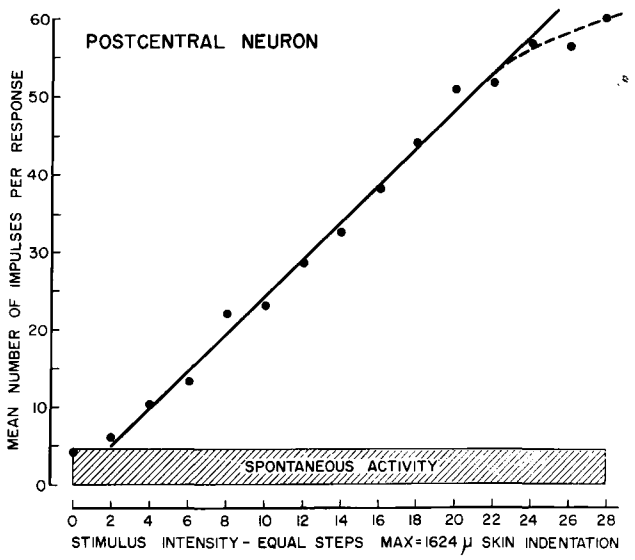


FIGURE 7 Results of an intensity series similar to those of Fig. 6, but carried out for a neuron of the postcentral gyrus of an unanesthetized monkey. The neuron was activated from a receptive field in the glabrous skin of the ventral pad of the contralateral forefinger. The field extended across four dermal ridges, and was completed covered by the 2 mm diameter stimulating probe tip. Stimuli were of 800 msec duration, delivered once in five sec. Total indentation of 1624 μ divided into zero and 15 equal steps (plotted on the abscissa as 0,2,4, etc.). A total of 160 stimuli were delivered in a random order. Points plot mean number of impulses for the responses evoked at each stimulus strength. The stimulus-response function is linear over a wide range of stimulus intensities, but for steps above 24 began to saturate. Level of ongoing spontaneous activity indicated by the lined area.

of about the same value.[8] Further, Figure 6-b illustrates that a thalamic neuron of the somatic afferent system (a third-order element), which was activated from a *single* touch corpuscle of the hairy skin, is related in a similar way to stimulus intensity. Two sets of data can now be compared: The first comprises the intensity functions for the first-order fibers innervating the glabrous skin of the monkey's hand and those for the central neurons upon which they project, together with the psychophysical functions obtained when human beings rate the stimuli delivered to their glabrous skin. The second set is composed of similar observations obtained when exactly the same stimuli are delivered to the hairy skin. The conclusion seems inescapable that *for one and the same afferent system, and dealing with the same attribute of the same sensory quality, quite different relations obtain between stimulus and response (both neural and behavioral), depending upon whether*

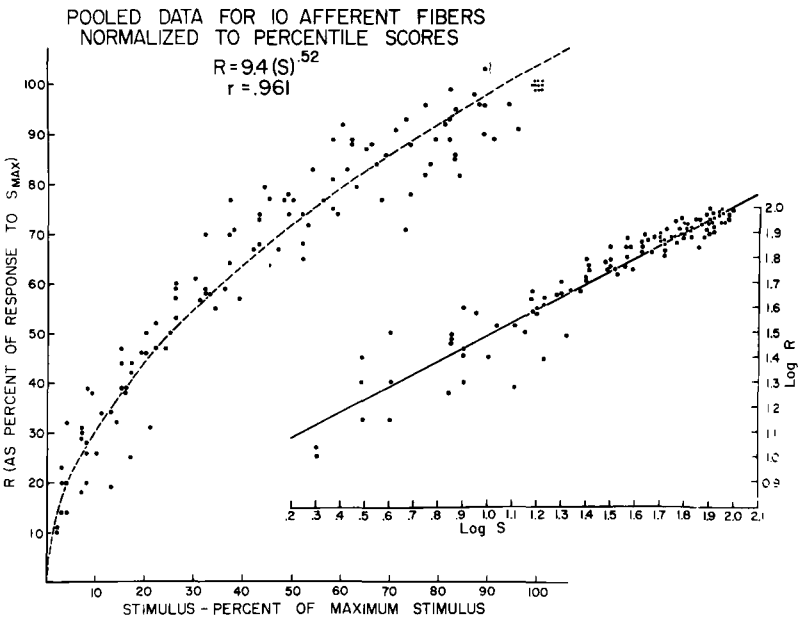


FIGURE 8 Plotted data for intensity series such as that of Fig. 3, but for tactile corpuscles of the hairy skin, normalized to percentile scores. Graph to the left plots the stimulus-response function in linear coordinates, that to the right in log-log coordinates The relation for each fiber fitted a power function of the form $R = K \cdot S^n$, with n's less than unity, with a high degree of certainty (P's=0.005, or less). Data for each fiber normalized by converting stimulus values measured in microns of skin indentation to per cents of maximal indentation, and response numbers to per cents of the response evoked by that maximal stimulus. In this procedure variations in value of the constant K from fiber to fiber are eliminated, and the trend of the entire population revealed. (From Werner and Mountcastle, Note 7)

glabrous or hairy skin is stimulated, and that these differences are uniquely set by the transforming properties of the two sets of receptors concerned. The brain operates upon either in a linear manner.

Observations of a similar sort are available for other sensory systems. For example, it has been known for a long time that the human estimation of light intensity can be described by a semi-logarithmic function, or, if you prefer, a power function with an exponent of about 0.3. A similar function describes the response of the retinal receptors to light—as measured, for example, by the electroretinogram. Recent experiments by my colleagues Drs. Poggio and Baker indicate that a similar situation holds for the relation of the response of lateral geniculate cells to the intensity of small spots of light shown upon the excitatory centers of their receptive fields. And, what is perhaps equally important, lighting of the surrounding inhibitory fields for these on-center cells subtracts response reciprocally in the same quantitative manner. While similar studies of the visual cortex are badly needed, it is already clear that in vision, as in somesthesis, the relation between the neural system response and light intensity, and for the human estimate of it, the governing transformation is affected at the level of the retina. The experiments of Desmedt[9] suggest that the same principle holds for audition, and Zotterman[10] has shown similar relations for the sense of taste. Professor Zotterman's experiments are particularly noteworthy because both the first-order fiber recordings and the psychophysical studies were made in human beings.

Information transmission

An exercise of some interest is to measure the information-transmitting capacities of a particular sensory avenue to the brain, and to compare it with that of human beings operating in that same sensory sphere and under the same experimental circumstances. The surprising finding has

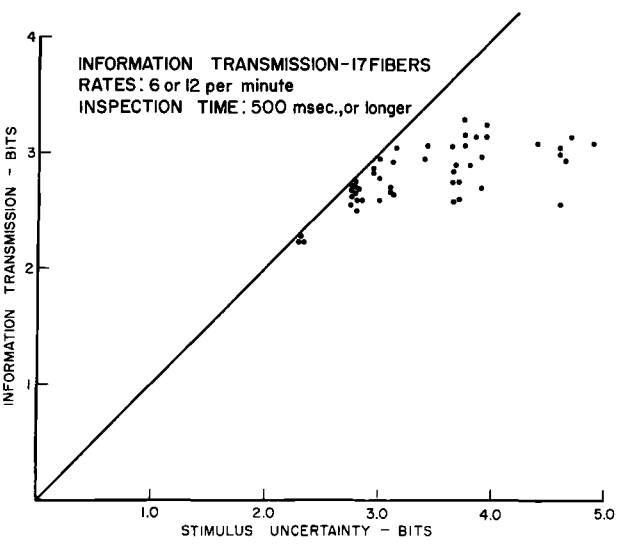


FIGURE 9 Plot of information transmitted over single myelinated mechanoreceptive afferent fibers innervating the glabrous skin of the monkey's head, in bits per response as a function of uncertainty in the stimulus intensity continuum.

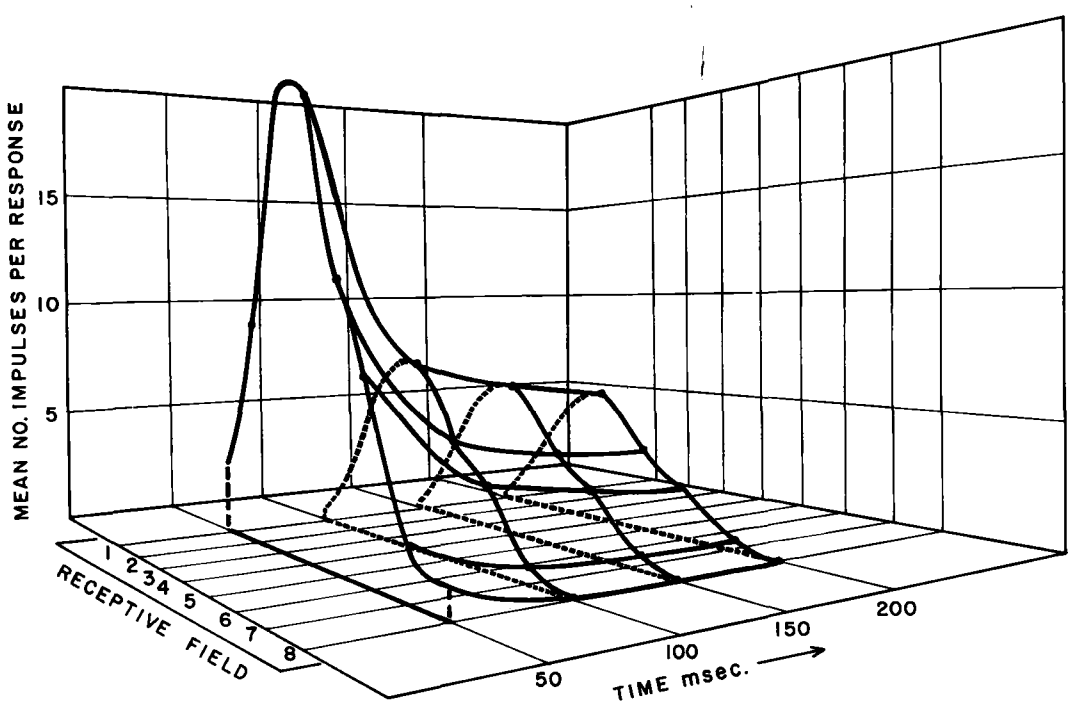


FIGURE 10 Results of a "field study" for a fiber of the median nerve of a monkey that innervated the glabrous skin of the hand. The fiber could be excited from a field extending across 7–8 dermal ridges of the skin of the distal pad of the thumb. A stimulating probe tip of 0.5 mm diameter was used, machined to a one-third spherical surface. For each point along the receptive field axis (the x axis) the probe tip was centered over a dermal ridge and a random-intensity series was carried out. Stimulus duration 200 msec. Counts of the number of impulses in each successive 50 msec period, evoked by the maximal stimulus used, are plotted along the y axis. Lines connect these points for each field point, for each time period. By a reciprocal interpretation, these contours are thought to represent the spatial distribution (within the neural population engaged) of the activity evoked by the stimulus were it delivered to the central, most sensitive point in the field, and thus the abstraction in neural space of the spatial contour of the stimulus. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

been made for a number of sensory modalities that when making subjective magnitude estimations of intensity the human observer transmits only about 2.5–3.0 bits per stimulus.[11] (Of course the addition of other dimensions will increase this transmission enormously.) This method of analysis[12] has been applied in the intensity studies of the two mechanoreceptive afferents discussed earlier; they are described in detail elsewhere.[6,7] The result for the mechanoreceptive afferents from the monkey's hand are shown in Figure 9: transmission is slightly less than 3 bits per stimulus for a single fiber. Comparison of this finding with that in man suggests two hypotheses that may be worth further exploration: (1) that the human CNS can, when attending, preserve all the information it receives over first-order fibers; and (2) that, in principle, information about the intensity of a stimulus could be conveyed by a single fiber.

Distribution of activity in a population: coding for spatial pattern

If there is any validity to the last statement, it leaves the second code—the distribution of discharge frequencies across a population of neural elements—free for signaling of spatial form, which is the central transform of the gradients of stimulus intensities across the receptor sheet. The result of an experiment planned to illustrate this distribution of frequencies in "neural space" as a method of coding is shown in Figure 10.[13] In this experiment, the stimulus was moved in steps along a line transsecting the peripheral receptive field of the fiber, a field of about 2 millimeters diameter, on the glabrous skin of the monkey's finger. At each position an intensity series of the type shown in Figure 4 was run—only the response numbers to maximal stimuli are plotted in Figure 10. By this

maneuver the fiber was made to occupy a series of different positions in the different populations engaged by the stimulus, e.g., from the edge of a population at point 1 to its center at about point 4, to the opposite edge at point 8. Thus, by combining all the results one can reconstruct that population, *post hoc,* without ever having observed all its members simultaneously. The contour in each sequential period of time represents the intensity contour of neural activity across the population at that instant. The onset transient and the decline to a quasi-steady state are obvious. It was of some interest that the stimulating tip in this case was machined to a one-third spherical surface, and the activity contour in the quasi-steady state appears to be a reasonable replication of it. The onset transient is, of course, a function of the rate of stimulus application, here very rapid. We have not yet performed this experiment with more complex forms as stimulating tips. One other implication from this experiment is that there is little redundancy in the total input signal, for all active fibers contribute to the central transform for spatial pattern.

Coding in the temporal domain: the sense of flutter-vibration

In what has gone before I have been concerned with coding for intensity and for spatial pattern during a short period of time when the stimulus is stationary—during the early steady-state discharge of the "slowly adapting" type of mechanoreceptive myelinated afferents from the glabrous skin of the hand. Now I wish to take up the question of coding in the temporal domain and to use the human sense of flutter-vibration as an object for study. Once again the problem has been approached by com-

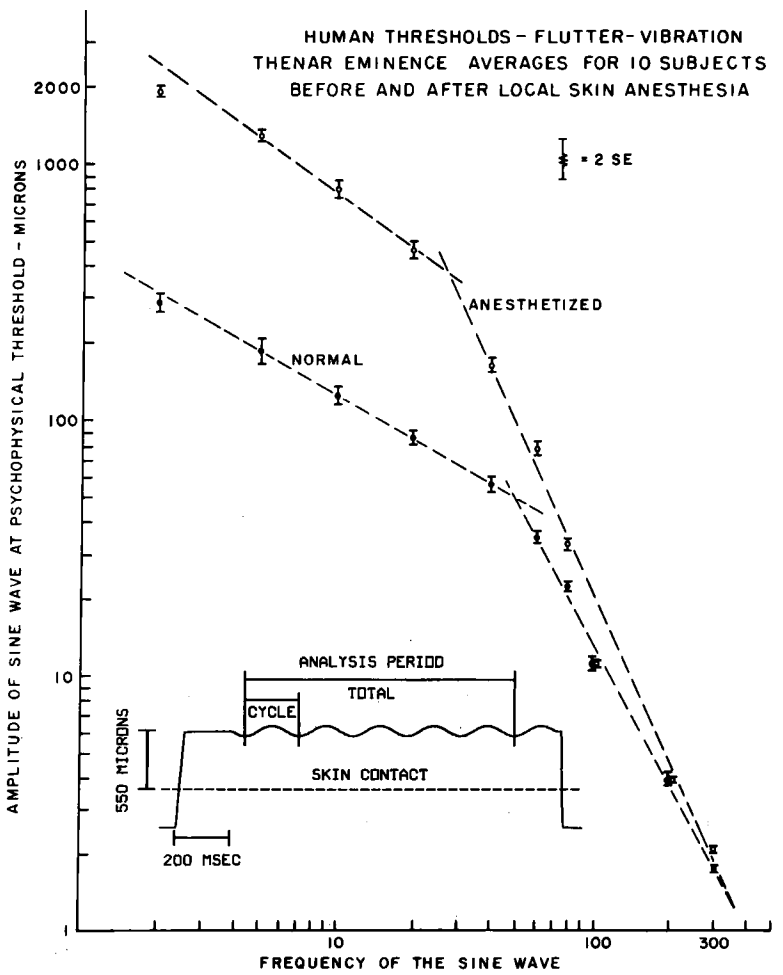


FIGURE 11 The relation between the amplitude of a sine wave oscillatory movement of the skin, delivered as described in the text, and the human threshold for the perception of movement, at a series of different frequencies. Points are means for ten adult observers; the lower curve for the normal state, the upper after anesthesia of the skin, by cocaine iontophoresis. The site of stimulation was the thenar eminence. Anesthesia elevated remarkably the threshold for perception in the low-frequency range (2–40 cycle/sec); the sensitivity which remains may be due to stimulus spread at high intensities. Sensitivity to high-frequency oscillation is little affected by skin anesthesia, which at the time of testing was complete for all cutaneous modalities.

Inset: Computer plot of the digitalized output of the displacement transducer measuring the position of the stimulating probe tip. In its rest position probe is more than 200 μ away from the skin. At the beginning of the stimulus pattern the probe is moved rapidly to a new position (rise-time 30 msec), which indents the skin approximately 570 μ. After a delay of 200 msec sinusoidal stimulation begins with initial phase a withdrawal of the probe from the steady state position. Duration of the sinusoidal stimulation 930 msec ($-0, \pm 1$ sine wave period). Total duration of the stimulus pattern is 1.4 sec. Detailed analysis of the nerve impulse response patterns confined to the period shown. The frequency and amplitude of the sine wave can be varied independently. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

paring observations made in both man and monkey by determining the frequency-threshold function for the first, and then seeking to discover which afferents from the monkey's hand might, by their dynamic functional properties, provide adequate signals. That is, an attempt was made to determine how oscillating cutaneous stimuli, which man can perceive as moving, are encoded in monkey first-order fibers.[14,15]

PSYCHOPHYSICAL OBSERVATIONS The sense of flutter-vibration has been studied many times before,[16] and we have repeated these only in order to compare results obtained in monkey and man by use of identical stimulus parameters. The stimulus pattern we have used is shown in the inset of Figure 11. It is a step indentation of the skin of about 500 μ, and about 1.5 seconds duration. After 200 milliseconds—after onset responses have subsided—a sine wave of a chosen frequency and amplitude is superimposed, lasting about 1 second. All parameters could be varied. For the monkey first-order fiber studies, and for some psychophysical studies, the pattern shown was used. For other psychophysical experiments, including that of Figure 11, a constant indentation of the skin was maintained, and the amplitude of the sine wave varied up to and down to threshold—the classical method of limits. Human observers were asked the question: "Is the stimulus moving or stationary?" The question asked the monkey first-order fibers was: "What neural signal of the stimulus frequency is there at the human threshold for the perception of periodic motion?" The results averaged for ten human subjects are shown by the lower curve marked "normal" in Figure 11. The function is double-limbed in log-log coordinates, fitted by two straight lines of different slopes, one for frequencies from 5 to 40 per second and another for those from 50 to 300 per second. We did not test above 300 per second, but the function is known to rise again precipitously for higher frequencies. We then repeated this experiment in the same subjects after anesthetizing the skin by local cocaine iontophoresis. The results are shown by the upper curve of Figure 11. They suggest, as does the double-limbed function, that the perception of movement depends upon two completely separate sets of afferents—one innervating the skin and most sensitive in the low-frequency range, a second terminating in deeper tissues, and sensitive in the high-frequency range.

TEMPORAL CODING IN FIRST-ORDER FIBERS The "quickly-adapting" myelinated mechanoreceptive afferent from the glabrous skin was, of course, the prime candidate for the cutaneous movement detector. Sample records obtained from such a fiber in the monkey's median nerve with the oscillatory stimulus at 40 per second, are shown in Figure 12; perfect entrainment occurred between 14 μ and 18 μ sine wave amplitude. Analysis of the distribution of the inter-impulse time intervals gives an answer to this question: For any given intensity, is there a definite neural signal of the stimulus period in the input trains? With the results, one can construct tuning curves such as that illustrated in Figure 13.

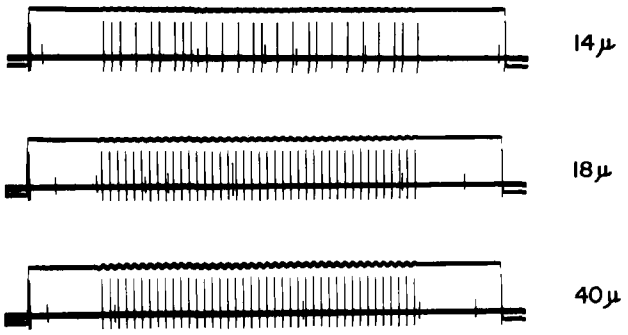


FIGURE 12 Sample records of impulse discharges in a single fiber of the median nerve of a monkey, innervating the glabrous skin of the palmar surface of the hand. Stimulus paradigm and data collection as for the inset of Fig. 11. Stimulus frequency 40 cycles sec. On and off transient discharges of this movement detector afferent obscured by movements of stimulus trace. The discharge of the fiber was perfectly entrained at a stimulus amplitude of 18 μ. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

All the facts recounted so far suggest that the high-frequency limb of the human function is served by an extraordinarily sensitive receptor lying in the deep tissues of the hand. The most likely candidate is, of course, the Pacinian corpuscle and its associated myelinated afferent. It is present in large numbers in the deep tissues, and well known from the work of Gray,[17] Loewenstein,[18] and Hunt[19] to be exquisitely sensitive to movement. This indeed turned out to be the case. Sample records from such an afferent driven at 150 per second, are shown in Figure 14. Tuning curves for 20 Pacinian afferents were constructed in the manner described above.

PSYCHOPHYSICAL - NEUROPHYSIOLOGICAL CORRELATIONS In order to make as direct a comparison as possible, we repeated the psychophysical studies with the stimulus delivered to the finger tip, for most of the peripheral fibers we have studied innervated the fingers. That function is shown by the heavy lines of Figure 15. The angle between the two limbs is less obvious than is

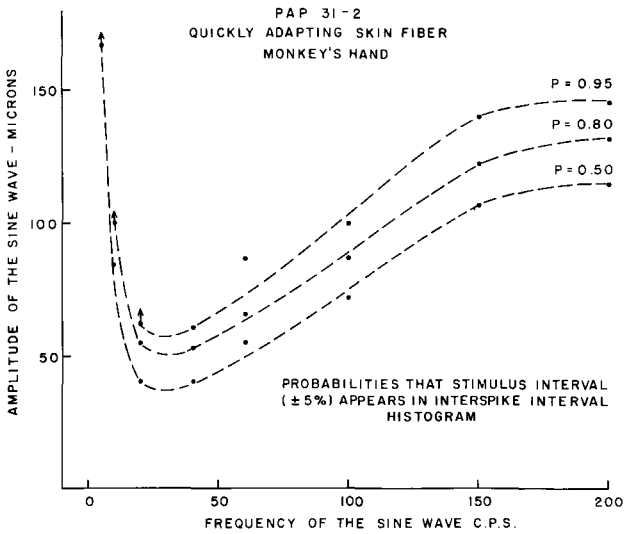


FIGURE 13 Construction of tuning curve for a movement detector fiber innervating glabrous skin of the monkey's hand. At each frequency tested the amplitude was determined of the sine wave cutaneous mechanical stimulus which was required to produce inter-impulse interval distributions containing the stimulus period, with the probabilities indicated, with an error of $\pm 5\%$. Arrows indicate that at no intensity could those requirements be met, for those frequencies.

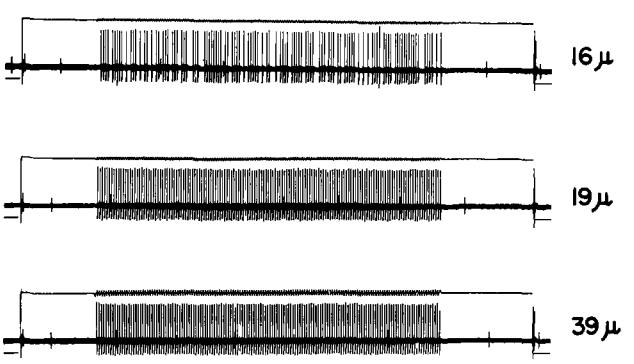


FIGURE 14 Sample records of impulse discharges in a single fiber of the median nerve of a monkey which was thought to terminate in a Pacinian corpuscle of the subcutaneous tissues of the hand. Stimulus paradigm and data collection as for the inset of Fig. 11. Stimulus frequency 150 cycles/sec. On and off transient discharges obscured by movements of the stimulus trace. The discharge of the fiber was perfectly entrained at a stimulus amplitude of 19μ. Among Pacinian afferents, this was a relatively insensitive one, and several were observed with perfect entrainment at best frequency with stimulus amplitudes below 2 μ of skin movement, even though Pacinian endings appeared to be several mm deep within the tissues of the hand. (From Mountcastle, Talbot, Darian-Smith and Kornhuber, Note 15)

that for the functions of Figure 11. The lighter lines plot the tuning curves for eight of the most sensitive cutaneous movement detectors studied in the monkey. The population of 65 we studied scatters from those levels upward along the intensity coordinate. It is obvious that their low-frequency halves overlap and could account for the human perception of movement in the low-frequency range. It is a reasonable assumption that similar fibers innervate the human hand (the hands of the two species are structually quite similar). They cannot account for the high-frequency human sensitivity, for at those frequencies they will be engaged only by very intense stimuli. The crosses on Figure 15, on the other hand, plot the tuning points for the 20 Pacinian afferents. When those for single fibers are connected they form lines roughly parallel to the high-frequency limb of the human function. The most sensitive of these fibers overlap that function, and could account for it on the same assumption given above.

Allowing that periodicity of movement is precisely coded in terms of impulse intervals, what reasons are there for assuming, as I do for a working hypothesis, that when de-coding for movement central mechanisms measure the length of the dominant period in the neural signal? After all, it might simply be the total number of impulses per unit time that is important; for signaling movement the more impulses there are the higher might be the perceived frequency, and the coding—de-coding mechanisms operate in a manner similar to that described above for intensity, i.e., the frequency code. There is one reason for thinking this is not true. A further increase in intensity beyond the tuning point may destroy the dominant interval while greatly increasing the number of impulses per unit time (see Figure 16). Yet, as Békésy has shown,[20] when this is done the subjective estimate of the frequency perceived by a human observer may *drop* by as much as one or two octaves.

If such a periodicity in first-order input is required for the perception of movement, we may next ask how central mechanisms discriminate between different frequencies. Humans can discriminate an increment of less than 5 cycles at 25 cycles per second, but that discriminable increment increases sharply in the high-frequency range.[13] Obviously, frequency discrimination cannot depend upon *which* fibers are activated by the two frequencies between which discrimination is sought, as is the case in audition, for the tuning curves of all are parallel, with best tuning frequencies nearly identical. Discrimination cannot depend upon the total number of impulses being different in the two cases, for given the probabilities required for tuning and the different sensitivities of different fibers, the total number of impulses could be identical in the two cases. What is left is the proposition that central nervous

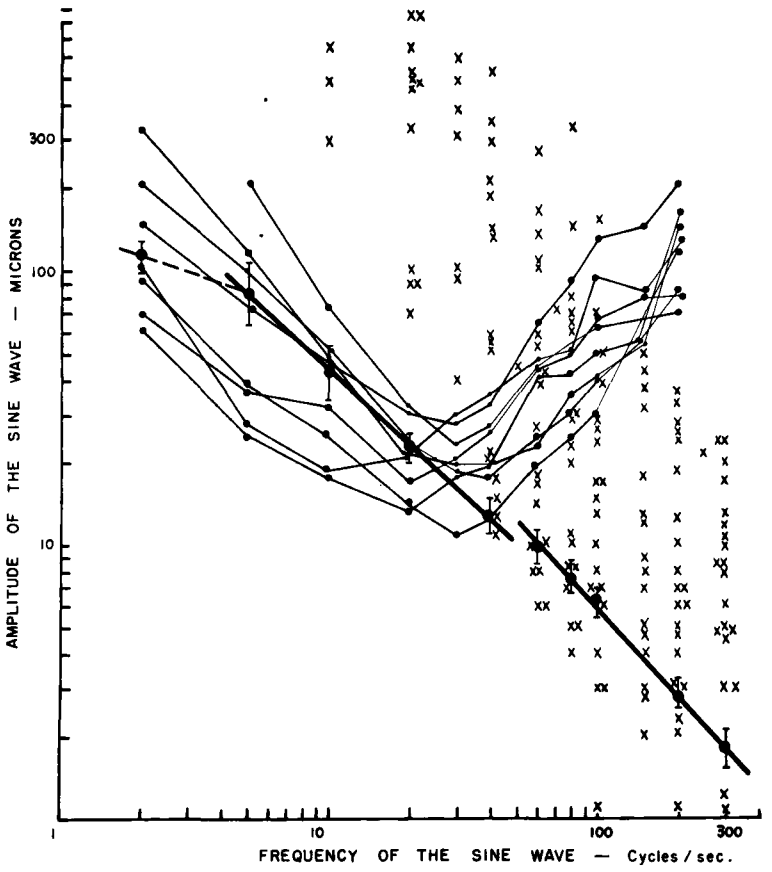


FIGURE 15 The heavy lines plot the human threshold function for the perception of sine wave oscillation of the skin when the stimulus is delivered to the volar surface of the distal phalanx of the finger, in the manner indicated in the inset of Fig. 11. Points are means for nine adult observers. The break between the two limbs of the threshold curve is less acute than it is when stimuli are delivered to the thenar eminence, as shown by the lower curve in Fig. 11. Lighter lines plot the threshold tuning curves for a number of myelinated cutaneous afferent fibers of the median nerve that innervated the glabrous skin of the monkey hand. Tuning points on these latter curves were determined as described in the text. These cutaneous "movement detectors" are the only myelinated afferents from the skin of the hand which, by their sensitivity to vibratory motion, can account for the low-frequency limb of the human threshold function. Crosses plot tuning points for a number of myelinated afferents ending peripherally in Pacinian corpuscles. When the points for any one fiber are connected they form tuning curves approximately parallel to the high-frequency limb of the human tuning curve, for which only they can account. (From Mountcastle, Talbot, Darian-Smith, and Kornhuber, Note 15)

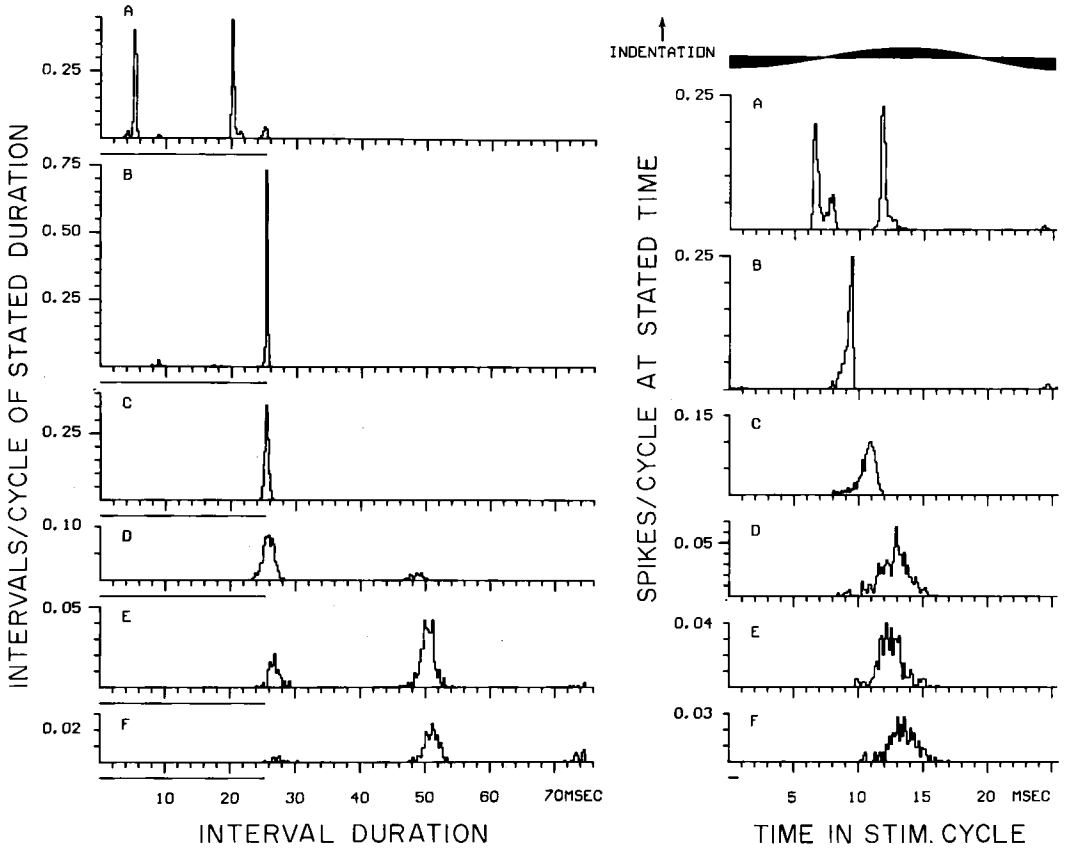
mechanisms can discriminate between the two dominant periods; between, for example, one of 40 milliseconds and one of 33 milliseconds.

The pervasiveness of convergence in even the most precise sensory system has made many neurophysiologists, including myself, reluctant to accept interval duration *per se* as a meaningful and measurable neural signal. I think we can escape it no longer. In recent experiments, for example, we have found the low-frequency dominant periods, which the cutaneous movement detectors signal with such precision, reproduced by cells in the unanesthetized monkey's postcentral gyrus, in spite of whatever convergence may exist at the three intervening synaptic transfer regions.

Simultaneous coding for frequency and intensity

Now I wish to compound the problem by asking how it is that a single type of fiber codes for more than one parameter. Some psychophysical studies showed us that the human subjective estimation of the intensity of flutter-vibration is a linear function of the amplitude of the sine wave stimulus, over a wide range of frequencies. Now, the quickly adapted afferents from the glabrous skin are the only ones we have discovered capable of providing a neural periodicity at 40 cycles per second in the intensity range required.[15] How do they also provide a neural signal for the intensity of that oscillation?

The results of a study aimed at the solution of this problem are given in Figure 16.[14] Cycle histograms are shown to the right, inter-impulse intervals to the left (see legend for definitions). At intensities below the tuning point, the discharge occurs regularly at sub-multiples of the sine wave length (F,E,D to the left). At and above the tuning point, the lock-in is nearly perfect (C,B). For a stronger stimulus, doubling occurs (A), and thus an increase in the total number of impulses. The signal for frequency is very clear from about 17μ to nearly 150μ stimulus amplitude. Now, where in this picture is the signal for the amplitude of the cutaneous oscillation, which can be judged so nicely by the human observer over this same range? We fell back upon the idea that it might be derived from the total population picture—a step we took somewhat reluctantly,



	A	B	C	D	E	F
SINE WAVE AMPLITUDE (IN MICRONS)	148	99	24	17	12	10
NUMBER OF CYCLES	614	624	608	624	609	621
NUMBER OF IMPULSES	1167	657	608	547	344	264

FIGURE 16 Results of a detailed analysis of the inter-impulse time intervals during an intensity series study of a "movement detector" afferent fiber of the median nerve of a monkey, which innervated the glabrous skin of the hand. Stimulus frequency 40 cycles/sec; probe tip 2 mm diameter. *To the right:* Histograms display the distribution in time of the occurrence of impulses, in relation to the cycle of the stimulus, displayed by the profile at the top; these are called *cycle histograms.* Correction made for all phase shifts in stimulating and recording equipment, but not for changes in setting up time as stimulus intensity is raised. Increases in intensity stabilize the discharge to a more narrow instant of time, but for A entrainement is broken by double firing with, on the average, nearly two impulses occurring per stimulus (see table below). *To the left:* Interval histograms, showing the sequence of events as stimulus intensity is raised. For weak intensities (F, E) the fiber discharges in a varying relation to the stimulus cycle, one-to-one, one-to-two, or one-to-three, but never randomly. For C and B it is nearly perfectly entrained at one-to-one, a relation disrupted when a much stronger intensity is used, as in A. (From Darian-Smith, Talbot, Kornhuber, and Mountcastle, Note 14)

for the population cross-section, at least for the steady state afferents discussed earlier, is a coding mechanism reserved for signaling spatial patterns. Nevertheless, we proceeded with such an analysis; the result is shown in Figure 17. The heavy lines running from upper left to lower right each outline a half of the population cross-section for a series of increasing intensities. Below 100 μ intensity these outline a neat contour of elements firing at the period of the stimulus. With further increases in intensity, however, the activity in the center of the population breaks this rhythm and grows in total numbers of impulses, but it is surrounded by a plateau band of elements that preserve the one-impulse-per-cycle relation. Is it possible that intensity is read from the growing center and frequency from this surrounding plane? To me this is not a very attractive hypothesis, and further studies are needed to elucidate this and other situations in which a single uniform population of elements must code simultaneously for more than one variable.

Summary

An important conclusion I wish to make is that the major nonlinear interface between our brains and the external world is at the level of receptor transduction and first-order neural encoding. Thereafter, the activity of neurons of the central nervous system and the over-all sensory performance bear the same relation to changes in stimulus parameters as does the input in the first-order fibers. That is, the brain operates upon its input in a linear manner.

A second conclusion is that different codes are used in different sensory elements, and that the same code may be used in quite different ways in different elements. For example, on the frequency code assumption, the nearly periodic discharge in the steady state mechanoreceptors of the skin fits all the code requirements we know as a signal of a certain intensity of stimulation; its gradation across a population of such elements is a signal of stimulus contour. In contrast, a nearly periodic discharge in the move-

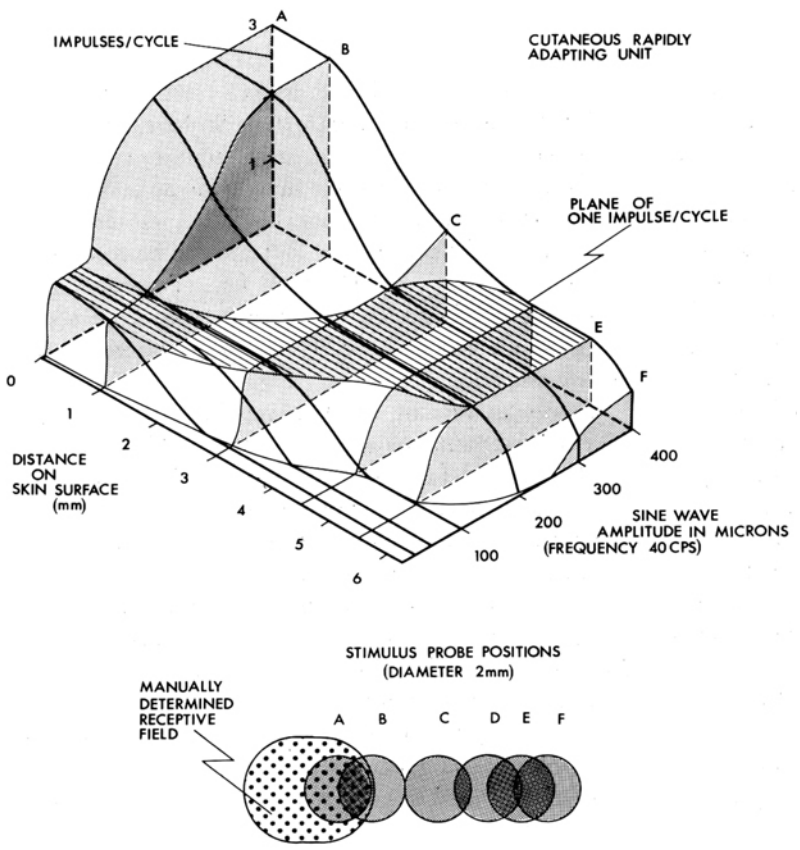


FIGURE 17 Results of an experiment of the type of Fig. 16, now aimed at population reconstruction. Receptive field of this movement detector fiber of the monkey's median nerve innervated a receptive field in the glabrous skin of about 4 mm diameter. The stimulating probe tip, 2 mm in diameter, was moved successively from position A, overlapping one half of the field, to F, some 6 mm away, and outside the field. At each point an intensity series was run at 40 cycles/sec., and the data obtained analysed in the manner of Fig. 16. The three dimensional drawing plots the contour of neural activity imagined to be occurring, in one half of the total population only: point 0 is in the middle of the population. At intensities of 100μ and below, the activity contours—shown as heavy lines running from left-upper to right-lower—outline a population entrained perfectly at its center and less certainly at the edge. With increases in the intensity, the entrainement is broken for elements in the center of the population, which now discharge an increasing number of impulses per stimulus cycle. This "hot center" of the population is surrounded by a plateau of elements that is still entrained perfectly, and that spreads laterally for the stronger stimuli. (From Darian-Smith, Talbot, Kornhuber, and Mountcastle, Note 14)

ment detectors signals oscillation at a certain frequency, and the decipherable signal appears to be the inter-impulse interval! What does this mean for central de-coding mechanisms? Is it possible that the code operant over a particular labeled line is a part of the label? Or, in the reverse sense, does a certain code in one line fit a certain key in the central machinery, while others open different channels in that machinery?

It should be emphasized that the codes I have discussed apply to first-order afferent fibers and their more-or-less direct links to the cerebral cortex. No one imagines that this is anything more than a first sampling of all possible neural codes. Of further interest is the question of how information must be re-coded for compression, both in numbers and in time. Of equal importance is the question of the codes used by those single neurons or relatively small populations that must be the final arbiters in decisions. Do they code by virtue of two stable states of discharge, each signaling a decision to executant assemblies—a decision such as go or no-go?

Peripheral encoding appears to be relatively simple, or perhaps it would be more accurate to say that there is nothing about it which, in principle, cannot be discovered through the use of current conceptual approaches and methodology. The larger problems lie in the central nervous system, and certainly our further understanding of its function will not exceed our knowledge of the way in which it codes and transfers information.

Postsynaptic Inhibition in the Central Nervous System

J. C. ECCLES

TO PRELUDE this story of inhibitory synapses, it is desirable to start with a discussion of excitatory synapses, because the role of the former can be understood only by reference to their antagonism to excitatory synaptic action.

Figure 1 is a general diagram illustrating the potential changes that are produced across the membrane of a nerve cell by activation of excitatory and inhibitory synapses, based on intracellular microelectrode recordings. Inhibitory synaptic action effects an increase of membrane potential, which is known as an inhibitory postsynaptic potential (IPSP). From a resting value of −70 millivolts, it might increase to −72 millivolts, and this change can occur in one millisecond. On the other hand, excitatory synaptic action gives the opposite change in potential, a depolarization or excitatory postsynaptic potential (EPSP), and by mixing these two synaptic actions in different time relations the inhibition is seen (in A) to counteract very effectively the depolarization produced by excitatory synapses. Under appropriate conditions, the inhibitory synaptic action can prevent the excitatory synapses from generating an impulse discharge, as can be seen in the recording from another cell (Figure 1B). So here, then, is the evidence that these excitatory and inhibitory synapses actually are contending for the membrane potential and, if that potential falls to the critical level, an impulse is generated inside the cell (Figure 1D) and can be recorded as it goes down the axon. Hence, as far as postsynaptic excitation and inhibition are concerned, we have a clear story of the antagonistic action on the motoneuronal membrane potential.

Increase in the number of activated excitatory synapses gives a corresponding increase in the EPSP (Figures 2, A-C). In the much faster pictures of Figure 2, D-G (note the millisecond scale), the EPSP also rises faster with increasing activation of excitatory synapses, but does not cause the firing of an impulse, as is seen in the top record. However, in the lower records it is obvious that an impulse is triggered, and this is an all-or-nothing response. The EPSP rises up to the critical level and fires the impulse; if there is a stronger stimulation, more of these excitatory fibers converging on the cell evoke an EPSP that rises more steeply and generates the discharge earlier, but the spikelike potential so generated is of the same size. When the inhibition is also recorded intracellularly, it is graded

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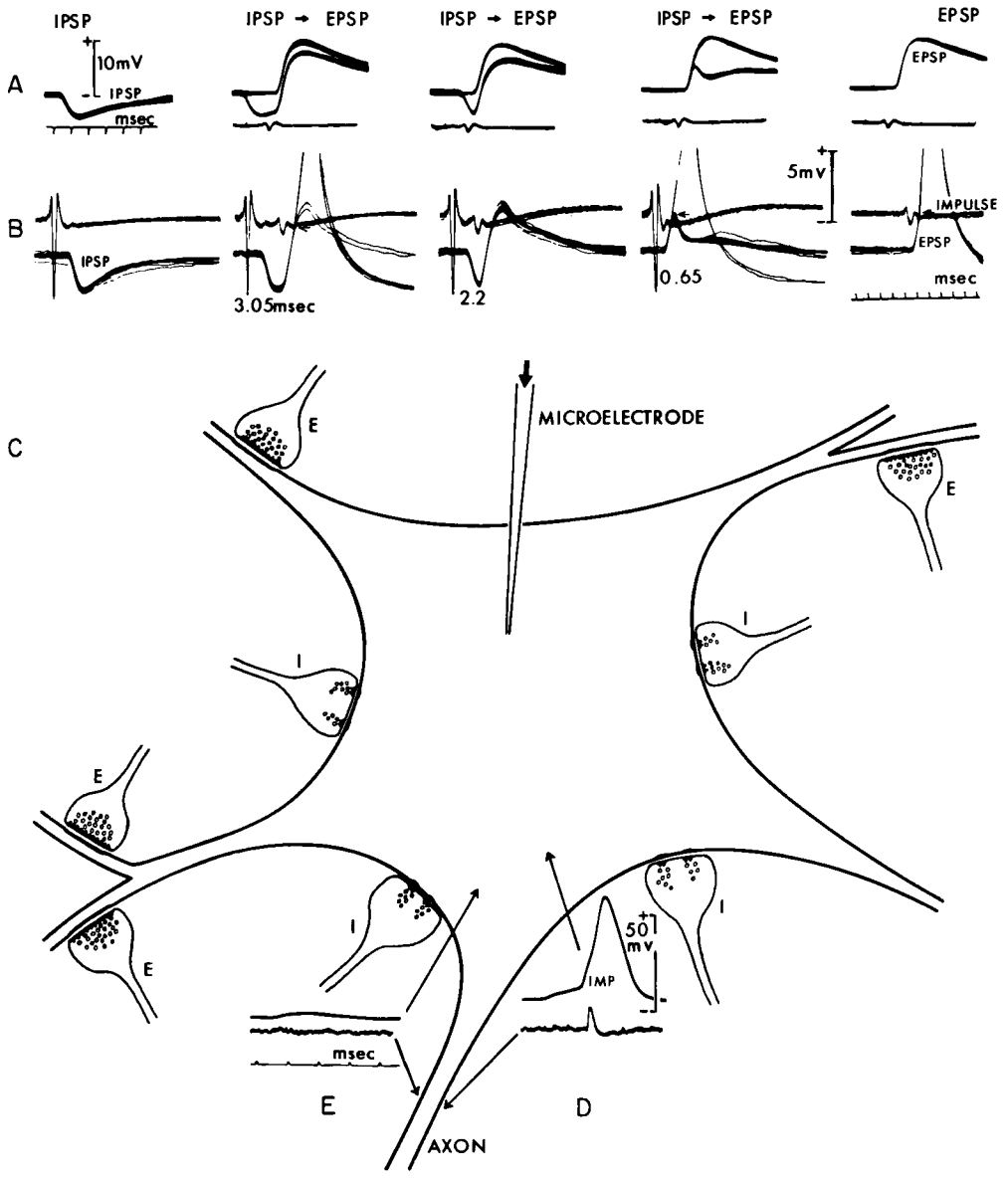


FIGURE 1 Diagram of neuron (C) with excitatory (E) and inhibitory (I) synapses and with two series (A and B) of specimen intracellular records above. D shows intracellular spike potential above and simultaneous record of that impulse in a ventral root filament; in E are the same two types of recording when the spike generation was prevented by inhibition.

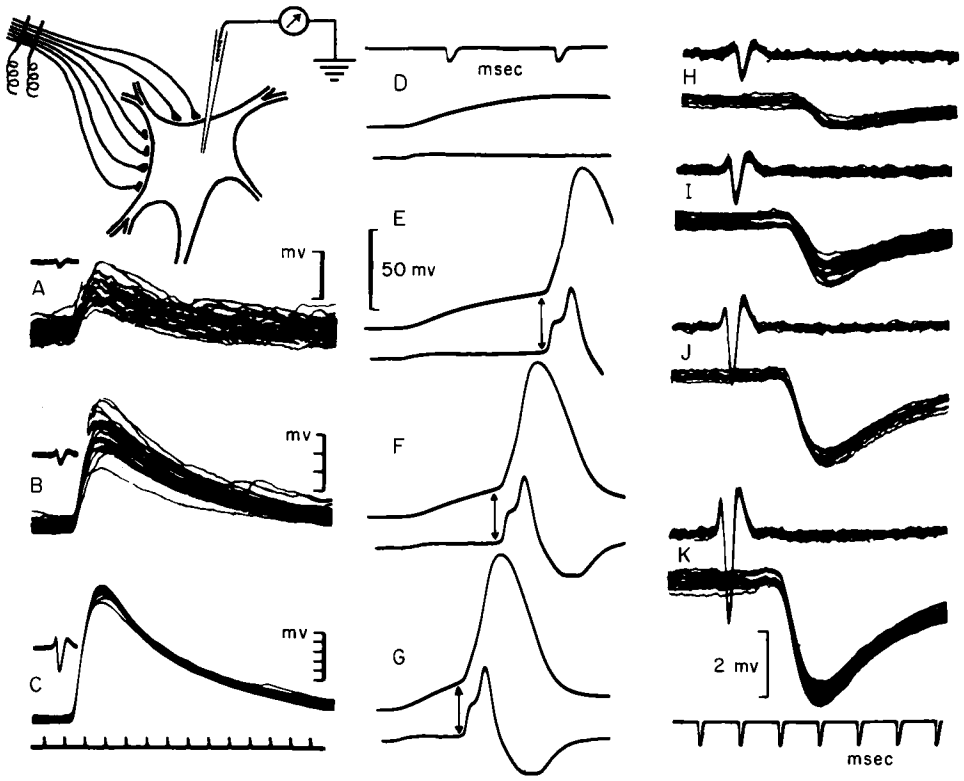


FIGURE 2 A to C: Excitatory postsynaptic potentials (EPSPs) obtained in a biceps semitendinosus motoneuron with afferent volleys of different size, the experimental arrangements being shown schematically in the inset diagram. Inset records (negativity downwards) at the left of the main records show the afferent volley recorded near the entry of the dorsal nerve roots into the spinal cord. Records of EPSPs are taken at an amplification that decreases in steps from A to C as the response increases. All records are formed by superposition of about 40 faint traces.[40] D to G: Intracellularly recorded potentials of a gastrocnemius motoneuron (resting membrane potential, − 70 mv) evoked by monosynaptic activation that was progressively increased. The lower traces are the electrically differentiated records; the double-headed arrows indicate the onsets of the IS spikes in E to G.[41] H to K: Intracellularly recorded IPSPs generated in a biceps-semitendinosus motoneuron by graded sizes of a quadriceps Ia afferent volley. (From Eccles, Note 42)

just as is excitation (Figure 2, H-K). The size of the EPSP depends on the number of activated synapses. These synapses are found, experimentally, to be almost additive in their effects on membrane potential—precisely what one would expect theoretically. As yet, there is no experimental test of this expected addition for unitary synaptic actions.

Figure 3 shows diagrammatically the manner in which excitatory and inhibitory synapses operate. The membrane potential is shown as −70 millivolts—the usual resting potential—and it is also approximately the chloride potential across the membrane, i.e., the electrochemical potential for chloride approximates −70 millivolts. The electrochemical potentials for sodium and potassium ions are about +60 and −90 millivolts respectively. Excitatory synapses tend by their action to bring the membrane potential to zero because of the opening of potassium–sodium gates caused by the action of the excitatory synaptic transmitter on the excitatory subsynaptic membrane. On the other hand, the inhibitory synapses tend to bring the membrane potential in the mammalian preparation to about −80 millivolts, the result of mixed potassium and chloride gates opening across the inhibitory subsynaptic membrane. In this way, the excitatory and inhibitory synapses pull the membrane potential in opposite directions and so counteract each other (D). As a consequence of their action, current flows from the rest of the postsynaptic membrane, depolarizing it in the case of the excitatory synapse (A), with the net inward current through the sodium–potassium gates. With the inhibitory

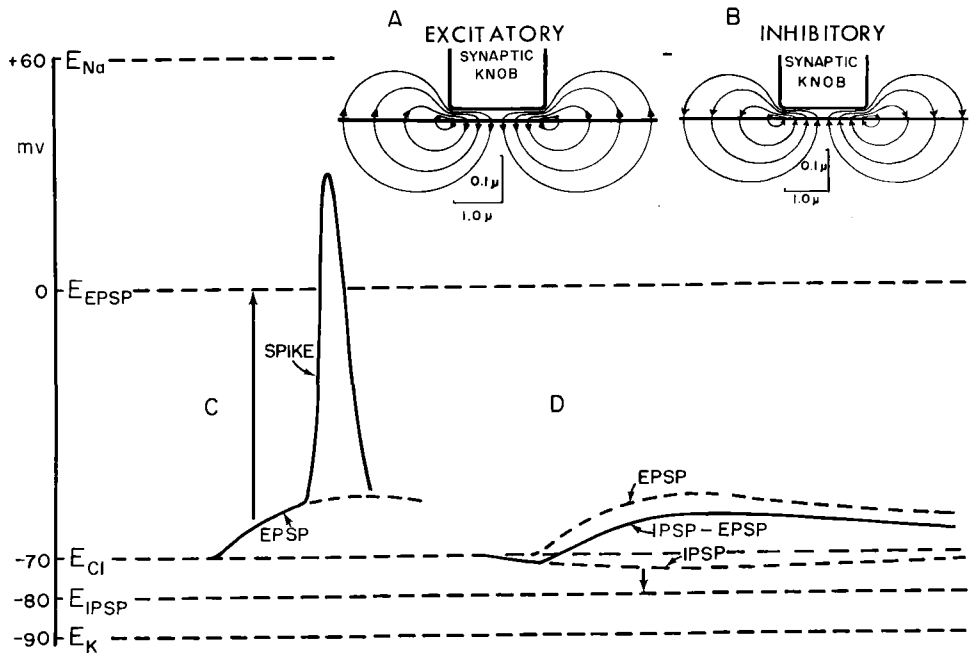


FIGURE 3 A: Diagram showing an activated excitatory synaptic knob. The synaptic cleft is shown at ten times the scale for width as against length. The current is seen to pass inward along the cleft and in across the activated sub-synaptic membrane. Elsewhere, as shown, it passes outwards across the membrane, so generating the depolarization of the EPSP. B: Similar diagram showing the reverse direction of current flow for an activated inhibitory synaptic knob. C: Diagram showing the equilibrium potentials for sodium (E_{Na}), potassium (E_k), and chloride (E_{Cl}) ions, together with the equilibrium potential for postsynaptic inhibition (E_{IPSP}). The equilibrium potential for the EPSP (E_{EPSP}) is shown at zero. To the left an EPSP is seen generating a spike potential at a depolarization of about 18 mv (see Fig. 2 E-G). To the right of the diagram an IPSP and an EPSP are shown alone (broken lines) and then interacting (continuous line). As a consequence of the depressant influence of the IPSP, the EPSP that alone generated a spike (left diagram) no longer is able to attain the threshold level of depolarization, i.e. the inhibition has been effective.

synapse, the net ionic current goes outwards through the potassium–chloride gates (B).

Inhibitory pathways in the spinal cord

Now, Sherrington's original idea on synaptic inhibitory action was very simple.[1] The same afferent fiber from the skin, as shown in Figure 4, forms synapses that directly excited the flexor motoneurons and inhibited the extensor motoneurons; complementarily, on the other side there was a reciprocal synaptic action. This figure gives the essentials of the diagram that was generally accepted before the chemical nature of synaptic transmission was recognized. That view lingered on even into the chemical-transmitter days. Thus, it was generally believed for many years that the central synapses from the same fiber could have opposite actions on different cells; now that has proved to be untenable, and there is no known example of this synaptic ambivalence in the nervous system, at least for the chemical synapses.

Figure 5 shows that there is an interneuron interpolated on the inhibitory line of even the simplest inhibitory pathways.[2] The impulses in the large afferent fibers (group Ia) from the stretch receptors of the quadriceps extensor are shown (Figure 5A) to be exciting the extensor motoneurons monosynaptically, so forming the pathway for the knee jerk. In addition, these group Ia fibers have axon collaterals that excite interneurons, which in turn send their axons across to the antagonistic motoneurons inhibiting them. In the monosynaptic excitatory pathway, as shown in Figure 5B, there is a very brief delay between the time of the impulse in the dorsal root and the onset of the EPSP of a motoneuron—only about 0.5 milliseconds. On the other hand, the inhibitory action on the antagonist motoneuron takes about a millisecond longer (Figure 5C). Moreover, just outside the antagonist motoneuron an im-

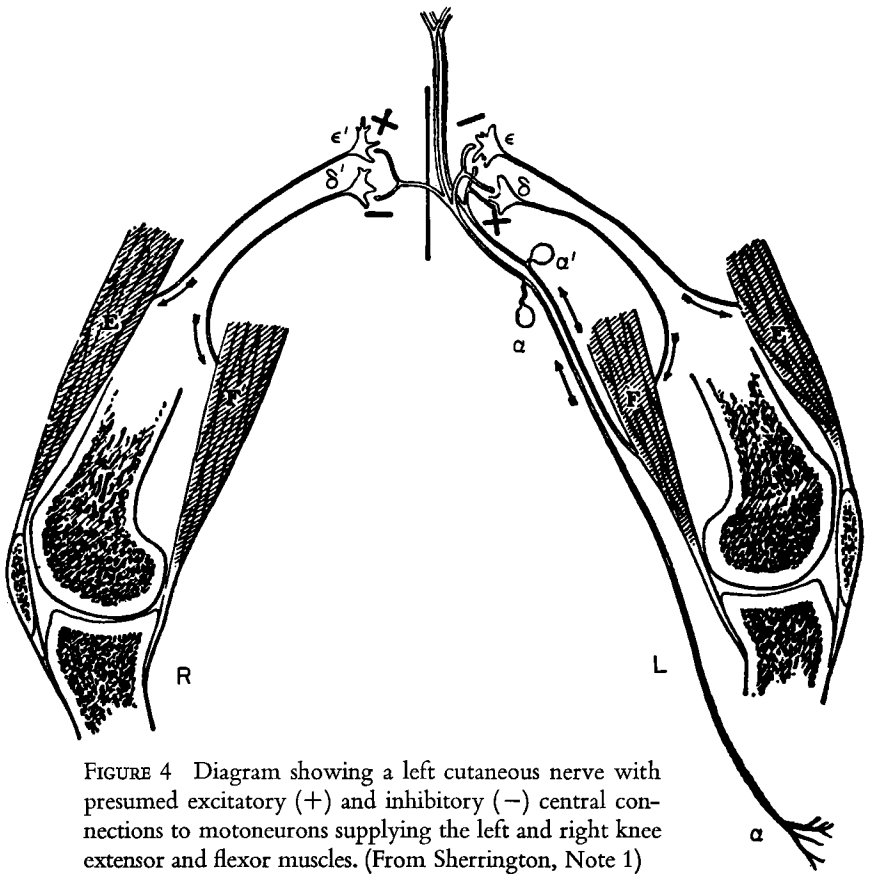


FIGURE 4 Diagram showing a left cutaneous nerve with presumed excitatory (+) and inhibitory (−) central connections to motoneurons supplying the left and right knee extensor and flexor muscles. (From Sherrington, Note 1)

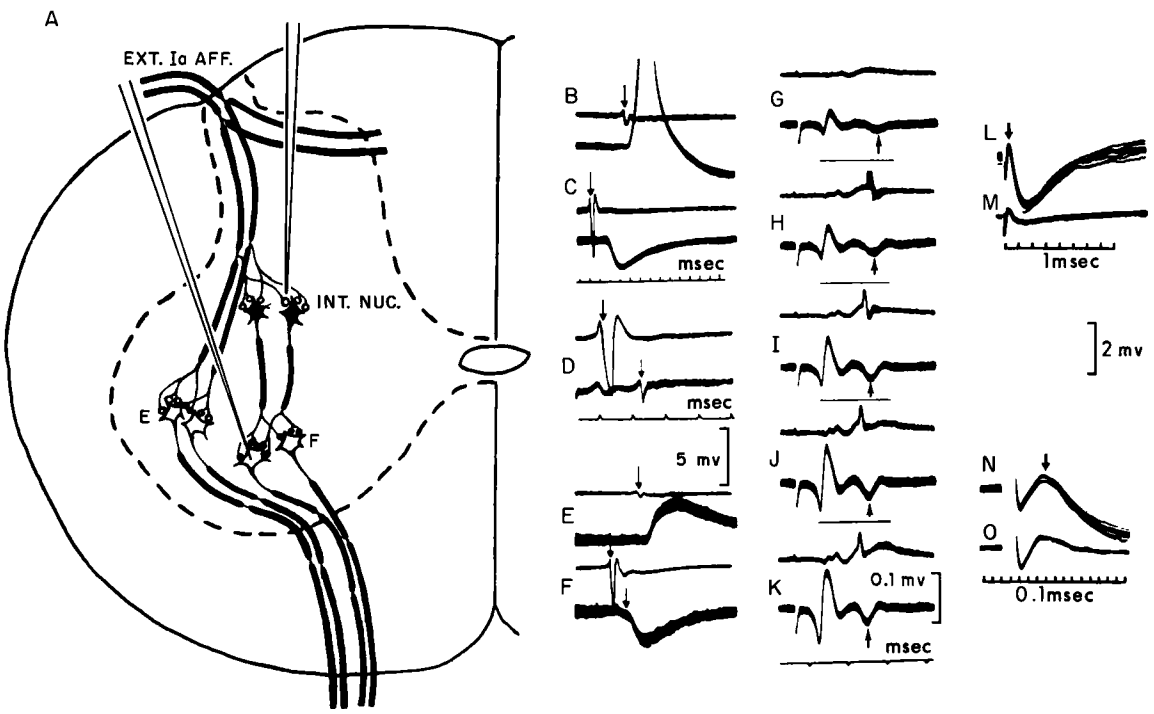


FIGURE 5 Diagram of transverse section of the spinal cord in the lower lumbar region. There is a full description in the text of the various specimen records of intracellular and extracellular potentials generated by Ia afferent volleys in B-K and recorded with one or the other microelectrode as shown in A. In L-O the stimulus was applied through the microelectrode in the intermediate nucleus in A. (From Eccles, Fatt, and Landgren, Note 2a, and Eide, Lundberg, and Voorhoeve, Note 3)

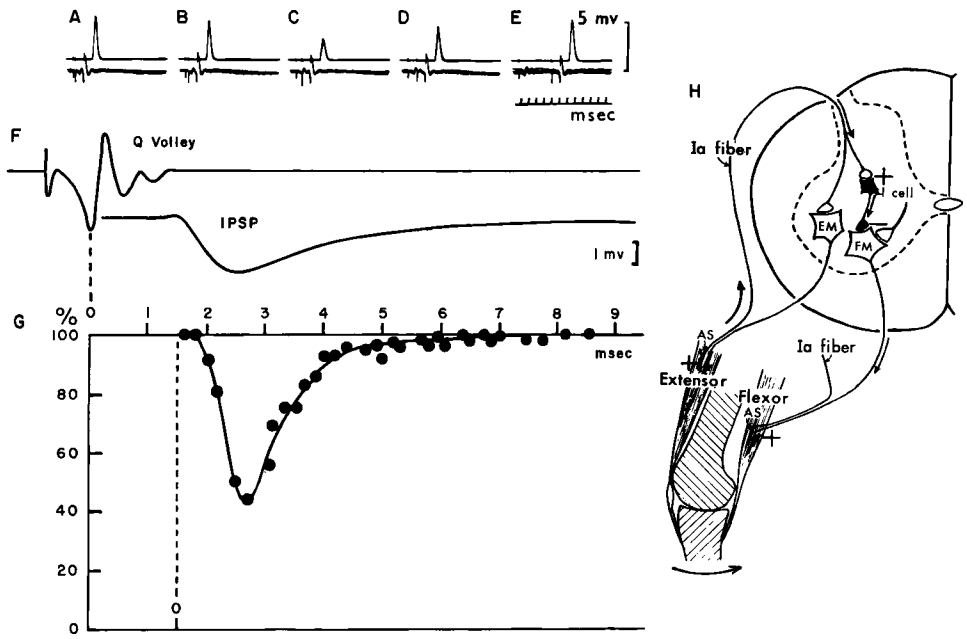


FIGURE 6 A-E: Inhibitory action produced on the monosynaptic discharge of flexor motoneurons (PBST) by a volley in the group Ia fibers from the antagonistic quadriceps muscle (Q), the pathways being illustrated in H. F shows the cord potential produced by the Q volley and the IPSP intracellularly recorded from a PBST motoneuron on the same time scale that also obtains for G, which gives the plotted points for the inhibitory curve derived from A-E. Further description in text. (From Araki, Eccles, and Ito, Note 4)

pulse can often be observed after a considerable delay and only about 0.4 milliseconds before the start of the IPSP (Figure 5D). Hence the longer delay of the IPSP results from a longer conduction time in the inhibitory pathway, and not from a longer intrinsic delay in inhibitory synapses. It is postulated that this longer conduction time is the result of an interpolated interneuron, as seen in Figure 5A. Everywhere throughout the nervous system, right up to the cerebral cortex, whenever there is an input line that branches and gives excitation and inhibition, the action of the inhibitory line exhibits a latency about 0.8 milliseconds longer. This is a kind of standard value required for the postulated extra synaptic relay. The time is occupied not only in the synaptic delay but also in setting up the impulse and in its propagation to the synaptic terminals.

If you record through a microelectrode in the intermediate nucleus, you often can see the responses of one of the interneurons that are postulated to be on the group Ia inhibitory path (cf. Figure 5A). Its spike response comes in with a fairly weak stimulus (H), which sets up only Ia impulses; and, as you stimulate more and more strongly (Figure 5, G-K), there is also a brief positive wave (arrows), which we now know to be generated by the whole population of interneurons firing impulses towards the motoneurons. This is diagrammed in Figure 5A.[2,2a]

If a stimulus is applied through the microelectrode in the intermediate nucleus,[3] these intermediate neurons can be stimulated directly, and the IPSPs of motoneurons have just as brief a delay as the EPSPs so produced; the latencies are only about 0.5 millisecond, as seen in Figure 5, L, N; M and O are the respective extracellular traces. There is no question whatsoever about the existence of these interneurons on the group Ia inhibitory pathway.

In Figure 6H, the pathways are fully diagrammed and are shown coming up from the extensor muscle, going directly to the extensor motoneurons, and through the interneurons to the flexor motoneurons. In all diagrams, the inhibitory neurons (i.e., those cells directly producing postsynaptic inhibition) are shown in black. Figure 6 also shows some of the ways in which this group Ia inhibitory action can be investigated.[4] Stimulation of the group Ia afferents of the flexor muscle evokes a monosynaptic reflex discharge that can be recorded as a spikelike potential in the ventral root (Figure 6, A-E). Stimulation of the Ia fibers of the antagonist extensor muscle will diminish this discharge to even less than half, as in C at the optimum interval. When the size of the test reflex as a percentage

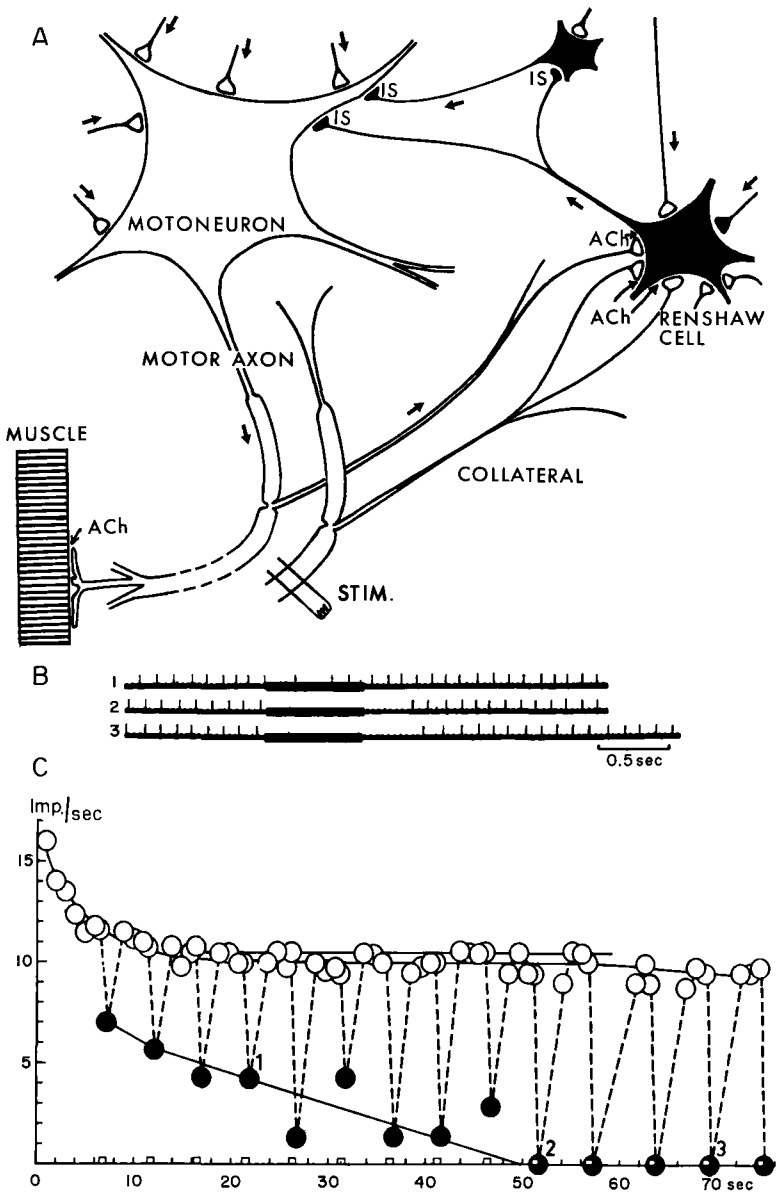


FIGURE 7 Inhibitory action mediated by negative feedback from motoneuron axon collaterals to Renshaw cells, the pathway being shown diagrammatically in A. In this and all subsequent figures the postsynaptic inhibitory cells and synapses are shown in black. Further description in text. (From Granit and Rutledge, Note 7)

of the control is plotted against the stimulus interval (Figure 6G), it is seen that the time course of the inhibition is very like the IPSP, which is recorded inside these motoneurons (Figure 6F, lower trace). In the curve of Figure 6G, the ordinates at each interval give the percentages of motoneurons that resist the inhibitory action. A comparison of the lower trace of F with G reveals a satisfactory explanation of this inhibitory action. To a large extent it is attributable to the increase in membrane potential, the IPSP, that is caused by the action of the synaptic transmitter (cf. Figure 3B). The time course of this membrane potential matches the inhibition of the neurons, and it can be concluded that the existence of the pathway is now well established.

Another inhibitory pathway is shown in Figure 7. In A is shown a motoneuron axon going to the muscle fibers and exciting them by the synaptic chemical transmitter, acetylcholine. This motoneuron also has axon collaterals within the spinal cord that form synapses with the so-called Renshaw cells, named after their discoverer, Birdsey Renshaw,[5] who died tragically when he was a young neurophysiologist in America. Renshaw cells are on the pathway of another example of inhibition. When the motor axons are stimulated by an applied electrode (Fig-

ure 7A), the impulses so evoked traverse the motor axon collaterals and excite Renshaw cells by acetylcholine.[6] The discharges of Renshaw cells pass to motoneurons and inhibit them by again putting a larger charge on the membrane and so counteracting excitation.

Granit and Rutledge[7] have demonstrated this inhibition nicely (Figure 7B) by causing a motoneuron to fire at a steady rate in response to a continued stretch and then stimulating motor axons, causing impulses to fire along the axon collaterals to Renshaw cells. As a consequence, the firing rate of the motoneurons to the applied stretch is slowed. As the firing rate of the motoneuron to the applied stretch gradually slows down with accommodation, interpolation of the same antidromic stimulation of Renshaw cells in its early stages merely slows the discharge but later the discharge is completely stopped (Figure 7C). Thus the Renshaw cell mechanism provides a most effective way of reducing or checking the firing of motoneurons.

The physiological significance of such inhibition via the Renshaw cell pathway can be derived from considerations of the manner in which movement is brought about by motoneurons firing down their axons and into muscles. All of these discharges are, at the same time, going back to Renshaw cells, which in turn inhibit motoneurons, thus reducing the frequency of their discharges or even silencing them. This action via Renshaw cells is a true negative feedback. It has no specific action in the sense of sculpturing a particular movement one way or another. (By sculpturing action, I mean a control of the spatio-temporal form of neural response operations.) All it does is to have a focusing action, in the sense that only the heavily excited motoneuron can continue to discharge and so cause muscle contraction; the others are silenced by this feedback.

The responses of the Renshaw cells were recorded with a microelectrode (Figure 8B) and were thus located in exactly the position (Figure 8A) definitively observed by Wilson,[8] who used dye marking. The technique gave confirmation of the sites that had been found by dead reckoning from tracking methods. When the dorsal roots are severed so that muscle nerve stimulation evokes only antidromic impulses in motor axons, the same Renshaw cell is fired by the motor axons of many muscles. If one records intracellularly from a motoneuron, antidromic impulses from many different muscles evoke inhibitory postsynaptic potentials by means of the axon collaterals and the Renshaw cells, as in an anterior biceps motoneuron (Figure 8C).

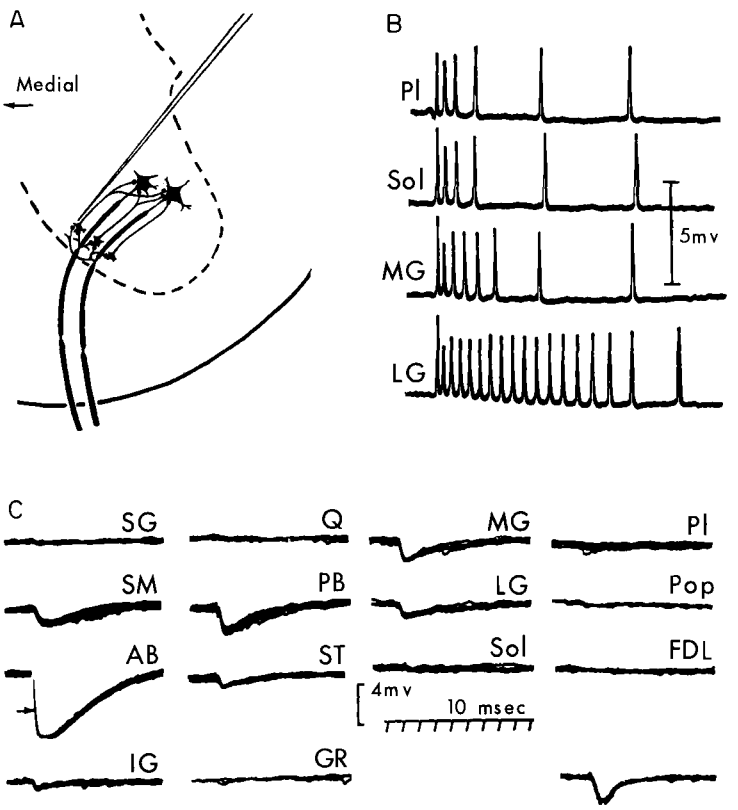


FIGURE 8 Recording from a Renshaw cell (A) shows (B) that it fires repetitively to single volleys in the motor fibers to four different muscles. C shows by intracellular recording that IPSPs of various sizes are produced in an anterior biceps motoneuron by single volleys in the motor fibers supplying eight different muscles of the same hind limb. SG, superior gluteal; Q, quadriceps; MG, medial gastrocnemius; Pl, plantaris; SM, semimembranosus; PB, posterior biceps; LG, lateral gastrocnemius; Pop, popliteus; AB, anterior biceps; ST, semitendinosus; Sol, soleus; FDL, flexor digitorum longus; IG, inferior gluteal; GR, gracilis. (From Eccles, et al., Note 43)

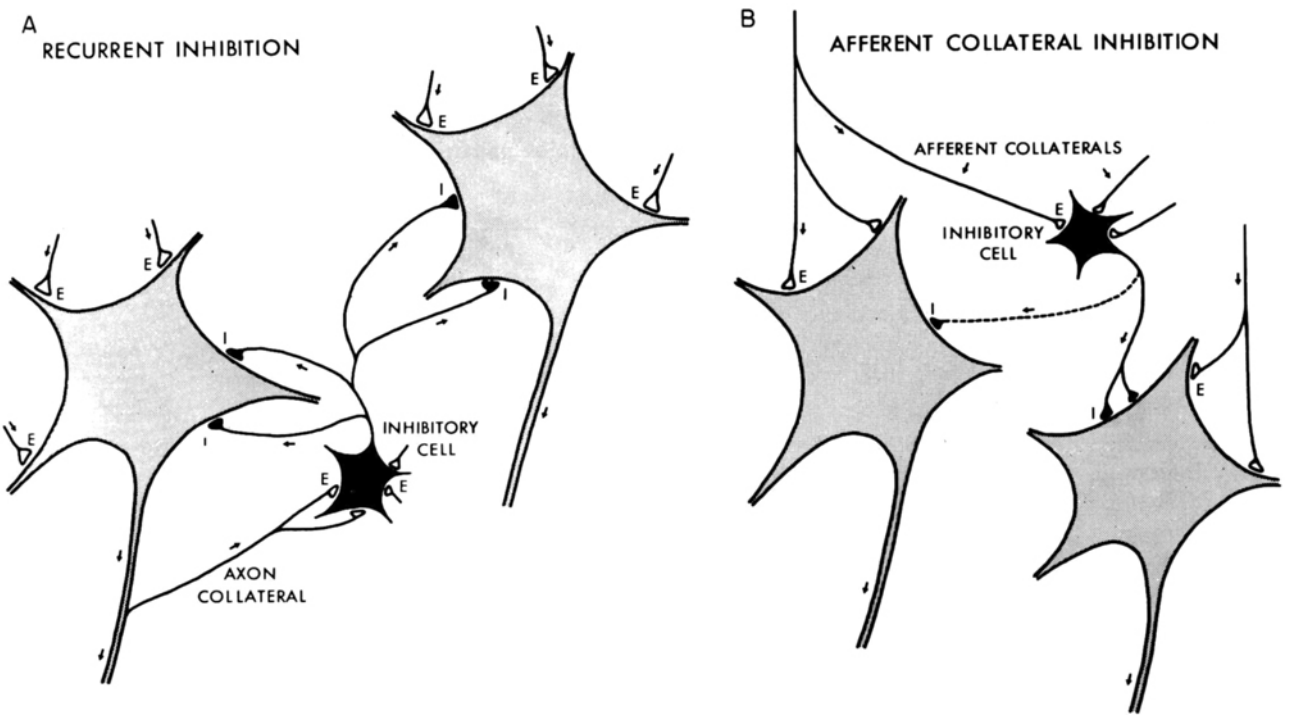


FIGURE 9 Diagrams of the two types of inhibitory pathways (inhibitory cells shown in black), as described in the text.

Design of inhibitory pathways

I can now diagram (Figure 9) two kinds of inhibitory pathways; the more complicated ones will be revealed as we probe into the manner of operation of more complex parts of the nervous system. The first (Figure 9A) is a pathway, such as that via Renshaw cells, where axon collaterals of a nerve cell effect an inhibition of this cell and its neighbors of like kind. This negative feedback usually has a kind of focusing effect by narrowing down the field of discharging cells. Such focusing is important, because there is a great deal of random spread of information in the synaptic transmission through successive relays of nerve cells. However, by the operation of the negative feedback control, those that have been weakly excited are silenced; only the strongly excited cells continue to discharge.

The other simple inhibitory pathway is the so-called feed-forward inhibition, diagrammed in Figure 9B. Impulses flowing along a pathway are partly diverted along collaterals that excite inhibitory interneurons. These interneurons inhibit cells that often have a function opposite to that of cells directly excited by the main operation channel of the input. This feed-forward inhibition, as it may be called, is perhaps a more sophisticated kind of inhibitory action than the feedback inhibition. It would exert a sculpturing action.

Inhibition in the hippocampus

Postsynaptic inhibition of hippocampal pyramidal cells is of particular interest because the inhibitory cells were first clearly identified here.[9,10] The hippocampus is a part of the cerebral cortex, the archicortex, and the same kind of inhibition also exists on the pyramidal cells of the neocortex. As shown first by Kandel, Spencer, and Brinley,[11] extremely large and prolonged inhibitory potentials are recorded from hippocampal pyramidal cells. Various inputs can be used to evoke these large IPSPs (Figure 10, A-C). It appears that the inhibitory pathway is of the negative feedback type, as illustrated in Figure 10F. Axon collaterals of pyramidal cells excite basket cells whose axons feed back inhibition onto the bodies of the pyramidal cells.

Ramón y Cajal first described the axons of basket cells that form a dense meshwork on the bodies of the pyra-

midal cells. Basket cells were, in fact, the first inhibitory cells to be identified. The identification was based on several lines of evidence. When you use a microelectrode to track from the surface down to the level of the somata of the pyramidal cells, a very large wave of extracellular positivity develops, as shown in Figure 10D. This wave diminishes rapidly at deeper levels as well as more superficially. When plotting its depth profile, measurements must be made at a fixed latency, as, for example, that given by the vertical broken line in Figure 10D; the measurements give the filled triangles, shown in Figure 10E. A similar profile is given by the positive waves evoked along this same electrode track by the two other inputs. All three profiles agree in giving the peak of positivity at the depth of the pyramidal cell bodies.

That peak means that a current is flowing from the peak of positivity, which functions as a source, to both more superficial and deeper sinks. These field potentials

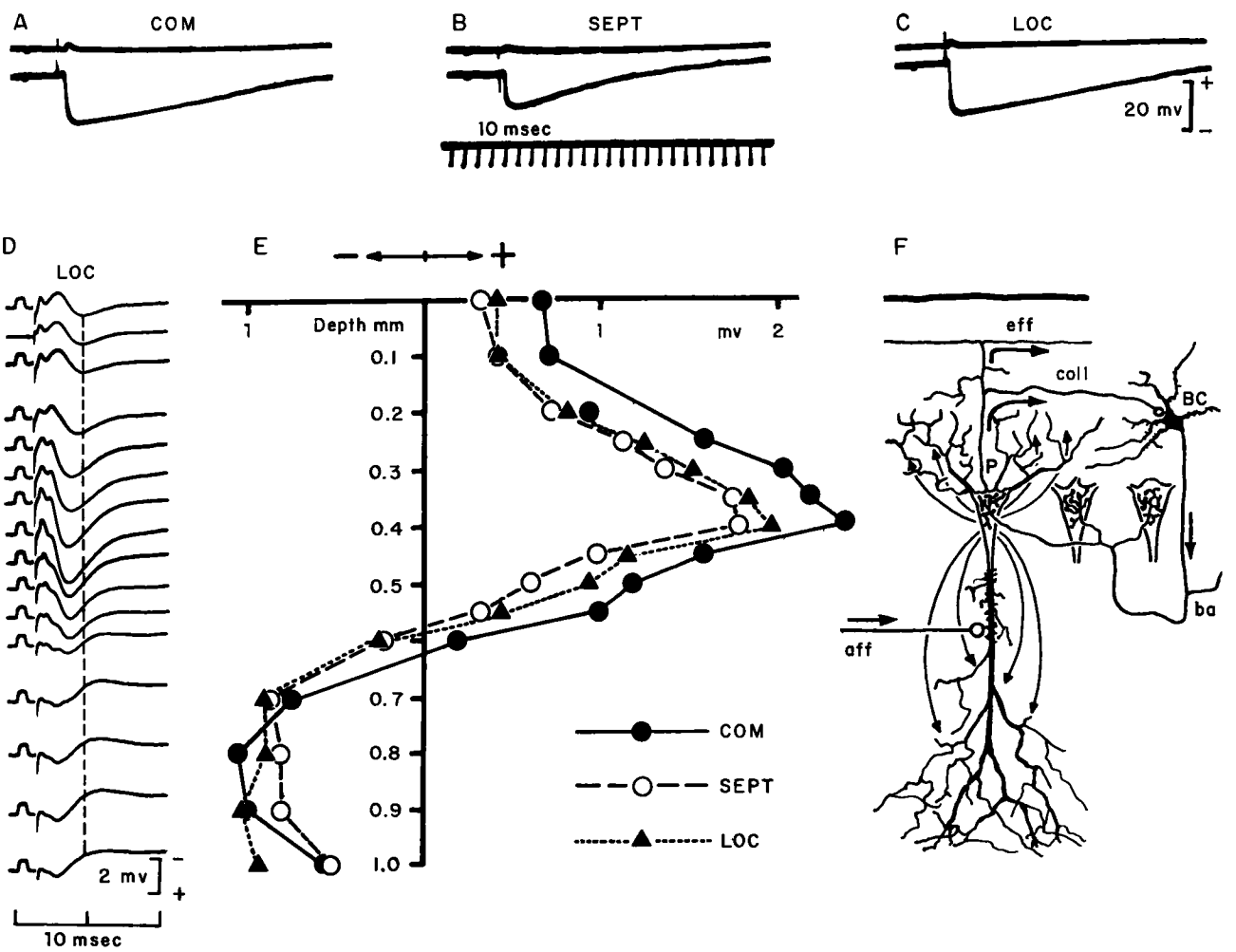


FIGURE 10 Potentials recorded intracellularly (A-C) and extracellularly (D,E) from the hippocampus as described in the text. COM, commissural stimulation; SEPT, septal stimulation; LOC, local stimulation. In F there is a diagrammatic representation of the recurrent inhibitory pathway from the axon collateral (coll.) of a pyramidal cell (P) to a basket cell (BC) whose axons (ba) feed inhibition back to pyramidal cell bodies. (From Anderson, et al., Notes 9 and 10)

are made simply by the ordinary ohmic law. The current flowing out from the lamina of pyramidal cell bodies produces the observed potential gradients in the extracellular medium; and, because intracellular recording from these cells demonstrates the large hyperpolarization of the IPSP (Figure 10, A-C), it is clear that the inhibitory synapses are exerting their hyperpolarizing action directly on the somata. This is precisely the location of the dense synaptic endings of the basket cells, and there are no other synapses on the somata; hence the situation is unambiguous.

In conclusion, it can be stated that there evidence of a powerful inhibitory mechanism on the hippocampal pyramids; in addition, it has been demonstrated that the basket cells are directly responsible for this inhibitory action. Furthermore, it seems that the basket cells are fired by axon collaterals, so this inhibition is an example of negative feedback.

Inhibition in the cerebellum

Figure 11 is a perspective drawing of a cerebellar folium.[12] The cerebellar cortex is a relatively simple and very stereotyped structure. There are only five kinds of cells in the cerebellar cortex. There are only two pathways in, quite distinctive and extremely different from each other, and there is only one way out—by the Purkinje cell axons. One of the pathways in, that of the climbing fiber (cf), has a 1:1 relationship, giving an extremely powerful synaptic excitation to Purkinje cells. The other line in is via mossy fibers (mf) which branch profusely to form excitatory synaptic connections in little glomeruli with the most numerous nerve cell in the brain, the granule cells (GrC). There are about 3×10^{10} of these cells in a human cerebellar cortex. Each mossy fiber excites about 1000 granule cells. Each granule cell axon ascends to the molecular layer, where it bifurcates to form a parallel fiber (pf) which runs along the folium of the cortex for approximately 1.5 millimeters in each direction. As the name implies, the parallel fibers run parallel to each other. They are densely packed, are only about 0.2 to 0.3 microns in diameter, and have clefts of only 200 Å between, so constituting most of the molecular layer that forms the superficial 300 μ of the cerebellar cortex.

The parallel fibers form synapses on the Purkinje cells and on two other kinds of cells that are of special im-

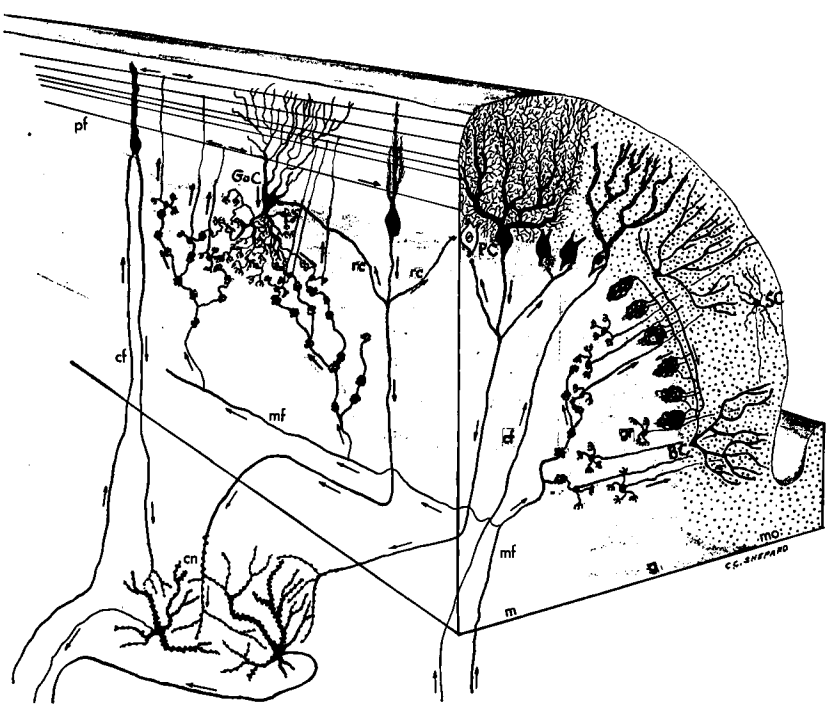


FIGURE 11 Schematic diagram showing in perspective drawing the various neurons and neuronal connections of the cerebellar cortex. Full description in text. (From Fox, Note 12)

portance for our present purpose: the basket cells (BC) and the Golgi cells (GoC) with their branching dendrites. There are about 200,000 excitatory synapses from parallel fibers on the Purkinje cell and perhaps 20,000 on thebasket cell, because the latter are not nearly so profuse in their branching dendrites. The basket cell axon runs transversely for about one millimeter in one or the other direction across the folium, and branches to give inhibitory synapses to the somata of the Purkinje cells en route.

The Golgi cell is also excited by the parallel fibers, but in addition it has deeper dendrites that are directly excited by the mossy fibers,[13,14] and its axon branches profusely in the granular layer to form an immense number of inhibitory synapses on the mossy fiber–granule cell synapses in the glomeruli.[13] Each Golgi cell inhibits a very large number—about 100,000—of these granule cells, and every granule cell receives Golgi cell inhibition.

A single volley from the superficial radial nerve evoked a repetitive discharge from a single granule cell (Figure 12B), and in the series C, D, E, this discharge was inhibited by a preceding stimulation of the parallel fibers by electrode (LOC) in A. This inhibition was complete for as long as 50 milliseconds, and even by 120 milliseconds (12E) there was not full recovery. This inhibitory action is also demonstrated in Figure 12G, where spontaneous discharges of several granule cells (12F) were completely inhibited for as long as 110 milliseconds by a LOC stimulus, and (in 12H) for 100 milliseconds after stimulation of the subcortical white matter through the electrode employed for juxtafastigial (JF) stimulation.

The only neuronal element that could be concerned in this inhibitory action on the mossy fiber–granule cell synapses is the Golgi cell, because it alone has synaptic endings in relationship to these synapses (Figure 12A). Parallel fiber stimulation by the LOC electrode presumably would excite Golgi cells via the crossing-over synapses given by the parallel fibers shown in Figure 12A as they cross the Golgi dendrites, and so produce the inhibition of granule cells demonstrated in Figure 12, C, D, E, and G. Furthermore, the inhibition produced by the JF stimulation is similarly explained, for this stimulus would directly excite mossy fibers that in turn activate granule cells and the parallel fibers stemming therefrom.

The inhibition of granule cells can also be observed by recording the negative wave (N_3) that a mossy fiber volley (fired by JF stimulation) evokes in the molecular layer and that is compounded of the spike potentials in the granule cell axons and the synaptic and spike potentials that they evoke in the Purkinje cells.[14] The N_3 wave of Figure 12I was diminished by a parallel fiber volley (at L in J and K), particularly the large volley of K, and this depression of N_3 is attributable to the inhibitory action of Golgi cells on the mossy fiber–granule cell synapse,[15] for it has been shown that a parallel fiber volley powerfully excites Golgi cells.[16]

Figure 13A illustrates some of the methods employed in investigating the physiological events evoked in the cerebellar cortex by an afferent volley in mossy fibers.[14,17-19] As we have seen, the first stage of transmission is from mossy fibers to granule cells and thence to the parallel fibers, so direct stimulation of the parallel fibers by a surface electrode provides a convenient way of simplifying the investigation by circumventing these earlier stages. The beam of parallel fiber impulses so produced directly excited the Purkinje cells that are on the beam; the synaptic depolarization leads to the generation of an impulse on the intracellular records from a Purkinje cell (Figure 13B). Following the spike there is a slowly developing hyperpolarization, which is the beginning of the large and prolonged inhibitory postsynaptic potential (IPSP). When the microelectrode records from a Purkinje cell that is lateral to the beam of excited parallel fibers, there is no initial excitatory phase, but merely the prolonged IPSP that is produced by the transversely distributed axons of the basket cells (Figure 13C). As would be expected, this IPSP effectively depresses all excitatory responses of Purkinje cells for 100 milliseconds or more. An example of this inhibition is shown in the extracellular recording of Purkinje cells in Figure 13, D-J. The test response is evoked by antidromic stimulation (cf. Figure 13A), which sets up an initial diphasic spike (control in D). In E-I the parallel fiber volley sets up a positive field potential, which in part is the extracellular counterpart of the IPSP; at the longer testing intervals of G to I there is depression of the negative (upward) phase of this antidromic spike, indicating that the antidromic invasion of many Purkinje cells is depressed by the IPSP. The time course of the depression is plotted in J and has a duration comparable with the IPSP.

Figure 14 shows a schematic drawing by Szentágothai[20] that attempts to give functional meaning to the transversely directed axons of basket cells. It is to be noted that the axons spread in one or the other direction transversely across the folium, not in both directions from one cell. The black cells centrally placed in A are basket cells with axons running transversely and giving synapses to the axonal poles of the Purkinje cell somata en route. A plan of a folium, i.e., as seen from above, is in B. The central group of parallel fibers indicates the longitudinal axis of the folium, while there are four basket cells in black with their transversely directed axons distributed to the Purkinje cells with somata indicated in the plan by circles and with the transversely directed dendritic expansions drawn thereon on a greatly contracted scale. The impulses of a

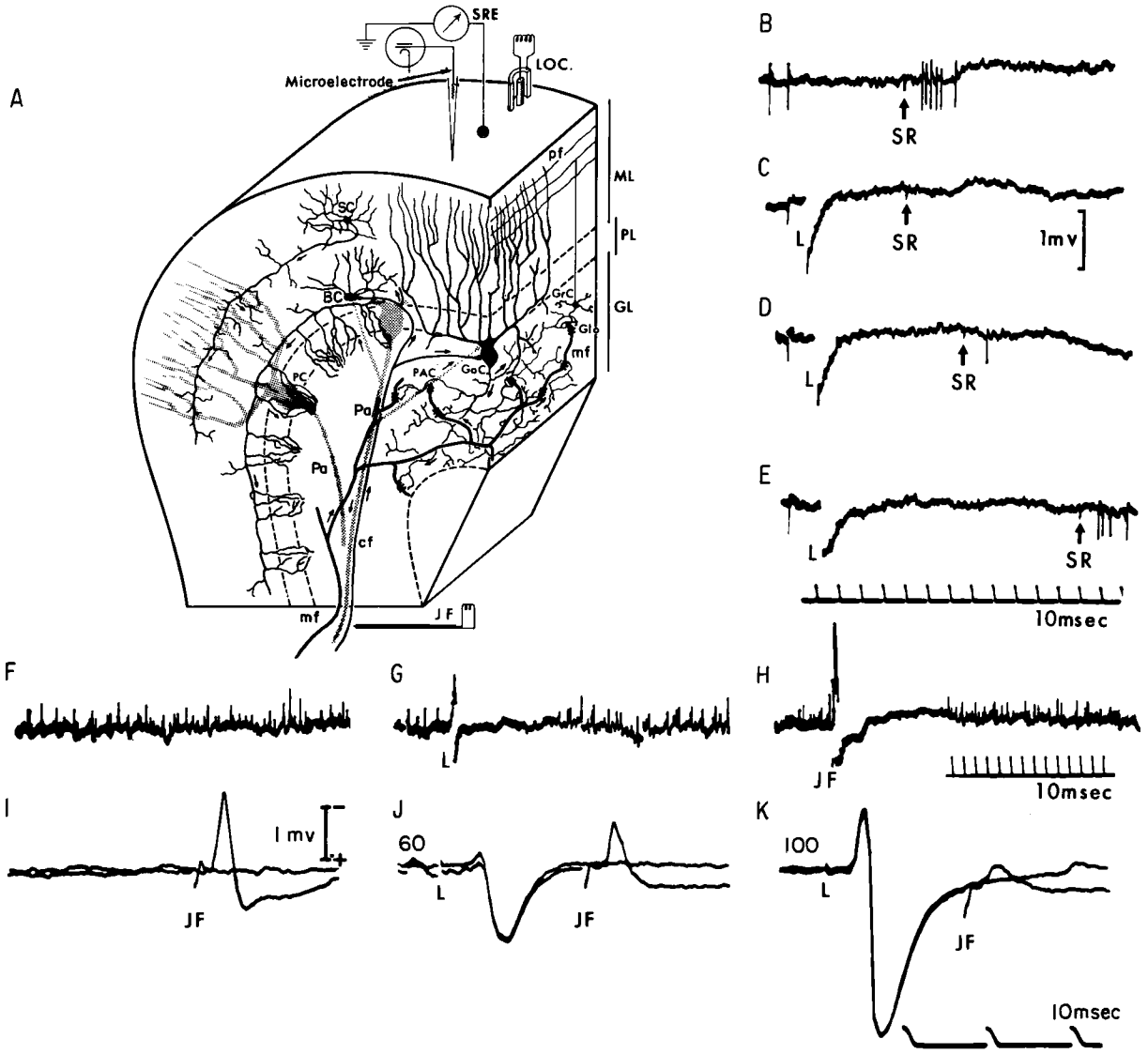
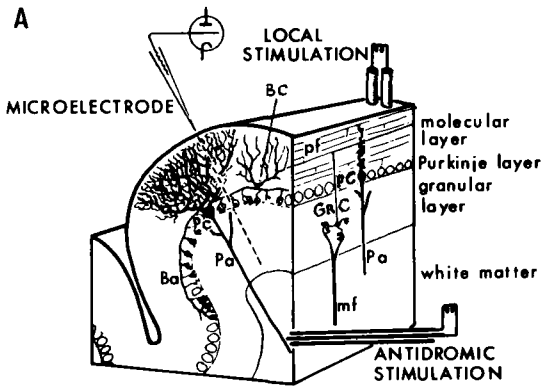
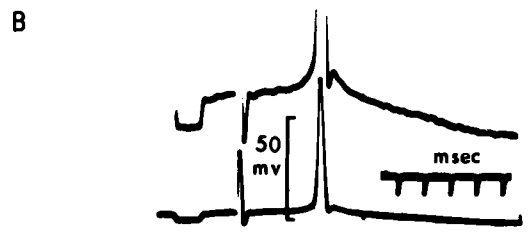


FIGURE 12 A: Perspective drawing of a cerebellar folium to show the anatomical relationships of the inhibitory interneurons and the experimental arrangements with respect to stimulating and recording electrodes. The cerebellar cortex is seen to be divided into three layers, the molecular layer (ML), the Purkinje cell layer (PL) and the granular layer (GL). The input to the cortex is by two types of fiber, the mossy fiber (mf) and the climbing fiber (cf). Single examples are shown of four types of interneurons, granule cells (GrC), Golgi cells (GoC), basket cells (BC) and superficial stellate cells (SC). Also shown are two Purkinje cells, one (PC) with its dendritic ramifications, and both axons (Pa), one with two collaterals (PAC) ending on the Golgi cell and the basket cell. The mossy fiber is shown with numerous branches and thickenings at the sites of its synapses on granule cell dendrites, so forming the glomeruli (Glo). Collaterals of the climbing fiber (cf) are shown making synapses on the Golgi cell and the basket cell. The axons of the granule cells bifurcate to give rise to the parallel fibers (pf) in the molecular layer. The electrode arrangements are described in the text. Arrows show directions of normal propagation in the mossy fibers, the climbing fiber and its collaterals, Purkinje axons and collaterals, and the axons of the interneurons BC, SC, and GoC.

B-H: A superficial radial volley (SR) evokes discharges from a single granule cell (B), which is inhibited by a preceding parallel fiber volley set up at L by local electrode (LOC) in A (C, D and E). In F, G and H, spontaneous discharge of several granule cells is inhibited by L and JF (juxtafastigial) stimuli.[25] I, J, K: Action of parallel fiber stimulation on responses evoked in molecular layer at a depth of 150μ showing inhibition of responses evoked by JF stimulation (I) by a preceding parallel fiber stimulation (L) of the strengths indicated in arbitrary units in J and K.





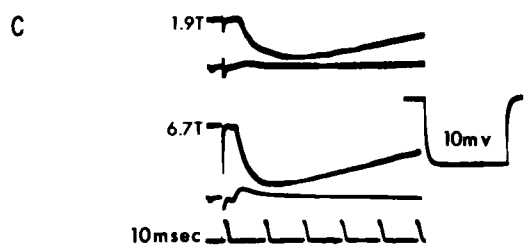
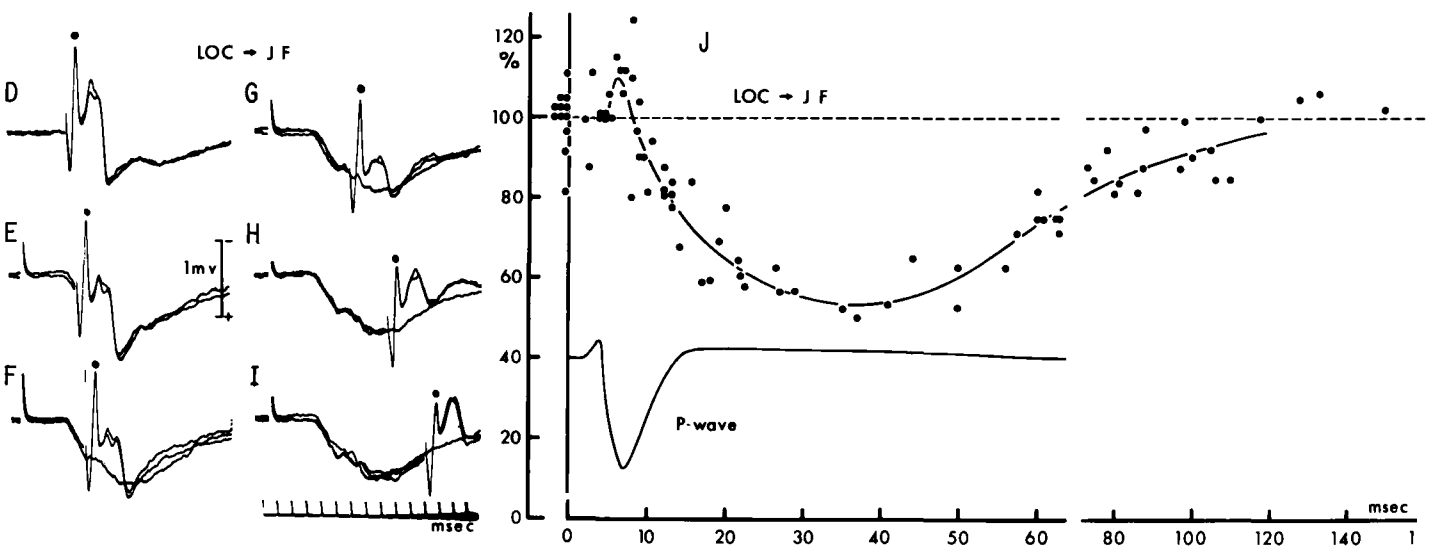


FIGURE 13 A: Diagram of cerebellar cortex showing experimental arrangement in relation to the essential structural features. PC, Purkinje cells; Purkinje cells with axons, Pa; mf, mossy fiber; GrC, granule cell; pf, parallel fiber; BC, basket cell; Ba, basket cell axon. Note antidromic stimulation of Purkinje cells by an electrode placed in the juxta-fastigial (JF) region, and parallel fiber stimulation by a local electrode (LOC in D-J). B: Intracellular recording at high and low amplification from a Purkinje cell, showing the response to a parallel fiber volley, an initial slowly rising depolarization leading to the generation of an impulse, followed by the very slow developing hyperpolarization, the IPSP.[19] C: Intracellular recording from another Purkinje cell that is out of line for the parallel fiber volley, showing the IPSPs set up by weak and stronger parallel fiber stimulation (1.9 and 6.7 times threshold). Immediately below each trace is the corresponding extracellular record.[24] D-J: Time course of the action exerted by a parallel fiber volley on the antidromic spike potential in the cerebellar cortex. D shows the typical complex potential produced by a JF stimulus in a normal cerebellum and recorded at a depth of 500μ. In E-I this response was conditioned by a parallel fiber volley at various stimulus intervals, there being superimposed in F-I a trace of the response to the conditioning volley alone in order to aid measurements of the size of the negative component of the antidromic spike (marked by dots above). The sizes of similar potentials in another experiment are expressed as percentages of the mean control responses and are plotted against the stimulus intervals in J. Note the change in abscissal scaling at the interruption of the base line at 70 msec. In J there is also a tracing showing on the same time scale the P wave that was produced by the conditioning parallel fiber volley as in E-1. (From Eccles, Llinás, and Sasaki, Note 18)



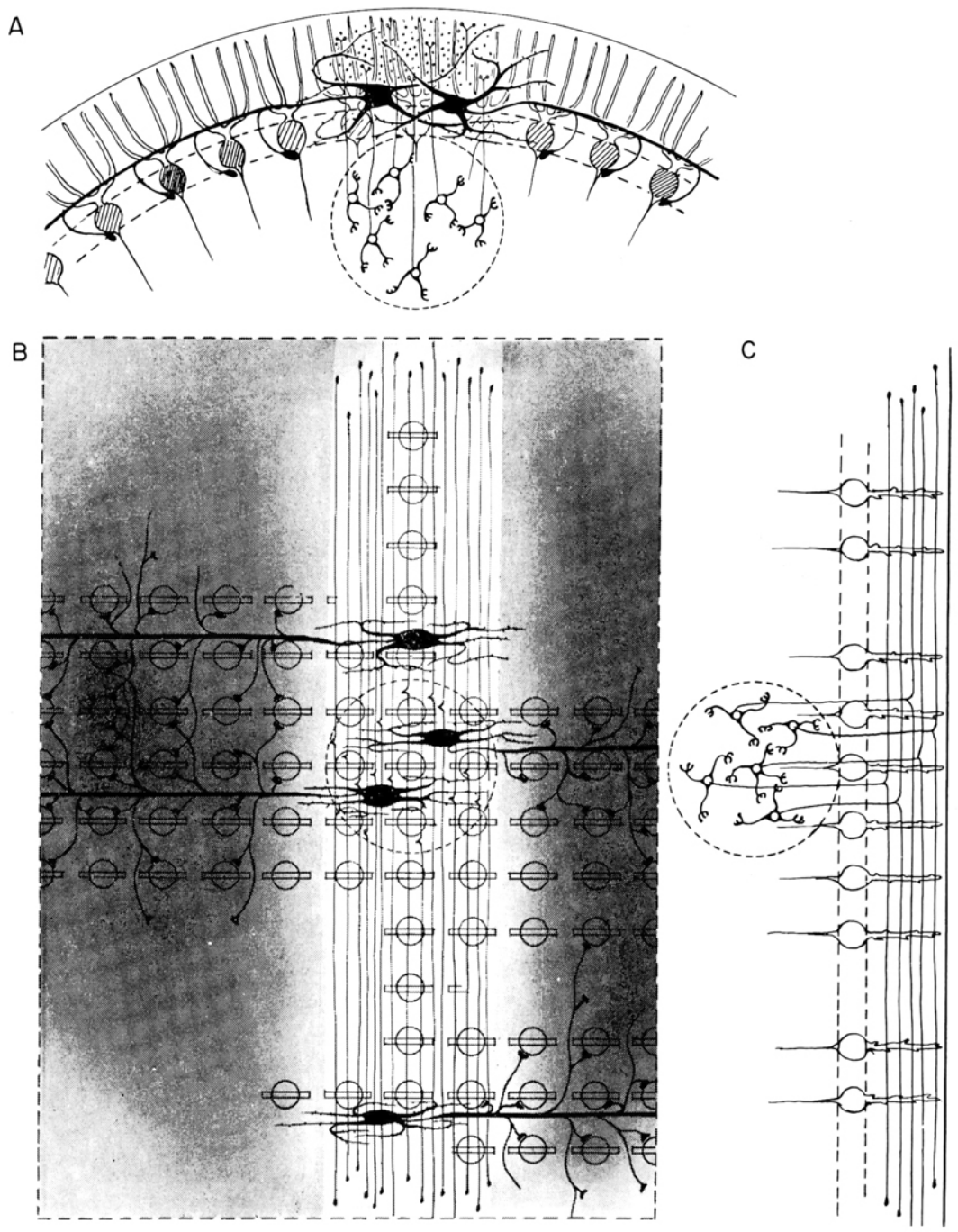


FIGURE 14 Diagram illustrating the concept of the higher order integrative unit of the mossy afferent-parallel fiber neuronal chain. Neuron matrix of folium seen in transverse (A) and longitudinal section (C) as well as from the surface (B). The assumption is made that all granule neurons inside the circles indicated are simultaneously excited and discharge impulses along their axons, the parallel fibers. In this case all Purkinje cells (indicated in B as small circles with overlying bars) along a longitudinal strip of about 3–4 mm length would be excited by the beam of parallel fiber impulses. A powerful inhibition would be exercised by basket neurons situated in the same strip of excitation on all the Purkinje neurons situated on either side of the excited strip. The degree of inhibition—as deduced from the number and strength of connections (convergence, size of terminal, etc.)—is indicated by shadowing in B and by the density of hatching of Purkinje cell bodies in A. The excited region is left white. Only a representative part of the neuron matrix is indicated, for the sake of simplicity, and because of limitation in space not the whole width of the inhibited side fields (10 rows of Purkinje cells, in reality) is shown. (From Szentágothai, Note 20)

beam of excited parallel fibers will excite synaptically the Purkinje and basket cells along this band; and sequentially, the basket cells (shown in black in A and B) will produce a wide and intense zone of inhibition flanking the central excited zone, as is indicated by the dark shading in B. Figure 14C gives in longitudinal section all the parallel fiber beam of B.

This diagram sketches the first suggestion of an operational pattern for postsynaptic inhibitory action. A sharply localized mossy fiber input into the cerebellum is converted into a narrow strip, 3 millimeters long, of excited Purkinje cells in the longitudinal axis of a folium, and it steeply grades into a deep and wide (1 millimeter) zone of inhibition on either side. Thus, an intense localized input via mossy fibers would suppress the Purkinje cell activation that had been produced by all the less intense inputs that were distributed laterally to the cell.

In a recent investigation,[21] a study was made of this transverse distribution of inhibition, using as a test the spikelike field potentials generated by the invasion of Purkinje cells by antidromic impulses (Figure 13, D-J). In addition there was a detailed study of the slow potential fields generated by a parallel fiber volley, and of this correlation with the action of excitatory and inhibitory synapses on the Purkinje cells. In general, these investigations have provided a remarkable corroboration of Szentágothai's operational diagram (Figure 14), but with the difference that even for Purkinje cells inhibition is dominant along the parallel fiber beam. Hence it can be envisaged that a powerful mossy fiber input through such pathways as the dorsal or ventral spinocerebellar tracts would cause a deep and widespread inhibition of Purkinje cells.

As illustrated in the three diagrams of Figure 15, a mossy fiber input to the cerebellar cortex activates three distinct types of inhibitory mechanisms.[13-15, 17-27] In A, the mossy fiber input excited granule cells to discharge impulses along their axons, the parallel fibers, and these in turn excite basket cells that exert a postsynaptic inhibitory action on Purkinje cells for as far as 1 millimeter in either direction transversely across the folium. In B the initial stage of the pathway is similar to that of A, the mossy fiber input exciting the granule cells to discharge impulses along the parallel fibers. The further stage is a negative feedback loop: the parallel fiber impulses excite the Golgi cells to discharge impulses that exert a postsynaptic inhibitory action on the granule cell dendrites by a negative feedback occurring in the same synaptic complex of the glomeruli as the mossy fiber–granule cell excitation. In C, the mossy fiber input excites the Golgi cell by synapses on the dendrites in the granular layer.[13,14] The inhibitory pathway is then, as in B, by the Golgi cell discharges to inhibit the granule cells. Thus, in C, the inhibitory action of the Golgi cells is of the feed-forward type and not feedback as in B.

The only neural pathway from the cerebellar cortex is provided by the axons of the Purkinje cells; the great majority of these axons terminate in the intracerebellar nuclei and the remainder almost entirely in Deiters nucleus. Ito and his colleagues have made the remarkable discovery that the Purkinje axons have a direct inhibitory

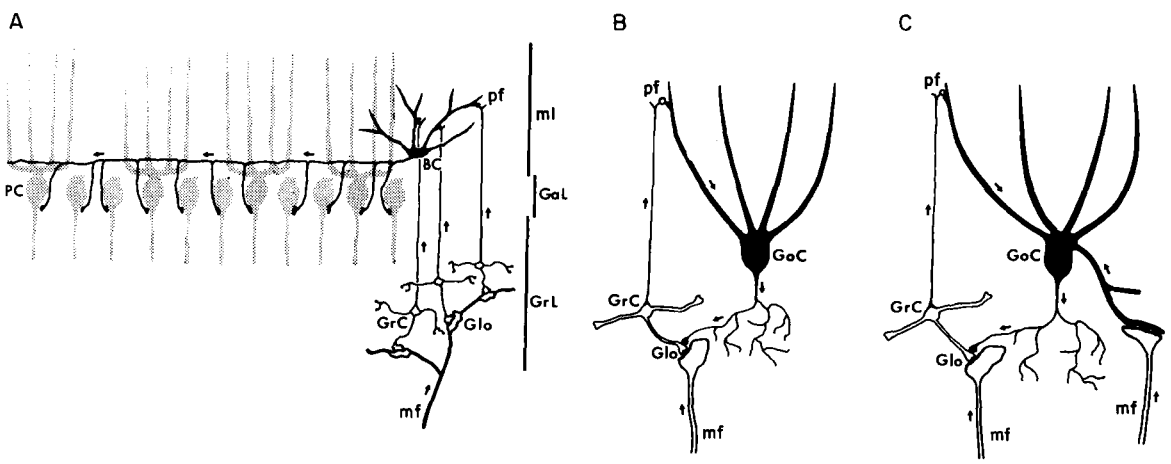


FIGURE 15 Diagrammatic representations of the three types of inhibition activated by a mossy fiber input to the cerebellar cortex. In a subvariety of A that is not illustrated, the superficial stellate cells, rather than the basket cells, are the inhibitory neurons. Mossy fiber, mf; granule cell, GrC; parallel fiber, pf; Purkinje cell, PC; basket cell, BC; Golgi cell, GoC; glomerulus, Glo; ML, GaL, and GrL are respectively the molecular, ganglionic and granular layers of the cerebellar cortex. Further description in the text.

action on the cells of both the intracerebellar nucleus and Deiters nucleus.[28-31]

For example, Figure 16A shows diagrammatically the stimulating electrode exciting the discharge of impulses down the Purkinje cell axons, and the intracellular recording from a Deiters neuron by a microelectrode. Also shown are the inhibitory postsynaptic potentials (IPSPs) evoked by progressively stronger stimulation. If the conduction time in the Purkinje axons is subtracted from the observed latency of about 1 millisecond, the synaptic delay for initiating the IPSP is about 0.4 millisecond, which establishes the monosynaptic inhibitory action of the Purkinje cells. With increase of the stimulus strength, there were later inhibitory potentials, and also, at times, stimulation of the climbing and mossy fibers in the cerebellum evoked EPSPs by axon collaterals, as indicated in Figure 16A. Figure 16F shows the IPSP at slower sweep speed with a later rebound depolarization, and in G and H this rebound evoked a prolonged (about 200 millisecond) repetitive discharge from the Deiters neuron down the vestibulospinal tract, even when, as in H, there was no background discharge.

Summary of cerebellar inhibitory pathways

It is possible to illustrate in diagrammatic form the essential features of the neuronal circuits that have been revealed by recent experimental investigations on the cerebellum. Figure 17A shows the simple and highly selective monosynaptic excitatory action exerted by a climbing fiber from the inferior olivary nucleus[32] on a Purkinje cell.[33] In Figure 17B, the mossy fiber input to granule cells is the initial synapse on the pathway by which parallel fibers excite Purkinje cells, but at the same time the parallel fibers excite Golgi cells, which feed back inhibition to the mossy fiber–granule cell synapse. This inhibitory pathway is a typical example of recurrent inhibition that provides a simple negative feedback control of granule cell activation.

Figure 17C shows the more complicated inhibitory pathways from basket cells. Again, this pathway is activated by mossy fibers via granule cells and parallel fibers, but the situation differs from B in that these cells exert their inhibitory action on Purkinje cells, so providing an example of what we may call feed-forward or ongoing inhibitory control. In this way the excitatory synapses of mossy fibers are controlled, not by antagonistic inhibition at the locus of their action, but by an inhibition exerted on the next stage of their excitatory action, namely the parallel fiber synapses onto Purkinje cells. As shown in Figure 17D, this inhibition is, of course, also effective against climbing fiber excitation.

The composite diagram in Figure 17D shows how the two inhibitory mechanisms lock together. It can be seen

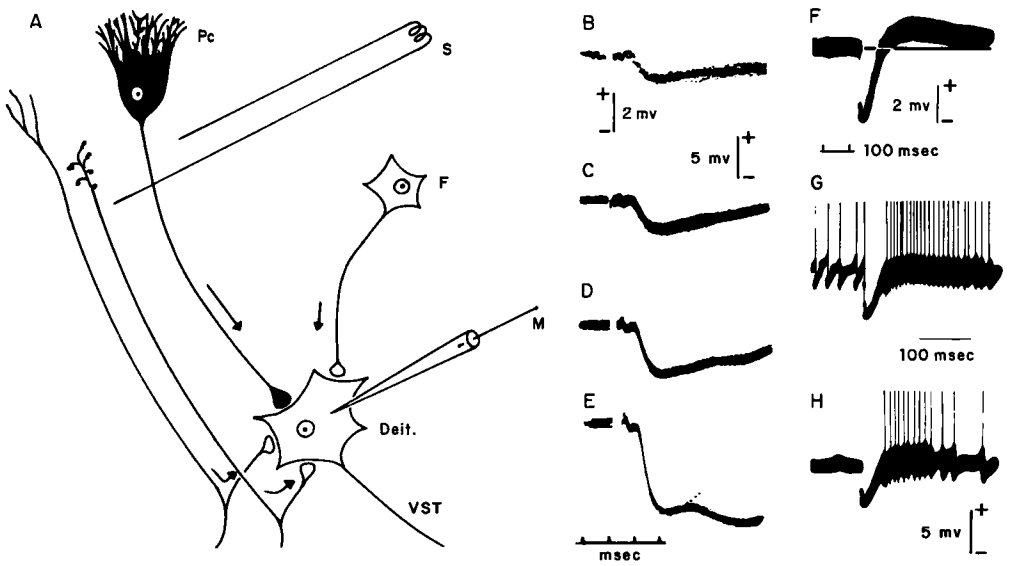


FIGURE 16 Inhibitory action of Purkinje cells on Deiters neuron. A shows diagrammatically the Purkinje cell (Pc) and the Deiters neuron (Deit) with intracellular recording (M). VST, vestibulo-spinal tract. Stimulation of the Purkinje axons by electrode S evokes the IPSPs of B and C, and with stronger stimulation (D,E) the IPSPs increase and have an added later component. F to H show the strong IPSPs at slower sweep speed with rebound discharges in G and H. (From Ito and Yoshida, Note 29)

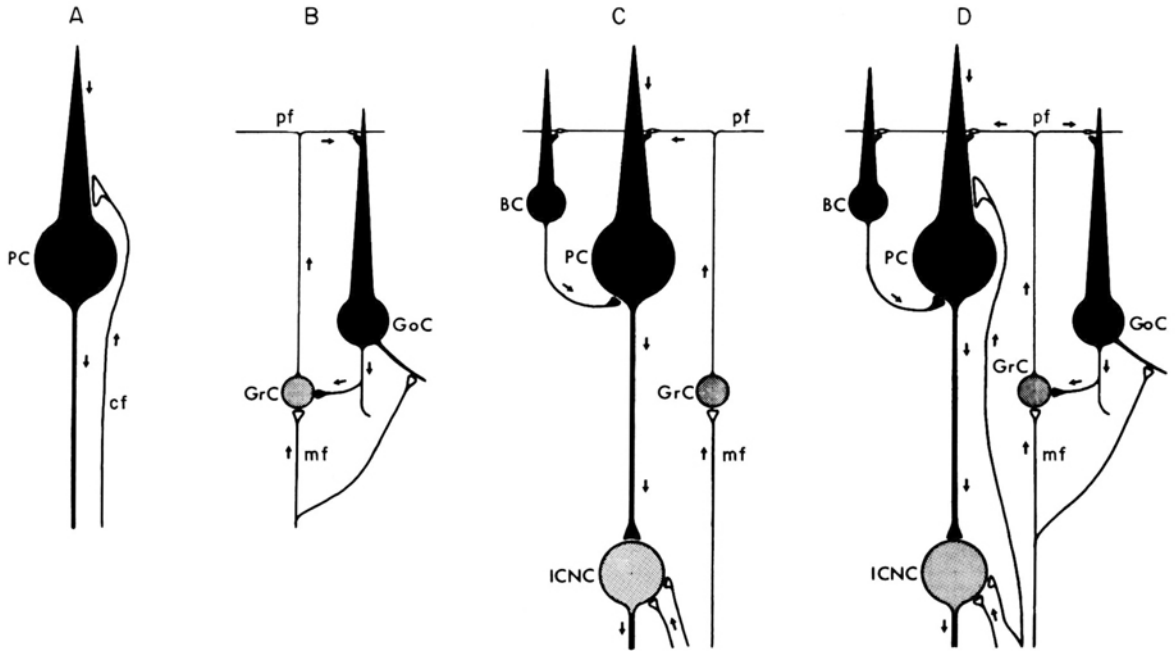


FIGURE 17. Diagrams of the most significant cells and their synaptic connections in the cerebellar cortex. The component circuits of A, B, and C are assembled together in D. Arrows show lines of operation. Inhibitory cells are shown in black. PC, Purkinje cell; cf, climbing fiber; GrC, granule cell; pf, parallel fiber; GoC, Golgi cell; mf, mossy fiber; BC, basket cell; ICNC, intracerebellar nuclear cell.

that the negative feedback via Golgi cells depresses all ongoing actions of granule cells via parallel fibers—not only the excitation of Purkinje cells, but also the feedback inhibition by Golgi cells and the feed-forward inhibition by basket cells and stellate cells. Finally, the Purkinje cells themselves, after receiving all this processed information, act by their discharges to inhibit the neurons in Deiters and the intracerebellar nuclei (ICNC), which are excited by various inputs from the brain-stem nuclei, including the axon collaterals of climbing fibers.

Principles of synaptic operation

For chemically transmitting synapses of the central nervous system, it is possible to state two principles to which there are no known exceptions. The first principle — that a cell acts by the same transmitter at all of its synapses — was originally suggested by Dale[34] and is derived from the metabolic unity of a cell, which has the same enzyme systems and has mitochondria down all its branches, no matter where they are or how diverse they may be. For example, a motoneuron gives synapses to a Renshaw cell in the spinal cord and to a muscle peripherally, and at both locations acetylcholine is the transmitter (Figure 7A).

The second principle is that, in all of the synapses made by the axonal branches of a nerve cell, the transmitters open the same kinds of ionic gates. These ionic gates may be either sodium and potassium ionic gates, in which case it is an excitatory cell, or they may be potassium and/or chloride ionic gates, which, of course, characterize an inhibitory cell. The only other possible kind of neuron in the vertebrate may be those effecting presynaptic inhibition. I assume that these are a third kind of cell, but there is as yet no significant information on this problem.

There would seem to be an exception to the second principle in some molluscan nerve cells, as illustrated in Figure 18, where the same transmitter, acetylcholine, excites at the synapses of a cell onto D-cells, and inhibits at the synapses on H-cells.[35,36] As it has worked out, the same ionic gates are opened, but they are doing different things.[37,38] The reason, I suppose, is that the mollusk has so few cells to play with. In a helix brain there are about 18 cells, so I suppose that it cannot afford inhibitory interneurons; hence it developed a clever device by which the same ionic gates (largely to Cl^-) effect opposing actions on the membrane potential. The chloride pump builds up a high Cl concentration in the D-cell and, as a consequence, when the chloride gates are opened by acetylcholine, chloride ions pass outwards across the membrane, which is thus depolarized. In the H-cell, on the contrary,

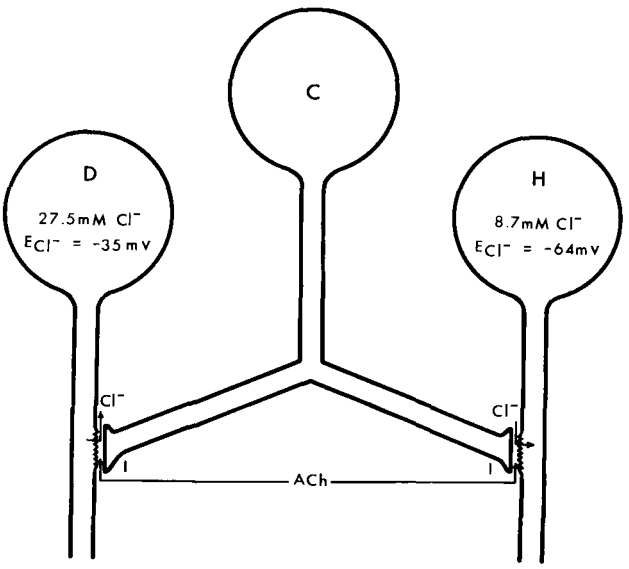


FIGURE 18 Diagram showing the average chloride ion concentrations and the chloride equilibrium potentials for the D- and H-cells of *Helix* as determined by Kerkut and Meech,[39] and the Cl^- movements across the ionic gates opened by acetylcholine.

there is an outward pumping of chloride, hence chloride ions pass inwards when the chloride gates are opened by acetylcholine, and hyperpolarization results.[39] Thus, these molluscan synapses actually provide excellent evidence in support of the second principle. The complex device of inward chloride pumps on the postsynaptic membranes of molluscans were developed in order to get excitation out of what is really an inhibitory synapse.

It is easy enough to understand the first principle, but the second principle is perplexing, because it is not clear how a cell can be constrained not to make synapses so that the same transmitter can open different types of ionic gates; that is, that the transmitter from any particular cell can open inhibitory gates at some synapses and excitatory gates at others. For example, acetylcholine does open excitatory gates at the vertebrate neuromuscular junctions and on Renshaw cells, yet it opens only inhibitory gates on molluscan neurons, both on the D- and H-cells. Evidently there is some principle of neurogenesis whereby cells are constrained not to develop different types of synapses, excitatory and inhibitory, on target neurons.

I make the initial postulate that, at an early stage of neurogenesis, cells are differentiated as being excitatory or inhibitory. They "know" what they are going to be, and even what kind of transmitter they are going to act by. In Figure 19A, an inhibitory axonal outgrowth is shown searching along the surface of another nerve cell; an excitatory site is already preformed on this cell with potassium–sodium channels available for opening by the right transmitter. The gates are right for excitation, but the axonal branch is repelled, presumably by a chemical sensing of a kind of aroma, so it moves on and finds a postsynaptic surface that it is attracted to. As a result, a large synapse is formed on this preformed inhibitory patch. So there is an inhibitory cell with an axon attracted only to preformed sites of inhibitory gates; if cells do not have the propensity to form such gates, the axon does not join up with them at all, and so on. To the right of Figure 19A is an excitatory cell that is already known as excitatory and that dislikes the inhibitory patch but is attracted to the excitatory patch on another cell. So the excitatory cell opens, joins, and makes synapses only where there are excitatory gates. That is one possibility. An alternative (B), which I do not like nearly so well, is that the inhibitory cell finds two cells; it does not like the aroma of one (nothing is preformed; it just has the wrong kind of chemical atmosphere) so the inhibitory cell moves on. (This is

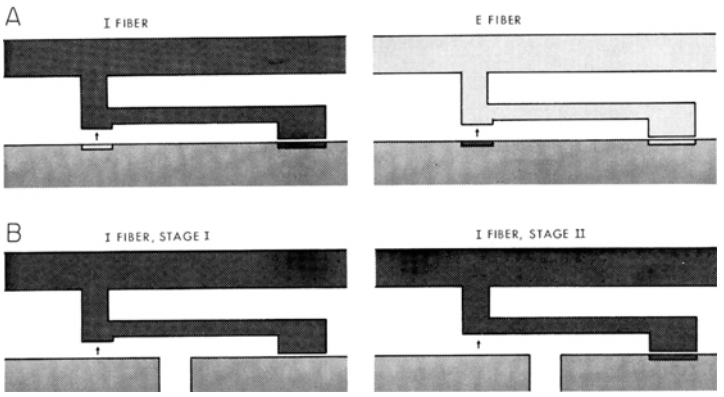


FIGURE 19 Diagram of two possible developmental procedures by which a nerve cell establishes either purely inhibitory or purely excitatory synaptic connections. Inhibitory cells are shown with dark toning, excitatory with light. In A the axonal outgrowth from an already specified inhibitory cell to the left rejects a contact with an already specified excitatory synaptic patch and grows on to find an acceptable inhibitory synaptic patch. The reciprocal situation is seen in A to the right. B illustrates an alternative postulate, but only for an already specified inhibitory cell, as described in the text.

what Weiss and others have observed in tissue culture experiments.) The outgrowing axon in B then nears a cell that makes a close contact with it and, in the fullness of time, makes an inhibitory synapse. This postulated manner of neurogenesis also gives the same specificity of connection with outgrowths from excitatory neurons.

Possibly it may be feasible to design experiments that distinguish between the alternative postulates whereby nerve cells make synaptic connections that are either exclusively inhibitory or exclusively excitatory.

Summary

There is first a description of two types of synaptic mechanisms, those of postsynaptic excitation and postsynaptic inhibition; and the mode of operation of postsynaptic inhibition is briefly outlined.

It is shown that a special kind of nerve cell, an inhibitory neuron, is interpolated on the two simplest inhibitory pathways in the spinal cord, namely that from Group Ia afferent fibers from muscle to the motoneurons of antagonistic muscles, and that from axon collaterals of motoneurons to adjacent interneurons. It is shown further that these are examples of two kinds of inhibitory pathway exerting a feed-forward and a feedback inhibition respectively.

There is a brief account of inhibition in the hippocampus, because this is the first instance in which the inhibitory interneuron has been identified with a histologically recognized cell, the basket cell. This is a further example of feedback inhibition.

The cerebellum provides a remarkable illustration of the complexities of inhibitory pathways in higher centers. It is shown that basket cells, Golgi cells, and Purkinje cells are all inhibitory, and the manner in which they interact in the processing of information in the cerebellum is outlined. The inhibitory actions of these cells are in part the feed-forward type and in part the feedback type, but some pathways are more complexly organized.

Finally, the principles of synaptic operation are discussed and two principles are defined. The first is that a cell acts by the same transmitter at all of its synapses. The second principle is that in all of the synapses made by the axonal branches of the nerve cells the transmitters open the same kinds of ionic gates. It is argued that there is no known exception to either of these principles.

The Adrenergic Synapse

IRWIN J. KOPIN

ON THE BASIS OF EVIDENCE that norepinephrine is stored in sympathetic nerves, is released in significant amounts during sympathetic nerve stimulation, and has effects that parallel those of nerve stimulation, it has been generally accepted that this catecholamine is the neurochemical transmitter that is released from sympathetic nerve endings.[1]

The processes of synthesis, storage, release, metabolism, interaction with a receptor, and termination of activity are all important to the concept of norepinephrine's function as a transmitter. Each has been studied in a variety of tissues in a number of species (e.g., cat, rat, dog, man). I will extrapolate and generalize the results in order to present a unified concept of a "typical" adrenergic neuron. Because of the lack of space, many details and much supporting evidence must be omitted. (For more detailed information on the various processes, see recent symposia, Notes 2 and 3.)

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Synthesis

Synthesis is the major source of acquisition of norepinephrine by the adrenergic neuron. Tyrosine, the precursor, is converted to norepinephrine by three sequential, enzymatically catalyzed reactions (Figure 1). Tyrosine hydroxylase, the enzyme that catalyzes the first of these reactions, has been demonstrated only recently. This enzyme is confined to neurons and specialized tissue,

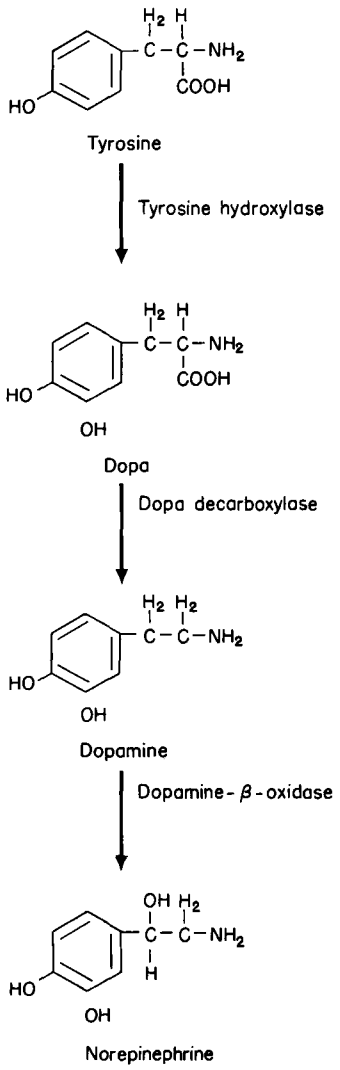
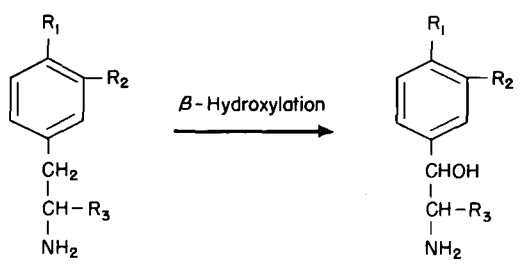


Figure 1 Pathways of norepinephrine synthesis.



Precursor	R_1	R_2	R_3	Product
Dopamine	OH	OH	H	Norepinephrine
α-Methyl dopamine	OH	OH	CH_3	α-Methyl norepinephrine
α-Methyl m-tyramine	H	OH	CH_3	Metaraminol
m-Tyramine	H	OH	H	m-Octopamine
p-Tyramine	OH	H	H	p-Octopamine
α-Methyl p-tyramine	OH	H	CH_3	α-Methyl p-octopamine

Figure 2 Substrates of dopamine-β-hydroxylase.

similar to that found in the adrenal medulla (chromaffin tissues), that contain norepinephrine, and its properties have recently been reviewed.[4] The hydroxylation of tyrosine, a branch point in tyrosine metabolism, is believed to be the rate-limiting step in norepinephrine synthesis; drugs that inhibit this enzyme cause makred decreases in endogenous norepinephrine levels.

Dopa decarboxylase, the enzyme that converts dopa to dopamine, is relatively nonspecific and will decarboxylate a number of aromatic amino acids.[5] Inhibition of this enzyme, which is present in high concentrations in a number of tissues, is not an effective means of diminishing norepinephrine synthesis.

Dopamine-β-oxidase catalyzes the last step in the conversion of tyrosine to norepinephrine. This copper-containing enzyme disappears from tissues after chronic sympathetic denervation and is probably present only in adrenergic neurons and adrenal medulla.[6] It is found in association with the particulate fraction of tissue homogenates and is thought to be bound on the synaptic vesicles of adrenergic neurons. It is relatively nonspecific and will catalyze the addition of a hydroxyl group to the β-position of a number of phenylethylamine derivatives (Figure 2). Some of the resulting compounds can replace norepinephrine at the adrenergic nerve endings and act as "false (or substitute) neurochemical transmitters."

It has been known for a long time that the degree of sympathetic activity does not influence the endogenous levels of norepinephrine; therefore, there must be some means whereby that level is maintained in the sympathetic nerve endings. Recently it was shown that stimulation of the sympthetic nerves in the vas deferens (deferent duct) of guinea pigs causes an increased rate of norepinephrine production from tyrosine.[7] The rate of norepinephrine synthesis also appears to be related to sympathetic nerve activity in vivo. If the sympathetic nerve impulses to the rat salivary gland are interrupted, there is a diminished turnover of norepinephrine. Recent experiments using tyrosine-C^{14} and dopa-H^3 have demonstrated that formation of norepinephrine from tyrosine is markedly accelerated during nerve stimulation. However, both the conversion of dopa to norepinephrine (Table I) and the levels of tyrosine hydroxylase are unaltered.[8] These results illustrate three major points regarding norepinephrine synthesis:

1) The sympathetic nerve has all the enzymatic machinery necessary for synthesis of its transmitter.
2) The rate of synthesis is limited by the first step in the sequence of conversion of tyrosine to norepinephrine.
3) When transmitter is released, synthesis is accelerated by a mechanism that does not involve alterations in

TABLE I

*Effect of sympathetic nerve stimulation on norepinephrine synthesis in rat salivary gland**

	(In Vivo) Norepinephrine from Tyrosine-C^{14} (cpm)	Dopa-H^3 (cpm)	(In Vitro) Tyrosine Hydroxylase (cpm DOPA)
Decentralized	68	410	3190
Stimulated	358	396	3170

*Data from Sedvall and Kopin, in preparation.

tyrosine hydroxylase levels. Presumably, some sort of feedback mechanism is available to control tyrosine hydroxylase activity.

Storage

Although catecholamines are rapidly inactivated when they enter the bloodstream, they are found in high concentration in the adrenal medulla, sympathetic neurons, and certain areas of the brain. In the adrenal medulla, they are held in granules that contain high concentrations of adenosine triphosphate (four moles of catecholamine for each mole of adenosine triphosphate), a specific protein (having a molecular weight of about 40,000), and adenosine triphosphatase dependent on Mg^{++} and Ca^{++}. These granules can take up radioactive epinephrine from solution by an energy-dependent process inhibited by drugs such as reserpine.[9] On the basis of these observations, it is believed that the catecholamines in the granules are bound in a protein–adenosine-triphosphate complex that may involve a bivalent anion.

Norepinephrine-H^3 injected into the bloodstream disappears very rapidly. A large fraction is metabolized, but a portion of the labeled amine is taken up and held in the tissues. If the sympathetic nerves degenerate, endogenous norepinephrine disappears and radioactive norepinephrine is not taken up, suggesting that the labeled amine found in the tissues is mainly present in the nerve fibers.[10] When homogenates of tissue are centrifuged in a sucrose density gradient, the norepinephrine-H^3 is found in the same particulate fraction as endogenous norepinephrine. Electron microscopy shows that this fraction contains vesicles similar to those seen in the sympathetic nerve endings. These vesicles contain adenosine triphosphate and protein; the ratio of adenosine triphosphate to catecholamines is 1:4. As in the adrenal medulla, the binding mechanism may involve adenosine triphosphate, a protein, and a bivalent metal. Radioautography has demonstrated that the radioactivity in the nerve ending appears to be associated with the dense-core vesicles. Because radioactive norepinephrine has the same distribution as endogenous norepinephrine and is susceptible to depletion by the same drugs, it is believed that endogenous norepinephrine is also present in the dense-core vesicles. Furthermore, drugs such as reserpine, which deplete norepinephrine, cause disappearance of the dense-core vesicles.

The chemical structures relevant to binding have also been studied. Norepinephrine has a catechol group (the two adjacent hydroxyl groups on the benzene ring), a β-hydroxyl group, and an amine group. To determine which chemical structures may be involved in the binding mechanism, related compounds that lack one or more of these groups have been studied. The amine group is necessary for binding. Phenylethylamine, which has only the amine group but none of the hydroxyl groups, is not bound; but phenylethanolamine can be bound by the vesicles to a slight extent. The β-hydroxyl group, therefore, appears to contribute to the binding. Dopamine, which lacks only the β-hydroxyl group of norepinephrine, is relatively strongly bound to the vesicles; thus, the catechol group appears to be involved in the binding mechanism. When either of the catechol hydroxyl groups of norepinephrine is lacking, as in m-octopamine or p-octopamine, the avidity for binding sites appears to be diminished in vivo, as well as in vitro.[11] This incomplete specificity of binding allows replacement of norepinephrine by other amines (false transmitters).

In summary, storage of norepinephrine in the adrenergic neuron appears to be in the dense-core vesicles, as seen by electron-microscopic examination of these nerve endings. The binding mechanism appears to be some type of complex with adenosine triphosphate, a bivalent metal, and a protein. All three hydroxyl groups seem to play a role in the binding of the amine. Vesicular binding also is apparently involved in mobilization of the transmitter for release. Only those amines that are bound in vesicles are released by nerve stimulation; and if binding in vesicles is prevented (e.g., by high doses of reserpine), even norepinephrine present in the soluble fraction is not released.

Release

The mechanism for release of catecholamines from binding sites was first studied in the adrenal medulla and has been reviewed.[12,13] Calcium ions enter the cells and appear to be the only ions required for release of catecholamines from the adrenal medulla following stimulation by any one of a variety of drugs or procedures. When the catecholamines are released, adenine and adenosine mono-

phosphate also appear in the effluent. The molar ratio (one mole of adenine with four moles of catecholamine) is the same in the effluent as in the adrenal medullary granules. The protein constituent of the granule is also released during catecholamine release. The ratio of the amine and the protein in the effluent is the same as that found in the adrenal medullary granules.[14] These findings support the concept that Ca^{++} is involved in the stimulus-release coupling and that with the release of catecholamines the whole complex is released, probably by extrusion. This mechanism would include membrane fusion (the vesicle membrane with the cell membrane) to allow the contents of the vesicles to be released.

In the adrenal medulla, acetylcholine is the physiological stimulus for release of catecholamines. It has been suggested that acetylcholine also plays a role in the release of norepinephrine from the sympathetic nerve endings.[15] However, this hypothesis has recently been critically assessed and the conclusion was reached that evidence for a cholinergic link is incomplete.[16]

The concept of quantal release of the transmitter was developed at about the same time vesicles were found to be present in axonal nerve endings.[17] The development of the attractive hypothesis that the contents of a vesicle constitute one quantum of acetylcholine is discussed in the chapter by Kravitz. There is also physiological evidence for quantal release of the transmitter in the adrenergic nervous system.

As mentioned earlier, only amines bound in vesicles are released by nerve stimulation. This provides additional evidence that vesicles may be the site from which norepinephrine is released. Because the binding capacity for each vesicle is probably limited, replacement of a portion of the norepinephrine by a "false transmitter" provides a means of altering the amount of active transmitter released by the nerve impulse (Figure 3).

Mechanisms for releasing a compound from nerve endings that do not involve binding in vesicles may also be present. Bretylium is a quarternary amine that interferes with release of norepinephrine from the sympathetic nerves. Although not bound in vesicles, the compound accumulates in sympathetic nervous tissue and is released during nerve stimulation. It is thus apparent that a substance need not be bound in vesicles in order to be released.

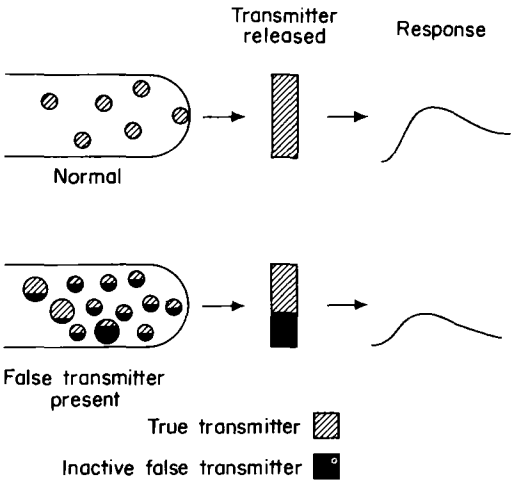


Figure 3 Diagrammatic representation of replacement of a physiological transmitter with an inactive "false" transmitter.

Metabolism

Until about a decade ago, little was known about the fate of epinephrine or norepinephrine. In 1957, it was shown that 3-methoxy-4-hydroxy mandelic acid, or vanillylmandelic acid (Figure 4), was the major metabolite of norepinephrine.[18] This compound is the result of two reactions: deamination and O-methylation (Figure 2). O-methylation was shown to be the major pathway for the metabolism of administered catecholamines.[19] On the other hand, drugs that inhibit monoamine oxidase elevate tissue levels of norepinephrine. Inhibition of catechol-O-methyl transferase, the enzyme that catalyzes O-methylation, potentiates the effects of administered catecholamines.

Intravenous administration of radioactive norepinephrine to rat, dog, man, and other mammals, is followed by an initial rapid rate of urinary excretion of radioactive compounds. The rate of excretion then diminishes, but radioactivity continues to appear in the urine for a long period of time. The initially excreted radioactivity is derived from norepinephrine, which is immediately destroyed, while that excreted later is derived from norepinephrine-H^3 that was taken up in the tissues and stored. Initially, O-methylated metabolites predominate, but later deaminated products make up the major portion of the metabolites. This shift in metabolic pattern suggests that while circulating active norepinephrine is metabolized primarily by O-methylation, norepinephrine in the bound stores is destroyed largely by deamination.

When norepinephrine is depleted from tissues by drugs, the physiological responses are variable. Tyramine administration results in replacement of norepinephrine in its stores by octopamine (the β-hydroxylated derivative of tyramine) and release of norepinephrine to the area of receptors, with resultant sympathomimetic effects. However, when tissue norepinephrine stores are depleted by reserpine, the expected physiological concomitants of nor-

epinephrine release are not seen. The metabolites of norepinephrine-H^3 released by tyramine and by reserpine also differ. When tyramine is administered, most of the released catecholamine enters the circulation as an amine and is destroyed mainly by O-methylation. If norepinephrine-H^3 is depleted by reserpine, however, less than 10 per cent of the released tritium enters the circulation before deamination. These findings, and those relating to other drugs, suggest that if norepinephrine is metabolized after being released onto the receptor, it will be O-methylated. Depletion (e.g. after reserpine) may also occur in the absence of a marked release of physiologically active norepinephrine, while deamination seems to occur mainly in the neuron.[20]

False transmitters

As indicated above, the vesicular storage sites for norepinephrine are relatively nonspecific, and phenylethylamine derivatives with a catechol or β-hydroxyl group can be bound. These compounds can be released by sympathetic nerve stimulation, and in replacing norepinephrine diminish the amount of the released physiological catecholamine. Most of these "false transmitters" are less active than norepinephrine, and their accumulation in the sympathetic nerve endings results in diminished sympathetic responsiveness (Figure 4). This mechanism of diminishing sympathetic activity has been invoked to explain how certain drugs can alter the amount of norepinephrine released. Thus, α-methyl dihydroxyphenylalanine (α-methyl dopa) is a precursor for α-methyl norepinephrine, because the enzymes involved in norepinephrine synthesis (Figures 1 and 2) are nonspecific. The α-methyl derivative of norepinephrine can be bound, replaces norepinephrine, and is a false transmitter. Its accumulation diminishes the amount of norepinephrine that is released. Similarly, following administration of a monoamine oxidase inhibitor, octopamine, which is the β-hydroxylated derivative of tyramine, accumulates in sympathetic nerves. Octopamine is also a false transmitter, and its presence is associated with an apparent partial adrenergic blockage.

Interaction with receptors

Norepinephrine that is released at the adrenergic nerve ending reaches an effector cell where it reacts with a specific site, the receptor, to initiate a sequence of events that leads to a response. Adrenergic receptors have been separated into two main types, α and β, based on the structure–activity relationships of various compounds (agonists) that interact with the receptor.[21] The order of potency at α-receptors (e.g. vasoconstrictors) is norepinephrine > epinephrine >> isoproterenol. At β-receptors (e.g. vasodilators) this order of potency is reversed. Although some exceptions do not clearly fit into one of these classes of receptors, the notion that there are two main types has been greatly strengthened by the discovery of drugs that specifically block one but not the other type of receptor. Little is known regarding the sequence of events between receptor–agonist interaction and the observable response or the action site of drugs that block adrenergic responses. The pharmacological basis for characterization of different adrenergic receptors has recently been summarized.[22]

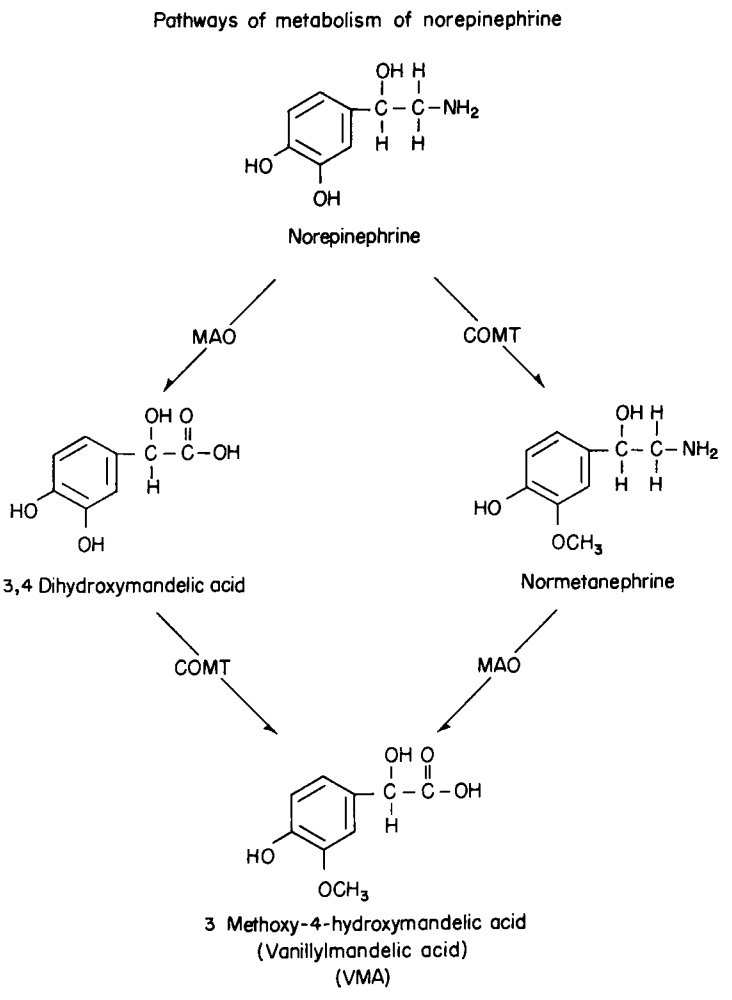


Figure 4 Pathways of norepinephrine metabolism.

In addition to responses that involve muscular contraction or glandular secretion, catecholamines have several metabolic effects (e.g. hyperglycemia). The mechanism of a number of these effects has been shown to be increased

formation of cyclic 3,5-adenosine monophosphate from adenosine triphosphate as a consequence of activation of adenyl cyclase. This enzyme appears to be present in many cell membranes and, in various tissues, is susceptible to activation by hormones other than catecholamines (e.g. vasopressin in the toad bladder, adrenocorticotrophin in the adrenal cortex, thyroid-stimulating hormone in the thyroid, etc.). Sutherland and his coworkers have reviewed various aspects of the properties, distribution, and role of adenyl cyclase in hormone action.[23-25] The relation of adenyl cyclase to the α- and β-receptors is unclear, but there is some evidence that the β-receptor is pharmacologically similar to the site of interaction that results in activation of adenyl cyclase.

Termination of activity

Although the metabolic fate of released norepinephrine appears to be O-methylation, and the inhibition of catechol-O-methyl transferase potentiates the effects of injected epinephrine, the effects of sympathetic nerve stimulation are not greatly altered when either this enzyme or monoamine oxydase (MAO) is inhibited. Cocaine, a drug that potentiates the effects of both administered norepinephrine and sympathetic nerve stimulation, does not inhibit either catechol-O-methyl transferase or monoamine oxidase, but it does interfere with uptake of administered norepinephrine into sympathetic nerve endings. Similar potentiation of administered norepinephrine is seen after chronic sympathetic denervation. From these and other observations it has been concluded that uptake into the neuron is the major means of physiologically terminating the action of released norepinephrine.

The process of active uptake of norepinephrine into sympathetic neurons occurs by a saturable mechanism that follows Michaelis–Menten kinetics and is inhibited by a wide variety of drugs.[26] The uptake process is also influenced by Na^+, K^+, and Ca^{++} ion concentrations. Several phenylethylamine derivatives are taken up by the same mechanism and are competitive inhibitors of norepinephrine uptake.

Conclusions and Summary

It is evident that the efficacy of neurochemical transmission depends on the rate of transmitter release, the accessibility of the transmitter to its receptor, and the responsivity of the receptor mechanism. Alterations in synaptic efficacy may be mediated by changes in any of these processes. The various factors influencing this efficacy are outlined in Table II.

TABLE II

Factors that may be involved in Alterations of synaptic efficacy

I. Rate of transmitter release
  A. Frequency of impulses
  B. Amount of transmitter per impulse
    1. Number of vesicles per impulse
    2. Amount of transmitter per vesicle
      a. Synthesis
      b. Storage
      c. Substitution of "false transmitter"
II. Accessbility of transmitter to the receptor
  A. Anatomical
    1. Interposition
    2. Dendritic or axonal growth
  B. Physiological inactivation
III. Response of the receptor at the same or at other receptor sites
  A. Quantitative changes
  B. Qualitative changes

The rate of transmitter release is the product of the frequency of nerve impulses and the amount of transmitter released per impulse. Alterations in the rate of synthesis (e.g. inhibition of tyrosine hydroxylase with α-methyl tyrosine) would change the amount of transmitter released. Similarly, interference with storage of norepinephrine by influencing the mechanism of binding (e.g. with reserpine) or by substituting a "false transmitter," as previously discussed, will diminish the amount of catecholamine released.

The accessibility to the receptor may be a consequence of anatomical changes such as dendritic or axonal growth, or alterations of interposition of glial processes between axonal ending and receptor. The inactivating mechanism (e.g. inhibition of uptake caused by cocaine) provides a functional means for altering the availability of the released transmitter to the receptor.

Each receptor probably occupies only a small portion of the total membrane surface, because a single neuron may respond to more than one type of receptor on the membrane. There may also be more than one type of receptor (e.g. α and β) for the same transmitter on a single cell membrane and the algebraic summation of the effects on the various receptors may determine the net effect of a transmitter on the effector cell. Alterations in the responsivity of the receptors or on their relative numbers may provide a means for controling synaptic efficacy.

Alterations in synaptic efficacy, by changes in the various processes discussed, could play a role in altering "switches" that are involved in the acquisition and storage of information by the brain.

Acetylcholine, γ-Aminobutyric Acid, and Glutamic Acid: Physiological and Chemical Studies Related to Their Roles as Neurotransmitter Agents

EDWARD A. KRAVITZ

WHEN THE MEMBRANE POTENTIAL OF A NERVE CELL is decreased to a certain critical level, the action potential mechanism is triggered. An action potential sweeps along the axon and invades the terminal portions, where excitation or inhibition is transferred to the next cell (or cells) in sequence. The transfer is mediated by the liberation of a minute amount of a chemical transmitter substance. This compound diffuses to the postsynaptic membrane, where it combines with specific receptor sites and initiates an ionic permeability change. The ions involved are characteristic of the transmitter compound and receptor sites at a particular junction. The transmitter substance is then removed from the synaptic region by specific mechanisms, and the entire process can be repeated up to several hundred times a second for brief periods.

If we concern ourselves primarily with the transmitter compound, it is possible to draw up a hypothetical scheme of the chemical events taking place at synapses (Table I).

This article will try to fill in this scheme with what is known of the chemistry of acetylcholine (ACh), γ-aminobutyric acid (GABA), and glutamic acid. It is necessary to select information from a wide variety of tissues to try to construct comprehensive pictures, as no single synapse has been studied in sufficient detail from chemical, anatomical, and physiological points of view. This is not a comprehensive review of the literature; a limited number of papers have been selected to illustrate particular points. (For detailed reviews, see books by Eccles and by Katz.[1,1a]

Acetylcholine

EVIDENCE FOR ITS TRANSMITTER ROLE Acetylcholine was the first specific chemical substance demonstrated as being released in response to nerve stimulation. The experiment was performed by Otto Loewi in 1921.[2] The vagus nerve innervating a frog heart was stimulated, and when the Ringer's fluid that filled this heart was transferred to a second heart, the latter immediately stopped beating. This suggested that the fluid contained an inhibitory substance that had been liberated in response to vagus nerve stimulation. In the next few years, Loewi and his colleagues characterized the material pharmacologically and to some extent chemically, and concluded that acetylcholine was the released substance. In the early 1930's, Dale and Feldberg and their collaborators established that acetylcholine was liberated at a wide variety of mammalian peripheral synapses. Acetylcholine release was shown in the autonomic nervous system, at preganglionic endings of both the sympathetic and parasympathetic divisions, and at the terminals of postganglionic parasympathetic fibers. Certain postganglionic sympathetic endings were shown to release acetylcholine. Finally motor nerve terminals on skeletal muscles were also shown to liberate acetylcholine. The entire period of the development of the concept of chemical transmission and the identification of acetylcholine as a transmitter compound was reviewed by Dale in 1938.[3]

To establish that a substance is a neurotransmitter compound, its release upon nerve stimulation must be demonstrated to be in a suitable amount to produce the observed physiological effect. While release has been shown in many

TABLE I

Transmitter chemistry in synapses

Presynaptic	Cleft	Postsynaptic
1. Metabolism		1. Receptor site
a) biosynthesis	diffusion	
b) degradation	across	
2. Storage	⟶	2. Inactivation
		a) enzyme
3. Release	diffusion	b) uptake
	away	
4. Reuptake	↓	

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cases, the comparison between the amount collected and the amount necessary to produce a given effect has not been successfully carried out, although attempts have been made in the case of acetylcholine.

A detailed review of the physiology of synaptic transmission at the neuromuscular junction was written in 1962 by B. Katz.[4]

Presynaptic events

METABOLISM: BIOSYNTHESIS AND DEGRADATION The interest in acetylcholine as a transmitter compound led directly to studies on its biosynthesis. Early studies demonstrated that brain slices[5] and brain homogenates[6] could synthesize acetylcholine and that a number of substances could affect the amount formed. However, the first real fractionation of the system was begun in the laboratories of Feldberg in England and Nachmansohn in the United States. The two groups of workers demonstrated synthesis with a synthetic system involving a soluble enzyme (choline acetylase) extracted from brain in various ways; a source of "acetate," such as citrate or pyruvate; adenosine triphosphate (ATP); and an unknown, heat-stable, dialyzable cofactor.[7-10]

The 1940's were a period of active investigation of two-carbon metabolism. Lipmann and Kaplan were studying another acetylation reaction—the acetylation of sulfanilamide prior to its excretion. Their system also had a heat-stable cofactor requirement. They tried their partially purified cofactor in the choline acetylation system and found that it worked; they then suggested that there was a common coenzyme for acetylation (coenzyme A, or coA).[11-12] This was substantiated; in the next few years the chemical nature of coA was worked out, and in 1950 it was demonstrated that the large number of compounds involved in the synthesis of acetylcholine could be reduced to acetyl coA, choline, and the enzyme.[13] The reaction is as follows:

$$\underset{\text{acetyl coenzyme A}}{CH_3C(=O)\text{—ScoA}} + \underset{\text{choline}}{(CH_3)_3N^+CH_2CH_2OH} \rightarrow$$

$$\underset{\text{acetylcholine}}{CH_3C(=O)\text{—O—}CH_2CH_2N^+(CH_3)_3} + \text{coASH}$$
$$+ \text{coenzyme A}.$$

The enzyme choline acetylase (also called choline acetate transferase) is widely, but selectively, distributed in the nervous system. Cholinergic neurons (those that liberate acetylcholine from their terminals) have a high level of the enzyme, while adrenergic (those liberating norepinephrine) or other neurons have a low activity. For example, ventral spinal roots (preganglionic autonomic fibers, motor nerves to muscle) can synthesize 10.8 milligrams of acetylcholine per gram of tissue per hour. Dorsal spinal roots (sensory fibers, transmitter unknown) can synthesize only 0.025 milligram of ACh per gram tissue per hour. (For this and other biochemical data, see the excellent review by Hebb.[14])

There has been no extensive purification of the enzyme until recently,[15] nor has the mechanism of the acetylation been carefully studied. In particular, the effect of factors that might regulate enzyme activity in vivo (such as acetylcholine itself) has not been explored in detail.

Acetylcholine is further metabolized by hydrolysis into acetate and choline by the enzyme acetylcholinesterase (acetylcholine acetate hydrolase). In contrast to the acetylase, this enzyme has been studied extensively. (The details of the mechanism of enzyme action will be presented when we consider postsynaptic events.) There is little doubt that the enzyme is also present in presynaptic terminals, so the reaction is given here:

$$\underset{\text{acetylcholine}}{CH_3C(=O)\text{—O—}CH_2CH_2N^+(CH_3)_3} + H_2O \rightarrow$$

$$\underset{\text{acetate}}{CH_3C(=O)\text{—OH}} + \underset{\text{choline}}{HO\text{—}CH_2CH_2N^+(CH_3)_3}.$$

The total pathway of metabolism of acetylcholine thus involves only two enzymes, the acetylase and the esterase. The search for possible sites of regulation of acetylcholine accumulation at the enzyme level should therefore be greatly simplified. Some results strongly suggest that acetylcholine can regulate its own accumulation, but the precise site, or sites, of control are unknown. The subcellular distribution of the enzymes of the metabolic pathway are now considered in the next section.

STORAGE AND RELEASE Two independent lines of investigation, one physiological and the other anatomical, focused attention on how transmitter was released and what the storage and release form of transmitter compounds might be. Physiological studies by Fatt and Katz[16] demonstrated that transmitter was released in packets (or quanta) of several thousand molecules, not as individual molecules. When recording with intracellular electrodes from particular parts of muscle fibers, spontaneous potentials (about 0.5 millivolt in size) with the time course and shape of normal end-plate potentials were observed. These miniature potentials were blocked by drugs that interfered with the combination of acetylcholine and the re-

ceptor site, and were potentiated by drugs that inhibited acetylcholinesterase. The potentials were always found at nerve endings on the muscle fiber and disappeared upon denervation. It was concluded that they were spontaneous, miniature end-plate potentials caused by a presynaptic release of acetylcholine. To test whether they resulted from single molecules of acetylcholine being liberated from nerve terminals, a constant low level of acetylcholine was flowed into a bath. There was no effect on the frequency of the miniature end-plate potentials—just a few-millivolt depolarization of the muscle-membrane resting potential, upon which the spontaneous miniature potentials were superimposed. This indicated that the mechanism could not resolve single molecular events. Thus, "packets" of acetylcholine must be producing the miniature potentials.

The relationship between this spontaneous release of acetylcholine and the normal release of transmitter upon nerve stimulation was studied by lowering the Ca^{++} content of the external medium. Calcium is known to be necessary for acetylcholine release from nerve terminals.[17,18] It was found that in low calcium medium the nerve action potential still released transmitter, but the size of the normal end-plate potential could be reduced to the size of the miniature potentials, or to an apparent multiple of the single unit size. A statistical analysis showed that the arrival of the action potential at a nerve terminal increased the probability of a quantum of transmitter being released.[19] Quantal release of transmitter, then, seemed to be the normal release mechanism, and it was estimated that about 300 quanta make up a normal end-plate potential.[20]

There was a possibility that the quanta might be related to some structural feature of nerve terminals. Electron microscopic observation showed that terminals had large numbers of 300- to 800- Å spheres packed in the area immediately adjacent to the postsynaptic membrane.[21-26] These were called synaptic vesicles,[25] and it was tempting to speculate that they might contain and release packets of acetylcholine.[23,24,26,27]

Biochemical studies attempting to isolate and analyze vesicles are presented later in this section, but it might be relevant here to consider the volume of a vesicle and the amount of acetylcholine that could be packaged in such a structure. A 500-Å vesicle could contain about 6×10^{-20} liters of fluid. It was computed that 62,000 molecules of acetylcholine chloride crystals could be packed into such a space.[28] The concentration of acetylcholine under these conditions would be 1.7 moles per liter (10^{-19} moles per 6×10^{-20} liters). This calculation ignores the actual wall thickness and would, of course, represent the maximum amount of acetylcholine that could fit into a vesicle. Physiological experiments have suggested that about 10^{-17} moles of acetylcholine are liberated at each nerve terminal per impulse.[29] If there are 300 quanta in an impulse, then 3×10^{-20} moles would make up a quantum, and the concentration of acetylcholine in a vesicle would be 0.5 mole per liter. Other experiments and calculations show somewhat different estimates of amounts of acetylcholine and volume of vesicles. The point of these exercises is to illustrate that little material can be contained in a vesicle and that if vesicles are the basis of the quanta, the acetylcholine within them would probably be hypertonic, and a specific uptake or binding process of some sort should be present.

Whittaker and De Robertis and their colleagues have been the principal workers attempting to isolate and analyze chemically synaptic vesicles from the nervous system (for review, see Whittaker[30]). Early in the studies an extremely interesting observation was made. Upon gentle homogenization of brain in isotonic sucrose and separation of the homogenate components by centrifugation in a discontinuous sucrose gradient (a layered series of increasing concentrations of sucrose), a particulate fraction containing acetylcholine could be obtained. This fraction, however, was not vesicles but apparently intact pinched-off nerve endings.[31-34] A bit of the postsynaptic membrane was often seen adhering to the particles, which were called "synaptosomes," and mitochondria and synaptic vesicles were packed within them. In turn, this fraction could be lysed by osmotic shock (by addition of water), and upon resedimentation in sucrose gradients a new fraction rich in acetylcholine was obtained. This appeared to be the synaptic vesicles.

While there is general agreement that a synaptic vesicle fraction rich in acetylcholine can be obtained in this way, there is considerable disagreement over the presence or absence of the choline acetylase. Some of this disagreement is undoubtedly because of species variation; in guinea pig brain a considerable portion of the acetylase is soluble after lysis of synaptosomes,[30] while in rat brain most of the enzyme is found in the vesicle fraction.[15,35] Various workers also question the purity of fractions obtained by others. The acetylcholinesterase is found in the synaptosome, but after lysis is reportedly associated with the broken membrane fraction rather than with the synaptic vesicles.[36] The average acetylcholine per vesicle has been estimated as about 300 molecules.[37] It is difficult to know the percentage of vesicles derived from the terminals of cholinergic neurons in a brain homogenate or if the acetylcholine content in vesicles is uniform, so this calculation may be meaningless. The preparation of vesicle fractions from autonomic ganglia should be superior in this regard.

The intact nerve-ending particles, the membrane fractions isolated from the particles, and the synaptic vesicles should prove of great value in future studies. With them, all of the machinery of the synapse is available in a readily

isolated, highly purified form. They should open the way to experiments that would yield considerable insight into the chemical processes that take place at synapses.

As has already been mentioned, the release of transmitter is dependent on the calcium content of the external medium.[17-19] Lowering calcium or raising magnesium[38,39] in the bathing fluid diminishes the output of transmitter, even though there is no effect on the arrival of the action potential in the nerve terminals.[40] Raising calcium above normal has the opposite effect, i.e., it facilitates transmitter release. The ionic changes alter the probability that a quantum of transmitter will be liberated without changing the size of the quantum. A direct injection of calcium into nerve terminals in a squid synapse did not cause a liberation of transmitter.[41] The mechanism of the calcium effect remains obscure.

Sodium ions have also been implicated in transmitter release. Birks and his colleagues have used a variety of techniques to alter the internal sodium concentration in nerve terminals.[42] Both the spontaneous liberation of acetylcholine and the size of the end-plate potential are affected by increases in intracellular sodium. Upon inhibition of the sodium pump, after the initial facilatory effects of sodium, transmission fails, and nerve terminals are found to be undergoing degeneration. A serious difficulty with these experiments is the inability to know with any degree of assurance the internal sodium concentration in terminals or if other ions, particularly calcium, may not also be changing. It may prove difficult to distinguish between primary effects of sodium ion and secondary effects of sodium alteration on a number of different processes taking place in terminals.

Finally, transmitter release can be completely blocked by the action of botulinum toxins. The botulinum toxins are a group of closely related proteins liberated by different strains of the anaerobic organism, *Clostridium botulinum,* and they are among the deadliest poisons known. Their effect seems to be specifically to prevent the release of acetylcholine from nerve terminals. The content of acetylcholine, the enzymes of its metabolism, and the action-potential mechanism are apparently normal in botulinum-intoxicated muscles.[43-46] The observed effect is the same as if the preparation were surgically denervated. The membrane component to which the proteins bind is unknown, but through the use of this material, it may be possible to label specifically an essential part of the acetylcholine release system.

It has recently been shown that the synaptic delay between the arrival of an action potential at a nerve terminal and the postsynaptic effect of the transmitter is primarily in the release mechanism, and not in diffusion or postsynaptic chemistry.[47]

Postsynaptic events

RECEPTOR SITE Acetylcholine diffuses across the synaptic cleft and combines with specific sites in the postsynaptic membrane. The effect of the combination of acetylcholine with a receptor site is to initiate an ionic permeability change that can either be excitatory or inhibitory to the postsynaptic cell. This depends on the receptor sites at a particular junction. In the heart, for example, the combination between acetylcholine and receptor produces an increased permeability to potassium.[48-50] Because potassium is at electrochemical equilibrium near the resting level of membrane potential, the net effect is to stabilize the membrane potential near rest and thus produce inhibition. At skeletal neuromuscular junctions, on the other hand, the permeability increase is to small cations in general.[51,52] Sodium is far out of equilibrium and rushes into the cell. The effect of its inward movement is to depolarize the cell above the threshold for excitation, so the cell fires. Acetylcholine, the same agent in both cases, has opposite physiological effects, clearly because of differences in the receptor sites at different junctions.

Receptor sites are accessible to acetylcholine from the outside surface of the cell. This is entirely reasonable, as the transmitter compound is liberated into the synaptic cleft. What is somewhat more surprising is that the receptor sites are apparently inaccessible to acetylcholine from inside the cell. Del Castillo and Katz demonstrated this by electrophoretic application of acetylcholine from micropipettes.[53] An acetylcholine microelectrode was positioned close to an end-plate region that was located with an intracellular recording electrode. Acetylcholine potentials were recorded with the intracellular electrode when acetylcholine was electrophoresed outside the cell but not when the acetylcholine electrode was inserted into the muscle fiber in the same location and acetylcholine electrophoresed inside the cell. Even with ten times as much acetylcholine liberated inside the muscle fiber, no potential change caused by the pharmacological action of acetylcholine was recorded.

Of course, it is of great interest to know the molecular nature of the receptor sites. One can assume that they are membrane proteins, and if they could be isolated it should be possible to relate conformational changes in the molecules to permeability changes in membranes. To date, however, there has been no convincing demonstration of their isolation. A report on the isolation of a protein[54] was subsequently claimed to be incorrect,[55] while another laboratory has reported the isolation of a mucopolysaccharide claimed to be the receptor site.[56]

It is unfortunate that so little is known of receptor-site chemistry, because the receptor sites are the only parts of

the acetylcholine system known to be capable of long-term changes in animals. Thesleff and Miledi and their colleagues have been studying a phenomenon that was known many years ago; if the nerve to a skeletal muscle is cut, the muscle becomes far more sensitive to acetylcholine.[57] Electrophoretic application of acetylcholine from a microelectrode (as above) showed that a normally innervated skeletal muscle fiber is usually sensitive to acetylcholine only in the vicinity of the end plate and at the muscle–tendon junctions. The rest of the fiber is virtually insensitive to acetylcholine. When motor nerves were cut or muscles were chemically denervated using botulinum toxin,[46] it was found that the area responsive to acetylcholine spread in both directions from the end-plate regions until muscle fibers were uniformly sensitive to acetylcholine (after one to several weeks). The original end-plate region was no more sensitive than in a normal muscle. It is as if available receptors had increased in number to cover the entire muscle surface following denervation.[58,59] Upon reinnervation, the area of acetylcholine response became reduced to normal size. However, this took place from the ends of the fiber into the original end-plate region.[60] It is difficult to devise a simple hypothesis to explain the spread of sensitivity out from the end-plate region and the shrinkage of sensitivity in from the ends of the fiber in response to removal or return of a nerve. Nonetheless, it is clear that the presence or absence of the functioning nerve regulates the phenomenon. A series of ingenious experiments have been designed to study this process in detail, and these have been reviewed by Miledi.[61]

ACETYLCHOLINESTERASE Acetylcholine in the synaptic cleft is inactivated by the acetylcholinesterase in the membrane of the postsynaptic cell. At this point, it is worthwhile to discuss in some detail what is known of the mechanism of action of the enzyme. It is the only part of the acetylcholine system that has been studied chemically in any depth; moreover, it is a classical case of how, from a study of the mechanism of enzyme action, an antidote to a particularly lethal type of poisoning was devised.[62,63]

The enzyme surface has two distinct sites, an anionic site capable of binding the quarternary nitrogen of the choline portion of the molecule and an esteratic site capable of donating electrons to the acetate portion of the molecule and cleaving it from choline. This leads to the transient formation of an acetyl–enzyme compound, which in turn can be hydrolyzed. The classical view of the enzyme surface and the sequence of reactions can be formulated as in Figure 1.

Pharmacological and physiological studies have led to the development and discovery of a wide variety of inhibitors of the enzyme. These are of many different kinds,

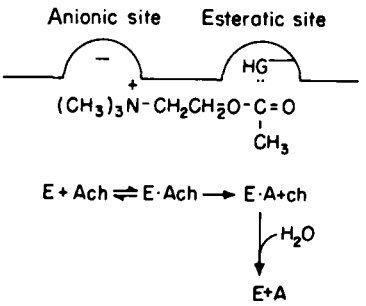


FIGURE 1 Acetylcholinesterase: enzyme surface and reaction sequence.

but only two types are discussed here. The first is acetylcholine itself and the second is a category of particularly toxic substances including the "nerve gases" and many insecticides.

In high concentrations, acetylcholine inhibits the esterase.[64,65] This appears to result when a second molecule of acetylcholine combines with the enzyme at the unoccupied anionic site prior to the dissociation of the acetyl–enzyme complex. This slows down the hydrolysis of acetate from the enzyme surface and thus inhibits enzyme activity[63]:

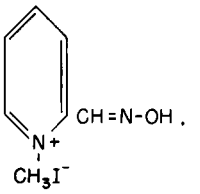
$$\mathrm{E{\cdot}A + ACh \rightarrow E{\cdot}A{\cdot}ACh \xrightarrow[\text{slow}]{H_2O} E + A + ACh}\,.$$

It is possible that acetylcholine, in high concentration, could regulate its own destruction by such a mechanism.

The compounds in the second group of inhibitors are all of the alkylfluorophosphate type. These compounds act as substrates for the enzyme and are cleaved, but the alkylphosphoryl enzyme compound that is formed is not hydrolyzed. The reaction sequence could be formulated as follows (R = alkyl group):

$$(RO)_2\overset{O}{\overset{\|}{P}}{-}F + E \rightleftharpoons E{\cdot}(RO)_2\overset{O}{\overset{\|}{P}}F \rightarrow E - \overset{O}{\overset{\|}{P}}(OR)_2 + HF\,.$$

An electron donor better than water was required to remove the alkylphosphoryl group from the enzyme surface. The anionic site was free and the critical interatomic distance between the anionic and esteratic sites was known, so it was possible to predict the sort of compound that would fit onto the enzyme and have a ready pair of electrons in precisely the correct place to remove the inhibitor from the enzyme. The predicted molecule was pyridine-2-aldoxime methiodide (PAM):

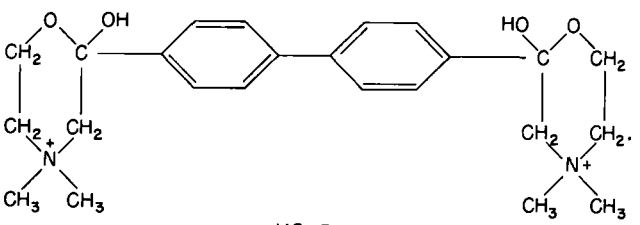


The compound was synthesized and, when incubated with a fully inhibited enzyme, rapidly restored enzyme activity.[66] A critical test was to see if it would then work in vivo as well. Animals were injected with lethal doses of di-isopropylfluorophosphate (DFP) or with DFP and atropine (to inhibit partially the combination of excess acetylcholine with certain receptor sites). When this was followed by an injection of PAM there was 100 per cent survival of animals.[67] PAM and compounds of this type are still used clinically to counteract alkylfluorophosphate poisoning.

The acetylcholinesterase and acetylcholine receptors are considered to be two distinct sites within the postsynaptic membrane. This is because they can be pharmacologically distinguished from each other. It is possible to inhibit one without affecting the other. This does not say anything about how far apart these sites are; they may even be on the same molecule. Until the actual molecular species have been isolated, it is not possible to draw any conclusions in this regard.

The physiological effects of interfering with the receptor or esterase can be summarized briefly. If the receptor sites are blocked by the use of agents such as curare (e.g., at the skeletal neuromuscular junction) or atropine (e.g., in the heart), transmitter combination with the receptor is diminished or blocked. The esterase still functions and quickly destroys available acetylcholine. Inhibiting the esterase with such drugs as eserine (physostigmine) or prostigmine (neostigmine) will reduce or prevent the destruction of acetylcholine, and there will be a potentiation of the acetylcholine effect. Now, presumably, diffusion from the synaptic cleft alone will remove the transmitter compound from its site of action, as there is apparently no acetylcholine uptake system. The combination between acetylcholine and receptor, however, does not lead to an infinitely long permeability change. There is a phenomenon known as desensitization, whereby the receptor will no longer function after prolonged exposure to transmitter compound.[68] Thus, inhibiting either the esterase or the receptor sites will block transmission at cholinergic junctions.

UPTAKE It is not known how the acetate from acetylcholine is metabolized. For choline, however, an uptake mechanism exists.[69] At least a portion of this uptake is back into the presynaptic nerve terminal where the choline can be used for acetylcholine synthesis. Uptake is apparently sodium dependent[42]; this could, therefore, be an active transport system utilizing the sodium gradient across the membrane to transport choline into the cell, as is the case for other transport systems in animals. The uptake of choline can be blocked with the drug hemicholinium, and interference with this process has rather drastic effects on synaptic transmission at certain junctions. The structural formula for one of the hemicholiniums, HC_3, is shown below:



HC-3

REGULATION OF ACETYLCHOLINE METABOLISM In a series of experiments aimed at studying the regulation of acetylcholine metabolism, Birks and MacIntosh perfused the superior cervical ganglion of cats with a variety of media in the presence or absence of drugs.[70] The acetylcholine content of the ganglia and the amount released with stimulation were measured and certain conclusions about the control of acetylcholine metabolism in the ganglion were deduced. The particular experiments described here are those in which normal cat plasma was the perfusion medium. Normal plasma contains choline, so perfusion was always in a choline-containing medium. With plasma perfusion alone and no drugs added, the acetylcholine content of the ganglion remained constant for some two hours or more. Upon stimulation at 20 pulses per second for the entire experiment, the acetylcholine content again remained constant, although considerable acetylcholine was liberated during the stimulation period. The amount liberated, however, could not be measured because the esterase destroyed all acetylcholine released by the preparation.

The same experiment was repeated in the presence of eserine to inhibit the esterase, and somewhat different results were obtained. With no stimulation the acetylcholine content of the ganglion doubled in about an hour and then remained constant for another hour. This would suggest that the level to which acetylcholine accumulated reflected some steady state of synthesis and destruction. As essentially all the acetylcholine in the ganglion is in the presynaptic axons and nerve terminals, it would also suggest that there is esterase in presynaptic neurons. When the preganglionic trunk was stimulated for the entire experiment in the presence of eserine, the ganglionic content of

acetylcholine still doubled. The output of acetylcholine could, however, be monitored in these experiments. There was a slight fall in output per unit time during the first few minutes; thereafter it was constant for the remainder of the experiment. The total amount of acetylcholine released could be some five times greater than the amount initially present, suggesting that synthesis and release were closely regulated, the one keeping pace with the other. It should be pointed out that in the hour during which acetylcholine in the ganglion was doubling, the output of acetylcholine was constant. Presumably, then, the physiological effect of preganglionic stimulation was not enhanced in spite of the marked change of acetylcholine in the ganglion. This should serve to caution against trying to relate change in transmitter levels in a tissue with behavioral changes in an organism without some other direct physiological correlates that show that the system in question has altered its functioning.

Finally, in perfusion experiments with eserine and HC_3 (to inhibit choline uptake), results different from either of the other two were obtained. With no stimulation there was a slight increase of acetylcholine in the tissue over about 5 to 10 minutes, and thereafter no further change in content appeared. With stimulation the acetylcholine content fell rapidly to very low levels and the output of acetylcholine decreased along the same time course. Yet in the preganglionic nerve trunk, a few millimeters away from the ganglion, there is a normal level of acetylcholine, and presumably choline as well. It would appear that an uptake of extracellular choline is necessary to maintain the level and output of acetylcholine during prolonged periods of stimulation.

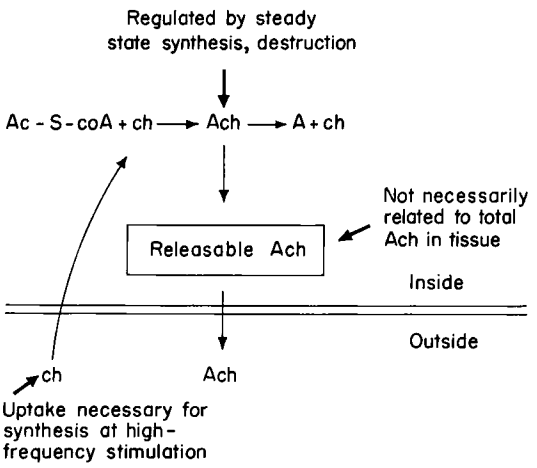


FIGURE 2 Presynaptic acetylcholine metabolism.

A schematic summary of these results is shown in Figure 2. It is now possible to return to the schematic diagram (Table I) and fill in some details of the events taking place in a cholinergic synapse (Figure 3).

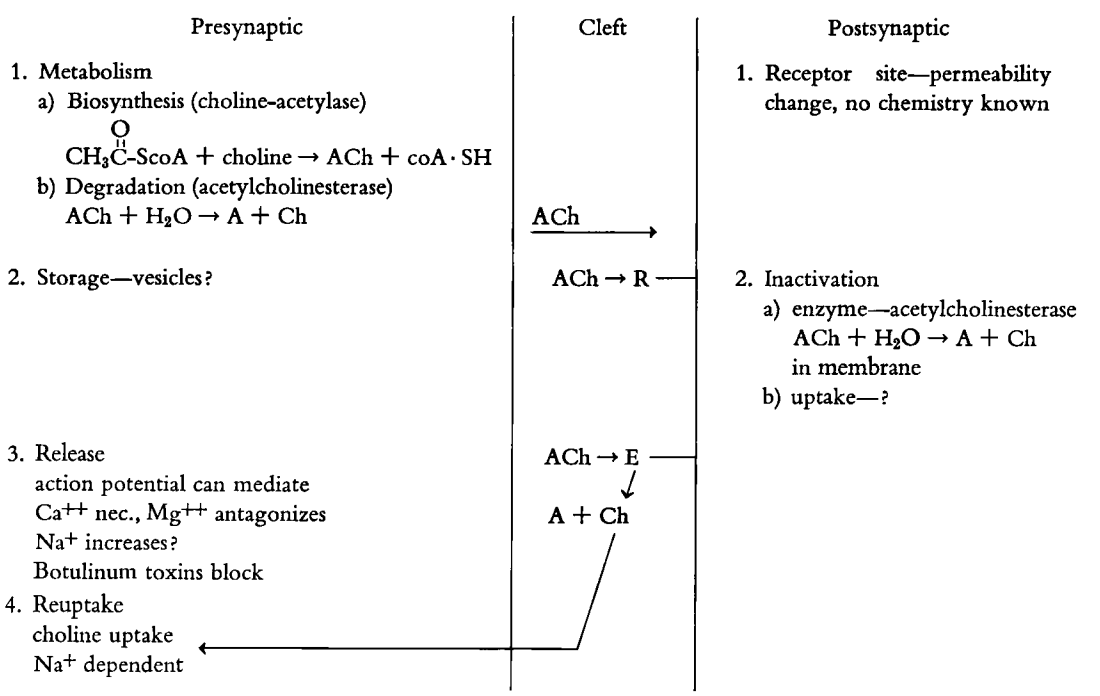


FIGURE 3 Synaptic chemistry of acetylcholine.

γ-aminobutyric acid (GABA)

BRIEF HISTORY GABA was first isolated from the mammalian nervous system in 1950.[71,72] Considerable attention was centered on GABA when it was found to be localized exclusively in the central nervous system, as was the enzyme that synthesized it, glutamate decarboxylase.[73] The entire pathway of metabolism was established, and there was a wide variety of speculations as to the physiological role of GABA in the central nervous system. Probably the experiment that most focused attention on GABA as a possible neurotransmitter compound was that of Bazemore, Elliott, and Florey in 1957.[74] These workers had isolated a brain substance (Factor I) that blocked the discharges in a crayfish stretch-receptor neuron (a single sensory cell with its dendrites entwined in a fine muscle bundle, usually with an inhibitory innervation). The discovery that Factor I was principally GABA prompted the speculation that GABA might be an inhibitory transmitter compound.

Much of the material in this article is from experiments with the crustacean nervous system. Crustacea have proved to be particularly favorable animals for studies on inhibition. The exoskeletal muscles have a dual innervation; excitatory and inhibitory axons innervate the same muscle. In certain muscles a single excitatory and a single inhibitory axon innervates the entire muscle by sending branches to the various muscle fibers. The detailed physiology of these axons and any interactions between them can, therefore, be studied in an easily isolated preparation. Moreover, the axons are large, and those serving the same function can be obtained repeatedly by dissection from different animals. A detailed chemical analysis can thus be made on a specific nerve cell of known function.

EVIDENCE FOR TRANSMITTER ROLE In lobsters and crayfish, externally applied GABA duplicates the effects of the neurally released inhibitory transmitter compound (for review, see Kuffler[75]). At the neuromuscular junction there are two sites of inhibition—a postsynaptic site[76] analogous to that described above for acetylcholine, and a presynaptic site on excitatory nerve terminals.[77] Both GABA and the inhibitory transmitter compound, when combined with postsynaptic receptor sites,[78] cause the membrane to become more permeable to chloride, an effect blocked by the drug picrotoxin.[75,79] At presynaptic excitatory nerve terminals, GABA and neural inhibition both cause a diminished output of quanta of excitatory transmitter,[77] and again this appears to be mediated by a chloride conductance increase.[80]

The GABA content of inhibitory neurons in lobsters is considerably higher than that of excitatory neurons; in axons the ratio is 100:1. The GABA concentration in inhibitory axons is 0.1 mole per liter.[81]

An essential piece of evidence for the role of GABA as a transmitter has recently been added. Stimulation of inhibitory axons to various lobster muscles causes a release of GABA into the bathing fluid. Excitatory nerve stimulation does not liberate GABA nor is GABA released when neuromuscular transmission is blocked by lowering the calcium content of the medium.[82]

Thus the evidence is very strong that GABA functions as an inhibitory transmitter compound in Crustacea. In mammals, GABA is confined to the central nervous system, where it is far more difficult to get any direct evidence on a possible transmitter role. A technique that has proved valuable, however, is iontophoretic application of GABA to single neurons in the central nervous system. A recording electrode, which can be fused in a variety of ways to a GABA electrode, is inserted into a cell. The tip of the GABA electrode is placed so that it remains outside the cell. The effect of iontophoresed GABA or other compounds are observed and are compared to the natural synaptic inputs to the cell. The ionic environment within the cell can be altered by electrophoresing ions into the cell, and the effects of this change can also be studied. The results are somewhat conflicting. Curtis claims that in the spinal cord GABA action and the natural inhibitory input to motor neurons produce different effects.[83] While GABA is inhibitory, it presumably does not bring the membrane toward the same equilibrium level as does the natural transmitter.

In the cerebral cortex and cerebellum, however, GABA appears to duplicate closely the effects of inhibitory synaptic activation. Krjnević, in studies of the cerebral cortex, has shown that GABA produces a hyperpolarizing potential change that is similar to a naturally elicited inhibitory potential[84]; if chloride is injected into cells, both the GABA and natural potential changes first diminish in size, then disappear, and finally reverse sign (become depolarizing), and GABA can excite cells as the internal choloride concentration increases. It has been suggested that the total output of the cerebellum (axons of Purkinje cells) is inhibitory.[85,86] Moreover, if GABA is iontophoresed onto Deiter's nucleus neurons, which receive Purkinje cell inputs, hyperpolarizing potential changes are produced (Obata and Ito, unpublished). These studies are not as detailed as those of Krjnevic, but nonetheless they suggest an important role for GABA as a transmitter compound in the mammalian central nervous system. Chemical studies have been carried out on the GABA content in various layers in the cerebellum[87,88] and on pooled single cells dissected from the cerebellum (Obata, unpublished), and in these cases there is considerably more

GABA associated with Purkinje cells than with other cerebellar structures. It should, however, be borne in mind that these chemical studies involve several cell types: the Purkinje cells themselves, the endings of other cells on Purkinje cells (which are thought to be inhibitory around the cell body), and glial material.

In the mammalian central nervous system, then, there is suggestive evidence that GABA may function as an inhibitory transmitter compound, but a variety of definitive experiments are lacking. Certainly in view of these recent results, the earlier work on the spinal cord should be reinvestigated.

METABOLISM: BIOSYNTHESIS AND DEGRADATION

GABA is synthesized in the central nervous system from glutamate in the presence of an enzyme, L-glutamate decarboxylase. This enzyme removes the α-carboxyl group from glutamate as CO_2 and requires pyridoxal phosphate as a coenzyme.[73,89]

The mammalian enzyme has not been extensively purified, although detailed studies on its properties have been carried out.[90] In lobsters the enzyme is found in both the central and the peripheral nervous systems.[91] It has been purified about two-hundredfold over a crude extract and shows a requirement for high concentrations of sulfhydryl compounds for optimum activity (Molinoff, unpublished). The lobster enzyme is inhibited by GABA at high concentrations, an effect that will be discussed further in considering the regulation of GABA accumulation.[92]

GABA is further metabolized to succinate in two steps. The first reaction is a transamination with α-ketoglutarate yielding succinic semialdehyde and glutamate (GABA–glutamic transaminase). The enzyme was first described in 1953[93,94] and recently has been extensively purified.[95] The transaminase also requires pyridoxal phosphate for activity. In contrast to the decarboxylase, the transaminase is not localized in brain and is found widely distributed in animal tissues.[96] In the lobster nervous system a similar enzyme is present, and detailed studies on its properties have been carried out.[97]

The oxidation of succinic semialdehyde to succinate in the presence of diphosphopyridine nucleotide (DPN)—also called as nicotinamide–adenine dinucleotide (NAD)—completes the metabolism of GABA to succinate. The enzyme succinic semialdehyde dehydrogenase, which has been partially purified, appears to be a typical aldehyde dehydrogenase.[98,99] As with the transaminase, it is found in various animal tissues (for purification of kidney enzyme, see Pitts et al.[100]) and is also found in the lobster nervous system.[101] The enzyme has a low K_m (Michaelis constant) and is usually present in excess of the transaminase, which probably explains why succinic semialdehyde does not accumulate in tissues.

The sequence of reactions for the synthesis and destruction of GABA are shown in Figure 4. The pathway can be seen to constitute a "shunt" around the oxidative decarboxylation of α-ketoglutarate to succinate (via succinyl-S coA). There have been numerous attempts to assess the quantitative significance of the GABA pathway in nervous tissues. It has been estimated that up to 40 per cent of the metabolism of α-ketoglutarate is through this pathway,[102] although the various estimates suffer from technical difficulties of one sort or another. The GABA pathway would be energetically less efficient than the direct oxidative pathway—3 ATP equivalents vs. 3 ATP equivalents plus 1 GTP (guanosine triphosphate)—and the effects of this difference on the metabolism of the cell are uncertain. It may also be important that the utilization of the entire GABA pathway does not produce any net change in the glutamate level of the cell, as the transamination restores glutamate that had been decarboxylated.

Various other GABA-containing compounds have been isolated from mammalian brain. Homocarnosine (GABA–histidine) and γ-guanidinobutyric acid[103] are found in brain as well as other tissues, and their synthesis has been described (Figure 4).[104,105] The levels of these compounds in brain is low and the significance of their presence remains obscure.

Finally, it has been reported that γ-hydroxybutyric acid exists in the central nervous system[106] and that it can be synthesized from succinic semialdehyde.[107] These reports

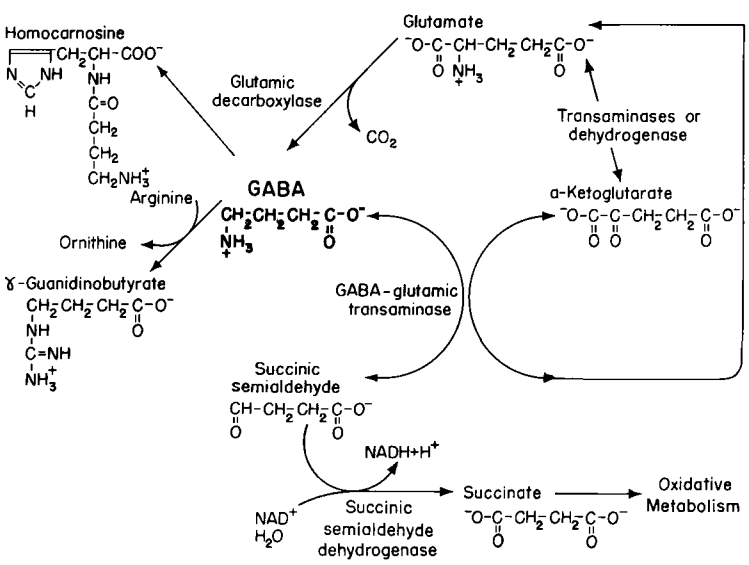


FIGURE 4 Sequence of reactions for the synthesis and destruction of GABA.

have not been substantiated in investigations by other workers.[108]

STORAGE AND RELEASE GABA and the enzymes of the principal pathway of its metabolism have been isolated in a synaptosome fraction obtained from the mammalian central nervous system.[109] However, lysis of this fraction did not yield a GABA-containing synaptic vesicle fraction. Moreover, the enzymes of the pathway are not found associated with the "vesicle" fraction. The decarboxylase is found to be soluble unless the calcium content of the medium is increased and the transaminase and dehydrogenase appear to be in a mitochondrial fraction derived from the synaptosomes. In the lobster nervous system all of the enzymes and GABA can readily be obtained in a soluble form (Hall, Molinoff, Kravitz, unpublished). The inability to isolate a vesicle fraction containing GABA may only reflect inadequate techniques for preserving GABA vesicles; it could also indicate that GABA is not a transmitter compound (although, as mentioned above, the evidence is increasingly strong that GABA is a neurotransmitter); or it could suggest that vesicles are not the release form of the transmitter. For example, release of transmitter from a medium of uniform, high concentration through specific channels opened for limited periods of time would also give quanta. In the lobster nervous system, in single inhibitory axons several centimeters away from their nerve terminals, the concentration of GABA is 0.1 mole per liter.[81] The concentration of GABA at nerve terminals in either the lobster or mammalian nervous systems is unknown, although the concentration in synaptosomes can be estimated. Again, as in the case of acetylcholine, the synaptosome fraction should be a valuable tool for studies of the GABA system.

The release of GABA has only been demonstrated for one synapse, the crustacean neuromuscular junction. The only agent known to be necessary for release is calcium.[82] Lowering the calcium of the external medium caused inhibitory synaptic potentials to disappear and prevented the release of GABA. Clearly, much study is needed to establish GABA as a transmitter compound at various junctions and to ascertain the details of the transmission process at such junctions.

Two further points are considered in this discussion—the chemistry of inhibitory receptor sites in Crustacea and a possible mechanism for terminating the actions of the inhibitory transmitter compound.

RECEPTOR SITES Postsynaptic receptor sites are not uniformly distributed along muscle membranes in crustacean muscle. The areas of greatest density of GABA receptors coincide with the location of inhibitory nerve terminals.[110] The presynaptic receptors for GABA on excitatory nerve terminals differ pharmacologically from the postsynaptic receptors.[111] While both produce conductance increases in response to GABA application, drugs such as β-guanidino propionic acid duplicate the GABA effect on presynaptic receptors but only combine with and block postsynaptic receptors without increasing conductance. Thus, in a single synaptic region (in this case, the crustacean neuromuscular junction), two different kinds of GABA receptors exist. The receptors do not show the phenomenon of desensitization described for acetylcholine receptors.[110] To date, there have been no chemical studies attempting to isolate and characterize this type of receptor site.

INACTIVATION There is no hydrolytic enzyme analogous to acetylcholinesterase, which destroys GABA. The only enzyme known to degrade GABA is the GABA–glutamate transaminase. This enzyme requires α-ketoglutarate for the metabolism of GABA, and it is therefore unlikely to be located at the outside surface of the membrane in synaptic clefts. Moreover, inhibitors of the transaminase do not prolong the action of the inhibitory transmitter at crustacean (Potter, unpublished) or mammalian (Ito, unpublished) synapses. There is, however, a specific GABA uptake process that has been demonstrated in lobster nerve-muscle preparations,[112] crayfish stretch receptors,[113] and in synaptosomes isolated from the mammalian central nervous system.[114] This is a sodium- and temperature-dependent process, and GABA is concentrated by the tissues against a gradient. In lobster nerve-muscle preparations, the process appears to be essentially unidirectional, but the site of uptake is not known. It is attractive to speculate that the presynaptic nerve terminals take up the released transmitter, but this remains to be established.

If uptake is the mechanism of inactivation of the inhibitory transmitter compound, then the acetylcholinesterase may be the exceptional mechanism for transmitter inactivation. At adrenergic synapses, there is a specific noradrenaline uptake system in presynaptic nerve terminals. Uptake processes exist at "GABA inhibitory" synapses and "glutamate excitatory" synapses, but there is only a choline uptake process at cholinergic junctions.

REGULATION OF GABA ACCUMULATION There have been numerous speculations on the possible mechanism of accumulation of GABA in the mammalian central nervous system. In general, these have been concerned with differential synthesis and destruction of GABA, but it is difficult to find an area of the mammalian brain in which clear-cut experiments could be performed to test these hypotheses. The lobster nervous system again provides advantages in this regard. Single axons serving precisely the same

function can be isolated repeatedly from different animals and can be analyzed biochemically. The distribution of the substrates (GABA, glutamate, and α-ketoglutarate) and enzymes (glutamate decarboxylase and GABA–glutamate transaminase) of the GABA pathway were compared in excitatory and inhibitory axons.[92] Of the substrates, only GABA was asymmetrically distributed, the concentration being 0.1 M in inhibitory and 0.001 M in excitatory axons. The transaminase activity was similar in the two kinds of axons, but the decarboxylase activity was about 10 times higher in inhibitory axons. Moreover, homogenates of inhibitory axons could synthesize more GABA than they could destroy; excitatory axon homogenates could not. However, in the presence of 0.1 M GABA, the decarboxylase was inhibited about 70 per cent and, in inhibitory axon homogenates, synthesis was approximately balanced by destruction. Thus, a hypothesis to explain GABA accumulation and maintenance was suggested. The decarboxylase difference between excitatory and inhibitory axons could be the key to the selective accumulation of GABA in inhibitory axons, and the GABA inhibition of the enzyme could determine the final level to which GABA would accumulate.

Clearly, there are many gaps in our knowledge of the synaptic chemistry of the GABA system, especially in mammals. One hopes that, as the evidence continues to grow that GABA is a transmitter compound in the mammalian central nervous system, more detailed chemical information will become available about the functioning of these synapses. An interesting observation in this regard is that in the lobster nervous system a high GABA level in a cell (cell body or axon) invariably labels that cell as inhibitory. If the same were true in the mammalian central nervous system, it might be a particularly valuable way to map certain inhibitory pathways. GABA would be the only transmitter compound with which this could be done, as both acetylcholine and noradrenaline can be either excitatory or inhibitory at different junctions.

Glutamate

The case for glutamate as a transmitter is considerably weaker than that for GABA or acetylcholine, so it will be considered here only briefly. The principal difficulties in accepting glutamate as a transmitter compound are that it appears to be ubiquitous in the nervous system and that it is an ordinary amino acid, apparently not specialized for transmitter use. However, these reasons seem inadequate for rejecting it as a potential transmitter substance. Indeed, insofar as it has been studied in both the mammalian and crustacean nervous systems, glutamate is an excitatory substance, similar in action to a natural excitatory transmitter compound.

Available studies of the crustacean nervous system are somewhat more complete than of mammals. Iontophoretic application of glutamate to muscle fibers has shown that glutamate "receptors" are not uniformly distributed along the fiber.[115] The regions of greatest receptor density are at the nerve terminals of excitatory axons. The application of glutamate causes a depolarization of the cell to a level of membrane potential near zero, but technical difficulties have prevented a detailed comparison of excitatory transmitter and glutamate effects. The receptor sites show desensitization to glutamate upon repeated application; this is also seen with the neurally released transmitter. Glutamate is the only excitatory substance isolated from the lobster nervous system,[116] but it is found in roughly equal amounts in excitatory and inhibitory neurons.[92] If it is a transmitter, either different release mechanisms would be needed at excitatory and inhibitory terminals or the postsynaptic membrane would be sensitive to glutamate only under excitatory nerve terminals. There is a sodium-dependent uptake mechanism for glutamate in lobster nerve-muscle preparations (Iversen, unpublished) but, as with GABA, it is not known if the uptake is primarily into presynaptic nerve terminals. Finally, there has been a report on the collection of glutamate when nerve bundles in certain lower forms (including Crustacea) are stimulated.[117]

In the vertebrate central nervous system, iontophoretic application of glutamate to nerve cells produces depolarization, which often leads to firing of cells. It has been reported, however, that glutamate does not bring the cell membrane potential to the same level as does the natural excitatory transmitter.[118] Serious technical difficulties stand in the way of such measurements, and other reports in the literature suggest that glutamate may indeed function as an excitatory transmitter.[119] If the latter is the case, it would be the only neurotransmitter not showing a marked asymmetric distribution between the cells that release it and those that do not.

Summary

There is no doubt that acetylcholine is a transmitter compound and the evidence for GABA is as strong. There is far less evidence for glutamate as an excitatory transmitter substance, but neither is there strong evidence against it. In all these cases, we know practically nothing of the chemical events taking place in membranes. We have no convincing chemical studies on the nature of the release mechanism, receptor sites, or uptake sites. The difficulty of working with membrane components is undoubtedly the

reason for our lack of knowledge, but hopefully this will be an area of intensive investigation in the future.

A good question with which to end this article might be, "Why do we care about this infinite sea of minor details on the events taking place in synapses"? The answer is obvious. As far as we know at present, it is only through the synaptic contacts on its surface that a cell in the central nervous system can be influenced by activity in other neurons. This, therefore, is the means by which a cell is informed of events occurring in the organism's environment. It is entirely likely, then, that both short-term and long-term changes that take place in the nervous system will involve changes in the functioning of certain chemical events taking place in synapses.

The Central Physiological and Pharmacological Effects of the Biogenic Amines and Their Correlations with Behavior

SEYMOUR S. KETY

THIS CHAPTER is the first of several that deal with behavior. It involves a major point of departure from most of the previous chapters, which have been based on rigorously established information and relationships acquired by the gradual accretion of hard fact upon hard fact. When we come to a subject as complex and multifactoral as behavior, we must work with hypotheses built upon hypotheses, even though many of them may not have been rigorously established. As you will see, in discussing the possible role of various drugs in the central nervous system, the evidence is much less direct and rigorous, and it is sometimes necessary to take two steps at a time. The accumulation of evidence, however, is compatible with certain hypotheses, and I will attempt a review of that evidence.

Localization of the biogenic amines in the central nervous system

Let us begin with a consideration of the anatomical relationships of the monoamines, especially the catecholamines and serotonin, in the central nervous system (CNS). In 1954, Martha Vogt[1] first described the gross distribution of norepinephrine in the mammalian CNS, and the range of results obtained by most workers for this and other amines is indicated in Table 1. You will note a wide range in the data; this represents values from different laboratories and from various species. The lower values are usually those found in man, and there are many artifacts that could cause this. The higher values are probably more significant. It is important to bear in mind that even within a given structure the distribution is localized and spotty, so anatomical regions are not the most precise means of defining the distribution. In any case, in terms of these gross distributions, norepinephrine is highest in the hypothalamus; then in the midbrain, pons, the medulla, the striatum and caudate nucleus, the hippocampus, the cerebral cortex, the cerebellum, and the spinal cord. The amine is quite low in white matter.

Shortly after Vogt's work, Bogdanski, Weissbach, and Udenfriend[2] reported the distribution of serotonin in the central nervous system, and this follows the same kind of pattern, except that there seems to be a tendency for serotonin to predominate in the invertebrates, to remain in the primordial parts of the brain, and to give way, so to speak, to norepinephrine in the most recently acquired portions of the mammalian central nervous system.

A more precise localization of the CNS monoamines

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has been made possible by an elegant histofluorescence technique developed by Hillarp and Falk and applied intensively by their students, especially Dahlström and Fuxe.[3] This technique is based upon a conversion of the monoamines by formaldehyde in dry protein films, in which the catecholamines go to isoquinolines and 5-hydroxytryptamine is converted to a highly fluorescent carboline. The catecholamine derivatives fluoresce with a green or yellow-green color and the serotonin with yellow. The differentiation of dopamine from norepinephrine by this technique is poor, and only on the basis of other pharmacological and physiological evidence have these workers been able to say whether a particular neuron contains norepinephrine or dopamine. Nor is it possible by this technique to exclude admixtures of other monoamines with a predominant one in the same cell, because

TABLE I

Distribution of norepinephrine, dopamine and serotonin in the central nervous system as determined by chemical (in dog, cat, man) and histofluorescent (rat) techniques.

	NOREPINEPHRINE		DOPAMINE		SEROTONIN	
	μg/gm	histo-fluor. degree 0–5+	μg/gm	histo-fluor. degree 0–5+	μg/gm	histo-fluor. degree 0–5+
Cerebral cortex	.03–.24	1–2	.00–.10		.04–.24	
Caudate nucleus	.04–.22		*3.1–8.0*	4	*.27–1.6*	2
Corpus striatum	.02–.25		*1.6–5.3*	4	.08–.23	2
Olfactory bulb	.05	*1–3*	—		.11–.35	
Hippocampus	.14–.20	*1–4*	.13		.64	1
Amygdoloid nucleus		2–3		2		2–3
Septum	.03	*1–4*	.04		*.03–1.5*	1
Hypothalamus	*.76–2.05*	*1–5*	.26–.75		*1.70–2.0*	1–2
Diencephalon less hypothalamus	.17–.37		.09–.16			
Thalamus	.13–.24	*1–5*	.01–.07		.22–.24	1–2
Mesencephalon					*1.0–1.7*	
Inferior and superior colliculi	.11–.16	1–3	.10–.13			1–2
Substantia nigra	.04	1	.40		1–4	2
Red nucleus	.22		.19			
Brain stem reticular formation	.35	*1–4*				1–2
Cerebellum	.06–.17	1	.03–.02		.27	
Pons	*.04–.41*	*2–4*	0–.11		*.19–.70*	*1–3*
Medulla	*.37–.72*	*1–5*	.13		*.63–1.20*	*1–3*
Area postrema	*1.04*				.26	
Locus ceruleus		*3–4*				1–2
Cerebral white matter	0	–	.42	–	0–.13	—
Ant. horn—spinal cord	.18	*1–5*				*1–5*
Post. horn—spinal cord		*1–5*				1
(Sup. cervical ganglion)	*6.8*					

From: McLennan, 1963 Synaptic transmission, Saunders Co., p. 70
Fuxe, 1965. *Acta Physiol. Scand.*, Vol. 64, Suppl. 247, pp. 37–85
Other reports in the literature.

fractional fluoroscopic examination has not yet been made and only the predominant color of the fluorescence has been reported.

Despite certain drawbacks, the technique has, to a considerable extent, revolutionized understanding of the distribution of the monoamines in the central nervous system. It has revealed that brain monoamines are intracellular and sharply limited to certain cells, where they occur in the cell cytoplasm, in the axon, and in highest concentration in certain varicosities of the axon that are synaptic terminals. After sectioning axons, one can demonstrate an accumulation of the fluorescence proximal to the lesion and a gradual distal loss of fluorescence. By these means, Carlsson, Dahlström, and Fuxe have estimated turnover of catecholamines within specific cell groups, have studied the action of drugs, and have demonstrated certain monoamine-containing pathways in the central nervous system. The techniques have indicated more clearly than has regional chemical analysis that the monoamines are ubiquitous in the mammalian CNS, but that their concentrations are especially high in regions where chemical studies had previously shown a high concentration.

In addition, however, there are fine monoamine-containing fibers, large in number but small in mass, in the cerebral cortex, hippocampus, and cerebellum, where previous chemical studies had not suggested their importance. By cutting various tracts and examining the increase or decrease of fluorescence, it has been possible to map out or postulate the existence of certain monoamine-containing tracts. Two of special importance have been described by Andén[4] and his colleagues—a nigro-striatal system of dopamine-containing neurons with bodies in the substantia nigra and with endings in the neostraitum, the caudate, and putamen. There also appears to be a serotonin-containing pathway that terminates in the same general region. Andén has also adduced evidence for an interesting system of norepinephrine-containing neurons; their cell bodies appear to be in the brain stem and they send axons via the medial forebrain bundle to the hypothalamus, the hippocampus, the limbic system, and throughout the entire cerebral cortex and cerebellum.

How well have particular monoamines been identified with presynaptic vesicles? The presynaptic vesicles occur in great profusion in the central nervous system. How sure are we that they contain monoamines? The evidence at present is still indirect. I have already indicated that the monoamine fluorescence is highest in the terminal axons of certain neurons. Cell fractionation techniques have been applied to the CNS, and have shown that the synaptosomes, where the concentration of vesicles is high, also have a high concentration of monoamines. Radioactive norepinephrine injected into the fluids that bathe the brain will accumulate in the synaptosomal fraction in a very high concentration. Radioautography does not offer conclusive evidence, because the resolution of these techniques does not permit localization to structures as small as vesicles. However, statistical studies by Aghajanian and Bloom[5] have indicated a high correlation between the presence of norepinephrine (as determined by radioautography) and the regions of the dense-core vesicles in the hypothalamus. Although there is no rigorous demonstration at present that any of these vesicles contain one or another of the so-called transmitters, there is certainly a wealth of evidence with which such a hypothesis would be compatible.

Synthesis and metabolism

Biochemical studies on the monoamines in the central nervous system have shown that all the enzymes for the synthesis of catecholamines and serotonin are in the brain, and the administration of labeled precursers results in the presence of more or less localized labeled amines. There are also specific intracellular mechanisms for the uptake of these monoamines in the brain. One problem here has been that the blood-brain barrier has prevented the entrance of monoamines directly into the brain from the bloodstream, but recently Glowinski,[6] et al., were able to bypass that barrier by injecting tritiated norepinephrine into the lateral ventricle.

The enzymes for the destruction of monoamines—monoamine oxidases and catechol-O-methyl transferase—are also present in the central nervous system, together with the respective products resulting from the action of these enzymes on the monoamine substrates. A number of different approaches, including studies of the decay of the labeled amines or measurements of their rate of fall after synthetic blockade, have indicated a rapid turnover of brain monoamines. These studies agree that norepinephrine has a rapid turnover—a half-time of two to four hours—more rapid in the cerebral cortex and the cerebellum than in the hypothalamus.[7]

Neurophysiological studies

Turning now to the physiological evidence for a role of monoamines in the CNS, we ask the first question: Is there evidence that these monoamines are released in association with synaptic activity, nerve function, or particular behavioral states? There are few direct demonstrations of the release of any of these presumed transmitters within the central nervous system. Under appropriate stimulation, acetylcholine has been recovered in the washings from the cerebral cortex in vivo. By using a so-called push-pull cannula, with which it is possible to perfuse small regions

of the central nervous system by pushing saline in and continuously removing it through micropipettes, McLennan[8] appears to have demonstrated that under appropriate stimulation acetylcholine and dopamine may be released from the caudate nucleus.

There is good but indirect evidence that these substances are also released with functional activity. Baldessarini and Kopin[9] have recently demonstrated that brain slices or isolated parts of the brain in vitro release striking amounts of norepinephrine when their neurons are depolarized with a mild electrical current or with potassium. Even more indirect, but much more extensive, is the evidence for release of these presumptive neurotransmitters that is inferred from a fall in their concentration in various parts of the brain in association with certain types of activity. Various workers have demonstrated that during severe stress, anoxia, exposure to cold, forced swimming, or electrical shock, a slight or a moderate decrease of one or another amine takes place in total or regional concentration in the brain. More interesting, perhaps, is the evidence of Reis and Gunne.[10] They stimulated the amygdala in cats and sometimes produced a rage response. At other times there was either sedation or no effect. When amygdala stimulation produced rage, it was associated in the telencephalon with a marked fall in norepinephrine but not in dopamine. When that structure was stimulated and no rage occurred, there was no change in the amine.

The direct application of possible transmitters to postsynaptic structures in the central nervous system has been fairly limited, but the results to date are compatible with some neurotransmitter actions. Most compelling was the demonstration by Curtis[11] of a physiological effect of acetylcholine at the Renshaw cell. This cell is monosynaptically excited by a recurrent collateral of the motor axon, and because acetylcholine is fairly well established as the transmitter substance at the myoneural terminal of the motor axon, it was a fair assumption that the recurrent collateral of that axon would also release acetylcholine. Curtis used micropipettes to inject acetylcholine around the Renshaw cell, and produced an excitation that corresponded in every important way with the excitation produced by motor nerve stimulation. Furthermore, the effects of certain drugs, such as prostigmine and β-erythroidine, were similar on this excitation whether produced by physiological stimulation or by local injection of acetylcholine. It could then be inferred that the motor neuron collateral on the Renshaw cell exerts a cholinergic function and that the synapse is a nicotinic synapse (i.e. dependent on the nicotinic properties of acetylcholine) similar to that in autonomic ganglia.

Although the Renshaw cell is the only central nervous structure in which physiological and pharmacological criteria for neural transmission have been reasonably well fulfilled, there is a fair amount of evidence for direct action of a number of these postulated transmitters on various cells in the central nervous system. Salmoiraghi,[12] among others, has obtained a great deal of this evidence, which can be summarized by saying that acetylcholine, norepinephrine, dopamine, or serotonin can be shown to activate or to inhibit particular cells at various places in the central nervous system. GABA appears to be predominantly inhibitory on those central neurons on which it has been studied, while glutamate and a number of amino acid anions appear to be predominantly exciting. Acetylcholine action on such neurons seems to be faster and shorter than that of other amines, such as norepinephrine, which is both slow and prolonged. Salmoiraghi and his associates have also obtained evidence of an interaction between norepinephrine and acetylcholine, in which the former appears to suppress the stimulation normally produced by the latter. All of this evidence does not conclusively demonstrate a neural transmitter role for any of the agents, but is certainly compatible with that possibility.

Evidence for a role of the monoamines in mental state and behavior

As we move along from the more rigorous physiological evidence to the behavioral effects, we unfortunately can find no crucial, direct evidence that an action on behavior is produced by any of these transmitters acting locally at specific sites. Most of the evidence depends upon a large number of pharmacological studies, which have utilized drugs as tools in studying behavioral effects of amines in the central nervous system. It is important to bear in mind certain drawbacks of this approach. No pharmacological agent produces only one effect; each has both the desired effect and a host of side effects. One of the difficulties is to separate that desired effect from the others.

The first entrance of pharmacology into this field probably occurred one April afternoon in 1943 when Hofmann, an organic chemist working in Basel, suddenly went berserk and had to be taken home from the laboratory. For the next several hours he experienced a number of strange hallucinations, illusions, and other psychotic symptoms. He was finally able to get to sleep with the help of a sedative and by the next morning was recovered. If he had been a psychiatrist, he might have wondered which aspects of his life situation could have precipitated such an episode, but being a chemist, he decided it was something he ate! He remembered that he had been working with a new compound LSD-25 (lysergic acid diethylamide), and thought it possible that he had inadvertently ingested a small amount. It was thus that the very important subjec-

tive effects of LSD were discovered. Hofmann returned to his laboratory and, in order to confirm his hypothesis, took a minute dose of the substance—a quarter of a milligram—in a glass of water, promptly went berserk, and again was taken home.

This strange compound, which has such potent effects in such infinitesimal doses (70 micrograms in a glass of water will produce an acute, toxic psychosis), stimulated a great deal of work, and it was not long before an objective, pharmacological effect of the drug was found. Gaddum in England and Woolley in this country independently discovered that in very low concentrations LSD would block the effects of serotinon on smooth muscle. They also speculated that as serotonin was present in the nervous system and LSD could block serotonin it might, in fact, produce its psychotic effects by blocking serotonin in the brain.

Shortly thereafter reserpine came into the picture as an important tranquilizing agent that produces a marked sedation in animals and tranquilization of overexcited states in man. In some individuals it produces a frank depression, which is hard to distinguish from clinical depression. Shore and his colleagues at the National Heart Institute examined the effects of reserpine and discovered that it almost completely depleted brain serotonin.[13] This depletion persisted until new serotonin was synthesized. Because reserpine was such a remarkable and rather specific depressant of mood, it seemed reasonable to wonder if its effect was somehow related to its action on serotonin in the brain.

Then, by another serendipitous route, iproniazid was discovered to have important side effects. This drug was first introduced as a treatment for tuberculosis, but while it appeared to halt the progress of the disease, it also produced psychic stimulation. The first reports of its success also told of patients dancing in the halls of the sanitorium, and this was not entirely because they had seen their X-ray films. Iproniazid is a potent excitant in animals and in large doses in man can be a euphoriant and even a psychotomimetic agent. In smaller doses it exerts an antidepressant effect, specifically on certain cases of clinical depression. It was soon learned that iproniazid is a monoamine oxidase inhibitor and elevates the levels of brain serotonin. This served as further support for the hypothesis that serotonin was *the* hormone involved in affective behavior in animals and man.

Unfortunately, some spoilsports came along and demonstrated that reserpine also depleted the brain of norepinephrine and dopamine and that iproniazid was capable of increasing the brain concentrations of these amines as well as serotonin. What had looked like a clear and simple hypothesis became, as it inevitably does, much more complicated. I think it is fair to conclude that the effect of reserpine and similar depressants and of iproniazid and other monoamine oxidase inhibitors upon mood or affect is clearly related to their effects on amines in the brain. When one prepares a series of congeners of reserpine, one finds that their effects on mood or affective behavior can be correlated with their ability to deplete brain amines. The high correlation between monoamine oxidase inhibition and antidepressant activity made a small fortune for the pharmaceutical industry, because all one had to do was develop a new monoamine oxidase inhibitor, make sure it got into the brain and was nontoxic, and one could be fairly certain that it would be a good antidepressant.

Which of these specific amines are related to mood or to any of the affective behavioral states is still an open question. At the present, to summarize a great deal of evidence and not attempt to suppress my own bias completely, the bulk of the evidence acquired since the early studies implicates norepinephrine as an amine in the brain that plays an important role in such mental and behavioral states. I want to point out, however, that considerably more work has been done on norepinephrine than on serotonin or on dopamine, and the evidence for norepinephrine involvement by no means excludes the participation of other amines in the same processes. Nevertheless, let me cite the evidence suggesting an involvement of catecholamines and especially of norepinephrine.[14]

If an animal in reserpine depression is treated with dopa, the catecholamine precursor, and one potentiates this with a monoamine oxidase inhibitor to favor the accumulation of the amines produced, one can rapidly reverse the depression. On the other hand, 5-hydroxytryptophan, the precursor of serotonin, does not have as specific or as dramatic an effect. Recently, two rather specific synthetic inhibitors have been discovered and applied in animal studies and in some human studies. The first of these is α-methyl-p-tyrosine, which blocks catecholamine synthesis specifically, producing a marked lowering of brain catecholamines with little change in serotonin. A number of workers have observed that when norepinephrine synthesis in animals was blocked by this compound, sedation followed. Sjoerdsma and his colleagues[15] have also found sedation in man. More interesting still, they discovered that when the drug is suddenly withdrawn, there appears to be an increase in anxiety and insomnia, as if the enzyme that had been inhibited is released with a rebound. Moore[16] has reported a suppression of conditioned avoidance behavior (but not escape behavior) in animals given α-methyltyrosine and has shown that the onset and duration of this behavioral effect closely parallels the change in brain norepinephrine. On the other hand, p-chlorophenylalanine appears to block tryptophan hydroxylase and

to inhibit the biosynthesis of serotonin, depleting the brain of this amine without having a striking effect upon norepinephrine. The drug has not yet been shown to produce any clear sedative effect or any other remarkable behavioral effects in animals. Reserpine still causes sedation, even though the serotonin in the brain is low.

Perhaps the most compelling evidence for the role of norepinephrine in emotion and similar behavioral states is that all the drugs with fairly specific effects on mood in man or in animals have also been shown to affect brain norepinephrine in ways that are compatible with its having an important role in these states. Earlier work was limited to measuring the gross concentration of the amines following the administration of the drugs. This has a serious drawback, as one would expect—a priori and on the basis of much evidence—that the effective concentration of an amine at a synapse represents a very small part of its total amount in the brain, most of it lying inactive in storage sites, such as vesicles. It is something like trying to correlate the whole animal concentration of serotonin with behavior, while realizing that as much as 98 per cent of the serotonin in the body is in the gut and not likely to be relevant.

I mentioned before that Glowinski labeled norepinephrine in the brain by injecting it into the lateral ventricle, thus permitting a study of its turnover. More recently, Schanberg, Schildkraut, and Kopin[17] have injected it more conveniently into the cisterna magna. Either route achieves a widespread distribution of the labeled norepinephrine in the brain. It is, of course, crucial to indicate whether the endogeneous norepinephrine is being reliably labeled by this technique; if not, one would simply be studying the effects of drugs on artificially administered radioactive norepinephrine and not upon endogenous epinephrine. After a few minutes, the injected norepinephrine distributes itself in fairly close correlation with the endogenous amine, although this is to some extent affected by the site of injection. More important, cell fractionation studies have shown that the labeled amine parallels the endogenous concentration, being especially high in the region where the vesicles are sedimented. Furthermore, as Iversen and Glowinski[7] have shown, the rate of turnover of tritiated norepinephrine thus administered in various regions of the brain is comparable to the rate of turnover of endogenous norepinephrine in these areas, as measured by its fall after blockade of synthesis. Hence, the effects of various drugs can be studied with some confidence that one is gaining an insight into what is happening to the amines in the brain.

Reserpine has been shown[18] to increase markedly the disappearance of radioactive norepinephrine in the brain with a concomitant decrease in normetanephrine and a marked increase in deaminated products. In his chapter in this volume Dr. Kopin mentioned that there are two pathways in the metabolism of norepinephrine—an O-methylating pathway, which appears to be mediated by catechol-O-methyl transferase (COMT), and a deaminating pathway catalyzed by monoamine oxidase. A great deal of evidence suggests that the monoamine oxidase is in the mitochondria and intracellular, while the COMT is at the synapse but postsynaptic; it is also present in the liver. Kopin and Gordon[19] demonstrated that when norepinephrine was released in the perfused heart by nerve stimulation or by a drug that mimics nerve stimulation, there was a simultaneous increase in its O-methylated derivatives; when the amine was released by reserpine there was an increase in the deaminated derivatives. This suggests that normetanephrine represents a release into the synapse and is therefore an active physiological release. The deaminated products, on the other hand, represent intracellular and presynaptic destruction of the amine. In any case, reserpine in the brain acts as it does in the heart, causing an increase in the deaminated products and a decrease in O-methylation; this would be compatible with a presynaptic destruction rather than a synaptic release of the catecholamine and with the depressant effects of reserpine.

Lithium recently has aroused considerable interest because of its rather specific effect upon mania. Without any reason to think that it might act through the catecholamines (rather, one generally thinks of its effects on membrane transport of sodium and potassium), Schildkraut, Schanberg, and Kopin[20] have examined the effect of this ion on the turnover and metabolism of norepinephrine in the brain. They found that lithium effects are similar to those of reserpine—an increase in deaminated products and a decrease in O-methylated derivatives—which again would be compatible with its sedative effects.

On the other hand, there are a number of drugs that may be classed as antidepressants or euphoriants. The monoamine oxidase inhibitors act predictably on brain norepinephrine; by blocking the deaminating enzyme they increase the concentration of norepinephrine and probably make more of it available at the synapse.[18] A marked increase in normetanephrine in the brain follows their administration; this is compatible with an increase in synaptically released and physiologically effective norepinephrine.

Imipramine, which was discovered later quite by accident, and which appears to be the most potent of the antidepressant drugs, is not a monoamine oxidase inhibitor and at first had no obvious effect on norepinephrine. It seemed to be an exception to the generalization that drugs affecting mood also affect brain norepi-

nephrine. Then, recent evidence uncovered a subtle but interesting effect. Imipramine appears to act both centrally and peripherally to block the reuptake of norepinephrine into the presynaptic terminal. This is probably a major means of terminating its action. Thus, imipramine would be expected to increase the concentration of norepinephrine at the postsynaptic receptor and potentiate its action there.

Finally, we come to the interesting drug amphetamine and its congener, dextroamphetamine, which have at least three actions relevant to our discussion. In the first place, they are important sleep-inhibiting drugs, producing wakefulness in the face of considerable fatigue. In large doses, especially in animals, they produce a marked increase in activity. They also cause a subjective euphoria in many individuals, so the drug is in slightly ill repute. Euphoria, being a subjective manifestation, is difficult to quantify, and therefore one seeks an animal analogue. This may have been found in the phenomenon of self-stimulation. If electrodes are implanted and stimulated in certain areas of the brain, especially the septal nuclei and the medial forebrain bundle, the animal will either seek repetition of the stimulus or will attempt to stimulate the area himself if he is provided with a suitable lever. The rate of self-stimulation can be quantified very neatly as Stein[21] has done. There is some evidence that self-stimulation is related to euphoria or reward. When similar areas are stimulated in man, the subjects often report a vague sense of pleasure and seek repetition of the experience. The administration of amphetamine markedly increases the rate of self-stimulation, and amphetamine is one of very few drugs that appears to have such an effect.

TABLE II

Actions of drugs on mood, behavior, and brain norepinephrine

Drug	Effects on mood (Man)	Effects on behavior (Animals)	Effects on brain norepinephrine (NE) and its metabolites: normetanephrine (NM); deaminated products (DP)	Interpretation of action in light of a norepinephrine hypothesis
reserpine	tranquilizer	sedation; depresses conditioned avoidance and self-stimulation	depletes NE; loss of "dense core vesicles"; increased disappearance of H^3NE; increase in DP; decrease in NM	*decreases* availability of NE at central synapses by presynaptic catabolism
tetrabenazine	——			
lithium salts	counteracts mania and hypomania	sedation (large doses)	no change in NE; increase in DP; decrease in NM	*decreases* availability of NE at central synapses by presynaptic catabolism
chlorpromazine	tranquilizer	sedation; depresses conditioned avoidance	no change in NE; no consistent effect on metabolites; inhibits uptake of H^3NE	*decreases* availability of NE at central synapses by interference with membrane permeability (?)
α-methyl-p-tyrosine	sedation	sedation; depresses conditioned avoidance and self-stimulation	blocks synthesis of NE; reduces levels of NE	*decreases* availability of NE at central synapses by blockade of synthesis
monoamine oxidase inhibitors	antidepressant; stimulation (large doses)	excitement; reverses reserpine depression	increases NE; decreases disappearance of H^3NE; decrease in DP; increase in NM	*increases* availability of NE at central synapses by preventing presynaptic catabolism
amphetamine	wakefulness; euphoria	stimulation; excitement; increases self-stimulation	slightly decreases NE in large doses; increased disappearance of H^3NE; decreased uptake of H^3NE; decrease in DP; increase in NM	*increases* availability of NE at central synapses by (1) facilitating its release; (2) inhibiting reuptake; also may have direct action on receptors
imipramine	antidepressant	reverses reserpine depression; potentiates amphetamine	no change in NE; inhibits uptake of H^3NE; slows disappearance of H^3NE; increases NM	*increases* availability of NE at central synapses by inhibiting reuptake

There is evidence that these effects are dependent on a release or potentiation of cerebral norepinephrine. In the first place, the amphetamines characteristically show a tachyphylaxis, i.e., a diminution in response with repeated doses. This is especially noted in their antisleep properties. One cannot do without sleep simply by taking amphetamine indefinitely; it works very well the first time, but the next night it does not work nearly so well, and by the third night it has no effect at all. Tachyphylaxis suggests that it is not the amphetamine itself that is producing the effect, because the same dose of amphetamine is applied each time, but rather that the amphetamine is releasing something else from a relatively limited store. If administered before amphetamine treatment, α-methyltyrosine, which blocks norepinephrine synthesis also blocks the effect of amphetamine on activity in animals, again suggesting that these effects are mediated by norepinephrine. If reserpine is given before amphetamine, the effect of the latter on self-stimulation is also blocked. The amphetamine still has its effect if it is given immediately after reserpine, when the level of reserpine is at its highest but it has not yet reduced the norepinephrine. After three hours, however, when norepinephrine levels are at their lowest point, the ability of amphetamine to accelerate self-stimulation is markedly reduced. In general, this ability parallels very closely the levels of brain norepinephrine. What does amphetamine do to the turnover and metabolism of norepinephrine in the brain? It has a rather complicated action, appearing to block uptake partially, to accelerate its release, and to be associated with a marked increase in methylated norepinephrine and a decrease in deaminated products.[18] This is also compatible with the idea that amphetamine releases norepinephrine at physiologically active sites.

Table II gives a summary of the drugs that affect mood in man and describes their effects on animals and their known actions on brain catecholamines.[14,17,18,22] Drugs that cause sedation or depression of mood cause an intracellular release and destruction of norepinephrine, so that less is available at the synapse. Drugs that elevate mood and increase arousal or activity appear to increase the effective concentration of norepinephrine at central synapses by one or more mechanisms: inhibiting the destructive deaminating enzyme, favoring release at physiologically active sites, or preventing its rapid removal from the synapse by reuptake through synaptic membranes. All of the drugs thus far examined, which elevate mood cause an increase in normetanephrine in the brain, suggesting that they increase the synaptic concentration of norepinephrine; the drugs that cause depression increase the deaminated metabolites, suggesting a presynaptic destruction of norepinephrine.

Summary

I think the notion is strongly supported, although by no means proved, that central norepinephrine plays an important role in affective and arousal states. This does not exclude the action of other amines in these states. It is extremely unlikely that emotional processes will ever have a simple molecular explanation. It is not so much that one amine determines emotional states as that several acting in concert in specific areas of the brain establish a primitive substrate that is elaborated upon and given significance by cognitive factors, which depend upon the particular situation and the past history of the individual. Although all of these may ultimately operate through molecules, their position and interaction with others in the tremendously complex network of structures we call the brain may be just as important as their chemical nature.[23]

Electrical Signs of Sensory Coding

F. MORRELL

IF ONE PLACES conductive electrodes on the surface of the scalp of the human or of other animals and amplifies the signal about a million times, a potential difference can be recorded between any two of the electrodes. This potential has the form of a more or less sinusoidal and regular oscillation with a dominant frequency of about 8 to 13 cycles per second in the adult of the species.

Intrinsic rhythms

In human electroencephalography, the frequency range just given has been designated the *alpha* rhythm; the range would have to be extended somewhat to include all those rhythms exhibiting similar physiologic behavior in other species or in the immature organism. Yet extending the frequency range only a few cycles in both directions would, with few exceptions, encompass all the dominant brain rhythms from all species and all ages thus far examined. For example, in Figure 1, the brain wave patterns of frog, guinea pig, rabbit, cat, monkey, and man are compared. The gross similarities across all species are plainly seen. It is equally evident that there are differences among these records. Yet the range of variation, at least with respect to dominant frequency, is extremely limited. These particular tracings were randomly selected from the various "preparations" used in our laboratory. Considering the gross differences in genetic makeup of the several species and the vast variations in life experience to which each had been exposed, it is difficult to believe that the frequency of brain rhythms might be directly related to the coding of sensory experience or to the information the brain contains. Indeed, the shifts in brain wave frequency in the same individual during the transition from a waking to a sleeping state (see M. Jouvet, this volume), or from waking to aroused state (see below), are far greater than are the differences among species, as shown in Figure 1.

The 8 to 13 cycles per second alpha rhythm under discussion may be described as the dominant pattern in the electroencephalogram. There are also subdominant rhythms characteristic of particular regions such as the *beta* activity of 18 to 24 cycles per second, which constitutes the spontaneous resting rhythm of rolandic motor cortex in man, and the 4 to 7 cycles per second *theta* rhythm of the hippocampus, which is best seen in the rabbit and cat although traces of it remain in man and monkey. Slower rhythms of 1 to 3 cycles per second called *delta* are normally present during sleep, but when present in the waking state are important diagnostic signs of intracranial pathology. Space limitations prevent any further consideration of these subdominant rhythms in this chapter.

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Activation pattern

One of the defining characteristics of alpha rhythms is that they are most prominent when the human or animal is awake and is *not* engaged in transaction of visual business. Figure 2 illustrates the classical "activation pattern"[1] or suppression of alpha rhythm and its replacement by low-voltage fast activity when a subject opens the eyes and looks at an object on a viewing screen. It is generally accepted[2] that the slower, higher-voltage, more regular, oscillatory rhythms are associated with decreasing levels of vigilance.[3] Exceptions to this general rule may be induced pharmacologically,[4,5] and are sometimes seen during transitional stages between wakefulness and sleep and during the rapid-eye-movement (REM) stage of sleep.[6] Nevertheless, electroencephalographers have repeatedly commented[7] upon the apparent paradox that higher-voltage electrical rhythms are characteristic of "idling" neurons, i.e., neurons which, at that moment, are not under specific stimulation. It has been presumed (for a thorough discussion of the history of ideas about brain waves see Notes 7–11) that nerve cells having nothing specific to do were somehow available to get into step with one another to produce a *synchronized* rhythm. On the other hand, under the influence of specific excitation, cellular firing patterns become highly differentiated, versatile, organized, and asynchronous with one another. The electrical pattern of the population (the EEG) then exhibits the low-voltage, high-frequency, and less regular features shown in Figure 2 (eyes open). Accordingly, the alpha "blocking"[12,13] or arousal reaction[14] or activation pattern[1] has also been called *desynchronization* to indicate the basic physiological mechanism that was thought to underlie it.[7]

Several forms of activation pattern or desynchronization have been identified. There is (1) a diffuse or *generalized* form affecting all regions of both cerebral hemi-

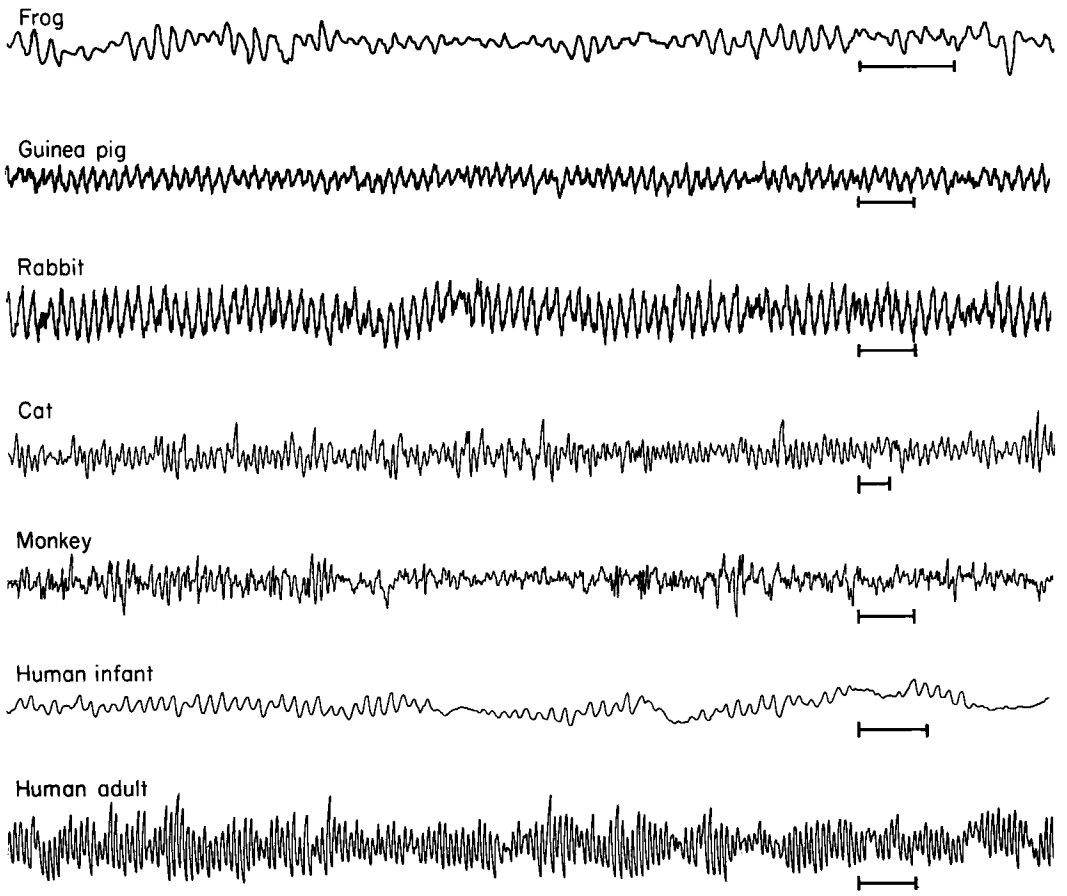

FIGURE 1 EEG tracings from various species as indicated. Calibration bar below each record equals 1 second. The human infant and adult records were obtained with scalp electrodes; all others were derived from electrodes on the surface of cortex.

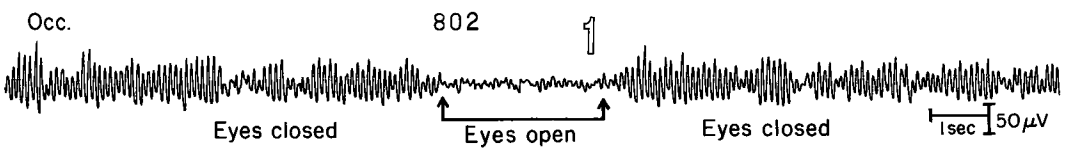

FIGURE 2 Activation pattern. Blocking of the human alpha rhythm is produced by eye opening in a normal human subject. Derivation is from right occipital electrode referred to linked ears.

spheres, and (2) a *localized* form limited to specific cortical regions. Furthermore, in the time domain one may distinguish between a form that is (3) *tonic* in the sense of maintaining wakefulness over long periods of time, and one that is (4) *phasic* and mediates sudden, brief shifts in attention in response to shades of sensory stimulation.

ANATOMY AND LITERATURE The anatomical substrate for all of the electrocortical reactions described above is not in the primary afferent pathways, but in the more medially coursing, multisynaptic reticular formations of the mesencephalon[14] and thalamus.[15,16] Although it has been argued[8] that the *generalized* and *tonic* reactions were attributable to the mesencephalic reticular system, while the *localized* and *phasic* forms depended on the thalamic system, there are circumstances in which such clear-cut distinctions are difficult to document. For instance, a sudden, unexpected, and startling stimulus delivered to any

sensory modality results in a generalized desynchronization of all cortical regions. This has been shown by Moruzzi and Magoun[14] to be mediated by the ascending mesencephalic reticular formation. If the stimulus is of no biological importance and is repeated many times, the generalized desynchronization rapidly wanes (habituation[17]), and eventually gives way to a localized desynchronization usually limited to the cortical region appropriate to the stimulus modality used. In this example, a diffuse activation known to be mediated by the mesencephalic system exhibits temporal characteristics that are phasic rather than tonic. In fact, this is the neural basis of the orienting reflex.

On the other hand, localized desynchronization habituates much less readily than the generalized form. Voronin and Sokolov[18] report that visual stimuli continue to induce localized blocking of the occipital alpha rhythm even after hundreds of presentations. Moreover, the topography of localized activation, although usually maximal in the cortical receiving area for the stimulus modality, is strongly influenced by the animal's prior associations with that

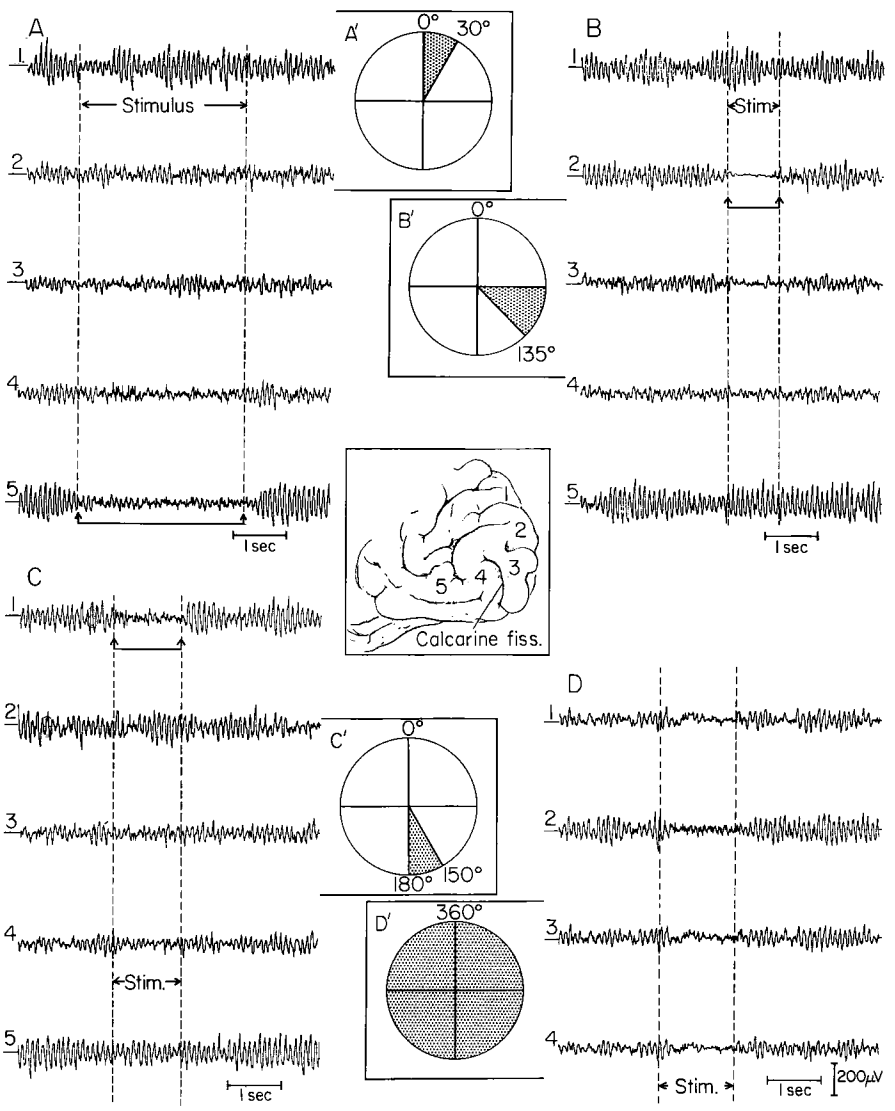

FIGURE 3 Electrocorticogram from left occipital cortex in an unanesthetized subject. Channel designations refer to electrode numbers positioned around the calcarine fissure, as indicated in the diagram. Patient was required to fixate upon a small red dot in the center of the viewing screen. Stimulation for 1 to 3 seconds was carried out in particular sectors of the contralateral visual field, as indicated in A′, B′ and C′, or with full field illumination (D′). The resulting desynchronization was limited to correspondingly small segments of occipital cortex (A, B, C). See upturned arrows.

stimulus, i.e. by conditioning. Thus, an acoustic signal or conditioned stimulus (CS), which had been repeatedly paired with a visual or unconditioned stimulus (UCS) may ultimately induce desynchronization mainly in the visual area.[19,20] This latter form of activation pattern has been shown to depend upon the integrity of the intralaminar reticular nuclei of the thalamus.[21,22] The response is certainly phasic, in that it does not outlast the duration of the stimulus. It is curiously persistent to repeated stimulation and, under proper recording conditions, lasts at least as long as a behavioral conditioned response, finally altering its electrical morphology (see following text) at about the stage when behavioral conditioned responses disappear because the animal had become satiated.

There have been numerous studies[23-30] dealing in great detail with the time course of habituation and of conditioning of alpha blocking, the various circumstances which influence it, and both the topographic and morphologic alterations in brain wave patterns that occur as a result of repeated stimulus presentation. Several reviews[26,31-33] will provide adequate coverage of the field for those particularly interested. Investigations have been carried out in human subjects[23,25,27,28,30] by recording electrical activity from scalp electrodes, and in animals having implanted electrodes in local brain regions.[29]

CLINICAL OBSERVATIONS Recently, we have had the opportunity to examine some of the characteristics of stimulus-induced localized desynchronization by recording directly from the surgically exposed visual cortex in a conscious human subject. Our findings have caused us to question the accuracy of the localizations previously obtained by use of scalp electrodes.

The observations were carried out in the course of a therapeutic procedure to relieve this patient of medically intractable epilepsy caused by the scar surrounding a previously drained abscess in the left posterior temporal region. As the cortical zone involved was adjacent to the area of cortical representation for speech, the operation was carried out under local anesthesia only. (Having the patient awake and able to converse during the operation makes it possible to map the speech area physiologically, and thus affords an extra margin of safety for the patient against inadvertent surgical injury to speech function. This is now standard neurosurgical practice, see Chapter XVIII in Note 7.) The local anesthesia entirely eliminates pain from the skin incision; the brain itself is not pain sensitive. The location of the lesion was such as to require a posterior bone flap, which was carried to the midline, thus exposing the entire occipital lobe of the left hemisphere. Cotton wick electrodes were placed upon the surface of the brain in many places and with many arrangements in order to define and map the area of electrical abnormality responsible for the convulsive seizures. Micro-electrodes were also employed to search for single cells having the abnormal electrical characteristics we have come to associate with epileptic discharge in experimental animals. Eventually, it was determined that the abnormal zone lay anterior to the occipital pole, sharply confined to the posterior temporal region. Thus, the observations reported here were derived from cortex, which appeared to be normal in every way measured, even though it was adjacent to (about 5 centimeters away) an epileptogenic lesion.

After distributing the electrodes above and below the calcarine fissure, as shown in the diagram of Figure 3, the patient was instructed to fixate upon a small red dot in the center of a viewing screen. Maintenance of fixation was monitored by previously applied periorbital electrodes and was, in fact, satisfactory for the duration of this test. Immediately upon fixation there was a generalized alpha blocking or desynchronization lasting several seconds, after which fairly prominent alpha activity recurred (despite continued maintenance of fixation) at all recording sites (Figure 3). Thereupon, various sectors of the visual field were illuminated (Figure 3A′, B′, C′) for 1 to 3 seconds. Figure 3a, b, and c illustrates the pattern of brain wave tracings that correspond, respectively, to each of the sector stimuli. Illumination of the upper 30° of the right visual field (Figure 3A′) resulted in a sharply localized desynchronization seen only in electrodes 4 and 5—i.e., below the calcarine fissure (Figure 3A). A stimulus subtending an arc from 90° to 135° (Figure 3B′) elicited desynchronization limited to electrodes 2 and 3 (Figure 3B) just above the fissure. Stimulation of the lowermost 30° of the field (Figure 3C′) gave rise to desynchronization only at electrode 1 (Figure 3C). Finally, full-screen illumination (Figure 3D′) yielded alpha blocking at all electrode sites (Figure 3D). These differential distributions remained consistent for some 10 to 12 replications for each sector. The particular localizations shown correspond exactly to the known anatomical distribution of fibers in the primary afferent pathway according to the specific retinal elements stimulated for each sector of the visual field. Moreover, lesions of those portions of striate cortex exhibiting the localized desynchronization illustrated in Figure 3 lead to visual-field deficits in sectors closely approximating those that were stimulated in the present study.[34] Thus, these data are in good agreement with both anatomical and behavioral evidence previously obtained by others for the primary visual pathway.

The degree of spatial discreteness exhibited by the localized activation patterns in this patient is comparable only to that reported by Jasper and Penfield,[35] who recorded

focal blocking of the rolandic beta rhythm in human motor cortex when the patient was instructed to move his fingers. Desynchronization or blocking of beta rhythm occurred in the area of representation for hand movement, while beta activity continued undisturbed in the nearby "face" area. These findings indicate a spatial selectivity and specificity in the activity of the thalamic reticular system (at least in man) not only between modalities, but even within the same modality. The anatomical system responsible for generating localized desynchronization must be capable of activation or of distributing its activation in a manner almost as specific and discrete as that of the primary sensory and motor pathways themselves. Jasper's suggestion[16] that the thalamic reticular system is indeed topographically organized (see also Note 36) is emphatically supported, as is his notion that this same neural network in normal physiological usage may be responsible for the capacity for precise focusing of attention on small, selected segments of the sensory field.

The cortical surface electrodes were equally spaced at 1-centimeter intervals. A localized desynchronization might occupy only 1 to 2 centimeters or less of cortical tissue. Because the usual scalp electrode records from not less than 3 centimeters, it is easy to see that such local responses might be "swamped" by the much higher-voltage, on-going, undisturbed alpha rhythm in surrounding tissue and therefore be undetected. In other words, reports of "habituation," i.e., disappearance of the alpha blocking response when based only upon records derived from scalp electrodes, might actually mean nothing more than that the response had become sufficiently localized to escape detection by that particular recording technique.

Alpha provocation

Figure 4B illustrates a stimulus presentation in which one could not clearly identify a desynchronization (compare Figure 4A). Figure 4B clearly demonstrates several morphological features that have been regarded as transitional stages between alpha blocking and alpha provocation.[27] The stimulus onset was not associated with any definite decrease in voltage, but after a latency of 250 to 300 milliseconds, the alpha frequency suddenly increased. This was followed by a spindle-like alpha "burst" (in electrodes 2 and 4), which was abruptly truncated upon cessation of the stimulus. Comparison of the response to stimulation in Figure 4A with that occurring on presentation of exactly the same stimulus (full-field illumination) in Figure 4B serves to emphasize that the activation pattern is a graded response; it is not all-or-none in character.

The details of any particular electrical response are related to some extent to stimulus intensity; a pistol shot, for instance, invariably produces maximal desynchronization. Except for such extremes, however, the form and duration of the activation pattern depends much more crucially on such things as novelty, prior associations with the stimulus, the biological significance of the stimulus, and, most importantly, on the state of vigilance of the subject at the time the stimulus impinges on the nervous system.[3] This latter, as was noted, is closely related to the background or "spontaneous" EEG patterns, drowsiness and sleep being characterized by an absence of alpha rhythm and either a predominance of low-voltage, 4 to 7 cycles per second activity (drowsiness), or higher-voltage waves at 2 to 3 cycles per second mixed with 14 cycles per second "spindles" (slow-wave or spindle stage of sleep). Presentation of a moderate stimulus in any of these states of lowered level of awareness may produce not an activation pattern, but rather a burst of high-voltage alpha rhythm. Interpretation of this phenomenon is still unclear. Some authors[7,37] prefer to think in terms of a continuum of consciousness extending from the excited, aroused (low-voltage fast pattern), through the waking but relaxed state (alpha rhythm) into drowsiness and then sleep (slower and slower rhythms). We ignore for this exposition the stage of REM or paradoxical sleep (Jouvet, this volume). Stimulus-induced alpha provocation would then be interpreted as a sign of arousal from a lower level of consciousness. Other workers[3,30] have suggested that alpha provocation is a sign of active "inhibition," and believe that the same stimulus may on one occasion induce "excitation," denoted by low-voltage, high-frequency activation patterns and, on other occasions, "inhibition," denoted by alpha bursts or alpha augmentation. Signal significance is regarded as at least as important as the subject's state of consciousness and/or the character of ongoing electrical rhythms at the instant of stimulus application.

Finally, if not already obvious from the figures, it must be mentioned that alpha rhythms normally have a periodic waxing and waning appearance (Figure 1, last tracing; Figure 2). Usually, the modulation is even and gradual. However, sometimes it is more extreme, and results in the appearance of spontaneous high-voltage, alpha bursts interspersed with epochs of very much lower amplitude, as illustrated in Figure 4C. If we now returned to our earlier statements concerning synchrony and desynchrony, we would be obliged to predict that the alpha bursts represent synchronous discharge of whatever elements give rise to the EEG, whereas the low-voltage periods in between should represent asynchronous firing of those elements. As mentioned above, microelectrodes were also used in the study of this same patient. The tracings shown in the next section are intracellular records of human nerve cells

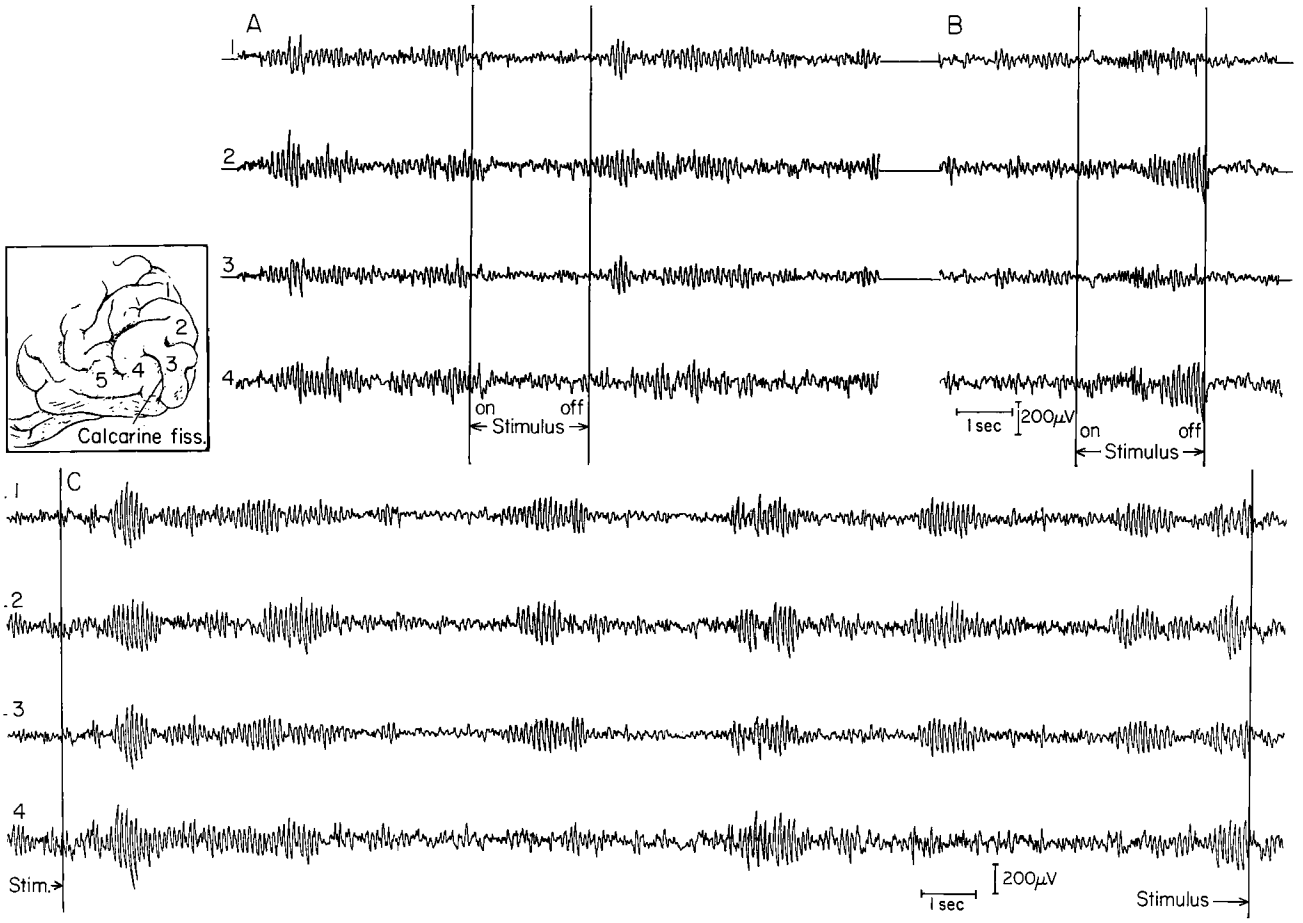

FIGURE 4 Electrocorticograms from the same patient as in Figure 3. Same electrode placement. Stimuli were full field illumination for the durations indicated in A and B. In C, the stimuli were 50 microsecond flashes. Various stages of "activation" are shown. 4A exhibits classical desynchronization. 4B reveals partial activation denoted by increase in alpha frequency and, then, alpha provocation exemplified by spindle-shaped burst of high voltage alpha terminated abruptly when the stimulus ceases. 4C illustrates spontaneous alpha burst pattern which alternates with much lower voltage, mixed frequency, "asynchronous" activity. On this latter background it was difficult to discern any effect of brief visual stimuli.

sampled simultaneously with the EEG of the cortical surface. They allow a direct test of the prediction stated above, and at least the beginnings of a cellular and neuronal interpretation of the brain wave patterns in man.

Sources of the EEG

Gas-sterilized glass capillary micropipettes were filled with 2 M K-citrate and had a tip resistance of 20 to 40 megohms in saline. A pressor-foot consisting of a clear plastic disk drilled with many tiny holes was placed on the cortical surface of the occipital pole and held in place by special clamps to the bony margins of the craniotomy. The pressure was not great enough to cause visible blanching of the cortical surface, although there was undoubtedly slowing of the surface circulation. A sterile micromanipulator (also attached to the bony margin) was used to lower the microelectrodes through the predrilled holes in the plastic pressor-foot. Many active cells were encountered, leading us to doubt that there was significant cortical ischemia. (Throughout this procedure the patient was awake, and continued to report correctly objects placed in his right visual field.) Five extremely stable cells were impaled and held for 5 to 8 minutes. Figure 4C is an excerpt of the electrocorticogram obtained during impalement. The electrocorticogram from the surface electrode nearest the penetration (electrode 2) and the amplified microelectrode recording were fed to an Ampex FR-1300 tape recorder

for later playback and analysis, and were also monitored on an oscilloscope. These cells exhibited stable resting potentials of 55 to 78 millivolts and normal spontaneous action potentials, excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). Among the many observations made, the ones pertinent to this report are the following:

1) There was very little correlation between the activity (either spikes or synaptic potentials) of single cells and surface EEG waves during periods of desynchronization or during the low-voltage activity between alpha bursts. Cross-correlation computations yielded values of 0.2 to 0.1.

2) During alpha-burst activity in the EEG, cross correlations were above 0.5 in all five cells (two were 0.5, one at 0.61, one at 0.69, and one at 0.78). However, there were tantalizing and inconstant phase shifts, with the cellular potential leading in some cases and the EEG in others. It

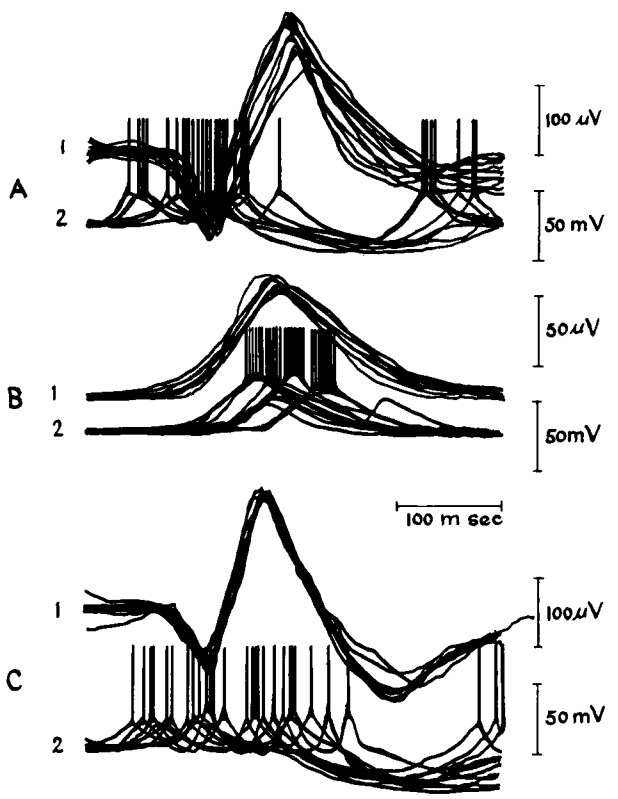

FIGURE 5 Superimposed line drawings of coherent wave-shapes (coherence judged by visual inspection) extracted from the surface record during spontaneous alpha bursts (channel 1), together with intracellular unit record (channel 2) corresponding to the EEG segment selected. Ten to twenty superpositions in each case. A, B, and C are three different cells. Further explanation in text. Negativity is up for channel 1 (surface record) and down for channel 2 (intracellular record) in this and in Figure 6.

was suspected, of course, that the reason for the observed jitter was the attempt to compare a unitary event with that of a mixed population; therefore, the alpha bursts in the EEG were scanned for single waves or coherent sequences.

3) In an analysis of single waves, three kinds of coherent waves were found: a diphasic positive–negative complex (Figure 5A); a monophasic negative wave (Figure 5B); and a triphasic positive–negative–positive sequence (Figure 5C). All waves of each type were then superimposed by line drawing so that closeness of fit might be estimated visually. For each trace, the corresponding intracellular record is shown on line 2. Parts A, B, and C of Figure 5 are each from different cells. When displayed in this manner it became clear that there was a regular and coherent relationship between the potential fluctuations of a single cell and appropriately selected waves of the EEG. For the sequence in Figure 5A, the initial positivity was, on all occasions, associated with EPSPs and cell discharge, while the larger negative component was related to a prolonged IPSP. The monophasic negative wave (Figure 5B) correlated with purely excitatory events in the cell, i.e., EPSPs and spikes near the peak negativity. However, there was a curious lack of membrane fluctuation, despite the rather large sample size (about 15 superpositions), in the period just prior to the onset of the negative surface potential. It was as though the membrane had been briefly "clamped" from without and then released as it was invaded by a depolarizing EPSP. This is probably the same event as was described by Creutzfeldt,[38,39] which he termed "synaptic silence." The third sequence (Figure 5C) was characterized by a sharper and narrower peak negativity with a steep descending limb culminating in a third positive deflection. Excitatory, depolarizing activity prevailed throughout the initial positive and negative phases, while the late positivity corresponded to an abrupt intracellular IPSP.

The monophasic negative waves (Figure 5B) correspond to the type II waves described by Spencer and Brookhart[40a,40b] and the Type B waves of Calvet, et al.[41] They are the result of postsynaptic depolarizations mainly on the apical dendrites of pyramidal cells. Biphasic positive-negative waves (Figure 5A) are the result of postsynaptic depolarization near the pyramidal cell soma, probably by the terminals of specific afferent fibers, as seen from an electrode on the "passive" end of the neuron in an electrotonic circuit, followed immediately by a more prolonged IPSP. A more prolonged depolarization, which outlasted the soma-dendrite delay in the longitudinal electrotonic spread of activity, would first be seen as a "reversed" potential by an electrode on the dendritic pole and then as a surface negativity (Figure 5C). The subsequent IPSP (Figure 5C) would then be seen as a surface positivity, as long

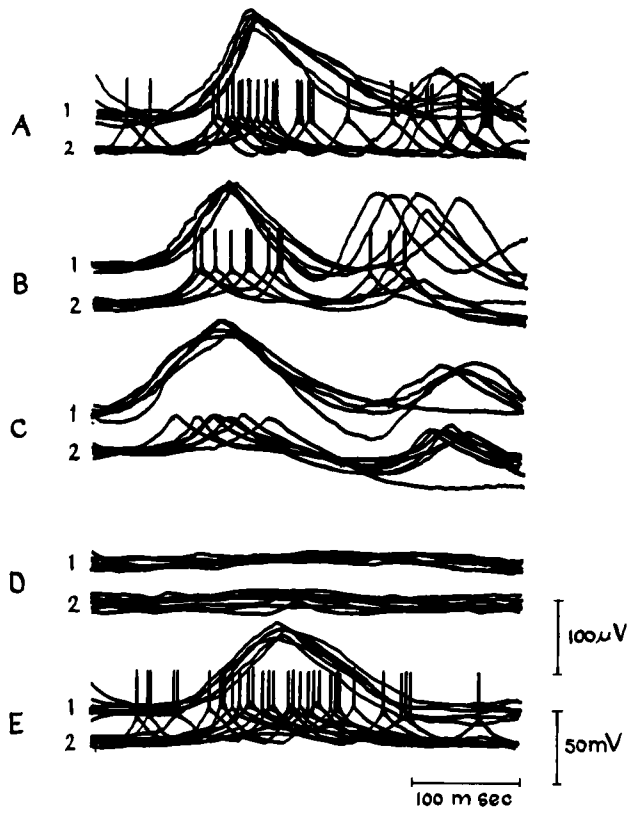

FIGURE 6 Superimposed line drawings of coherent wave-shapes in the EEG of another patient (channel 1) and the corresponding intracellular unit records from a microelectrode (channel 2). In this case, A, B, C, D, E are all from the same cell (membrane potential 69 mv; action potential 73 mv) continuously held for 20 min during intravenous administration of methohexital. A illustrates the relationship between intracellular activity and EEG wave-shape before administering drug. B, after approximately 200 mgm; C, after 400 mgm; D, after 500 mgm. Infusion of drug was then stopped and the tracing labeled E was obtained 5 min later. Unit spikes in this and the preceding figure have been retouched and truncated where necessary for clarity of photographic reproduction. Further explanation in text.

as the polarization of the apical dendrites was less pronounced than that of the soma. The fields for both of these latter waves (Figures 5A and C) correspond with the Type 1 of Spencer and Brookhart[40a,40b] and type A of Calvet et al.[41]

Quite apart from a complete interpretation of the manner in which these complex interrelationships sum and finally emerge as the surface-recorded EEG, there can be little doubt that the synaptic potentials of nerve cells are the elements that give rise to brain rhythms. The firmness of this conclusion stands in striking contrast to the uncertainty of early workers[42-45] who were limited to extracellular records of cortical neurons. That the slow waves were not simply the envelope of all-or-none CNS single-unit action potentials was clearly demonstrated by Li and Jasper,[42] who recorded EEG and single units extracellularly and found that with asphyxia and with deep barbiturate anesthesia, all units stopped firing at a time when it was still possible to record spontaneous EEG waves as well as slow potentials evoked by thalamic shock.

We have had the opportunity to obtain intracellular records from nerve cells in the temporal cortex in one additional patient who was undergoing deep barbiturate narcosis with a new, ultra short-acting barbiturate as part of the diagnostic evaluation of his temporal-lobe seizures.[46] The procedure was undertaken after implantation of intracranial electrodes through burr holes in the skull and, on this occasion, the microelectrode was inserted through the small burr hole. Intracellular recording was obtained in one cell maintained in "healthy" condition (stable membrane potential of 65 millivolts) for 20 minutes. During this time, simultaneous EEG and intracellular recordings were made (Figure 6) before the intravenous introduction of methohexital (Figure 6A), during induction that was carried to the point of EEG silence (Figures 6B, C, D), and after a substantial amount of recovery (Figure 6E). The entire induction and recovery occupied less than 15 minutes. We observed, as others have,[47] that action potentials disappeared at levels where EEG waves could still be detected (Figure 6C). However, the EEG waves were invariably associated with slow synaptic potentials in the cell soma. When the EEG became isoelectric (Figure 6D), synaptic potentials failed also. Infusion of the drug was immediately stopped and within 20 seconds there was recurrence of EEG waves; within 5 minutes (Figure 6E) cellular discharge reappeared.

From this and from observations in many other laboratories on experimental animals (see Purpura, this volume)[48-52] a consensus is emerging that regards the slow oscillations of the EEG as the algebraic sum of hundreds of thousands of synaptic potentials in the many thousands of neurons which lie within the "field" of a gross-recording electrode.

The detailed electrophysiology of cortical neurons under study in many laboratories has already gone far to substantiate the original model of Eccles[53] based upon the spinal motor neuron. Given what is now known of the biophysical properties of cortical pyramidal cells and the differential localization on the membrane surface of inhibitory and excitatory synapses from specific, unspecific, and intracortical terminals, as well as the careful analysis of intracortical potential fields by Spencer and Brookhart[40a,40b] and Calvet, et al.,[41] it is now possible to explain in detail the mechanisms that generate most of the wave

shapes contributing to the EEG. The "pacing" of mean cortical neuronal firing and of EEG frequencies depends mainly on pacemaker cells in thalamic nuclei,[52] although it is also clear that recurrent inhibition also exists in cortex[54,48] and may contribute to the roughly 100-millisecond cycle of excitability. The model proposed for the thalamus by Andersen and Eccles,[52] in which the firing of each afferent axon simultaneously excites via recurrent collaterals a wide-spread, IPSP-mediated, "surround" inhibition lasting approximately 100 milliseconds, fits the available data extremely well. It is wise to be cautious, however, remembering that investigators thus far have succeeded in impaling only that 1 per cent of cortical neurons with the largest cell soma. Nevertheless, it is particularly exciting to see that observations on the human nervous system are in such good accord with that being obtained from animal experimentation.

There is now sufficient evidence to indicate that the terms "synchrony" and "desynchrony" are no longer merely plausible assumptions of the electroencephalographer. The assumptions have been confirmed by direct observation of the behavior of nerve cells and the intercorrelation of their activities with the concurrent EEG.

EEG and sensory coding

Two important conclusions relevant to the problem of sensory coding may be adduced from the evidence thus far. The first is that the most reliable sign of active neuronal processing of sensory information is differentiation and diversification of cellular firing patterns, as expressed by desynchronization of the EEG. Diversity, in fact, is the hallmark of cortical nerve cells. Even under most extreme drives—such as epileptic discharge, which itself imposes brain wave synchrony (Figure 7, channel 1)—two simultaneously monitored cells (Figure 7, channels 2 and 3), less than 100 microns apart may show oppositely directed behavior.[43]

The second major conclusion takes cognizance of the ubiquity of brain waves, the narrow range of frequencies they occupy throughout all species, and now the mechanism of recurrent inhibition[52] responsible for their timing. Collectively, these facts argue for the operation of genetically determined mechanisms and render it extremely unlikely that specific sensory coding has anything to do with the frequency of brain waves. Changes in EEG frequency relate more to the balance between cellular synchrony and desynchrony than to the specific information content of a signal. If recorded with adequate resolution, they may indicate *where the action is,* but not *what* the action is all about. These comments apply only to the gross frequency of the spontaneous EEG. We have shown in the previous section that selected wave shapes are, in fact, highly correlated with the graded synaptic activity in particular cells. Creutzfeldt[38] and Landau (this volume) point out that such high correlations are also found for evoked potentials. It is quite possible, therefore that stimulus-bound or time-locked wave shapes extracted in some way (averaging, autocorrelation, etc.) from the ongoing EEG may be related to information coding. (More is said on this subject by E. R. John, this volume.)

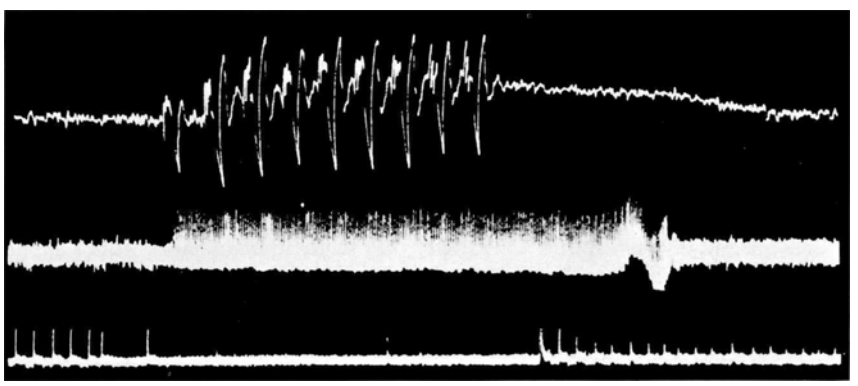

FIGURE 7 Two nerve cells (channels 2 and 3) recorded extracellularly (tip separation of the two microelectrodes $> 100\,\mu$) in cerebral cortex of the cat during the occurrence of paroxysmal epileptiform discharge and slow potential shift (channel 1). One cell (channel 2) was clearly excited while the immediately adjacent one (channel 3) showed inhibition of spontaneous discharge. (From Morrell, Note 43)

Sensory coding in single neurons

The mammalian visual system is especially well suited for an analysis of sensory coding. The elementary details of stage-by-stage connectivity have been intensively studied.[55-66] An extraordinary degree of order and specificity has been found not only at lower levels but also extending into the cortical regions and even beyond the primary receiving area.[67] Single cells have been shown to be extremely selective in their stimulus preferences, and the required stimuli are generally quite complex.[66,67] Multisensory interactions at single units have been shown by Jung and coworkers[68] and by Murata, et al.,[69] to be a very common and pervasive feature of visual physiology.

Our investigation was designed to provide information on the following three questions:

1) Do cells that respond to specific and complex stimuli exhibit equally specific response patterns, which constitute a neural signature for that stimulus?

2) In cells that respond to more than one stimulus configuration or more than one sensory modality, are there detectable differences in response pattern for each stimulus?

3) Are response patterns completely fixed or can they be modified as a result of experience?

The experimental animal was the curarized, unanesthetized cat. Prior to the experiment, the animals were fitted with an implanted nylon receptacle that could be opened, when necessary, for insertion of the microelectrode, and with a cap of dental cement attached to the skull and specially molded to receive the ear bars of the stereotaxic instrument. The receptacle was 5 to 6 millimeters in diameter and extended from the interaural line (Horsley–Clarke 0) anteriorly over the lateral gyrus. Thus, all penetrations were made in the zone designated as visual area III by Hubel and Wiesel.[67] This area is considered analogous to Brodmann area 19 or parastriate cortex in man. During experimental sessions, an endotracheal tube was inserted for artificial respiration and the animal was immobilized with Flaxedil. The head was securely fixed in the stereotaxic apparatus by means of the cement cap, the body was kept warm and supported by elastic bands, and all injections were made through an indwelling femoral cannula. Thus, there were no pressure points, and great care was taken to assure that the procedure involved no stress for the animal. A one-diopter contact lens was fitted to each eye to assure a fixed focus and to protect the cornea from drying. Pupils were dilated with atropine.

Single-unit records were obtained with tungsten microelectrodes.[70] After suitable amplification, the records were monitored on an oscilloscope and fed to a tape recorder for later playback and analysis.

All visual stimuli were projected on a viewing screen located 30 centimeters in front of the eyes. Stimuli could be delivered to each eye separately. Stimulus duration was always 50 milliseconds. Acoustic stimuli were 10-millisecond clicks repeated for 50 milliseconds; tactile stimuli consisted of weak electrical shocks to the contralateral hind limb. All stimuli were delivered on a random schedule with a mean intertrial interval of 22.5 seconds. A prepulse was put on tape 50 milliseconds before each stimulation.

Data analysis was performed on a LINC computer. It was programmed to compute and display summed post-stimulus-time (PST) histograms (binwidth manually selectable) out to 250 milliseconds after stimulus onset or 300 milliseconds after the prepulse.

These experiments were carried out over a five-year period. During that time there were changes in experimental technique, recording apparatus, and even in strategy. Furthermore, each cell had a unique preferential or adequate stimulus (or stimuli), as well as a unique response pattern. Such particular preferences were sought out by the experimenters and were determining factors in the design of each experiment. Finally, variations in our ability to hold on to the unit and to maintain it in "normal" condition determined the duration of each experiment and the number of replications and controls that were possible in each case. Taken together, all these factors resulted in a lack of strict standardization of procedure from one experiment to the next. Nevertheless, as will be shown, every effort was made to provide internal controls with which experientially determined changes in response pattern might be compared.

RESPONSE CHARACTERISTICS Receptive fields were carefully plotted for each cell encountered and the "preferred" stimulus configuration was determined. The latter were usually complex, consisting of edges, bars, or lines of various lengths and orientations. When the optimal stimulus was used, the mean cellular response was usually quite stable (as measured by PST histograms of sums of 20 trials), even though there was considerable trial-to-trial variability and scatter of latencies and sometimes omission of some components. Figure 8 illustrates the first 12 trials out of the total of 20 used to compute the histogram shown in the lower part of the figure. Two bursts of activity are evident when the tracings are displayed in this way and, of course, two peaks in the histogram. By examination of single tracings, the early burst would not have been detected in trials 3, 5, 8, and perhaps 7.

A more complex response is shown in Figure 9, where trial-to-trial differences in latency are prominent. An inhibitory interval may be recognized in the histogram;

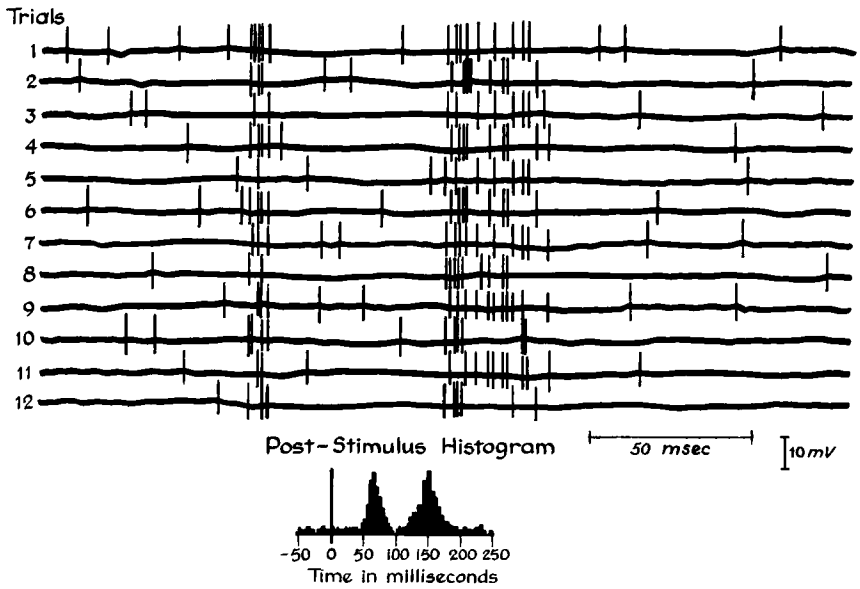

FIGURE 8 Single unit extracellular records of the first successive 12 trials of the 20 trials that were summed to form the PST histogram illustrated in the lower half of this figure. Each sweep was triggered by onset of stimulus. Preferred stimulus for this cell was a dark bar on a light background oriented on a line from 5:00 to 11:00, "stopped" on the left, but extending out of the receptive field on the right. It was 4 mm wide. Duration was 50 msec. Tracings were displayed on a storage oscilloscope by stepping down the vertical beam for each trial and were then photographed. The PST histogram displayed below was triggered by the prepulse which had been placed on the tape recording 50 msec before stimulus onset. It, therefore, illustrates the prestimulus level of activity (–50 msec to 0), as well as the cellular response to stimulation. When displayed simultaneously in this manner, it is evident that there are two distinct bursts of activity (perhaps corresponding to onset and cessation of stimulation) which are reflected in the two peaks of the histogram. However, if the single traces had been examined separately, the early component might have been missed on trials 3, 5, 8, and possibly 7. Binwidth equals 5 msec in this and all other histograms displayed in this paper. The calibration bar at time zero in this and all other histograms equals 20 spikes.

after the first burst, the activity drops below the baseline of "spontaneous" activity (−50 milliseconds).

A total of 890 cells of visual area III responded to "preferred" stimuli in the manner shown. A change of stimulus configuration—from only contralateral eye to both eyes simultaneously, or a change in the orientation of a line—resulted in a different response pattern. Eight hundred seventy-one of these cells also responded to tactile and/or acoustic stimuli with response patterns different from those elicited by visual stimuli. The "preferred" visual stimulus was then combined with other visual or other sensory stimuli, yielding histograms having an extremely complex form rarely attributable to a simple linear summation of the firing pattern for each separate stimulus. Following this procedure (usually 2 blocks of 20 trials each), the original visual stimulus (test stimulus) was again presented alone. In the great majority of cells, response patterns were identical with those elicited prior to the paired-trial experience. However, in 102 well-studied cells, the subsequent test stimulus evoked stable response patterns with a marked resemblance to those elaborated by the combined stimuli.

RESPONSE MODIFICATION IN "POLYMODAL" CELLS

Figure 10 demonstrates the histograms along with single-trace examples of the response to the visual stimulus (Figure 10, L), to a shock to the contralateral hind limb (Figure 10, S), to the two stimuli combined (Figure 10, L +

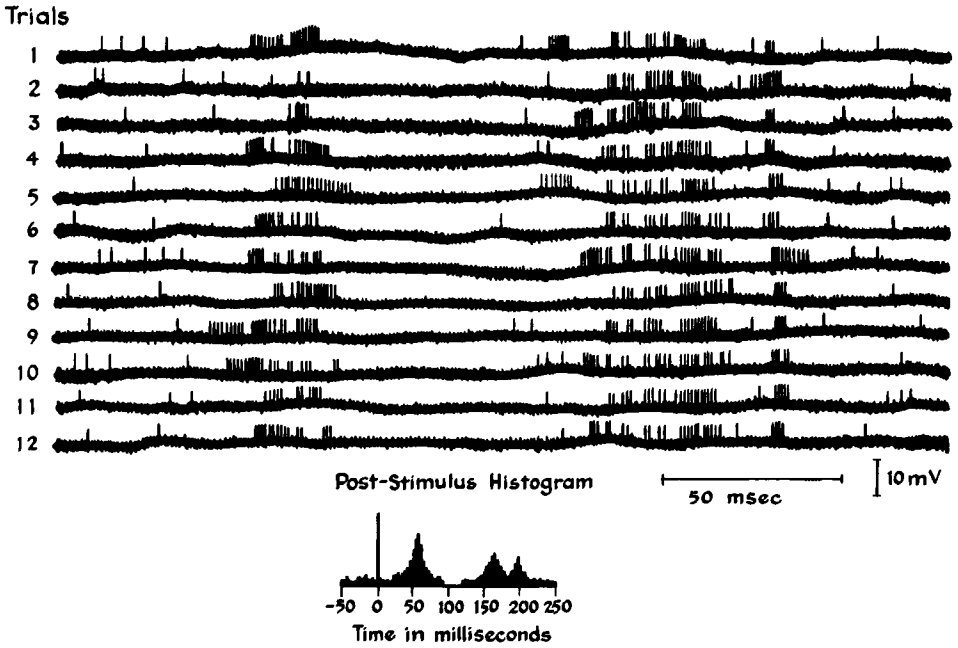

FIGURE 9 Single unit extracellular records of the first successive 12 trials of the 20 trials which were summed to form the PST histogram illustrated in the lower half of this figure. Three peaks are recognizable in the single tracings (except trial 2), although the "latency" varies from trial to trial. The "scatter" of these "latencies" may be visualized as the width or negatively accelerated slope of the histogram peaks. Note also how the histogram displays an inhibitory interval between the first and second peaks. The stimulus in this case was a dark corner in the right upper quadrant of the visual field.

S), and to the visual stimulus alone after the paired trials (Figure 10, L, lower). An appreciation of the true time course of this effect may be gained by viewing histograms of each successive sum of 20 trials for the entire stimulated output of a nerve cell throughout the period of its observation. Cell 63-294 delivered a moderately high-frequency, rather prolonged burst to a light line at about 2:00 in its receptive field (Figure 11, trials 1 to 20). A second sum of 20 trials (trials 21 to 40) gives some indication of the stability of the histogram. The cell also responded to acoustic stimulation with a brief burst peaking somewhat earlier than the peak of the light-evoked discharge (trials 41 to 80). Stimulation with the light line was then resumed; the resultant histogram (Figure 11, trials 81 to 100) showed that neither the interposition of acoustic stimulation alone nor of time itself had modified the response to light. Next, the light and click stimuli were presented simultaneously for two blocks of 20 trials each (trials 101 to 140), yielding PST histograms of very different composition from those for either stimulus individually. A reorganization or a completely new organization of response pattern emerged. Note that it took time; there are differences between the first (trials 101 to 120) and second (trials 121 to 140) sums of 20 trials of paired stimulation. Furthermore, following this "experience," the light alone elicited a complex patterned discharge very similar to that elaborated by the combined stimuli (Figure 11, trials 141 to 200). The reorganization thus established did not persist indefinitely but began to decay during trials 201 to 220. An attempt to re-establish it by interposing a series of click stimulation alone (trials 221 to 240) was unsuccessful, the subsequent testing with light (trials 241 to 260) yielding a histogram indistinguishable from that of the original response to light (trials 1 to 20). Note also that the response pattern to click (trials 221 to 240) was unchanged from that in the control period (trials 41 to 60). However, a series of 20 trials of combined light *and* click stimulation (trials 261 to 280) did have the effect of restoring the modified pattern, which then was persistently elicited by light alone (trials 281 to 320) for as long as the cell could be held.

RESPONSE MODIFICATION WHEN NONPREFERRED STIMULUS IS INEFFECTIVE Figure 12 illustrates sequential

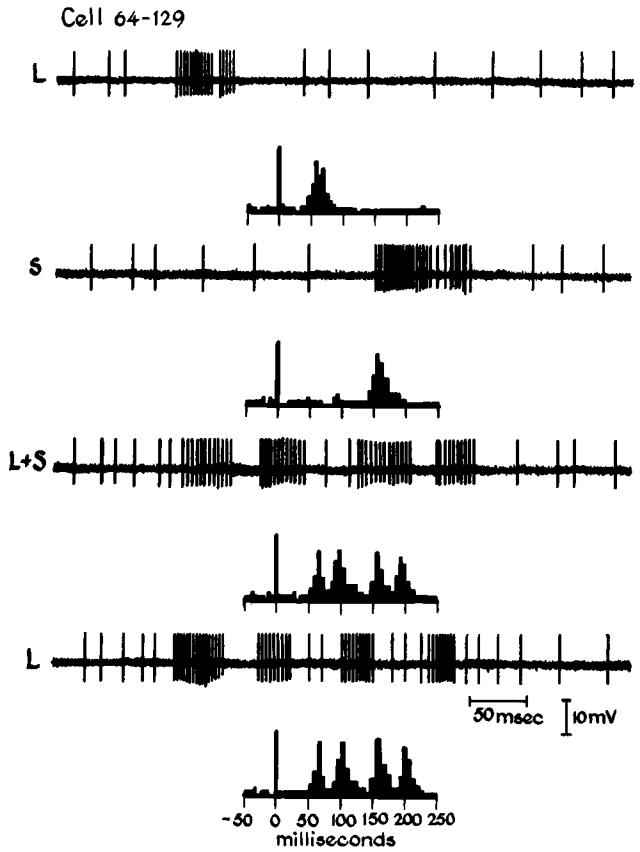

FIGURE 10 Experientially-induced modification of response pattern. Cell responded to a dark, horizontal bar at 3:00 (L) and also to electric shock to the contralateral hind limb(S). Combining these two stimuli (L + S) resulted in a histogram very different from that which might occur from simple linear addition of the two separate responses. Furthermore, after 40 trials of such paired stimuli, the original visual stimulus (L) was presented alone. It elicited a pattern much more like that elaborated by paired stimulation than like that which it produced prior to pairing.

The histograms are, again, sums of 20 trials. The single traces are those most representative of the over-all pattern in each group of 20.

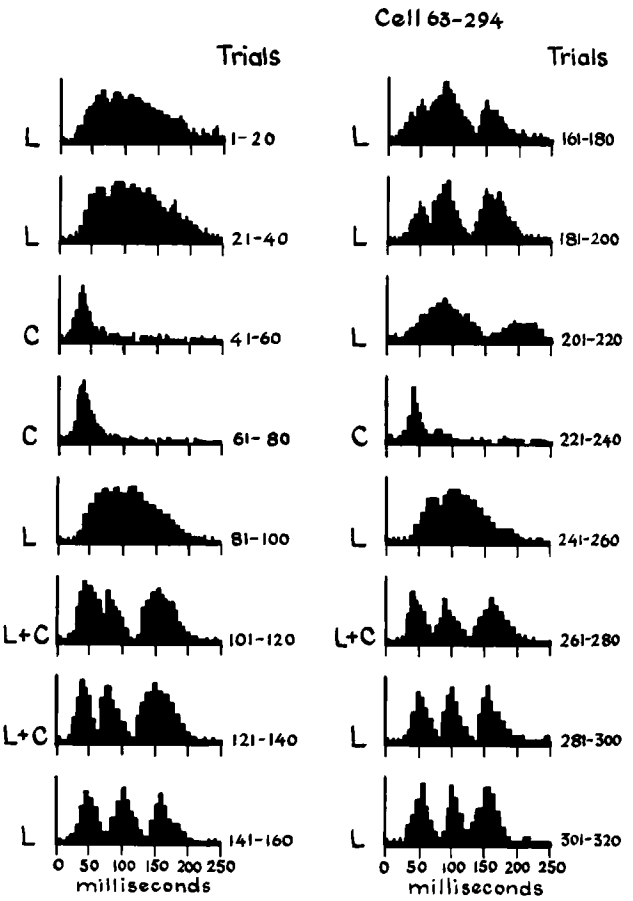

FIGURE 11 Response modification in a polymodal cell. "Preferred" visual stimulus (L) for this cell was a light line at 2:00 in its receptive field. Click stimulus (C), 30 db above human auditory threshold in open field conditions, was also effective, although with a different pattern. This figure illustrates the PST histograms obtained throughout the entire course of observation of this cell. L + C indicates "preferred" visual and acoustic stimuli combined. Kind of stimulus is indicated on the left and trial numbers to the right of each histogram in this and all subsequent figures. Further explanation in text.

PST histograms in a cell that responded only to illumination of its receptive field in the contralateral eye (Figure 12, trials 1 to 20) with a brief burst followed by an inhibitory interval. Ipsilateral eye stimulation (trials 21 to 60) produced no alteration in spontaneous firing rate or pattern. Yet, when the two eyes were stimulated simultaneously (trials 61 to 100), a reorganization of firing pattern occurred, consisting mainly of the appearance of two late peaks and perhaps some increased scatter of the early component, such that it encroached upon, and thereby shortened, the inhibitory interval. Now, however, when the "preferred" stimulus to the contralateral eye was reintroduced, the response pattern retained the two additional components contributed by or elaborated during paired stimulation (Figure 12, trials 101 to 140). The new pattern began to decay during the third postpairing block (trials 141 to 160) and was gone by the fourth block (trials 161 to 180). Stimulation of the right eye alone was still ineffective (trials 181 to 200), and did not result in restoration of the modified response to the left- or contralateral-eye stimulus (trials 201 to 220). Stimulation of both eyes simultaneously again generated a complex histogram con-

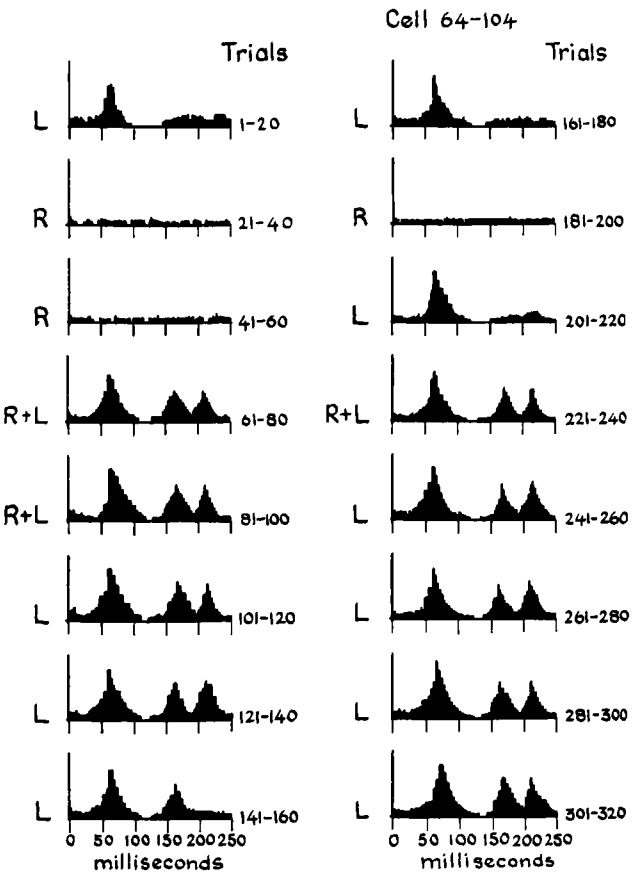

FIGURE 12 Response modification in the case where the nonpreferred stimulus was ineffective. This cell responded to a light line 2 mm wide and 3 cm long only when the stimulus was presented to the left eye (L); stimulation of the corresponding receptive field area with the same stimulus to the right (ipsilateral) eye caused no alteration of spontaneous firing pattern (R, trials 21–60). However, simultaneous binocular stimulation produced a response pattern (trials 61–100) different from that to left eye alone (trials 1–20). PST histograms of successive sums of 20 trials are shown. Further explanation in text.

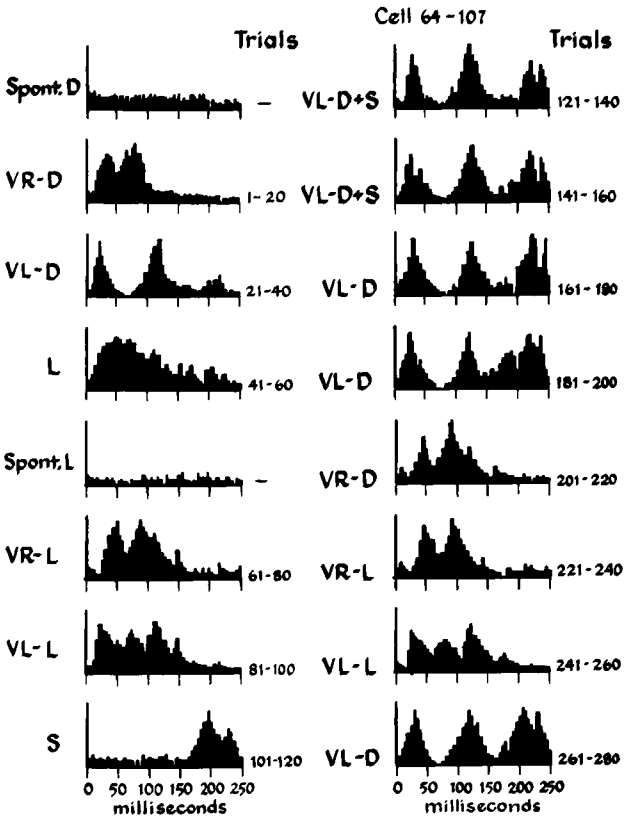

FIGURE 13 Further differential specificity. This cell had an extraordinarily rich response repertoire. It gave different response histograms to each of the following stimuli: a vertical bar 3.6 cm in length moving from left to right in a dark room (VR-D), the same stimulus moving in the opposite direction (VL-D), same stimulus moving from left to right with the room lights on (VR-L), and the same stimulus moving from right to left with the room lights on (VL-L). The cell also responded simply to diffuse illumination of the room (L) and to an electric shock to the contralateral hind limb (S). One of these stimulus configurations (VL-D) was paired with shock (S). The others served as controls for specificity. Histograms labeled "Spont. D" and "Spont. L" represent sums of randomly chosen, 250 msec, segments of record when the cell was unstimulated either in the dark room (D), or with the room lighted (L). See text for further explanation.

taining the two late components (Figure 12, trials 221 to 240). Following this "reinforcement," stimulation of the contralateral eye alone reproduced the modified pattern for the unusually long period of about 40 minutes comprising four successive blocks of 20 trials each (trials 241 to 320) before the cell was lost.

The cells in Figures 11 and 12 both illustrate a differential specificity for the combined stimulus configuration. Stimulation by the nonpreferred member of the pair, whether it itself elicited a response, as in the case of the click (Figure 11), or did not, as in the case of ipsilateral eye stimulation (Figure 12), was not effective either in producing the response pattern caused by paired stimulation or of restoring the capacity of the "preferred" stimulus to elicit the modified response.

FURTHER DIFFERENTIAL SPECIFICITY Another cell affords a striking example of differential specificity. PST histograms of cell 64-107 are shown in Figure 13. This cell had a response repertoire that was more varied than most.

It responded best to a vertical bar, 3.6 centimeters long, moving from left to right or right to left across its receptive field. It also responded transiently to diffuse illumination of the room. But the most important characteristic of the cell was that response patterns to the vertical bar moving to the right and to the left, respectively, were different, depending on whether the testing was done in the dark or in a dimly lighted room. Thus, one could distinguish four stimulus configurations to which the cell was differentially responsive: (1) visual stimulus moving to the right in the dark (VR-D); (2) visual stimulus moving to the left in the dark (VL-D); (3) visual stimulus moving to the right in the light (VR-L); and (4) visual stimulus moving to the left in the light (VL-L). The corresponding histograms are illustrated in Figure 13, VR-D, trials 1 to 20; VL-D, trials 21 to 40; VR-L, trials 61 to 80; VL-L, trials 81 to 100. The cell also responded to an electric shock delivered to the contralateral hind limb (Figure 13, S, trials 101 to 120) with a long latency. One of these four configurations, VL-D, was paired with the shock for two blocks of 20 trials each (trials 121 to 160). Following this "experience," the VL-D-elicited response histogram was modified by the addition of a late component, which resembled that contributed by shock stimulation (Figure 13, trials 161 to 200). Testing of each of the other stimuli was then carried out. VR-D, trials 201 to 220, may be compared with its control, trials 1 to 20; VR-L, trials 221 to 240, may be compared with VR-L, trials 61 to 80; VL-L, trials 241 to 260, with VL-L, trials 81 to 100. Finally, it was possible to restimulate with VL-D, trials 261 to 280, and note that the histogram modification was still present as compared with the control VL-D, trials 21 to 40. In this instance, the modification persisted for 60 minutes after pairing, of which 30 minutes were devoted to testing other stimuli and therefore not to stimulating with VL-D. It seems possible that the shorter duration of the effect seen in most cells (*circa* 20 to 30 minutes) may be a consequence of continuous testing, which, since it is "unreinforced," may result in a process analogous to extinction.

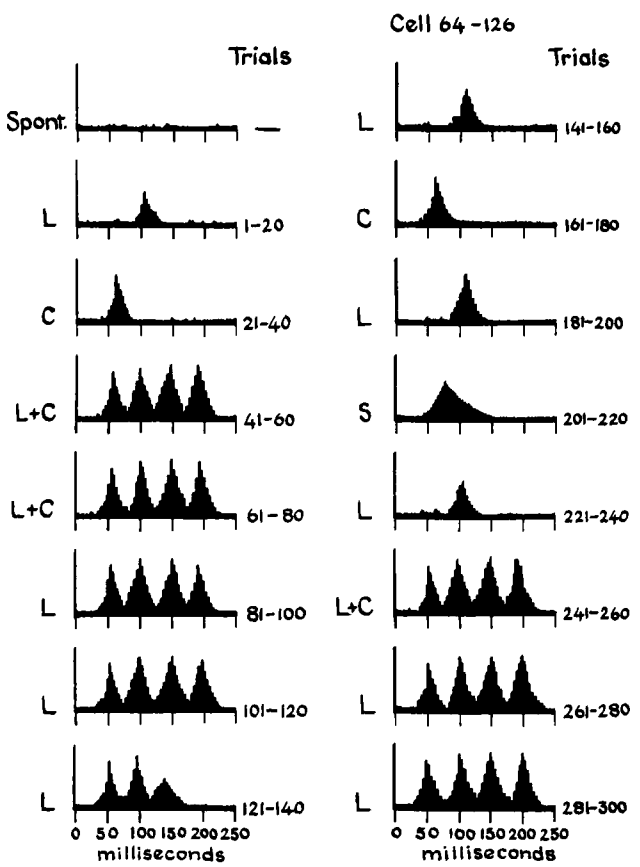

FIGURE 14 Cell was responsive to visual (L), acoustic (C), and tactile (S) stimulation. Illustrates PST histograms over the duration of experiment. Electric shock was used late in the experiment as a "novel" stimulus and did not result either in "dishabituation" or response restoration. Note that each of the three modalities of stimulation produced a different histogram. See text.

SOME CONTROLS Cell 64-126 (Figure 14) responded to acoustic (Figure 14 C, trials 21 to 40), as well as to visual stimulation (Figure 14 L, trials 1 to 20). Combining the two stimuli resulted in a complex, rather rhythmic histogram, which was certainly more than a linear transformation or addition of the two independent responses (Figure 14, trials 41 to 80). Upon testing with the "preferred" stimulus alone, the modified or new response persisted for some time (Figure 14, trials 81 to 120) and then began to decay (Figure 14, trials 121 to 140 and 141 to 160). After it had decayed, it was not restored by exposure to the nonpreferred stimulus (Figure 14, trials 161 to 180 and 181 to 200), nor by exposure to an electric shock (Figure 14, trials 201 to 220) introduced here as a novel stimulus, even though the shock succeeded in evoking a response from the cell. Again, only the specific pairing of the two stimuli restored the effect (Figure 14, trials 241 to 260), which then persisted as long as the cell could be held.

In contrast, cell 65-203 fell into what we now designate as a "sensitization" group. It responded best to a light line directed toward 7:00 o'clock ("preferred" stimulus), but also to an electric shock to the contralateral hind limb (Figure 15 S, trials 21 to 60). Combining the two stimuli yielded response histograms (Figure 15, trials 61 to 100), which indeed seemed nothing more than a linear addition of the two independent responses. Nevertheless, the new response persisted when stimulation was resumed with

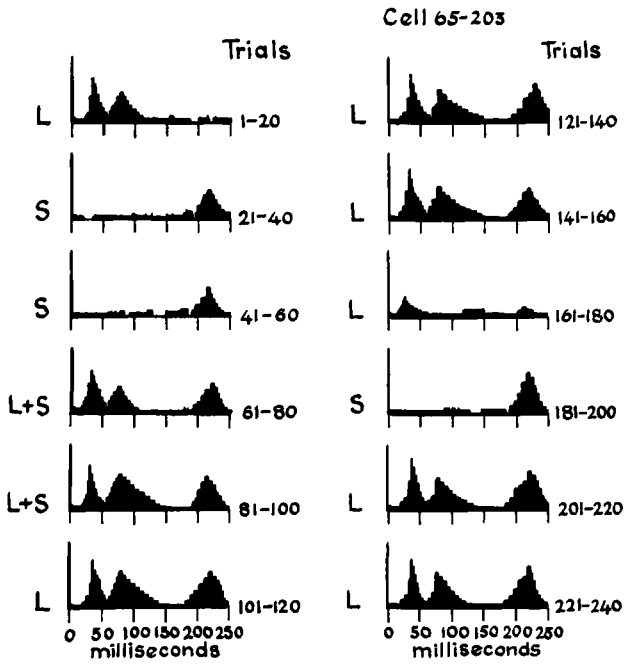

FIGURE 15 "Sensitization" cell. This cell was responsive to a light line directed toward 7:00 o'clock (L) but also to electric shock (S). The experiment differs from previous examples in that following decay of the modified response pattern (trials 101 through 180), presentation of shock alone, *without pairing,* resulted in restoration of the modified response histogram. See text.

the "preferred" stimulus alone (Figure 15, trials 101 to 160). The usual response decrement occurred to a level even lower than that of the control or original response. However, upon presentation of the shock alone, *not paired stimulation,* and even though the shock alone did not elicit the response pattern of the paired stimuli (Figure 15, trials 181 to 200), there was restoration of the modified response pattern when tested by light stimulation alone (Figure 15, trials 201 to 240).

DISCUSSION The 102 cells that generated records of the type illustrated herein stand in striking contrast to the great majority of 769 cells wherein multimodal responses were also demonstrated. In this latter group, the response pattern elicited by pairing did not show any persistence whatsoever when tested with either preferred or nonpreferred stimuli. Their response patterns were surprisingly constant, and did not even show habituation or any appreciable variation over long time periods of several hours and many hundreds of stimulations, at least as measured by PST histograms of sums of 20 trials.

The complexity and specificity of the stimulus required to excite all of these cells, their receptive field patterns, ocular-dominance distribution, and columnar organization emphatically confirm the observations of Hubel and Wiesel.[67] The latter workers did not report a testing of multisensory convergence, but our observations on that score are in accord with those of Murata et al.[69] and Jung.[68] In fact, Murata et al.[69] even examined their results by means of PST histograms, although apparently they did not look for evidence of modifiability.

The general stability of cellular responses in visual cortex makes those cells exhibiting response modification stand out clearly. In fact, there was never any ambiguity over which cells would and which would not exhibit this property. The phenomenon was either clearly present or the cell manifested no sign of modification, even after hundreds of trials.

The distribution of modifiable cells was not random; it was quite specific. The cells were almost always encountered in groups, and such groups were invariably found in columnar penetrations of the microelectrode.

Given the data already on hand concerning multisensory convergence (see also Horn[71]) it is not really surprising that stimulation from other modalities might modify the response to "preferred" visual stimuli, although at best this might be expected in only a minority of cells, or there would not be as much constancy to our visual world as common observation insists there is.

Yet we have no way of judging whether the proportion we have found (roughly 10 per cent) represents a realistic estimate of that portion of the population having "plastic" properties. There are, indeed, several reasons to consider that this estimate may be erroneously low. Thus, the recognition of pattern required the summed PST histogram method of data analysis. This, in turn, required that the same cell be held for several hours to provide all the necessary controls and comparisons. Needless to say, we may not have hit upon the appropriate or adequate stimulus in many cells. Moreover, if we lost a cell before adequate controls had been established, it was placed in the unmodified category. Hence, only cells in which adequate controls had been established were included in the modifiable group.

However tempting it may be, we do not yet believe it is wise or useful to label this phenomenon with the term "conditioning," even though many of the criteria for conditioning are met and many of the subsidiary terms used in behavioral conditioning, e.g., extinction, etc., may be appropriate. We understand the phenomenon described herein as a transient modification of response pattern that is specific to a particular past experience. The duration is limited, but is of the same order of magnitude as the "con-

solidation" time,[72] or short-term memory, and the heterosynaptic facilitation found by Kandel and Tauc in *Aplysia*.[73] Behavioral conditioning requires the laying down of a permanent memory trace, and we particularly do not have evidence that the microstructure of a cellular firing pattern is the physical substrate of enduring memory. It is quite unlikely that recognition or recall requires the cellular re-creation of these temporal patterns of discharge. Recognition and recollection do not require 250 milliseconds of time, as anyone who has worked with tachistoscopic presentation of signals can testify, and as Lindsley[74] has shown in an elegant physiological experiment.

These experiments have, however, provided answers to the questions set forth at the beginning of this section. There are clearly substantive differences in the response pattern of each single cell when it is activated by different stimuli. Thus, a cell may be responsive, for instance, both to a visual and to an acoustic stimulus, but this does *not* mean that modality information is lost. These cells must have their connectivity arranged in highly specific and organized ways, so that fibers carrying acoustic information to two different cells in visual cortex may modify the output of the cells differentially. Similarly, Figure 14 illustrates that a single cell, even though capable of activation by visual, acoustic, and tactile stimuli, maintains stimulus-specific discharge patterns. These findings allow strong inference that the microstructure of the response as exhibited in PST histograms does, in fact, constitute the neural code for that particular experience. On the other hand, it is also true that the histograms exhibit varying degrees of "scatter" about the mode. On any single trial the response of a particular cell is probabilistic rather than absolutely deterministic with respect both to timing (see Figure 9) and pattern (note omission of the early component in some trials illustrated in Figure 8). Therefore, on any given trial, the response pattern of a single cell cannot uniquely specify the nature of an experience.

In relating these observations at the cellular level to the behavior of an organism, one must bear in mind the probability that information is processed in parallel in thousands of cells, so that the organism need not depend on the reliability of any single element for identification of an experience. These parallel chains need not all carry exactly the same information and, strictly speaking, therefore may not necessarily be redundant. It is only necessary that the nervous system receive enough information about an experience to identify it even if some aspects are left out or are distorted. Furthermore, it is likely that on first exposure to a stimulus, the nervous system specifies it less precisely than after many exposures. Ultimately, as was noted above, the code must be transformed from one based upon a discharge pattern through time to one that is more stable, i.e., immune to electrical interference,[75] more disseminated, and susceptible of very much faster readout.

The term "probabilistic" is used herein merely to describe the nature of the observed relationship between input and output. It does not imply any particular conclusion about the fundamental nature of the "noise" in the system. It is quite possible that a completely deterministic system, but one in which many variables are unknown, would appear probabilistic to the limited viewpoint of a microelectrode sampling the output of one single cell. Nor, of course, do these considerations exclude the possibility of some fundamental stochastic property operating on the process of synaptic transmission. All that can be said at this stage is that it is not necessary to introduce any fundamental indeterminacy in the system to account for all the available data.

At first glance it seems especially appropriate that cells exhibiting "plasticity" should be found in these higher reaches of the visual system. Such cells all receive inputs from hypercomplex cells and are higher-order hypercomplex, to use the terms introduced by Hubel and Wiesel.[67] As they were not randomly scattered, but were organized quite specifically on a columnar plan, these cells would be in a position to make use of all the detailed information built into the connectivity of the visual system. They simply add another stage of complexity onto the levels already described by Hubel and Wiesel.

Certain other considerations lead us to question the proposition that "plasticity" might be a property evolved only in higher-order cells. Thus, David Lindsley and K. L. Chow[76] have observed similar transient modifications of firing pattern in lateral geniculate neurons. This occurred in approximately the same proportion of total cells sampled, i.e., 10 per cent, and the cells were also grouped together. Similar observations have been made by Yoshii and Ogura[77] in reticular formation and by Kamikawa, McIlwain, and Adey[78] in the thalamus. Moreover, Weingarten and Spinelli[79] have shown changes in the receptive fields of retinal ganglion cells as a result of auditory and somatic stimulation, and Adkins, Morse, and Towe[80] have reported analogous observations in cuneate cells.

These related findings suggest rather that "plasticity" may be built in all along the neuraxis. There may be a specifically organized system of cells, developed during maturation, having a genetic endowment for the appropriate connectivity for intermodality interaction and short-term maintenance of change.

Whether the permanent traces that underlie learning take place in these cells or in still other more specialized ones with which these are presumably connected, is a matter for future investigation.

Summary

We have reviewed some aspects of the electrical activity of the brain with respect to what relevance they might have for the problem of sensory coding. Discussion included the intrinsic rhythms of the EEG, what is known of their control, and the influence of sensory input upon them. Next, in the context of an opportunity to record intracellularly from human nerve cells, the relationship between synaptic potentials in single units and the grossly recorded EEG was defined and work from other laboratories reported. All these led to the conclusion that the EEG does indeed represent the summed synaptic potentials of the hundreds of thousands of neurons within the "field" of a recording electrode. Current notions concerning the timing or pacing of brain waves were reviewed, and the conclusion reached that it was most unlikely that brain rhythms themselves were used in sensory coding. Finally, the problem of sensory coding in single neurons of the visual system was directly approached with a relatively prolonged monitoring system, and a small subsystem of cells was identified. This subsystem had an exceedingly rich repertoire of responses and response patterns that could be modified by past experience. Some implications of these observations were discussed.

Evoked Potentials

WILLIAM M. LANDAU

OUR EMPHASIS upon cerebral cortical phenomena is, in general, geared to the faith that there we may find the roots of what we modestly call our higher functions. In support of this faith: this is as far as one can go without hitting bone; the volume of gray matter in cortex is much larger than that in any other part of the brain; and its modifiable and variable spontaneous activities seem to be a worthy substrate for the complexity of behavior—at least if one follows Lashley's axiom that the physiological substrate can be no less complex than the behavior it produces.

Conversely, the assignation of decision making, discriminative, and abstract behavioral attributes to a little man inside the phylogenetically older brain structures not only misses, but actively escapes the necessity of confronting the nervous system as an integrated, as well as an integrative organ of adjustment. Hughlings Jackson,[1] drawing his data from human clinical material, put it simply: "The more gray matter, the more movements." By this he meant that cortex offers the largest repertoire for adaptive behavior or of increased degrees of freedom for fine environmental adjustment. C. J. Herrick,[2] emphasizing new dimensions of behavior associated with the evolution of neocortex, reached a similar conclusion using data from comparative neurology. Obviously, our research tools are crude for the analysis of a structure to which is assigned the function of behavioral finesse. But like Mr. Micawber, or the drunk searching under the street lamp for the wallet lost farther down the street, we persistently expect that "something will turn up" under the oscilloscope light.

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General concepts and history of evoked potentials

The stimulus-initiated, synchronized volley directed through an afferent channel is a useful investigative tool because it combines the convenience of a time lock in the test procedure with the advantage of activating many neuron units in parallel. Before it was possible to make recordings from single units, the behavior of individual cells was necessarily inferred from that of the synchronized population. This latter, grouped activity is still more convenient for evaluating certain integrated responses than is the use of a computer that must summate the unit potentials of selected cells laboriously recorded one at a time. The DC (direct current) amplifier exposed a new universe of long-duration phenomena,[3] which I leave for Dr. Rowland to present in the next chapter in this book.

To provide perspective, I shall take a quasihistorical approach. In their original report[4] of evoked response in visual cortex, Bartley and Bishop state:

"As to what these two responses might mean in terms of vision we cannot hazard a guess at present. It is highly improbable that it requires a second of time for a rabbit to see a dog; it might take that much time for him to complete his whole visual activity with respect to what he 'saw' more immediately. A more serious obstacle is offered by the fact that the cortex does not respond continuously, but is refractory or depressed for some time after a single stimulus; the rabbit presumably gazes upon his environment unembarrassed by the periods of cortical blindness suggested by such findings. It is possible that our procedure of stimulating all of the fibers of the optic nerve at once, adopted as a means of simplifying the record of the response, has rendered it too simple to be interpreted in terms of vision, although still too complex to be analyzed in terms of nervous pathways. A further possibility is that the mode of action of the cortex is different enough from that of lower centers and of peripheral nerves, so that the concepts of neurological functions founded on a study of lower levels do not apply to cortical activity. At any rate, any concrete interpretation offered at this stage could only bias a future investigation, and further conclusions are therefore postponed."

And later[5]:

"In general, it is not necessary to infer that each individual impulse traveling up a fiber from the retina arrives as a unit impulse at the cortex, and registers there as such. Rather we would look upon the cortex as being in constant activity, the physiological activity of the whole network of neurons bearing some direct relationship to the 'present state' of the animal's complex behavior, which is sometimes referred to as his 'mental state.' Impulses coming in from the periphery, by one route more or less directly, by a second which reflected or interpreted the general state of activity of the thalamus as conditions are modified by such impulses, would then take effect by modifying the cortical activity continuously going on, and the *change* in the whole picture of such continuous activity, rather than the specific set of impulses sent in, would correspond to visual perception. Such activity would then tend to revert, gradually and possibly with rhythmic disturbances still persisting, to its previous state of activity. Failure to return completely would correspond in a sense to memory; rhythmic disturbances persisting after the exciting impulses ceased would have an analogy at least in such phenomena as after-images.

"This imaginary picture already far outdistances our experimental findings, but that is the privilege of hypotheses in general. We may at least rest assured that such a picture does not err on the side of complexity, however improbable as fact."

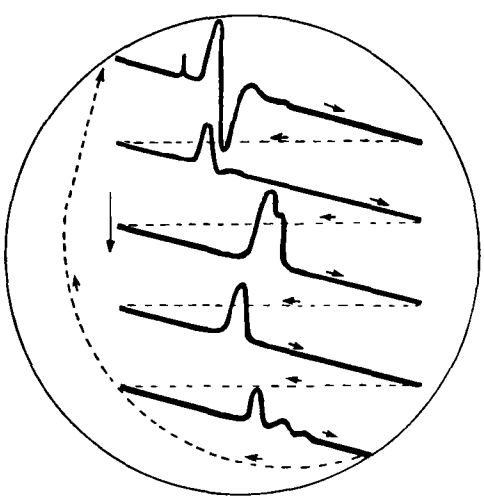

FIGURE 1 Diagram of rabbit optic cortex response to stimulus to opposite optic nerve. Each solid line represents a successive segment of approximately one-fifth second. Surface positive up. (Adapted from Bartley and Bishop, Note 4)

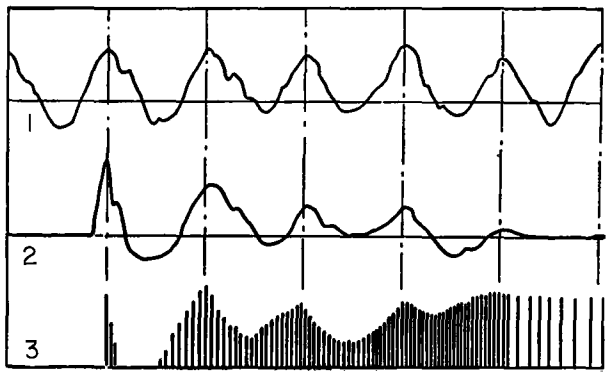

FIGURE 2 Diagrammatic representation of: (1) Spontaneous alpha rhythm at 10 cps; (2) Cortical response to single peripheral stimulus. Surface positive up. (3) Excitability cycle for the second of two stimuli. Immediately after the onset of the primary response there is a period of marked refractoriness, then responsiveness during its later surface positive phase, depression when the surface goes negative, etc. (Adapted from Bartley, Note 8)

Note the emphasis upon the brevity of the evoked response. Later, Bartley[6-8] was to show that stimulus parameters critical for some short-term perceptual phenomena in human vision could be correlated with certain electrical responses of the rabbit optic tract and cortex. Prolonged responses to continuous stimulation have been studied by a few workers,[9] but, by the usual definition of evoked response, what goes on beyond some milliseconds after the stimulus ends is not stimulus-bound, and is lost in the baseline noise.

One can distinguish at least five major lines of investigation of evoked potential over the last quarter century, all to some extent interrelated. (1) The interaction of evoked responses with spontaneous activity and with other responses evoked by the same or different pathways. (2) The spatial distribution of evoked potentials as an anatomical technique for tracing synaptic pathways. (3) The exten-

sion of microelectrode mapping studies to define the functional relations and restrictions of the synaptic connections of individual cells. (4) Defining the origin and mechanism of the evoked potential at the levels of tissue, neurons, and portions of neurons. (5) Single-unit evoked responses and their correlation with the integrated tissue potentials.

Bartley and Bishop's diagram of the response of rabbit optic cortex to electric shock applied to the opposite nerve[4] is seen in Figure 1. (The short latency response in top trace is all that, in today's conventional usage, would be considered primary evoked potential.) The entire reaction in the rabbit lasted a whole second; it showed some variation from animal to animal and also according to anesthesic level and stimulus intensity. Obviously, something goes on long after the time needed for conduction in fiber pathways along orthodox routes.

Interactions of evoked response with other cortical activity

The early discovery of the prominent interaction of the evoked wave train with the "spontaneous" cortical waves is shown diagrammatically in Figure 2.[8] Stimulus intensity is a significant variable; a large volley may drive through at any time of the cycle and actually reset the rhythm of the baseline pattern, while a weaker one, depending upon its timing, will have larger or smaller effects upon the ongoing wave pattern. In preparations with high-voltage, sustained, spontaneous activity, the evoked rhythm may be relatively less intense, and the net effect may be one of abolishing or tending to suppress spontaneous rhythm.[10,11] Recent microelectrode studies often correlate the depression phases with prolonged intracellular hyperpolarization (inhibitory postsynaptic potentials, or IPSPs).

Bartley introduced another level of complexity by moving the stimulus distally to the primary receptor.[7] Figure 3 shows the optic nerve response to a brief flash of light. This response has a sharp wave front, but it also has a prolonged, irregular, repetitive barrage not seen with synchronous electrical stimulation. These summated unit responses have complex forms, as the total retinal impact to the central nervous system lasts a tenth rather than a thousandth of a second. The patterns reflect the first levels of end organ—neural interaction in the retina. (The encircled late secondary waves in the rabbit nerve correlate with intensity-duration stimulus parameters that evoke in man the perceptual illusion that two flashes occur when only one is presented.)

The response evoked at the rabbit end organ was followed through to the visual cortex (Figure 4) to produce

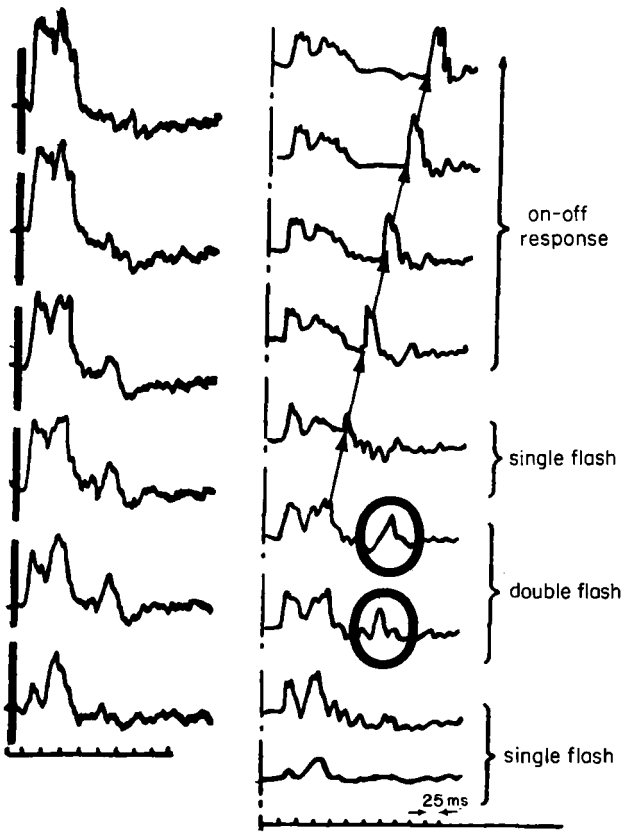

FIGURE 3 Records of optic nerve discharge in rabbit. In left column, intensity increases from below upward. In right column, duration alone increases from below upward. Bottom record is just supra-threshold. Encircled secondary waves correspond to perceptual illusion of double flash. Calibration: 25 msec. See text. (Adapted from Bartley, Note 7)

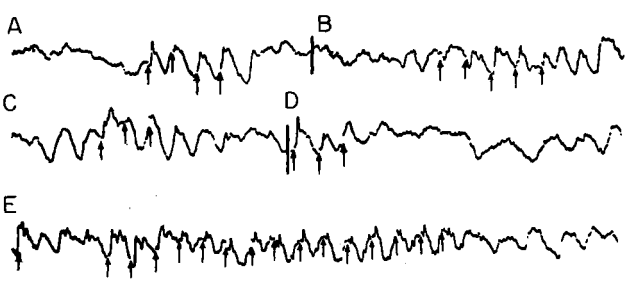

FIGURE 4 Rabbit optic cortex response to visual stimulation (arrows). Rate of flashes is 6 per second. Surface positive up. A: Flashes during quiet interval produce large waves. B: Flashes during irregular waves—less effective. C: Flashes during large waves—still less effective. D: Flashes may produce waves of higher frequency than the spontaneous ones. E: Repetition of flash brings about better synchrony. See text. (Adapted from Bartley, Note 11)

the expected complexity of response variation.[11] Thus cortical activity may be relatively flat in dim light; irregular, slower activity may appear in darkness; and a high-frequency, low-amplitude response will often result from rapid, repetitive brief flashes. With long flashes, the retinal "off" response (Figure 3) may also be seen in cortex, sometimes more prominently than the "on." A slow flash frequency may break up the alpha cycle or, conversely, the size of the primary evoked response may vary when the spontaneous waves are more durable. If stimulus and background waves are "phase locked," the evoked responses tend to fall upon the same portion of the wave cycle and are accordingly more constant. Such relationships between evoked and spontaneous waves are still being discovered.[12]

Bartley[6,8] suggested that such a critically timed series of flashes produces an average perceived brightness higher than that produced by the same illumination intensity when it is continuous (now called the Bartley effect). He also suggested that sensory fusion of repetitive stimuli occurs when the majority of the neural population can no longer follow the stimulus rate, while individual subgroups of neurons follow alternate stimuli.

A computer analysis of unit behavior[13] confirms that responding neurons tend to have "favorite" and cyclic latency preferences (Figure 5). Discharge in each unit is most likely during the integrated primary response or an ensuing, major alpha wave; it may fire more than once per stimulus. There is a high degree of correlation between the field potential derived from many units and one cell's firing probability conditioned by a cyclically variable level of excitation.

Evoked responses are by no means restricted to the afferent pathways from the periphery, but can be produced by arbitrary electrical stimulation of neurons afferent to any test region. The closer the stimulus to the response area, especially if no synaptic relay intervenes, the less temporal dispersion there will be in the volley triggering the postsynaptic evoked potential. Furthermore, cortical responsivity can be measured directly by

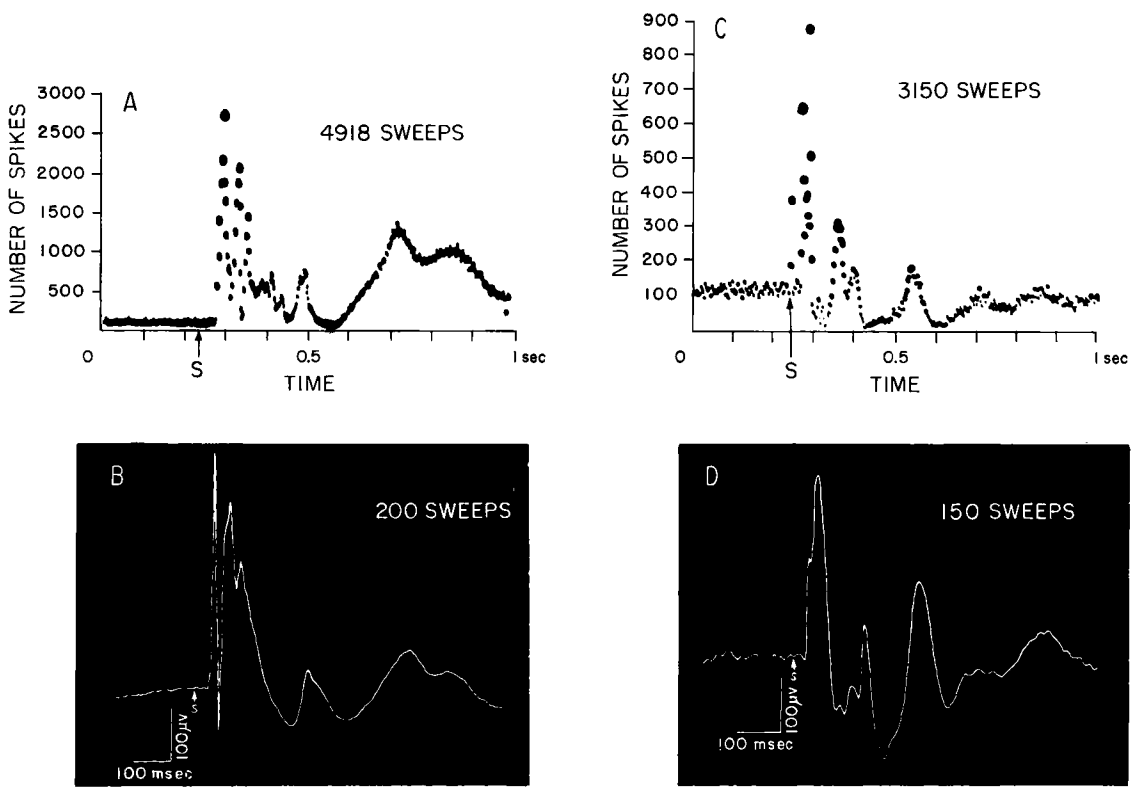

FIGURE 5 The relation between probability of firing of a single cell of cat visual cortex (upper records) and the evoked potential wave form of the same locus after cell death (lower records). A and B are one experiment, and C and D, another. Photic stimuli. (Adapted from Fox and O'Brien, Note 13)

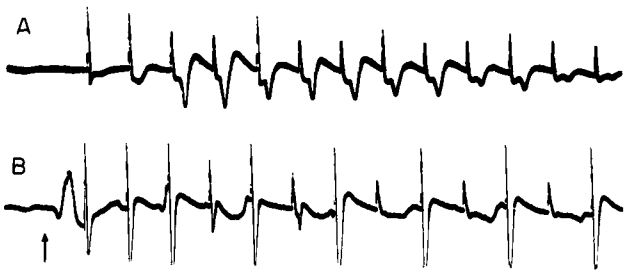

FIGURE 6 Cat visual cortex response to 8/sec shocks to geniculocortical radiation axons. Surface positive up. A: Diphasic positive-negative primary evoked response followed by late surface negative augmenting waves from later stimuli. B: Tetanus (100/sec) to medial thalamus at arrow before same optic radiation stimulation as in A. Notice increase of primary response and abolition of augmenting waves, probably by occlusion. See text. (Adapted from Landau, Bishop, and Clare, Note 14)

repetitive stimulation when the serial excitability changes of relay nuclei are removed from the test situation.

Various inputs produce different evoked response patterns in the cortex. All varieties of interaction may be seen, ranging from none, when it can be assumed that different populations of neurons in the same tissue are activated independently, to either facilitation or depression of one response by another.[14] Test response depression may occur because the elements are already activated by the conditioning input (occlusion), or by a mechanism of true inhibition, post- or even presynaptic.

Figure 6 is a complex example; repetitive optic radiation shocks evoke primary responses plus the spindle of late waves called augmenting waves. High-frequency stimulation to the medial thalamus produces potentiation of the primary response, while the later augmenting waves disappear. The alternation of larger primary responses as stimulation continues demonstrates the "alternation of units" hypothesis proposed by Bartley and Bishop; most neurons cannot continue to follow the stimulus frequency.

The "evoked potential club" was exclusive during the 30's and early 1940's. While Bartley, Bishop, and O'Leary, and Marshall focused upon temporal and spatial patterns of neuronal excitation, Dempsey and Morison studied the nature of reactions to direct stimulation of thalamic nuclei, and Adrian discovered unit responses.

Anatomical mapping

Over a period of many years Woolsey and his collaborators developed the use of evoked potentials as a tool for the definition of the anatomical projection of localized sensory fields onto the cortical surface. Similar techniques were also used for mapping a variety of subcortical relay levels and the cerebellum. Woolsey's group has given greatest attention to the somatosensory system in many species, but have also made auditory and visual maps.

As the method evolved, it became clear that adequate sampling often required one or more days of experimentation, the brain being depressed by deep anesthesia and exposed. Obviously, this technique suppresses spontaneous activity, and it may be safely inferred that under such conditions the evoked responses that make their way through several relays to cortex derive from pathways in which there is the highest degree of spatial as well as temporal summation.

Under such conditions, less well-focused paths, which might give small or subthreshold effects in other cortical areas, are not seen. Thus, if the cortical area responding to a peripheral point stimulus is considered to have the configuration of a mountain, the mapping technique shows only the areas above timberline, while overlapping slopes and foothills are omitted.

Qualitative response specificity in single cells

Extension of the concept of mapping into the microdimension of single-unit recording has, however, produced data of considerably more functional importance. Mountcastle showed[15] that, under light anesthesia, vertical columns of cells in the cortex tend to respond to the same modality and region of somatosensory stimulation. One column may be activated by superficial stimuli and an adjacent one by deep limb stimuli or by input from different regions of the same limb. Since thalamocortical afferents ascend vertically in cortex, it may not be too surprising that any population of axons capable of firing one cell in a column will also fire others, but the modal specificity could not have been predicted. Further, at several levels of afferent relay, patterns of focal excitation and inhibition in surrounding neurons sharpen the contrast for most modalities of sensation.

Hubel and Wiesel's studies of light-evoked units at prethalamic, thalamic, and cortical levels show further increasing degrees of "tuning" complexity both to the position of the peripheral stimulus and to its shape, configuration, and direction of movement.[16] They describe at least three levels of complexity in primary and adjacent visual cortex. What they call a hypercomplex unit may respond optimally to a target moving in a single axis, not at all when it moves in the opposite direction, and best when the stimulus target is neither too narrow nor too wide. Such complex conditional responses in single cells bear out very old concepts of a comparative analytic "percept" function for paravisual cortical areas.

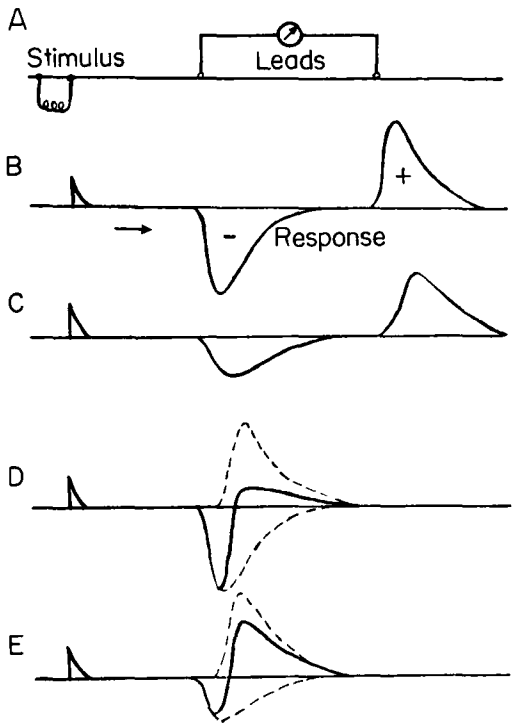

FIGURE 7 Diagram of records from nerve suspended in air. A: The baseline potential of the leads is between two regions on the outside surface of the resting axon membranes (positive charge outside, and negative inside). This is like recording from the anodes of two batteries hooked up in parallel to a series high resistance. B: As the action potential conducts from the left to the first electrode, the resting membrane discharges. The duration of the all-or-none action potential is self limited and the membrane is recharged. As this region recovers to the resting level, the activity conducts to the other electrode; as it goes negative, following the same time course, the recorded trace is the electrical mirror image of the first impulse passage. This is called a diphasic record of a monophasic response wave. If the nerve is crushed between leads, conduction does not reach the distal lead, and one records a single monophasic wave. C: It is suggested here that the region of the first electrode is partially depressed, or one might postulate that the axons are smaller at this region, or that the electrode contact is less close than at the right electrode. D: If the recording leads for B are placed closer together along the conducting fibers, the active wave length will begin to pass under the second electrode before it has left the first. The potential difference then recorded (heavy line) will be the algebraic sum of the mirror image waves (dash lines), out of phase by the conduction time between the two leads. This diphasic record distorts both duration and wave form, and amplitude is reduced. E: Record for condition C with electrodes closer together, as in D. One observes another distorted form of summated potential difference with predominance of the second phase. (Adapted from Bishop, Note 17)

One should hesitate to extrapolate, from brief observation of such units under anesthesia, that the neuronal pattern is rigidly restricted over long periods of time. Even though a neuron favors a particular stimulus pattern, it may still be able to respond to other patterns and other intensities under other circumstances. In any case, the reciprocal patterns of excitation and inhibition among the small populations of neurons studied by such techniques obviously have significance in discrimination, even if we do not know how the little man inside the head perceives the unit percepts. It is significant that the crude, massive stimulus of the ordinary evoked potential entirely obscures these fine relationships.

While anesthetics minimize the potentiality of evoked responses, the converse is true of excitant drugs such as strychnine, picrotoxin, metrazol, and chloralose, which also has anesthetic properties. To be sure, even the potentiation of synaptic transfer by a drug cannot produce responses if connections do not exist, but there is danger of overemphasizing the normal significance of channels demonstrated by this technique. There are enough pathways in the nervous system for one to find a way to fire almost any neuron from any other. The problem of physiology, however, is not to find alleys, but to locate and understand the traffic pattern on the highways where the action is.

Physiological derivation of integrated evoked responses

I have taken for granted that gross cortical evoked potentials *can* be recorded. The neural basis for the extracellular currents that give rise to the evoked potential remains complex, even with the recent accumulation of intracellular records.

It may be simplest to start where the physiology of 1931 started[17] with conducted action potentials of peripheral nerve suspended in air between two electrodes (Figure 7).

The nerve suspended in air has a monophasic action spike because the extracellular current pathway along the thin fluid layer of the nerve sheath has high resistance. Significant outward current is therefore limited to the area immediately adjacent to the active region.

If this external resistance is made low by placing the nerve in a volume conductor such as the two-dimensional model of a nerve on a wet blotter (Figure 8), current flows out of the resting axon (the source) both before and behind the active region (the sink) of inward current. Thus, the classical volume conductor wave is a positive wave of approach, negative of presence, and positive of retreat.

If both electrodes in the medium are near the active tract, the triphasic wave may be recorded twice with

further wave distortion when both sites are simultaneously active. The triphasic form may be recorded widely in the volume conductor if the leads are far enough apart to be on different isopotential lines.[18] For example, the shock-induced action spike conducted from eye to thalamus may be readily recorded between one electrode on the visual cortical surface and another outside the cranium.[18]

Whenever rigid electrodes are inserted into active tracts, local injury effects may distort the recorded activity. This phenomenon is called the "killed-end effect."[19] An electrode on crushed nerve records a steady negative potential relative to the normal outside fiber potential because the electrical battery of resting membrane continues to pass current through the killed tissue and back into the axoplasm (Figure 9A). The lead on killed tissue is not at the same potential level as one inside an intact axon. Instead it lies along the tissue resistance (IR drop) pathway from external to internal normal resting membrane level (Figure 9B). This lead from killed region to normal axon surface records the demarcation potential of classical physiology.

When the propagated spike in normal membrane comes up to the killed margin, the generator of the demarcation potential is turned off with the time course of the action spike, and is recorded as a *positive* deflection from the baseline negative level of the *injured* region. Just as the action potential of uninjured nerve is the transient negative variation of resting potential, this is the transient positive variation of the steady negative demarcation potential. Note that this action potential is the same in form and direction as would be derived from a microelectrode inserted into an uninjured axon.

Therefore, an electrode in a central white tract may be expected to record both killed-end positive spikes from axons in closest contact with the electrode, and triphasic spikes of passage from nearby uninjured axons. The monophasic killed-end form dominates the picture to a remarkable degree, possibly because the extracellular space is tight in cerebral white matter—the tissue impedance is six times greater than that of physiological salt solution.[20] Chronically implanted electrodes may be expected to produce increasing local tissue damage as time passes. Positive cell unit spikes recorded externally with fine electrodes in gray matter also relate to local damage. Furthermore, a killed-end equivalent lead may occur without tissue damage when the conducting elements end between recording electrodes. Thus, geniculocortical axons come to their synaptic termination at a mid-cortical level, and their field potential is recorded as a spike that is positive at the cortical surface.

Only a small part of most potentials evoked in gray matter is represented by conducted axon spikes, but it was

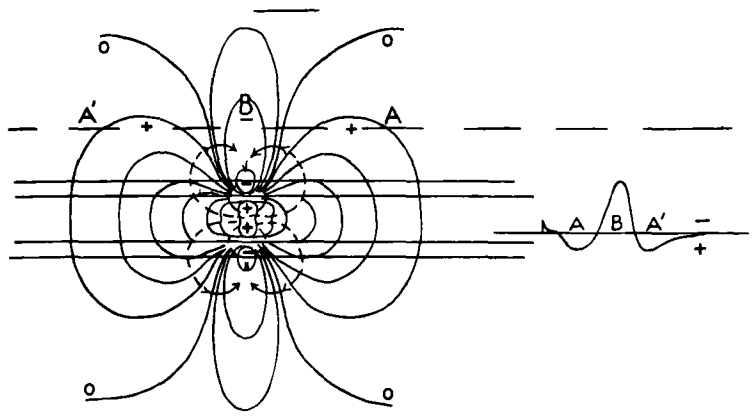

FIGURE 8 Left, theoretical static potential and current field of active locus of nerve in a volume conductor. The parallel lines are the axon membranes. Active locus under point B; impulse conducting from left (A') to right (A). The full line isopotential lines are crossed perpendicularly by curved dash lines representing current flow. Although the negative peak B should reflect the true time of peak action at the critical electrode, the point at which the recorded potential crosses the 0 potential reference baseline does not necessarily indicate when activity arrives, because the summation of positive and negative waves is a function of the approximation of electrode to membrane surface. Note that the O potential lines, which extend to the distant reference, diverge laterally; thus, an electrode coming toward the nerve in the vertical plane to one side of the dipole center goes from negative to positive isopotential area without moving along the horizontal axis. Note that only *extra*cellular current flow paths are recorded by gross extracellular leads. Right, derived form of conducted action potential recorded in volume conductor. See text. (Adapted from O'Leary, Note 18)

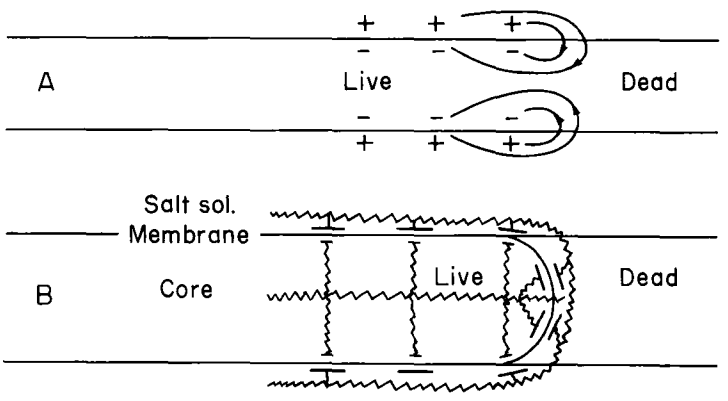

FIGURE 9 Diagram of situation when one electrode is placed upon live and one upon crushed nerve in air. See text. (Adapted from Bishop, Note 19)

this very difference that impressed early workers, for the amplitude and long durations of cortical potentials were another order of magnitude from those expected from nerve physiology. Indeed, Bartley and Bishop[21] implanted a vagus nerve in the active rabbit cortex and plotted the distribution of its action potentials in the nearby tissue. Starting with the number of fibers in the nerve and its maximal action potential, they calculated that there could not be enough nerve fibers in a given area of cortex to sustain the recorded evoked potential, even with repetitive firing. They concluded that the cellular components of cortex have longer response durations and different electrical characteristics than could be accounted for by an axon model.

It was evident at the outset of investigations in this area that the major anatomical orientation of cortical neurons lay in the vertical plane. Analysis of the evoked potential field across active cell layers showed a dipole field—essentially a standing wave—for a given component distributed over a vertical depth measured in fractions of millimeters. The large axons from lateral geniculate that activate visual cortex conduct 0.5-millisecond spikes at a rate of 60 meters per second, or 60 microns per microsecond. Such swift conduction through the 2-millimeter thickness of cortex would mean that the entire neural membrane would be discharging almost synchronously. Obviously, only much slower conduction can be invoked reasonably for cortical waves.

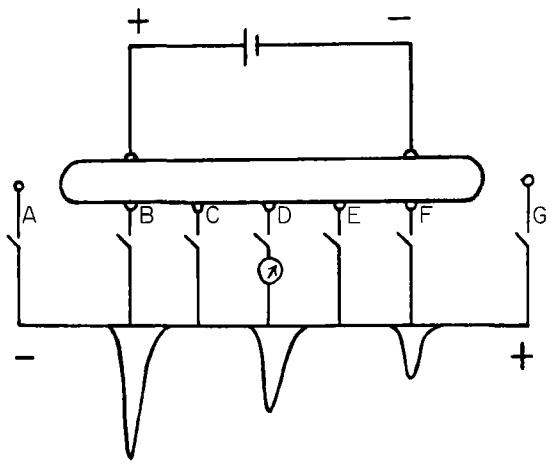

FIGURE 10 Model of nerve cell discharging with larger local (transmembrane) magnitude on left (B) than on right (F). Field potential between points A and G would be recorded as positive on the right. Upper circuit represents external polarizing battery applied to active cell, the cathode on right. This would further depress resting and action potentials at F, while increasing them at B; the potential recorded from end to end of the element would increase. Reversing the polarizing battery would have the opposite effect, depressing and even reversing the action potential between A and G. See text. (Adapted from Bishop and O'Leary, Note 22)

Consider the entire neuron (Figure 10) as an elongate sphere or cylinder.[22] If the action potential variations recorded with fine microelectrodes were indeed of equal size over the neuron's surface, there would be no extracellular potential gradient, and extracellular evoked potentials could not be recorded. It follows that activity within the soma-dendrite complex *must* be localized, or at least be unequal at different regions. Extracellular current then flows from an inactive or less active region toward the more active region. (Transmembrane resting potential may also be nonuniform.)

A plane of such dipole generators in parallel would have many of the general electrical characteristics of a single unit membrane itself, as if the giant squid axon membrane were spread out as a sheet (Figure 11). For example, the relatively simple evoked potential of the superior colliculus is essentially a surface-negative wave that reverses as the critical electrode penetrates the cell layers to produce an even larger positive potential.[23,24] With small electrodes, local killed-end effects are negligible in relation to the standing dipole wave.

It was early recognized[21] that the phenomenon of the static dipole layer raises a technical difficulty in determining which tissue is active. Any generating cell layers between the recording electrodes have a fair chance of affecting the recorded potential difference unless the electrodes are closely spaced directly across the tissue being studied (Figure 12).[25] One cannot record single potential levels, but only potential differences; so what has been called the monopolar lead for brain potentials is as meaningless physiologically as it is physically.

This problem of locating the action has become especially important now that computer averaging measures such small potential shifts. Thus in the experiments of Kelly, et al.,[26] similar somatosensory responses of various forms could be recorded over most of the monkey cortical surface. In most situations, leads from surface and subcortical white matter to a distant electrode gave identical responses. Indeed, the surface lead gave the same response when the cortex was removed and wet cotton was substituted. Only where the potential reversed between electrodes on surface and in subcortex was the active region defined; the amplitude was also largest there. The operant axiom is that *only* potential reversal across a cell layer gives assurance that an evoked field potential is other than a distant, volume-conducted response. (Stimulus-locked unit responses recorded with fine electrodes, as in Figure 5, can also localize the active region.)

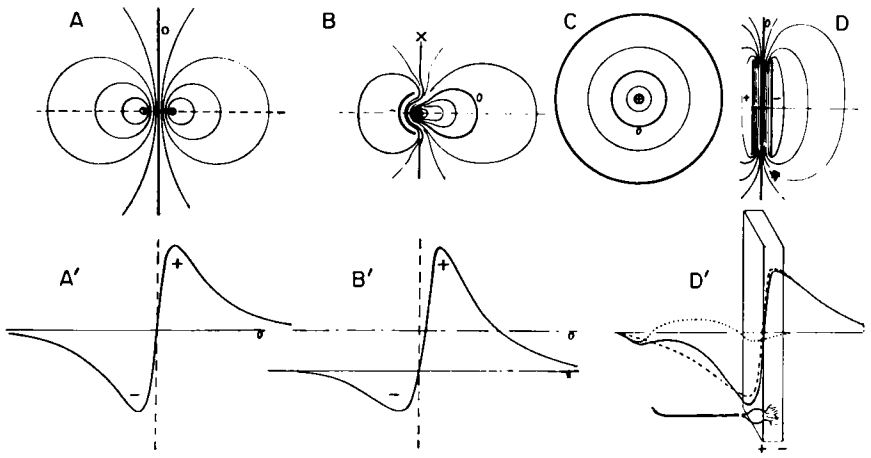

FIGURE 11 Field diagrams of potential distribution in conducting medium about various active structures. A: Isopotential lines of a simple dipole field. A′: Potential level recorded along dash line axis of A. B: Intermediate circumstance between A and C—A hemispherical, radially oriented cell-layer arrangement like superior colliculus; the outside of the hemisphere negative. The reference electrode on the infinite isopotential line X is biased toward the negative side of the true O isopotential line which is a closed ellipse. B′: Potential plot of a probe electrode referred to a distant lead and inserted from the left along the dash line in B. Negative and positive components are not equal, because the electrode passes the reference isopotential line before the true O isopotential. For such a distorted field, even the position of the reversal point along some axes may be skewed from the true position of the curved cell layer generator. C: Theoretical closed field for a spherical layer of cells oriented radially and active simultaneously over the entire surface. This is electrically similar to the situation for a spherical single cell membrane that is active simultaneously and equally over its entire surface. The only action currents would be generated along the radii; the potential field is self-contained. Thus, unlike the situation in A, where a distant reference electrode is at the O isopotential level midway between dipole peaks, a similar distant reference for C would still be at the same level as a lead applied close to the spherical surface. D: Electrical field of a plate of parallel cell units as in A, e.g., the midregion of the lateral geniculate nucleus. The dotted line represents the axon response, which sums with the dash-line cell potential to produce the recorded full-line picture. D′: Recorded action potential of D in relation to tissue layers as in A. See text. (Adapted from Bishop, Note 23)

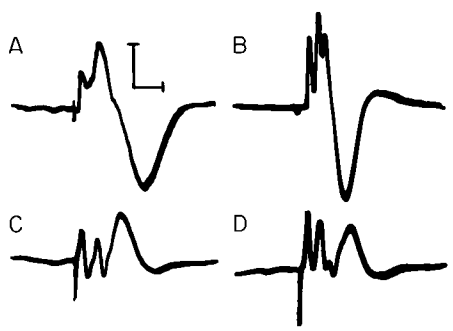

FIGURE 12 Shock to cat ventral medulla eliciting antidromic pyramidal tract and dromic medial lemniscus stimulation. A: Antidromic transcortical response (surface to subcortical white) in anterior sigmoid gyrus. B: Dromic transcortical posterior sigmoid response to same stimulus. C: Record from same surface lead as A against frontal sinus "indifferent" lead. Circuit now also includes intervening other active tissue, which distorts record as shown. D: Lead from the posterior sigmoid surface as in B against the frontal sinus. Response is a complex mixture of dromic and antidromic responses. Surface positive up. Calibration 200 microvolts and 5 msec. (Adapted from Landau, Note 25)

Relations among evoked potentials and single unit responses

Primary evoked cortical potentials to a highly synchronized stimulus come in a variety of inconvenient forms and sizes (Figure 13). Recruiting and direct responses are relatively simple activities in superficial neuropil, while callosal and sensory radiation responses begin with early activity in a deeper level, at or near the somata.

The recruiting wave was originally described in cortex activated by slow repetitive stimulation of the medial thalamus, but similar forms can be evoked from other basal structures and other cortical regions as well. It is characteristically a 15-millisecond, surface-negative, graded response, localized within the upper layers of cortex where dendrites are the predominant neural tissue.[27,28] Intracellular recording during recruiting waves[29] shows that depolarization in the deep soma is associated with the surface-negative wave (Figure 14). Thus the model of an axial unit active to varying degrees over its length is affirmed; in this case, extracellular current flows from less depolarized soma and perhaps basal dendrites toward more depolarized apical dendrites.

A similar superficial graded response can be evoked by direct stimulation near the recorded locus of cortex. It probably is a mixture of direct activation of dendrites and of presynaptic axon elements en route to axodendritic synapses. When these surface-negative waves are recorded between leads subtending the upper portion of cortex, companion electrodes subtending the lower portion show current flowing upward from the inactive or relatively inactive region toward the surface[30] (Figure 15).

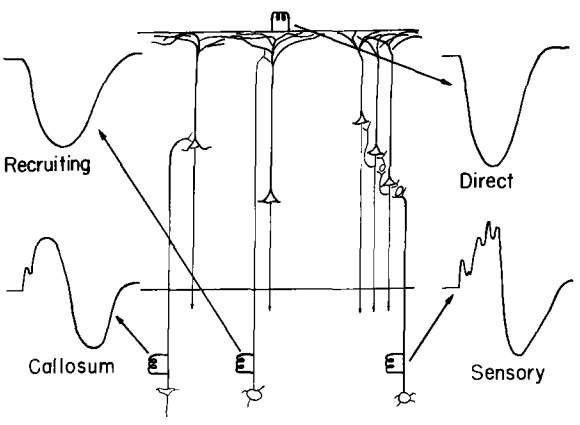

FIGURE 13 Diagram of varieties of primary cortical evoked responses in cat. Surface positive up. Duration of traces is *circa* 15–20 msec. See text.

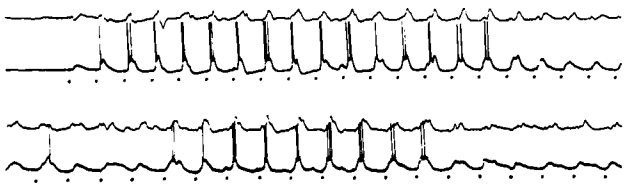

FIGURE 14 Correlation of surface cortical waves (upper trace, surface negative up) and simultaneous intracellular potentials (lower trace, depolarization up), during recruiting waves from 8/sec medial thalamic stimulation in cat. See text. (Adapted from Creutzfeldt, Watanabe, and Lux, Note 29)

Artificial hyperpolarization of the membranes of the surface region results in a larger depolarizing active response and, accordingly, more current flowing upward from below. Conversely, depolarization of the surface with cathodal current results in the opposite effect. With strong currents the response may even reverse, indicating a more active depolarizing response in the deep region below the surface.

The antidromic response to pyramidal tract stimulation (Figure 16) offers a certain model of primary activity at a deep pyramidal cell cortical level, spreading upward. (Postsynaptic effects via retrograde axon collaterals may complicate this picture.[31]) It also provides interesting evidence about the spindles of rhythmic cortical waves that are readily triggered by a shock to thalamus or cortex, and that also occur spontaneously. To maintain normal rhythmic activity there must obviously be a mutual dependence of excitation and inhibition in thalamus and cortex, whether or not there is a true rhythmic reverberation. An antidromic volley will also regularly set off such spindles,[30] and they have been recorded intracellularly by Jasper and Stefanis.[32] Although thalamic involvement via pyramidal tract collaterals has yet to be excluded from this phenomenon, it suggests the heresy that autonomous oscillatory rhythm in cortical neurons may be a significant factor in the evoked response.

The primary sensory projection response (Figure 13) is set off by a volley in the largest of thalamocortical afferents, those that comprise only 2 to 4 per cent of the population in subcortical white matter.[33] The first axonal radiation spike is followed by a series riding on a positive wave, followed by a surface-negative wave. The serial spikes arise at successively higher levels, but certainly include some repetitive unit firing as well. The major axosomatic synaptic connection is probably via both star cell interneurons and some direct axonal contacts. It is also probable that interneurons are involved with axon collaterals in the serial pyramidal cell unit pathway. The negative

wave, which begins in the depth, appears to propagate upward along dendrites, but later responding synapses on dendrites may also be involved; in young animals with prominent axodendritic synapses[34] only a surface-negative wave is seen.[35] A slower negative wave originally related to a dipole deep in cortex[36] may be associated with late postsynaptic potentials in the depth.[29,37]

The response to corpus callosum stimulation[38] is an intermediate type (Figure 13). The primary axon spike is not followed by synchronized serial unit discharge, although unit responses can be recorded. As shown in laminar recording, the slow-wave sequence behaves as if it were an initial active locus in midcortex that propagates slowly upward.

Polarization effects upon diphasic responses are also consistent with the view that even complex evoked potentials represent neurons active to different degrees at one end from the other. Thus the surface-positive and negative components of the visual projection response are affected reciprocally[14,30] (Figure 17 B,C). These effects are quite different from those produced by neural synaptic interaction; all visual cortex components except radiation spike are increased by medial thalamic stimulation (Figure 17A).

Within wide limits, the immediate cortical output via

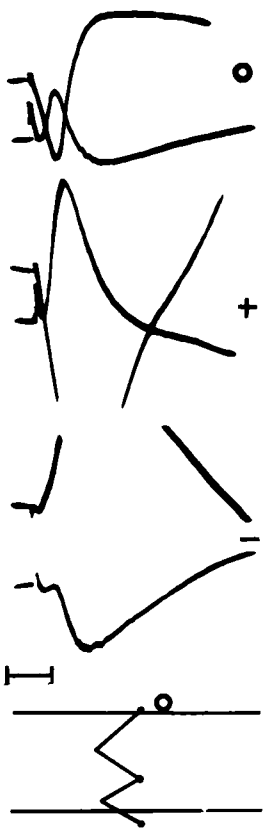

FIGURE 15 Direct cortical response in cat. Diagram indicates upper oscilloscope trace from upper laminae of cortex; bottom, from lower laminae; circle is polarization electrode. Top pair of traces shows superficial negative wave (down) in upper cortex, the current flowing up from deep source at lower level. Middle pair show exaggeration of normal response in upper segment during surface-positive polarization and correlated decrease of lower segment response. Lower pair shows reversal with surface-negative polarization. Time base, 10 msec. (Adapted from Landau, Bishop, and Clare, Note 30)

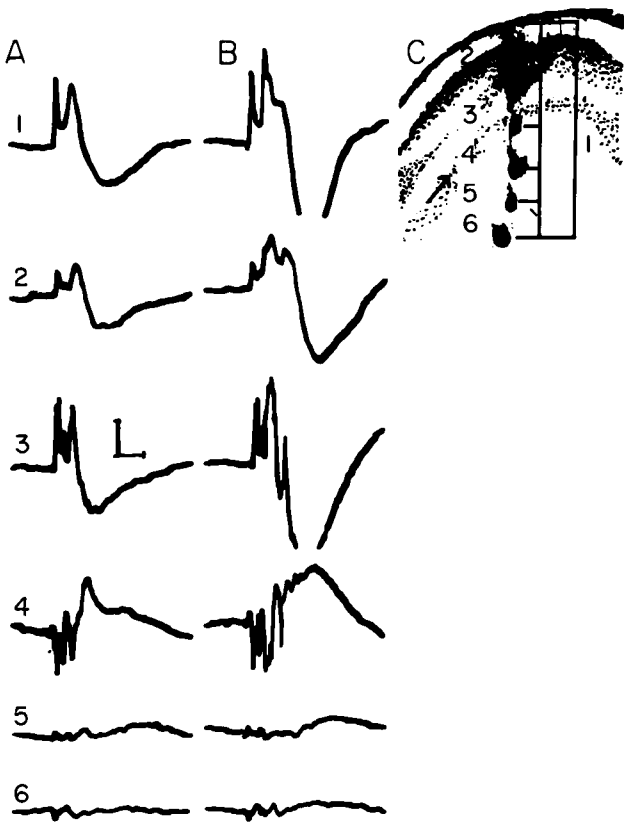

FIGURE 16 A: Antidromic pyramidal tract response in cat anterior sigmoid gyrus recorded between 0.5 mm spaced electrode pairs, as shown by numbers in histological section C. Surface positive up. A-1 is total response from surface to white matter. Initial spikes represent two populations of conduction rates in the pyramidal axons. Later surface-negative wave implies propagation upward. A-2 and A-3 are segments above Betz cell layer (arrow in C); A-4 is below; A-5 and A-6 are in white matter. B: Exaggeration of surface-negative wave with two shocks at 2 millisecond interval. Calibration: 100 microvolt, 5 msec. (Adapted from Landau, Note 25)

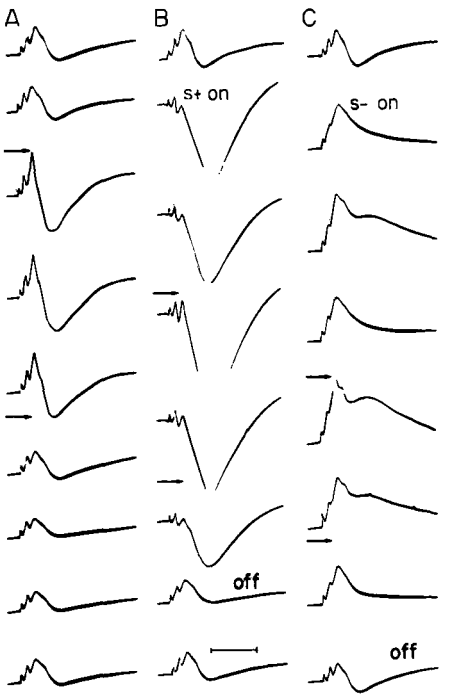

FIGURE 17 Cat visual cortex responses to constant 3/sec. stimuli to optic radiation. A: Potentiation by 100/sec. tetanus to medial thalamus between arrows. B and C: Surface-positive and surface-negative polarization as indicated, plus thalamic tetanus as in A, between arrows. Surface positive up. 10 msec. time base. See text. (Adapted from Landau, Bishop, and Clare, Note 14)

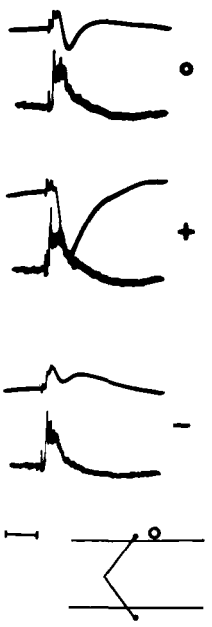

FIGURE 18 Transcortical cat sensorimotor cortex response to thalamic radiation shock (upper trace, surface positive up), and projected pyramidal tract axon response (lower trace). Even with gross distortion of cortical response during polarization (surface positive in middle pair; negative in lower pair), projected response is essentially unchanged. See text. Calibration: 10 msec. (Adapted from Landau, Bishop, and Clare, Note 30)

axons is remarkably independent of these field potentials, normal or distorted by polarization[30] (Figure 18). In similar experiments, the degree of safety factor in single cells has been shown directly by Purpura.[39] At current intensity that grossly distorted the evoked potential, excitatory postsynaptic potential (EPSP) and spike discharge were virtually unaffected (although they could be modified by very large currents). This implies that the local postsynaptic membrane changes near the site for triggering all-or-none axon spikes are relatively prepotent and independent of extracellular current flow patterns along the soma–dendrite axis.

I have been emphasizing the degree of rigidity of all-or-none cortical output in relation to artificial distortion of cortical field currents. However, simultaneous studies of spontaneous and thalamic evoked intracellular and surface potentials in normal cortex, some previously cited, have often shown significant correlations; yet unit discharge may have a variable relation to the polarity, form, or even presence of the field potential (Figure 19). Also with strong local cortical stimulation,[40] unit spikes in the soma sink are recorded as surface-positive field spikes, the surface acting as the source. But with a smaller stimulus the soma EPSP relates to the primary surface negative wave as in the recruiting wave; depolarization for this component must be diffuse. Similarly, a later prolonged, deep soma hyperpolarization (IPSP) correlates with a prolonged surface after-positivity.

Such hyperpolarization adds new complications to the interpretation of field currents, depending whether effects on the neuron are diffuse or localized. For example, with medial thalamic stimulation (Figure 20) the IPSP inhibition of cell spikes relates to surface-negative waves in the surface recording.[37] Extracellular current thus flows from hyperpolarizing IPSP in soma toward the relatively less active (or depolarized) superficial dendrite region. Conversely, in convulsive after-discharge, rhythmic surface-positive waves are localized by laminar recordings 0.1 to 0.2 millimeters below the pial surface; presumably these are derived from dendrites.[41] Thus, the direction and amplitude of vertically oriented field potentials are determined by variations of *kind, degree,* and *distribution* of postsynaptic activity affecting the entire soma-dendrite complex.

Summary

Cortical neurons have somata and dendrites distributed in many patterns, and their entire surfaces are covered with synaptic globs whose presynaptic connections come from multiple sources. Both local injury effects and the placement of both electrodes of a recording pair in relation to

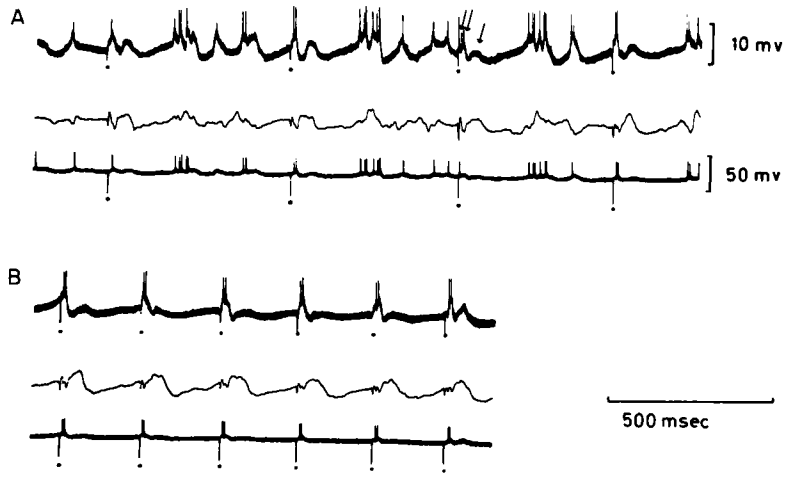

FIGURE 19 Correlation of intracellular (upper trace, high gain; lower trace, low gain; depolarization up) with gross surface cortical responses (middle trace, surface negative up) in sensorimotor cortex following stimulation (dots 2/sec. in A, 4/sec. in B) of cat thalamic nucleus VL. Note general correlation of EEG and intracellular oscillations. Stimulus evoked positive spikes at surface in B relate to EPSP plus spikes in the soma; thus localized deep activity draws current from above. But slower "spontaneous" cell depolarizations with spikes between stimuli in A are related to surface-negative waves; this suggests diffuse depolarization, more superficially (Cf. Fig. 14). See text. (Adapted from Creutzfeldt, Watanabe, and Lux, Note 29)

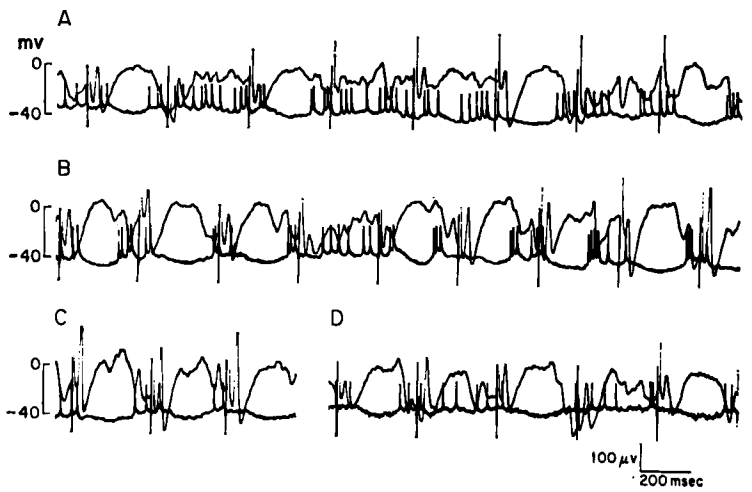

FIGURE 20 Stimulation in cat medial thalamus. Slow surface negative (up) cortical waves (upper trace) associated with spike inhibition and late intracellular hyperpolarizing (down) IPSP (lower trace). See text. (Adapted from Pollen, Note 37)

active tissue elements are significant determinants of potential amplitude, form, and polarity. In spite of such complexities, we may accept that cortical evoked potentials are made up of the following: (1) graded dendritic activity of about 15-millisecond unitary duration: this activity conducts electronically, and in some circumstances as a regenerative active process; (2) all-or-none 1 msec. soma-axon hillock spikes; and (3) 0.5 msec. axon spikes, probably of least significance in the integrated field potential. Polarizing and depolarizing synaptic actions take place on both soma and dendrites, and even on some axons.

The possibilities for addition and subtraction of action currents for so many derivations make it clear that a single physiological unit of which the gross evoked potential is the integral must be a constantly changing will-of-the-wisp. This is not to say that an evoked potential is not a function of the numbers and rates of discharge of single cells, but rather that the function is a difficult one to solve precisely, especially when different members of the population may be simultaneously active in different ways.

There are many unanswered questions. I have mentioned the possibility of spontaneous potential oscillation in neuron membranes. Is this significant in cortical dendrites? Is it safe to presume that all depolarizing shifts represent EPSPs and all polarization IPSPs? What about effective synaptic transmission with minimal or no PSP? Of what significance are rates of change of potential in relation to amplitude, location on the neuron surface, and the payoff of spike discharge? Does the adaptation phenomenon of peripheral nerves relate to central neuronal excitation? To what extent are late or summating components of evoked potentials derived from glia? Which

synapses are more or less potent because of size or location on the membrane surface? How much is our view distorted because only giant cortical neurons, which comprise less than 1 per cent of the population, have been studied? Finally, with what validity can even the most reliable measure of complex evoked field potentials recorded from scalp, cortical surface, or single brain loci be translated into local field and unitary nerve cell dimensions?

I submit that our present lack of answers for such elementary queries indicates the unwisdom of simplistically extrapolating evoked potentials into a mentalistic or behavioral metaphysical never-never land.

Steady Potential Phenomena of Cortex

VERNON ROWLAND

THE GENERAL LACK of understanding in a given area of investigation is reflected in the semantic variability in communications on the subject. This variation is sufficiently great in the area of slow potential phenomena as to warrant a clarification of terms currently used.

Steady potential

The best-defined phenomena are represented by the terms steady potential and steady potential shifts. Steady potential, or SP, refers to measurements taken on the cortex and should be used in place of direct current (DC) potential because of the many electrical circuit connotations of DC which have uncertain biological relevance. The use of DC amplifiers and DC or nonpolarizing electrodes undoubtedly contributes heavily to our referring to the data these devices produce in cortical macroelectrode recording as DC potential. But one critical difference of the SP of cortex is that, although we have approximations toward an absolute reference in the spatial sense, we do not yet actually have one and we must content ourselves by combining a spatial relationship between two electrodes with a temporal criterion of their constancy, or, as the preferred terminology indicates, their steadiness over time. The lack of an absolute reference spatially is compensated by the temporal criterion of steadiness and by the production of relative SP shifts to environmental stimuli, allowing such shifts to be used as a dependent variable in the neurophysiological analysis of behavior.

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By using cortical surface electrodes referred against a ventricular, a periosteal, or a killed cortex electrode in many unanesthetized rabbits, O'Leary and Goldring[1] find the range of values to lie between 0.5 and 5 millivolts, surface positive; but higher values of 20 millivolts have been reported by Bures[2] and 30 millivolts by Aladzhalova.[3] Values of 5 to 7 millivolts are reported for human cortex. Despite the tenfold range among subjects reported by O'Leary and Goldring, the potential over hours is reported by them to vary not more than half a millivolt. Although this half millivolt value is small as viewed from establishing a stable recording circumstance, it represents the maximum value of most of the dynamic changes in SP, that is, the relative SP shifts seen in response to environmental stimuli.

Large SP shifts in the 5- to 10-millivolt range, which characterize spreading depression, and related smaller SP shifts in clearly identified ictal phenomena,[4] are arbitrarily excluded from this discussion because they represent dynamics generally considered pathologic. Although such large shifts may have much to tell us about neural function, they have not yet been reported in chronic implanted preparations exposed to stimuli in the physiologic range.

SOURCES AND INTERPRETATIONS OF SP As O'Leary and Goldring[1] emphasize, the surface macroelectrode records a mixture of many factors in the genesis of potential of possible glial, vascular, humoral, and metabolic, as well as neural, origin. Neurophysiological analyses have led them to infer, however, that the SP represents the potential gradient recorded across the thickness of cortex relevant to the radial orientation of apical dendrites.

The cytoarchitecture of mammalian cortex is thus seen as especially favorable to the study of SP. The concept of

cortex as a polarized sheet of nerve cells[5] is cited. However, recent studies[6] have demonstrated SP shifts from the surface of the pigeon brain, which lacks cortex and has an entirely different nonradial structure. These studies led to the conclusion that mammalian cortical structure with its radial orientation is not an essential prerequisite of SP dynamics.

A number of investigators, notably O'Leary and Goldring,[1] have shown SP shifts as longer-enduring summated potentials building out of the transients of evoked responses, direct cortical responses, and recruiting phenomena, making the neural (especially the postsynaptic) origin of the SP shifts a particularly inviting concept. Correlations with action potentials, however, have been studied only in an extremely limited way, and no over-all agreement has yet emerged. One approach has been to correlate the appearance of extracellularly recorded action potentials with spontaneous surface slow rhythmic activity in the one-cycle-per-second range in unconscious animals. Fromm and Bond[7] and Robertson[8] independently found surface positivity linked with appearance of action potentials, and surface negativity with reduction or absence. Cortical subsurface electroencephalagrams (EEGs) were not recorded, so that it is not known whether, in this situation, the surface negativity reflects positivity (hyperpolarization) in the soma region or local negativity of the dendrites. If the latter were the case, the dendrite activity would be reciprocally related to the cell bodies—their activation being inhibitory to the cells.

A third interpretation[9,10] assigns surface negativity to presynaptic fibers that ascend in the cortex. Surface positivity is assigned to postsynaptic dendritic influence. This concept arises from a formulation of two steady potential, oppositely oriented dipoles in cortex, with the pattern of the EEG being "the result of a regulated balance of [these] two oppositely directed D.C. potential sources oscillating normally around a medium level." It has been proposed,[11] on the basis of modulation of the direct cortical response by SP shifts and by observing some correlation between EEG and SP patterns in sleep and waking, that the EEG represents an oscillatory modulation of a single dipole represented by the SP. Our own experience in conditioning SP shifts has shown us as many, if not more, examples of independence of SP shift from EEG rhythms as dependence (Figure 1). Sufficient data do not yet exist from conscious, unrestrained animals to integrate and simplify our understanding of the mechanisms involved in these patterns.

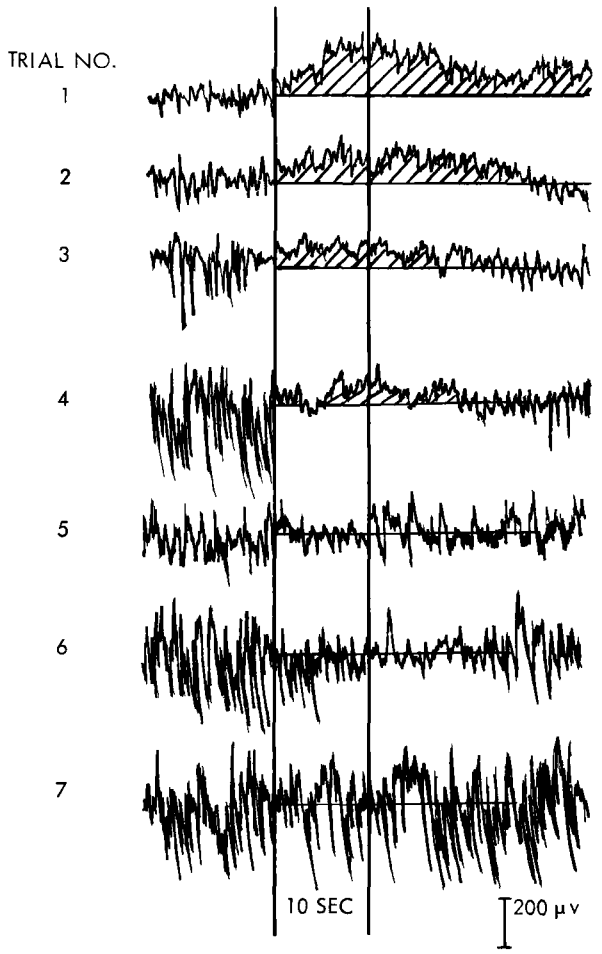


FIGURE 1 Progressive diminution in amplitude and duration of SP shift to light of moderate intensity, with simple repetition of 10-second trials applied during darkness and an average intertrial interval of 2 minutes. Records taken with implanted electrodes of 0.5 mm tip diameter placed between a .4 mm caudal to the anterior pole, 2 mm left of midline on rat cortex, and a reference 1 to 2 mm immediately subjacent. In trials 5 and 6 note the persistence of the transition to electrographic arousal without a concomitant SP shift. Positivity is up in this and all subsequent figgures.

SLOW POTENTIALS Both Fromm and Robertson refer to their surface recorded activity as "slow potentials." These differ from SP shifts because, even though their period is irregular, they are rhythmic or oscillatory, whereas SP shifts, as commonly studied in relation to stimuli, are not. For this reason, it is essential always to qualify spontaneous slow potentials as rhythmic and SP shifts as nonrhythmic.

Oscillations of an amplitude characteristic of the EEG range are rarely if ever seen slower than 1/2 to 1 cycle per second. The slow aftermaths of evoked responses are usually lost in the background SP in a half second or less, and repetition of them to provide summation and SP shift in the absence of any motivational situation (i.e., condition-

ing, cf. below) must usually be faster than two per second. This suggests that, although a differentiation of SP shift from very slow oscillation on the basis of duration must be somewhat arbitrary, a shift must endure 1/2 to 1 second or longer to be clearly an SP shift. This does not mean that its rise time cannot be abrupt, for it can be as fast as that of an evoked response. Perhaps when the underlying generators of SP and its shifts are clearly identified, this temporal definition will have to be modified, but it is useful at present.

Returning to action potentials and their correlations with SP shifts as distinguished from slow rhythmic potentials, we find no studies of the waking, unrestrained animal, indicating what these cortical relations may be in respect to SP shifts evoked by environmental stimuli. Caspers[11] has correlated rising activity of brain stem reticular units with cortical negative SP shifts as the implanted rat arouses from sleep. But the question that has yet to be answered is whether the SP shifts endogenously produced in any way modulate cell function.

Polarization studies

This leads to a second approach to the problem of SP shift and unit activity, in contrast to the correlational studies already mentioned. A number of investigators have applied low-level DC (this is a correct use of the abbreviation) to cortex with very definite modulation of unit potential firing patterns. It has been shown that the great enhancement of surface dendritic potentials with weak surface anodal polarization appeared not to affect the spike pattern of cortical cells until the current was tripled at the the surface, allowing transmission to invade the soma region directly.[12] Other investigators[13-16] have shown modulations with currents from 10^{-6} to 10^{-9} amperes, but the imposed current densities and voltage gradients are viewed by some as exceeding the range of endogenous potentials. Morrell (personal communication) suggests that the endogenous fields may well modulate activity at large numbers of synapses in a localized region. This vexing and complex problem of possible electrotonic coupling has been viewed[1,17] with interest because it may represent an important mechanism. I merely want to emphasize that variant interpretation is still lively, and even if SP shifts do not modulate cellular actions directly, they do indicate important chemical dynamics of longer time course and effect than those reflected by the rhythmic EEG. A number of polarization studies show effects long outlasting the polarization. Strumwasser,[13] applying a nanoampere through a microtip to a frog cerebellar cell, demonstrated a clear acceleration of action potential during a one-second polarizing stimulus. When this was repeated every few seconds, there developed over minutes a gradual change of the interpolarization firing pattern that he called entrainment. It consisted in the progressive and retained dropping out of potentials during the interval between polarizations. Morrell[14] has shown a number of applications of the enduring effects outlasting an applied low-level (10-microampere) anodal polarization. This technique has shown a confirmation of the so-called dominant focus for some associational mechanisms. If one so polarizes in a motor area and then adds an extraneous environmental stimulus, only the extremity whose cortical headquarters were polarized shows an electromyographic response, and this effect outlasts the polarization by some 20 minutes before fading away. Morrell has applied this technique to visual cortex, polarizing anodally at the surface and showing enhanced responsivity of subjacent cells to light flashes, the effect outlasting the polarization by many minutes and gradually fading. He has shown similar effects in enhancing the retention of an otherwise evanescent conditioned EEG rhythmic pattern. Cathodal polarization at the same level, on the other hand, produces a profound but reversible impairment of a visually discriminated shock avoidance performance.[18] A number of other reports show the preferential action potential driving with anodal direct current and inhibition with cathodal direct current. But none of these can yet be taken as evidence that the brain's endogenous SP shifts do the same thing.

The long-enduring aftereffects of low-level cortical anodal polarization are not clearly established as an exclusive property of applied direct current as contrasted with pulsed currents. However, they raise many interesting challenges concerning endogenous SP shifts. Are they related to phenomena that outlast the occurrence of the shift and could they represent some link between the millisecond phenomena of electrophysiology and the seconds, minutes, hours, and days of various degrees of experience retention? I know of no direct information on this problem, but shall present some behavioral studies that appear to bear on it indirectly.

Methods

The general method involves the chronic implantation in cat and rat cortex of silver–silver chloride, agar-bridge electrodes with long-term stability. The animals are then recorded in the waking, unrestrained state in a sound-resistant recording chamber with masking sound (background noise to obscure adventitious sounds). A reference electrode is placed in white matter, or on a cortical surface that has been found, on specific testing, to be unreactive. No region is taken as an inactive reference until it has been shown by test to make no contribution to an SP shift. The

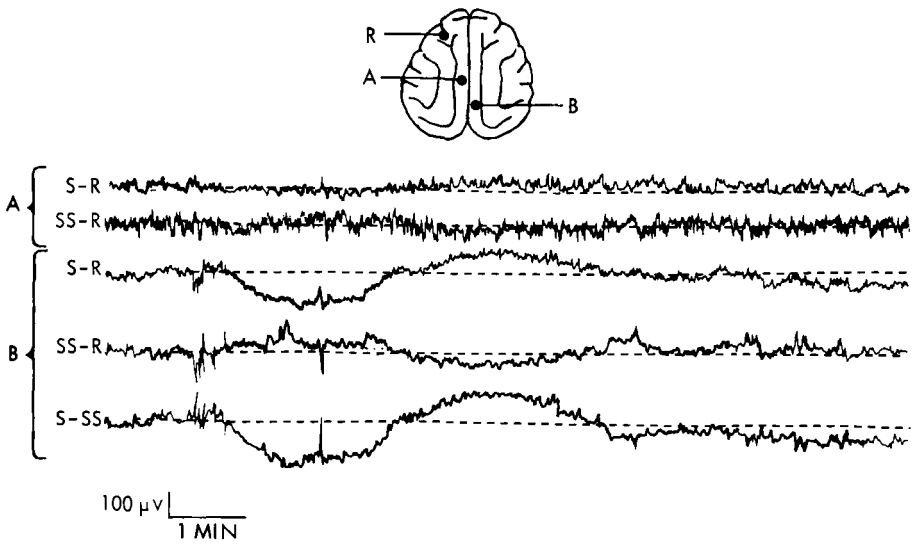


FIGURE 2 Mirror-image relations between surface (S) and subsurface (SS) leads at point B of cat cortex; minimal reactions are seen at point A during simultaneous recording. Amplitude summation is seen when common reference (R) is eliminated by recording S-SS at point B, which confirms that activity is not occurring at R.

As cat laps milk-fishmeal homogenate, surface-negative, depth-positive shifting takes place; after reaching maximum (as feeding ends) it returns to pretrial baseline. An inverse rebound takes place as animal settles down and licks chops. (Note slow time base.) Record is filtered to half-amplitude frequency response at 15 cps, and rhythmic activity is attenuated.

best method of recording is not always available in a chronic preparation, but it has been achieved on a number of occasions and demonstrates mirror-image SP shifts between cortical surface and a point 1 to 2 millimeters immediately subjacent (Figure 2). If we obtain a completely symmetric reflection between surface and subsurface leads, both recorded against the same remote reference, we do not need to exclude reference as a contributor to the shift. But usually the reflection is asymmetric and reference could be involved. By recording between the surface and subsurface derivations, we can exclude it and get the algebraic difference of the surface and subsurface contributions; that difference appears as an amplitude summation.

Transmission of retinal potential can be confused for cortical SP shift,[19] but in many instances it is readily distinguishable. We are now instituting continuous monitoring of retinal potential and usually find that the eye motions related to visual search are abrupt-step functions in contrast to the smooth ramps that characterize many of the SP shift responses. Furthermore, in visual cortical recording, lambda waves serve as useful markers for eye movements and enable us to observe the independence of SP shifts and at least those retinal potentials generated by saccadic (jerky) movements of the eyes.

Another source of cortical SP shift seems, at times, to arise from the movement of the head in space, suggesting the possibility of an influence from the labyrinths (Figure 3). This is presently under study from two points of view —one, to determine if it represents a true cortical response to labyrinthine influence and, two, to discriminate it from the SP shifts in which we are primarily interested. Posterior sigmoid response to rapid passive extensions of the head is produced by grasping the muzzle and moving the head from the horizontal to the vertical plane, holding it for the duration indicated by the subjacent line, and at the end restoring it to the horizontal plane. The tactile input alone does not produce this change. The subject shown in Figure 3 had eyes removed and cannulae implanted by direct vision on each eighth nerve. On the left side the cannula slipped away during cementation and was found at autopsy to have missed the nerve by 9 millimeters. Although it missed its target, it provided a control for the right side placement, which terminated directly on the nerve. Only when procaine was injected on the right side was there produced the profound (at 2 and 9 minutes) but reversible (after 20 minutes) removal of the head extension effect shown in Figure 3. This unilateral procedure produced nystagmus (when the subject had eyes) and loss of the righting reflex, which recovered in about the same

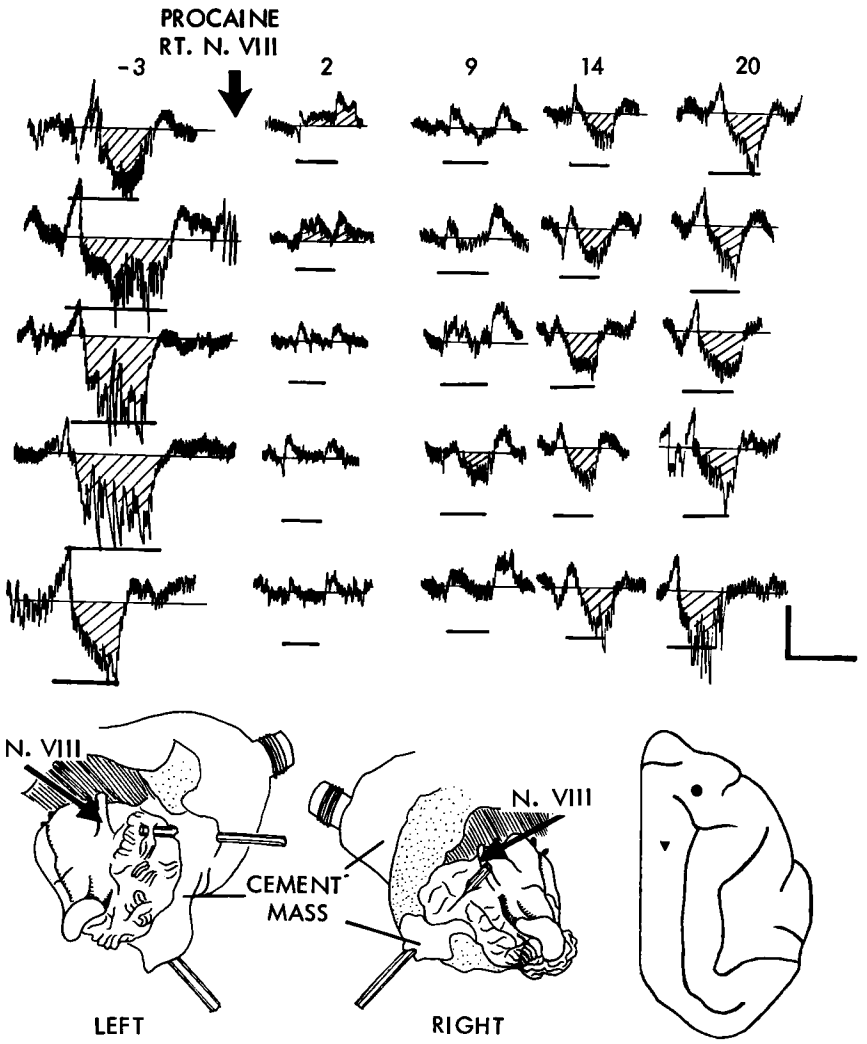


FIGURE 3 Reversible disappearence of steady potential shift (related to passive head extension) is produced by procainization of right N. VIII. Vertical columns represent consecutive trials that occurred at 3 minutes before and at 2,9,14, and 20 minutes after 0.5 cc of 1 per cent procaine had been injected through the right cannula. Dot in drawing (bottom right) indicates the stigmatic electrode; triangle is the reference in white matter. Calibrations: 100 microvolts, 1 second.

time course as the extension response depicted. The shortened durations of the extensions in the procaine period are not a significant stimulus variation in this test. Injection of normal saline to the right eighth nerve was without effect. The reversible removal of the approximately one-second shift from head extension by the eighth nerve block suggests that the shift is indeed related to labyrinth input.

In another study a cat was restrained in a horizontally rotating cylinder and trained to limit rotation by repeated bar-pressing (Figure 4), the press or release serving only to change the direction of rotation. We observed SP shift in the posterior suprasylvian gyrus by the surface-subsurface recording technique. The third channel in Figure 4 shows the swinging SP correlating with the timing of the press and release of the lever. This could represent a combination of visual and labyrinthine effects along with motor response, but has not yet been further analyzed. This last response looks like a violation of the concept that the SP shift shall not be found rhythmic; the concept should be modified to state that SP shifts shall not be found *spontaneously* rhythmic.

Despite the concern that head motions may contribute

to SP shifts, we often see smooth, continuous shifts of SP while the head is in a relatively stable, constant position, as when the cat is lapping milk, and, furthermore, SP shifts occur in the flaxedilized (chemically paralyzed, but conscious) preparation, as we[20] found some years ago and as has been observed by others.[21] Also, spontaneous head motions are often unaccompanied by a shift. The foregoing deals with some of the major technical problems, and I shall mention others as I proceed.

SP shifts in behavior

Moving now to some more psychological considerations, we can utilize the commonly applied but oversimplified concept that stimuli are conventionally divisible into nonreinforcing and reinforcing categories. Reinforcement usually refers either to reward or punishment, and induces some form of overt approach or avoidance behavior, respectively. Reinforcing stimuli involve, rather directly, activation of motivational systems.

Learning proceeds most readily, insofar as we can detect it in animals, when a stimulus of the former category, now called the conditional stimulus, is linked with one in the latter category, now called the unconditional stimulus. Our experience to date, along with that of others[21,22] shows that SP shifts to nonreinforcing stimuli—usually light, touch, and sound—are most likely to occur with novel presentation, and readily subside on subsequent repetition. That is, as shown in Figure 1, the shift appears to correlate with orienting response in the unrestrained animal and to subside with loss of novelty. On the other hand, our experience with reinforcing stimuli—food, aversive electrical stimulation, rewarding electrical stimulation, and sex—is consistent with the concept of nonsubsidence of shift with repetition, except as a function of drive and drive reduction. Here it is not a function of loss of novelty.

Furthermore, the pairing of a reinforcing stimulus with a nonreinforcing one will, in many instances, lead to the acquisition of an SP shift to the nonreinforcing signal, or conditional stimulus. It is therefore suggested that the SP shift is a significant neurophysiological criterion for defining so-called reinforcing stimuli. Perhaps this will be clearer with some examples.

SP Responses to Nonreinforcing Signals I have already shown an example (Figure 1) of subsidence of response to a moderate intensity light in the rat. Lickey and

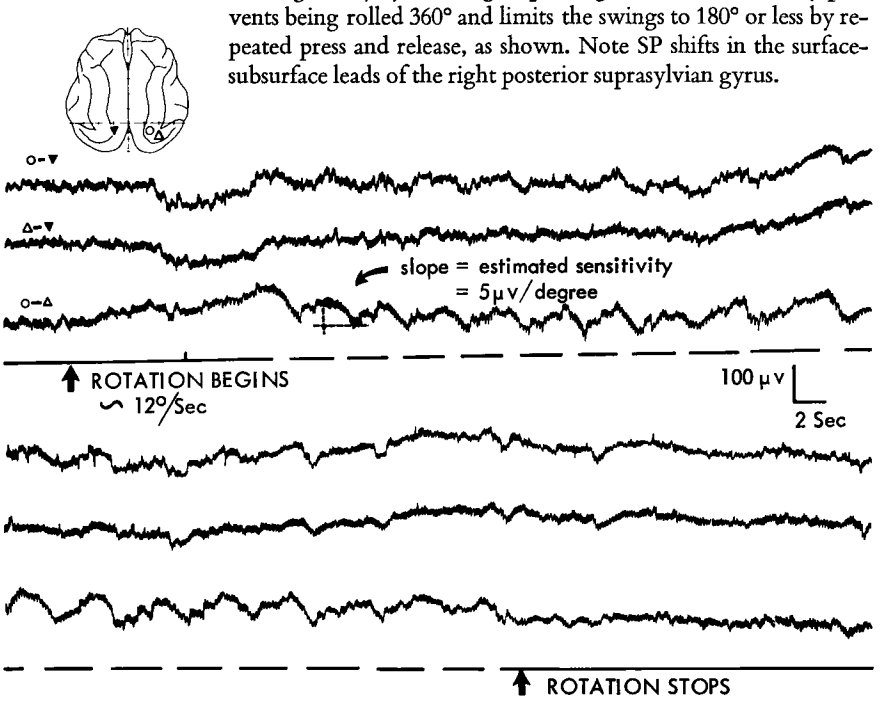


Figure 4 Rhythmic SP shifts related to cat's rotational acceleration around its own longitudinal axis, which is in a horizontal plane. Cat controls direction of rotation (away from or toward a vertical to the ground) by releasing or pressing the lever. Cat usually prevents being rolled 360° and limits the swings to 180° or less by repeated press and release, as shown. Note SP shifts in the surface-subsurface leads of the right posterior suprasylvian gyrus.

Fox[21] have observed SP response in the flaxedilized cat to all three modalities—touch, light, and sound—and, in addition to the loss of response to unreinforced signals, have observed an interesting set of relations to which they have given the name "primary negative rule." This means that the primary receiving area for one modality shifts to the negative when that modality is stimulated, and becomes positive or less negative when other modalities are stimulated. All three specific receiving areas of the cortex showed this effect. We have seen partial confirmations of this in the waking, unrestrained cat. But whatever the absolute or relative polarity of the shift, the unreinforced signal quickly failed to elicit the shift response on simple repetition.

SP responses to reinforcement

FOOD Turning to reinforcing stimuli: first, response to the cat's lapping of milk-fishmeal homogenate has shown sustained and large shifts in several animals. When the animal has been fed to satiety after a 24-hour deprivation of food and water, a magnitude of 2 to 3 millivolts has been reached at some loci. More usually the shifts are on the order of 100 to 500 microvolts, occurring in both positive and negative directions (Figure 5). The slope is greatest at onset, progressively declines with satiety, and begins to return to the pretrial baseline as the cat settles back from eating. If we divide the feeding into 25-gram portions (Figure 6), we see the shifts but in reduced amplitude and duration. At one locus (1st column, trial 7) a conspicuous positive rebound appears. When the animal, after satiation, sees the food but does not approach it, we see only a positive shift. This shift with lapping was totally new to us, but we discovered from Mary Brazier's[23] accounts that we had long been scooped by no less a pioneer than Richard Caton,[24] who, in 1875, gave a verbal description of SP shift with eating. He implanted in awake animals nonpolarizing electrodes leading to a galvanometer apparently sensitive enough to register brain waves. I know of no other report in the intervening 90 years.

If we cocainize the mouth, we can block the lapping-induced SP shift, showing that the motor side of this response is not itself responsible for the shift (Figure 7). The procedure, effective in three cats, slows down the rate of ingestion because the animals frequently interrupt their lapping to inspect the cup and surroundings. Taste and oral sensation are, of course, the feedback on consummatory behavior, but the cocainized cat must substitute vision. After all, how does it know if it is getting food back if it has lost its feedback? The block is reversible, as shown by the recovery at 30 minutes, and then the animal is sated. The slowing of the ingestion rate is not the cause of the loss of shift, as shown on two subsequent days, where the rate of presentation of the food was controlled to simulate the normal and protracted rates. The shifts were not blocked by the slower rates. In these runs (right column, Figure 7) only the superposed SP shifts are shown; the rhythmic electrocorticogram (ECG) is deleted. The 4 cubic centimeter per minute rate was considerably slower than the spontaneous rate of ingestion and equivalent to the rate under cocaine, but the shifts were clearly preserved. Intraperitoneal injection of cocaine in the same dosage used for oral cocainization was without effect in blocking the SP shift to lapping three minutes after injec-

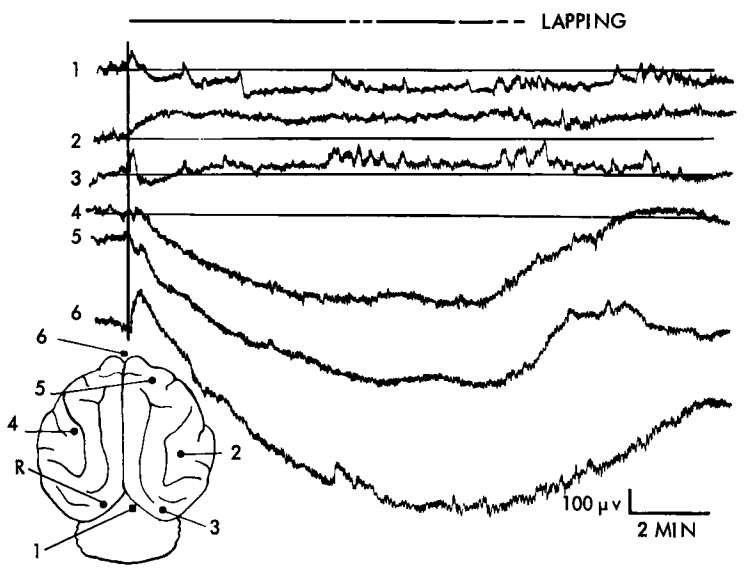


FIGURE 5 Sustained cortical SP shifting accompanying lapping of high-incentive evaporated milk–fishmeal homogenate by cat deprived of food and water for 24 hours. Total volume consumed: 266 cc (according to the pattern labeled *lapping*). Oppositely directed shifts (leads 1 and 2) testify to quiescence at reference. Marked shifts in leads 4 to 6 show progressive loss of slope from onset. Circles are pial implants; squares are surface implants in skull.

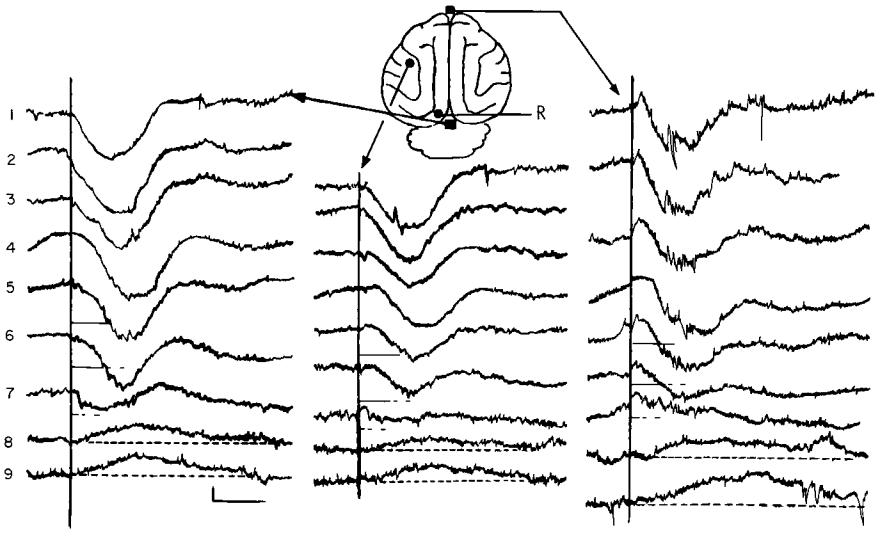


FIGURE 6 Responses of same subject as that in Fig. 5 to multiple, 25-g feedings. Simultaneous recordings from three sites show shifts as trials proceed in time from top down. Lapping pattern is the same for first 5 trials and is shown by horizontal line under fifth trial. Partial satiety resulting from intermittent feeding is shown in next two trials. Last two trials were accompanied by shift of opposite polarity. This occurred when sated animal responded by looking at food arriving in cup but not approaching it. Calibrations: 100 μv, 2 minutes.

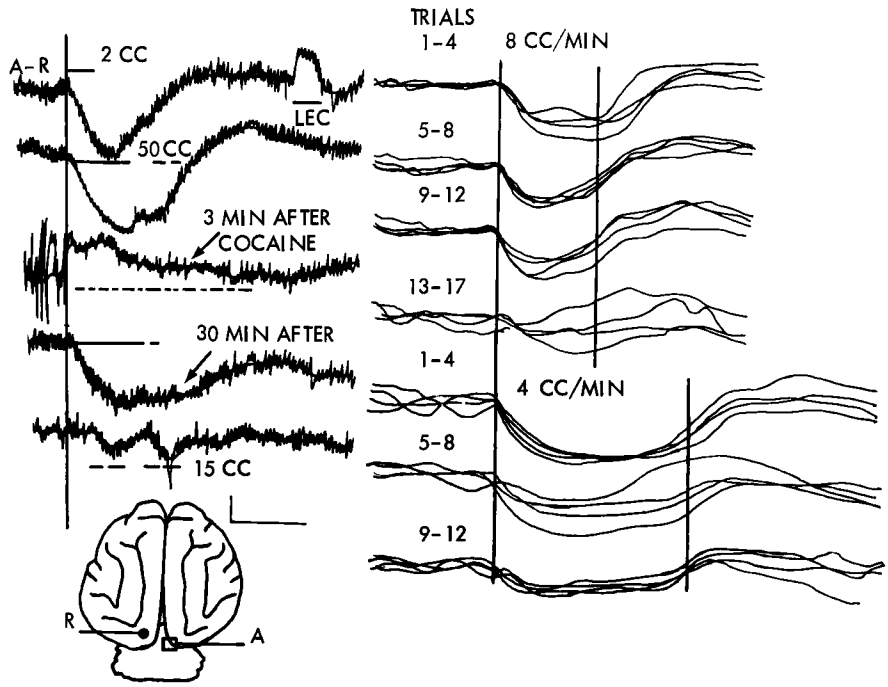


FIGURE 7 Left column: Serial feedings, as shown in Fig. 6, with oral cocainization three minutes before the third trial, produce loss of shift followed by lapping and restoration 30 minutes later. Top tracing shows large shift to very small (2 cc) feeding in the subject deprived of food for 24 hours. Bottom tracing shows smaller shift, accompanying lapping of 15 cc of food, an inverse effect attributed to partial satiety. LEC in first tracing shows positive-going shift as cat licks empty cup. Broken horizontal line under third tracing reflects intermittent lapping, caused by frequent interruptions for visual inspection of cup and surroundings.

Right column: Single-line reductions of steady potential shift are superimposed, showing all trials by groups on a single day under a controlled rate of food inflow – 8 cc per minute in the upper series and, on a subsequent day, 4 cc per minute in the lower series. Note independence of the shift amplitude from rate of food input, but the relation of the shift duration to rate of input. Note also loss of shift in late trials of each session. Calibrations: 100 μv, 2 minutes.

tion, thus eliminating central action of cocaine as a mechanism of the block. Swabbing of the mouth with other substances—water, saline, weak quinine—did not block the appearance of shift three minutes later. Thus it was the peripheral anesthetic action of the cocaine that was essential to this effect.

We can also see here the marked shift that took place when the animal was given a small (2 cc) feeding when it was very hungry following a 24-hour deprivation. The consumption of 7.5 times that amount of food when it was near satiety produced a much smaller shift. On the other runs of Figure 7, one can see the decaying shift response with satiety. We conclude, therefore, that the shift reflects an interaction of both drive and oral feedback. Other studies show that the shift is sensitive to the qualitative value of the food, i.e., the incentive. Because this is detected by oral sensation, we can relate it to the drive-oral feedback interaction. The SP shift response appears to build in a manner suggesting a regenerative feedback system; that is, each motor response, each lap, is immediately

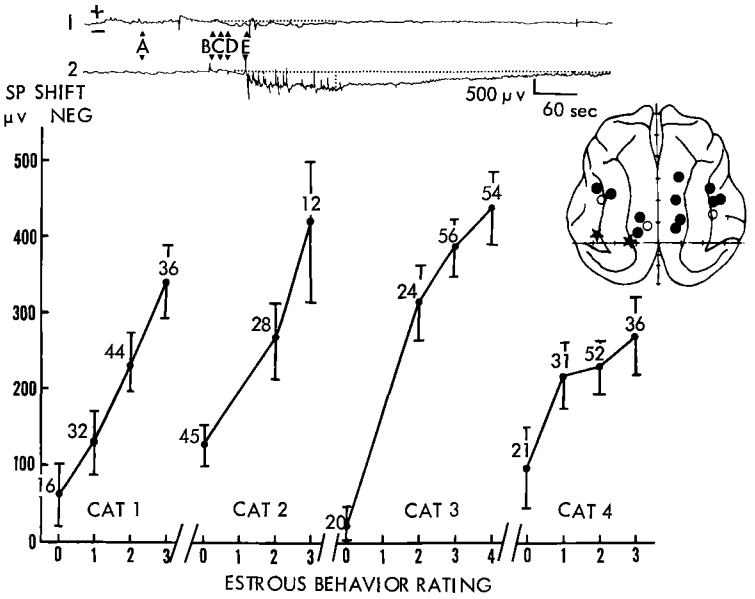


FIGURE 8 Variation of steady potential shift with estrous response to genital stimulation in four female cats. Tracings at 1 and 2 are filtered to one-half amplitude frequency response at 3 cycles/sec. Method of taking maximum amplitude measure is shown in the second tracing (dotted vertical). At 1, a small shift accompanies a behavioral response of magnitude 1. At 2, a 500 μv shift accompanies a behavioral response of magnitude 3. Letters indicate technique of opening cage door (A), grasping scruff (B), rubbing perineum (C), inserting glass rod in vagina with rhythmic stimulation (D), releasing cat and closing cage door (E).

Cortical points for all four cats are indicated on the brain schema, maximum response occurring at loci shown by stars, intermediate response by solid circles, and minimal response by open circles. The maximum amplitude of response for each site was measured, and the average of these for each trial was taken as the measure of cortical response for each cat. Number of trials for determination of each point on the curves is shown, with vertical lines representing two standard errors of the mean, above and below the mean.

associated with a high-incentive gustatory reinforcement, which triggers another lap. This is attended by a summating SP shift until the satiety mechanisms come in. Such a resonant relation between lap and taste is a compelling linkage that leads to invariant, maintained, and stereotyped behavior. If the animal has to substitute a more derived feedback path, such as vision, the lapping behavior is much more variable. A specific test of imposed conflict on the consummatory response has yet to be tried as a possible modulator of the SP shift to lapping.

The development of high-voltage, slow activity in the conventional ECG during lapping has been shown,[25] and we also have observed this in some of our animals. It is most likely to appear after the onset SP shift has been well established, and it sometimes appears to relate to eye closure during lapping. This is tentatively interpreted as a relative inhibition of cortex during a stereotyped behavior under subcortical control. Systematic studies have not been done on this interesting combination of patterns.

Our cumulative experience with lapping responses leads to a map derived from 12 animals in which no general trend is clearly established. One sees admixtures of both positive and negative shifts of varying amplitude; also, as one might expect, many sites show no shifts. The lack of localization is attributed to the complexity of the gustatory, drive, and consummatory mechanisms involved.

SEX Turning to a second motivational system, the sexual, we have found SP shifting to occur in scattered cortical sites in varying degree, correlating in amplitude and duration with the degree of sexual response as determined by a behavioral rating scale (Figure 8). Four female cats were made artificially estrous by estrogen injection and were followed over a six-month period through three or four such cycles. Estrous response was rated by assigning a zero value to warding-off behavior or no reaction during stimulation; a value of 1 to a response of tail deflection, presenting behavior, and treading; a value of 2 when, in addition to 1, the animal showed characteristic rubbing and rolled on its back at least one or two times in the so-called after-reaction period; a rating of 3 if the after reaction was accompanied by 3 to 9 rolls; and a rating of 4 for over 10 rolls.

Only one cat attained a 4 rating; the other three reached the level of 3. The four electrode positions on the cortex of each cat were randomly chosen, and the reactivity of the animals was sufficiently different to allow little generalization about more or less reactive cortical regions. Most responses occurred in the negative direction, and when the animals were anesthetized and artifically rolled, no shifts were produced, ruling out movement artifact.

The results in Figure 8 show that averaged maximal amplitudes of all sites, over many trials and during three or four cycles in and out of estrus, give a rising monotonic effect in correlation with the behavior. Other rhythmic stroking, along the side of the chest, did not evoke these

shifts, although when the animal was at a level 3 estrous behavior, or even sometimes lower, such stimulation would produce rubbing behavior, and smaller shifts could be observed in relation to this.

Here again, as postulated for the lapping of food, in which sensory input and motor response go into a resonant build-up of activation, it appears that hormonally induced sexual arousal leads to motor response that enhances specific stimulation; a regenerative cycle between behavioral output and sensory inputs leads to the stereotyped behavior characteristic of high motivation and an attendant neural correlate of the incrementing SP shift.

REWARDING HYPOTHALAMIC STIMULATION In addition to food and sex, hypothalamic electrical stimuli of both the reward and punishing types induces SP shifts. I believe it quite possible that early electrophysiological studies demonstrating cortical SP shift to subcortical electrical stimulation[26,27] may reflect the involvement of motivational systems. This becomes apparent when similar stimuli are used in the behavioral situation.

Figure 9 shows medial hypothalamic trains in the rat that the rat itself starts. Three rats were trained to self-stimulate when a cage light came on for short intervals. A faster time scale (at top of Figure 9) shows an immediate shift to the electrical stimulus and the latency of a second or so until the aortic blood pressure started to rise. This pressure record was added in order to determine whether the shift might be a trivial concomitant of recording electrode-tissue interface change with blood pressure alteration. Although brain blood pressure is not recorded in the figure, general systemic pressure rise and cardiac deceleration came in a second or two after the SP shift, and was evidence of independence of the SP shift from systemic hemodynamics.

Further, a series of self-stimulations produced a sustained SP shift, while the blood pressure showed an oscillatory pattern. Sharp dips in the ECG record are stimulus artifacts, which reversed in direction on polarity reversal of the stimulus. The baseline shift polarity remained unchanged. An interesting hypotensive rebound occurred after the animal discovered that it was no longer being reinforced, but the return of the SP shift followed an independent time course. The shift response to the "light on" stimulus is an acquired one and reflects conditioning.

PUNISHING HYPOTHALAMIC STIMULATION One animal, with an extreme lateral hypothalamic placement,

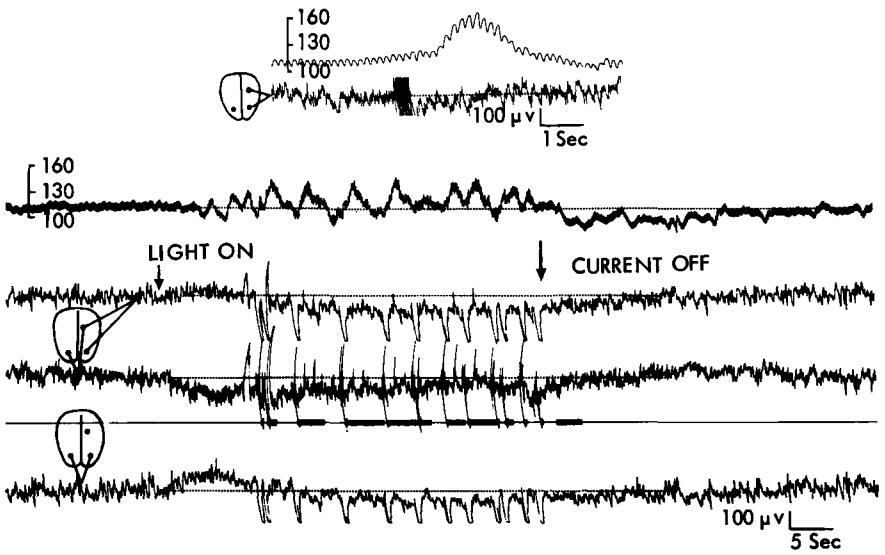


FIGURE 9 Rat blood pressure and cortical steady potential shift response to intracranial self-stimulation at medial hypothalamus. Top two tracings show immediate steady potential shift to the electric stimulus and a delayed bradycardia and pressor response recorded from the abdominal aorta. Lower four traces were recorded simultaneously on a slower time base and show oscillatory pressor response to a series of self-stimulations delivered only at the onset of each bar press (duration of bar press is indicated by length of black rectangle on signal line). "Light on" causes rat to approach bar and "current off" leads to cessation of response. Note sustained steady potential shift despite oscillatory blood-pressure pattern.

showed only aversive response to stimulation applied under a wide variety of parametric conditions (Figure 10). Using John Lilly's[28] terminology, we were clearly in a stop system rather than a start system, as shown in the preceding study.

In responses to a wide variety of electrical parameters, the animal showed only aversion and would stop the stimulus with a latency that was a function of the energy in the stimulus. As he responded faster, the onset slope of the SP shift became greater. This again suggests that (at least for punishing reinforcement) the greater the urgency demonstrated behaviorally, the more apparent the SP shift in cortical records.

Conditioning

Turning now to conditioning, we find that coupling a reinforcing stimulus with a nonreinforcing signal produces an acquisition of an SP shift to the formerly inactive nonreinforcing signal.

We have shown that cortical steady potential shifts are conditionable in the unrestrained animal by coupling food reinforced stimuli to light or sound signals.[29] Many cortical loci did not shift, but those that did were clearly seen to be modulated by the drive state of the animal. The conditional shift diminished as the animal approached satiety and was enhanced when the animal was hungry. The shift reduction was a function of the food ingested and not the number of trials. Thus the shifts to an environmental signal that quickly disappeared on simple repetition were reestablished to that signal when it was paired with a reinforcement. Discrimination reversal and modulation by drive level constituted controls for the possibility that the reacquisition of shift to a nonreinforcing signal was nonspecific.

More recently, we have begun a comparison of opposite

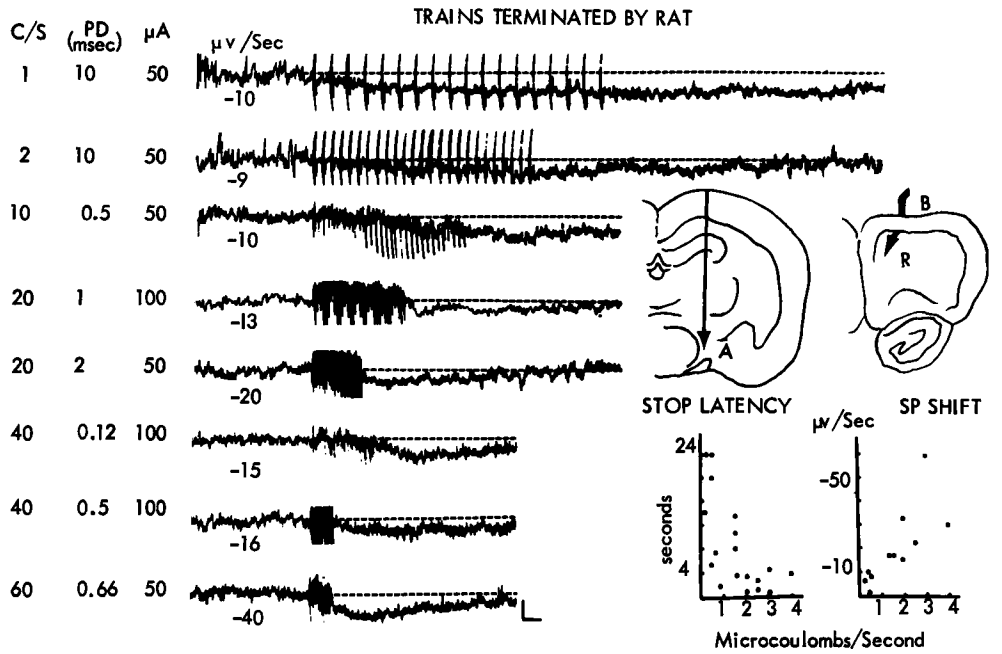


FIGURE 10 Inverse relation between stop latency and slope of steady potential shift to lateral hypothalamic electrical stimulation started by the experimenter and terminated by the rat. Representative samples of the electrographic responses and the accompanying stimulus patterns are shown with the onset slope indicated in μv/sec (determined by the time required to reach the maximum amplitude of steady potential shift). Table at left indicates frequency (C/S), pulse duration (PD), and current (μA) used for the associated tracing. Point A, in sketch, right, shows terminus of stimulating electrode, 1 mm above optic tract in region of extreme lateral hypothalamus. No overt behavioral response to any stimulus condition was observed to result from internal capsule stimulation. Recording electrode on pial surface at B referred to a penetrating white matter electrode (R). Plot, below left, shows declining tolerance (stop latency) of stimulus as a function of increasing energy (microcoulombs/second) calculated from parameters shown at left. Right plot shows increasing onset negativity in steady potential shift (μv/sec) as a function of the energy in the stimulus input. Points on the plot represent averages of three trials. Calibrations: 100 μv, 1 second.

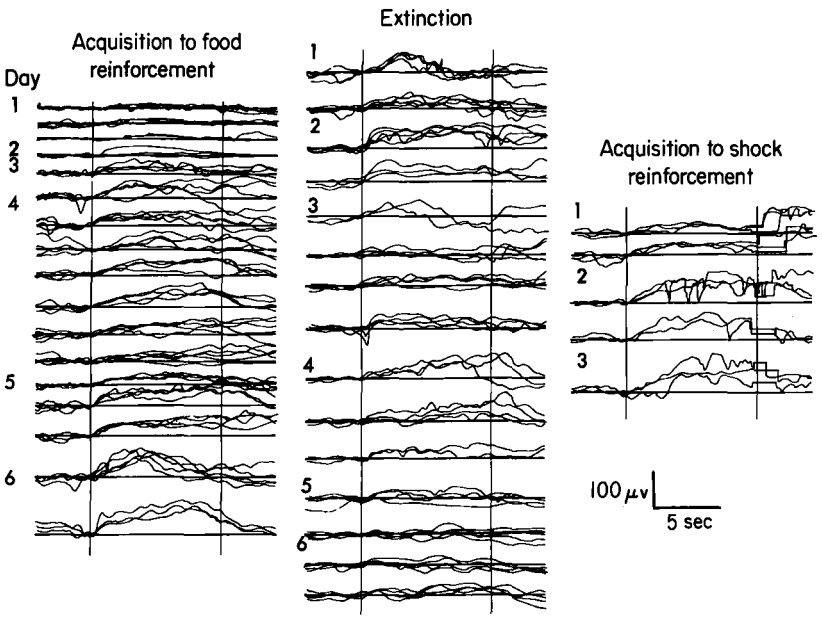


FIGURE 11 Comparison of food and pain reinforcement on conditioned steady potential shift to a light flash signal delivered for 10 seconds, as shown by vertical lines. Each column of tracings shows separate period of training. Days within each period are indicated by numbers. Calibrations: 100μv, 5 seconds.

kinds of reinforcement to the same signal at different training periods in the animal's history. Figure 11 shows superimposed baseline traces (the accompanying ECG removed) for acquisition to a 1.2-per-second light flash of 10 seconds' duration. Food reinforcement was presented at the end of the 10-second interval. Marked positive-going elevations in a posterior suprasylvian subsurface lead were acquired from the third to the sixth training day. The pattern of rapid rise followed by decline in the first set of trials of the sixth day was associated with, respectively, orienting to the signal, approaching the cup, and, as the potential began to diminish, the cat licking the empty cup. In the second set of trials, the peaking of the response appeared later in association with a more leisurely approach to the cup and increasing satiety. During the next six days, acute and chronic extinction took place while both the signal and food were presented, but without close temporal relationship. Acquisition to aversive shock reinforcement of 3 milliamperes to subcutaneous coils implanted under the scalp was more rapid, polarity of the shift was unchanged, but amplitude and time course were somewhat different. In this second training period, the animal retreated from the light source, and the numerous changes in SP were related to slow head turning and cringing behavior. They are not movement artifacts, for many motions of greater magnitude between trials are unattended by such shifts. Our present hypothesis concerning them is that the animal's reaction related to avoidance may temporarily lower the level of activation, but when such motion is ineffective in terminating the stimulus, the higher activation level is restored. Figure 12 presents samples from this study of related findings in the same animal.

Evidence is available to show that steady potential shifts can be acquired relevant to offset of a signal and be maintained over many seconds of no specific signal input in relation to the animal's expectation of food reinforcement. Figure 13 shows, at the upper left, the last trial of a two-per-second click of 10 seconds duration to which a stable visual cortical steady potential shift has been demonstrated. It is the first time the animal has been put into the unusual extinction situation of the clicking continuing without interruption. This elicits a momentary response of the cat licking the empty cup (LEC). The left column then shows a series (every other trial) of presentations of unreinforced 10-second interruptions of the click to which there is no generalization either behaviorally or in the SP shift. Brief transient shifts appear related to orienting. Lambda activity is present without relevant shifts, a control eliminating retinal potential as an origin of shift. The right-hand column, after full acquisition to reinforced trials of 10 seconds of silence, shows shifts like those shown to the inverse stimulus situation and subsiding with satiety (trials 23 and 25). These

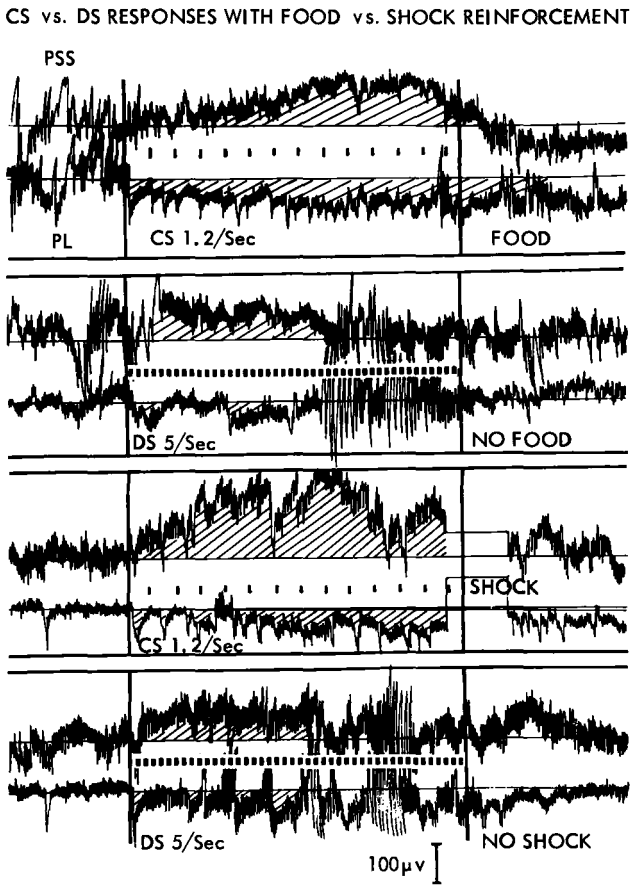


FIGURE 12 Details of the electrographic responses from the two acquisition periods of Fig. 11 from a posterior suprasylvian (PSS) subsurface lead and a posterior lateral (PL) surface lead, both recorded against the same reference. CS-UCS interval: 10 seconds. CS and DS are light flashes at the frequencies indicated. The top pair of records shows negative shifting at a posterior lateral (PL) surface placement recorded against the same reference used for the posterior suprasylvian (PSS) lead summarized in Fig. 11. PL lead shows local differences in time course and polarity of the shifts from PSS and, in addition, superimposed lambda activity, which serves as an indicator of independence of the shift from retinal potentials generated by eye motion.

The second pair of tracings shows the response to a discriminated nonreinforced 5/sec flash, which is intrinsically more activating, but to which all generalized approach to the bar had subsided. It characteristically showed an accompanying slight shifting to the first half of the signal, but a marked inhibitory spindling in 90 per cent of its applications during the second half. This two-phase pattern was interpreted as an intitial activation with orienting and generalization followed by a countervailing inhibition relevant to discrimination.

The lower two pairs show the CS and DS conditions during the flash pairing with shock, in which similar effects are seen.

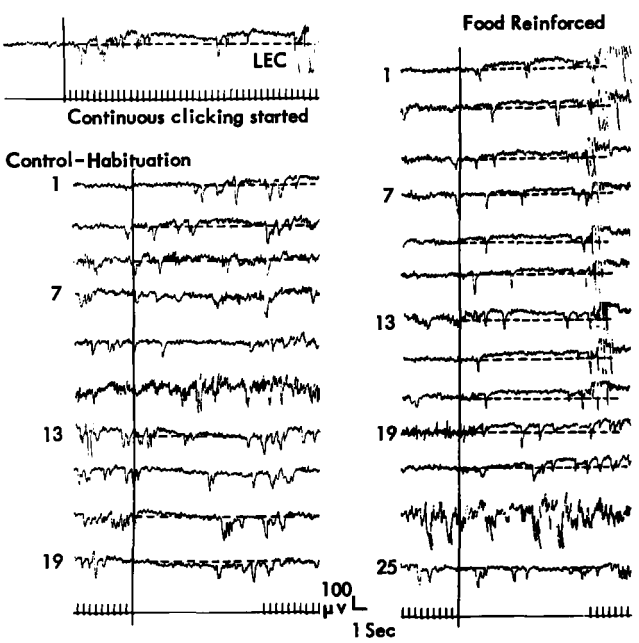


FIGURE 13 Independence of steady potential shift from a sustained environmental stimulus. Top left tracing shows transition from a conditional stimulus or 2-per-second clicks to extinction. The SP shift was that characteristically acquired by this animal in visual cortex. The left column shows every other trial during introduction of 10 seconds of silence into otherwise continuous clicking without reinforcement. Only a few brief shifts are observed and there is no indication of accompanying behavioral generalization to the inverted stimulus conditions. Right column shows a similar sampling of responses after two weeks of training with good acquisition of shift to silence with hunger, subsidence with satiety. Downward deflections and negative spikes appear with each movement of the eyes, and indicate independence of the steady potential from eye movement.

represent, then, independence of sustained input to a specific system.[30] It is presumed that the shift is generated by mechanisms of arousal or activation that are drive-relevant and are related to the anticipatory behavior of the animal independent of sustained specific environmental cuing. The reticular activating and limbic systems would appear to be of primary interest in this respect.

Summary

SP shift represents cortical activation of greater degree than the electrocorticographic transition from high-voltage slow to low-voltage fast activity.

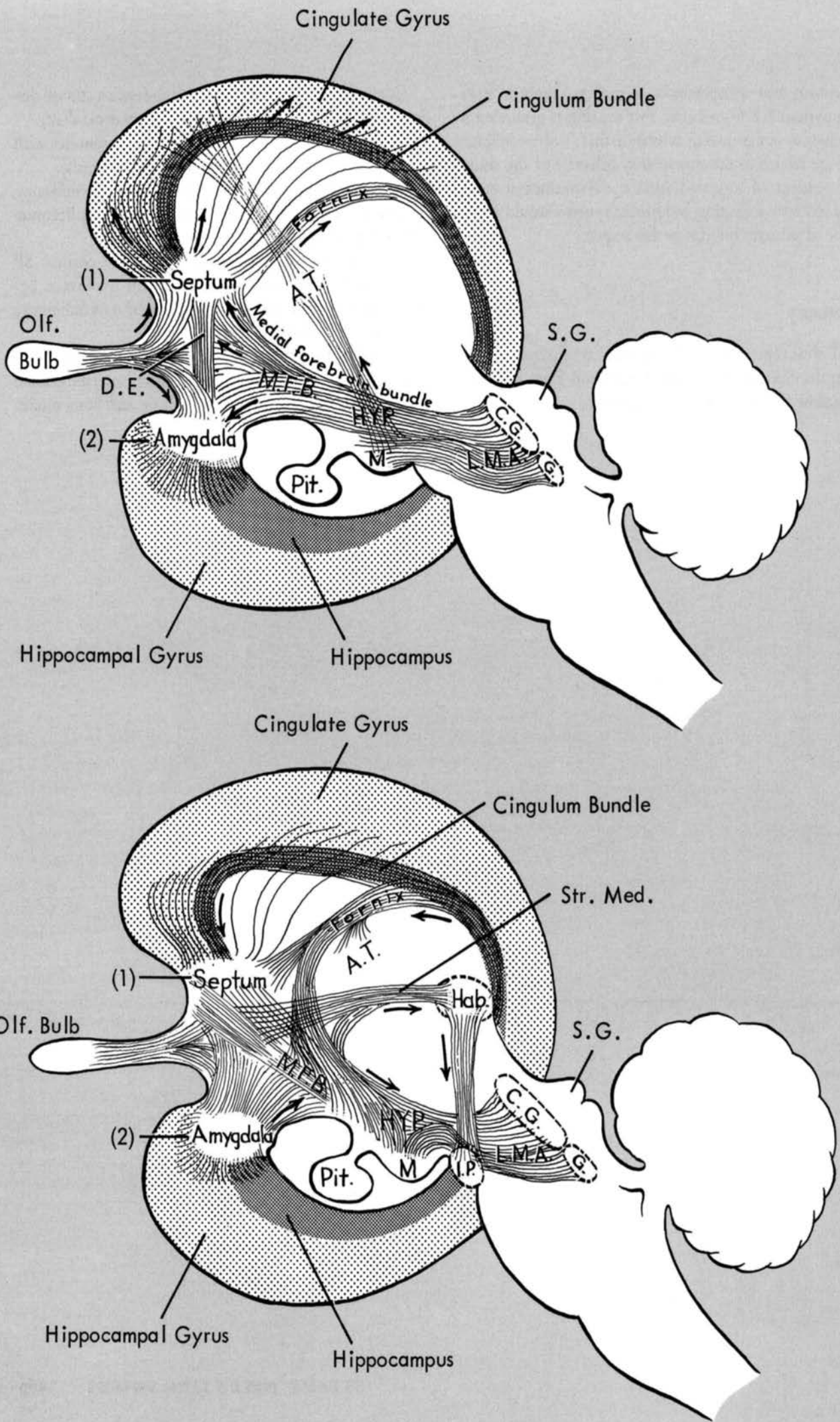
Cortical SP shift response to nonreinforcing stimuli disappears with loss of novelty (information dependent).

Cortical SP shift to reinforcing stimuli diminishes with drive-reduction (dependent upon biological needs).

In learning, the lost SP shift response to a nonreinforcing stimulus is acquired in some cortical loci and becomes drive-dependent.

In initial studies, polarity of conditional cortical SP shift is independent of approach versus avoidance behavior during the conditional signal, but shows differences in time course and amplitude.

SP shift is most likely to occur under conditions of innate or acquired response urgency in relation to the general mechanisms subserving drive-reduction and homeostasis.


Cingulate Gyrus
Cingulum Bundle
Fornix
(1) Septum
A.T.
Olf. Bulb
Medial forebrain bundle
S.G.
D.E.
M.F.B.
H.Y.P.
C.G.
(2) Amygdala
M
L.M.A.
G.
Pit.
Hippocampal Gyrus
Hippocampus
Cingulate Gyrus
Cingulum Bundle
Str. Med.
Fornix
A.T.
(1) Septum
Hab.
Olf. Bulb
S.G.
M.F.B.
C.G.
H.Y.P.
(2) Amygdala
Pit.
M
I.P.
L.M.A.
G.
Hippocampal Gyrus
Hippocampus

BRAIN CORRELATES OF FUNCTIONAL BEHAVIORAL STATES

"Feelings provide the 'go/no go' switch for all behavior." LIVINGSTON, PAGE 505. *The limbic system, here illustrated with a diagram from P. D. MacLean, is believed to contain mechanisms involved in the switching from one major type of behavior to another,* PAGE 506.

INTRODUCTION

ROBERT B. LIVINGSTON, M.D.

Brain Circuitry Relating to Complex Behavior

WE CAN BE certain of two things: none of our direct ancestors died before reaching reproductive age, and all of them succeeded in reproducing something.

The continuity of replicating organisms, for which we stand as biological witness, goes back beyond the oldest civilization, beyond the most ancient kingdom, beyond domestication of animals, beyond tree-climbings and ground-gropings, to beyond the time when some creatures slithered out upon the mud flats, bailing this chancy atmosphere in and out of their new-found lungs. Our survivorship goes back all the way to a cluster of fateful catalysts in some primeval sea.

During these few billion years, an increasingly complex and diverse accumulation of capabilities has emerged as a result of slight incremental innovations, heated in the slow fire of protoplasm and hammered out on the anvil of evolution in the compelling test of survival. The grand aggregate, achieved gradually over this long period, provides a legacy of ready-made apparatus that is capable of a wide variety of goal-seeking actions. Successful biological mechanisms are inherent in cellular and intercellular systems of organization; they are inherent in the chassis of surviving individuals. These patterns for survival are

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abundantly safeguarded, at molecular, cellular, and behavioral levels of organization, for their transference to succeeding generations. During billions of years, there has never been a failure among our direct ancestors at any of these levels of organization.

Some goal-seeking systems at the molecular level can be identified by physical-chemical techniques. Other goal-seeking systems at the level of brain circuitry can be identified by neurophysiological techniques. At each level, parts of these systems are concerned with the appetites and satisfactions that govern behavior. All of these goal-seeking systems originate in and are intrinsic to protoplasmic materials. Many such systems are peculiarly specialized and are located in particular nervous and endocrine systems. Evolutionarily elaborate organisms possess appetites and satisfactions, not only to fulfill vegetative needs; not simply for the obligate cooperations required for sexual union, the rearing of young, and the safeguarding of food, family, and territory; not just for the adaptive behaviors essential to meet successfully the vicissitudes of environmental change; but also for extra energies, strivings, and outreachings—the extravagances that go beyond mere survival.

Feelings belong to the center stage of the nervous system. Feelings shape all experience and behavior. Feelings are related to seeking and fulfilling appetites; they are also related to positive joy, to internal satisfactions springing from the discovery of novelty, from creative expression and constructive adaptation, including the scientist's singular esthetic satisfaction derived from appreciating the symmetry, simplicity, order, and beauty of nature. The central objective in the fourth section of this book is to depict the fundamental, underlying biological processes governing sleep and wakefulness, feeling, appetite, satisfaction, and consciousness, and to depict the dynamic organization of brain circuitry essential for learning and memory.

Neuroscientists are drawn to this field both by interests attaching to the biological material itself and by a desire to learn more about ourselves as *human beings*. Our interests are quickened by the unrealized but realizable potentialities of mankind, by the awkwardness and tenuousness of the human experiment. What, indeed, are human limits? Human opportunities? What might be the opportunities if only we knew more about perception, judgment, and action? What might become of man if only he could discover more about learning and memory?

Conscious awareness of man's full potentialities would, more rapidly and certainly than anything else that science can offer, facilitate the creation of a more hospitable world. The brain is a product of evolution, just as are teeth and claws; but we can expect much more of the brain because of its capacities for constructive adaptation. Neuroscientists can take as their long-range objective the understanding of the fullest potentialities of mankind in order to help humanity become more fully self-aware and to illuminate man's nobler options. Above all, it is the human brain, with its capacities for memory, learning, communication, imagination, creativity, and the powers of self-awareness, that distinguishes humanity.

We are engaged in activities that hold great promise for the future expansion of human consciousness and conscience. We live in a society indulgent to science, at a moment of urgent, worldwide need for useful insights and applications stemming from our professional role. Whether we like it or not, we are involved in public administration. Herrick wrote, "Man's capacity for intelligently directed self-development confers upon him the ability to determine the pattern of his culture and so to shape the course of human evolution in directions of his own choice. This ability, which no other animals have, is man's most distinctive characteristic, and it is perhaps the most significant fact known to science." President Kennedy, with his flair for memorable contrast, declared in his campaign that "ours will be known as the generation that turned this planet into a flaming pyre or that safeguarded succeeding generations from the scourge of war"; and, in his Inaugural Address, that "... man holds in his mortal hands the power to abolish all forms of human poverty and all forms of human life." For prior generations no equivalent alternatives were available for either benefit or disaster. For our generation these choices are scarcely imaginable in their full import.

It remains for us to do what we can. Professor Schmitt has made the clarion call for expeditious advancement of all fields of knowledge of brain and behavior. The Neurosciences Research Program is interested not only in the biological substrates of higher nervous system process, but, indeed, in all means by which knowledge in this area will permit men to embrace more constructive options and to realize fuller potentialities in ways heretofore inconceivable because of our incomplete understanding of brain mechanisms.

We must bear in mind that the brain is simultaneously the integrater of physiological, psychological, and social processes. Cross-cultural misunderstandings are ofter deeply rooted in inadequate cultural presuppositions concerning human perception, judgment, and action. Some of these presuppositions are already known to be unlikely or entirely wrong. Solutions to problems of ill health and achievements of insight into molecular and individual integrative processes cannot provide the outer limits of our interests: we need urgently to comprehend all of the fundamental processes underlying human social behavior. There is "a clear and present danger" that ignorance of

these processes may encourage continuation of the kinds of primitive behaviors that at any time now may lead to premature termination of the experiment of being human.

Nervous systems are built for actions

Life is, above all, a wellspring for actions. The nervous system at birth manifests considerable innate behavior that is largely governed from the brain stem level. Thus, an anencephalic monster (Figure 1) is nearly as well equipped as a newborn babe with mechanisms for visceral survival.

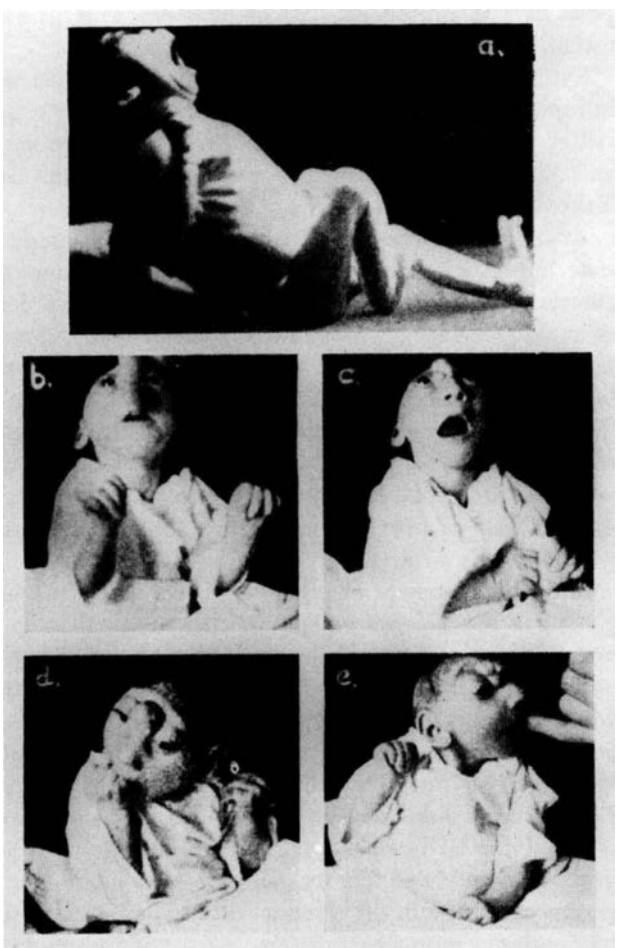


FIGURE 1 Innate behavior exhibited by Gamper's mesencephalic infant. The child had no cortex or basal ganglia. The brain stem and cerebellum were considered intact. Reflex sitting (not illustrated) was accomplished when light pressure was applied to the legs. a: Yawning with spreading of arms. b: Oral, visual head and arm reflex following of finger touching upper lip. c: Coordinated gaze and lip smacking when finger removed. d: Spontaneous sucking of fist. e: Oral, visual, head and tonic neck reflexes of trunk and arms exhibited when finger touches outside corner of mouth. (From Gamper, Note 41)

He possesses rooting reflexes that enable him to grasp and hold the breast and to suckle. His respiratory and cardiovascular controls are quite competent. His temperature control mechanisms are adequate for sheltered survival. Infants premature by a few weeks are manifestly less competent. But the newborn's nervous system possesses more generalized capabilities than are reflected in these individual housekeeping actions and their feedback loops. Rooting, suckling, swallowing, cardiovascular, respiratory, and thermal regulations are all interdependently integrated. These, in turn, are intermittently subordinated to waking, sleeping, laughing, crying, vomiting, defecating, and other activities. The nervous system establishes an overall unity of performance for the individual; it orchestrates the entire muscular and glandular apparatus and provides coherence among all potentialities for action; it magnificently subordinates parts for the purposes of the whole.

The nervous system is a source for activity as well as integration. The brain is not merely *reactive* to outside stimuli; it is itself spontaneously active. When we analyze the learning paradigm, for example, we tend to emphasize stimulus leading to response (S → R). We are inclined by this schema to fix our attention on the evoked nature of the process, on the conditional stimulus (CS), and the unconditional stimulus (UCS), which, when applied appropriately, yield a conditional response (CR). We must remind ourselves that these processes occur within a larger framework. Certain conditions are prerequisite before any stimuli can lead to learning. A stimulus (CS or UCS) becomes important only when the nervous system is properly oriented and appropriately receptive toward the stimulus.

Brain cell activity begins in embryonic life and probably contributes to organizational development. Brain development occurs most rapidly prior to birth and for a few months thereafter. Following this period of exuberant growth, the rate of development decreases markedly; yet, even in the adult, there is no point beyond which development ceases, beyond which the capacities for reorganization following disease or injury disappear.

The human brain comprises about two per cent of body weight, but it consumes twenty per cent of the body's oxygen at rest.[1] The brain consumes oxygen at a rate comparable to that of active muscle. Active muscle can sustain such a rate of oxygen consumption for only a short period, but the nervous system continues its high rate for a lifetime, awake or asleep, from before birth until death.

Professor Schmitt has stated that beginning shortly after birth, nerve cells rarely replicate, and has suggested that this may result from an absence or defect of the reductase mechanism for replicating DNA. Cessation of cellular replication during early infancy may represent a teleologi-

cal advantage. Replication of nerve cells after the early postnatal period of rapid development could be embarrassing. It would be accompanied by loss of whatever experiential advantages had already been acquired. Conel's work shows that during the first few months of life brain circuits are modified in optically noticeable cellular details.[2] Assuming that individual experiences might modify some of these growth processes, if cells were to replicate after this period there would need to be some mechanism for endowing succeeding cellular generations with idiosyncratic experiential acquisitions. These are not presumed to be introduced into the nuclear DNA, and therefore they would be lost to any replicated cells. Moreover, during the period of rapid growth, local dynamic influences of differentiating and growing parts on one another would presumably be unreproducible. Thus, there may be a sound basis in brain circuitry for the disappearance or defect of the reductase mechanism for replicating DNA.

Actions are directed toward goals

Spontaneous electrical activity of brain cells can be detected via electrodes implanted into brain regions and into nerve cells in tissue culture. Any modest amount of nervous tissue possesses activity, whether within or outside the brain. There is an interdependence between neurons and glia: pure cultures of glia have been obtained, but culture of neurons has been unsuccessful when glia are absent from the explant. Nerve cells continue to be biologically and electrically active in tissue culture.[3] They show nuclear rotation, protoplasmic movements, axonal flow, and marvelously dynamic growth cones. Growth cones, as they extend a long, fibrous tendril outward, display a fringe of mobile membranes, waving like the veils of a slow dancer. These motile membranes at the growing tip apparently have selective chemical affinity toward or repulsion from making attachments to certain other cells. Glia wander over nerve cells and from one nerve cell to another, engulfing materials with their fibrillary and fanlike undulating membranes. Some glia show rhythmic contractile pulsations as well as general motility. Myelinization of nerve fibers has been seen in tissue culture to be contributed by glia.

During development of the nervous system, primordial motor cells migrate from the central canal earlier than do sensory cells. Motoneurons extend processes out to the body wall to establish contact with muscle cells and to provide functional transmission prior to the development of the sensory half of the reflex arc.[4] This implies again that the nervous system is built for action prior to reaction. For the duration of life, patterns of action are generated and controlled according to the internal state of the neuraxis, and are only modulated by sensory input.

Motor systems involving what Yakovlev calls *visceration* (Figure 2[5]) are continually active. We can readily appreciate the interdependence of sensory and motor coordinations in these regulatory activities. A priority of physiological goals involves control of respiration, temperature regulation, water balance, nutrition, provision of sleep—in that order—and other goals relating to sex, esthetics, curiosity, novelty, and freedom (absence of restraint). Unmet needs challenge the priorities of all other goals. At any instant, our behavior exhibits the result of a flux of purposes held in provisional balance by social patterns for eating, sleeping, work, recreation, and privacy.

During the waking state, motor mechanisms are active in *expression* of bodily attitudes—gestures, pupillary dilatation, perspiration, vasomotor and respiratory patterns, and other means for exhibiting mood and emotion.[5] As Yakovlev pointed out, the word emotion comes from *e-* or *ex-*motion, motion out from and reflecting the internal state. Indeed, it is difficult to mask our real feelings, moods, emotions. We must resort to deliberate subterfuge in order to avoid revealing expressions that coincide with our actual internal state. It takes a consummate actor to distort his facies and alter his bodily attitudes of expression sufficiently to provide verisimilitude. It may be that the best actors learn not only how to imitate well, but also how voluntarily to evoke in themselves a true change of internal state that will provide a valid source for the mood and emotion to be displayed.

In the development of other expressive skills, such as playing a musical instrument, practice commands improvement of perceptual habits, acoustic and proprioceptive, as well as motor habits. Perceptual habits against which the immediate performance is measured are indispensible for the controlled release of musically adequate sequential patterns of body, limb, and finger performance. Both perceptual skill development and motor training are required for all learned motor coordinations, whereas genetically endowed sensory and motor integrations accommodate most of the activities of visceration. Sensory rehearsal and motor performance are equally important and form a composite skill. As Bartlett wrote, "The common belief that 'practice makes perfect' is not true. It is practice *the results of which are known* that makes perfect."[6] Sensory rehearsal yields an increasingly efficient access and control between sensory input and motor performance.

We have long known that our motor systems are organized toward goal-seeking performances of visceration, expression, and effectuation. It is now becoming increasingly clear that our sensory systems are similarly goal seeking in their organization. Perception, as well as motor

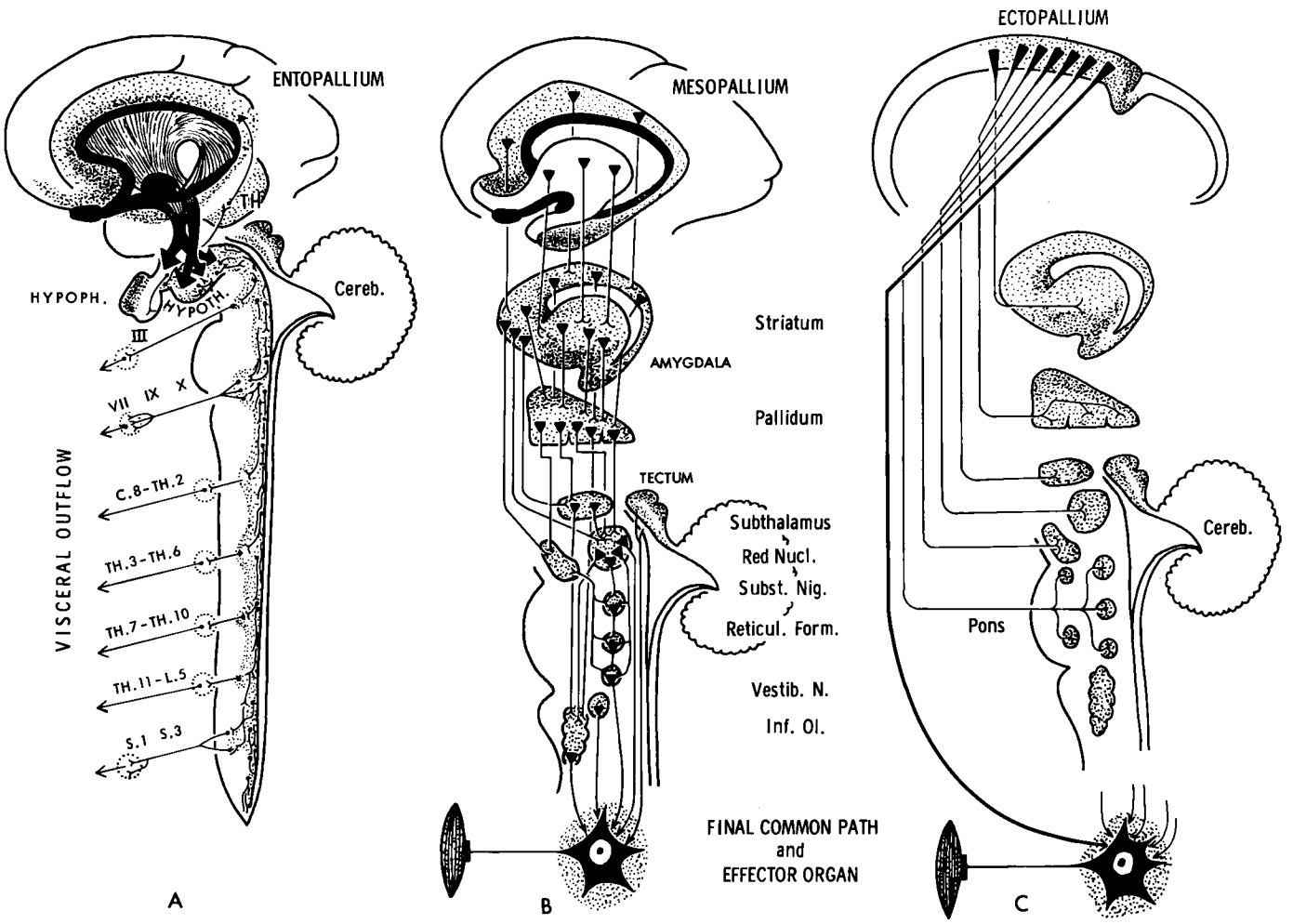


FIGURE 2 Yakovlev's motor system schemata for *visceration* (A), *expression* (B) and *effectuation* (C). Visceration is accomplished by the innermost and adjacent tissues surrounding the cerebral ventricular system and spinal canal. Highest representation of this system is identified as *entopallium*. Expression is controlled mainly by the limbic system, basal ganglia, and brain stem extrapyramidal organization. Highest representation of this system is *mesopallium*. Effectuation is conceived as dependent on the relative integrity of the two former systems and specifically on the integrity of corticifugal projections to brain stem and spinal cord. Whereas the two prior systems control a succession of motor nuclei by connections *in series*, the corticifugal connections lie *in parallel*. The highest representation of this system is *ectopallium*. (Redrawn from Yakovlev, Note 5)

skills, become organized according to past experiences, expectations, and purposes.[7]

The third system of motor activity, which Yakovlev calls *effectuation,* involves manipulative motor performance.[5] A university, its buildings, and furnishings represent the results of effectuation; the clothes we wear, the automobiles, trains, and aircraft by which we arrive at that university are all products of effectuation. Effectuation emerges on top of the functional integrity of underlying motoric components for visceration and expression.

The sphere of human effectuation is impressively extensive as compared with that for reflection, rational thinking, and creative and esthetic endeavor. Man may justifiably be called *Homo faciens,* the doer or manufacturer, rather than *Homo sapiens,* the thinker. For most of his existence man has been occupied with brutish muscular activity. Now that immense power and conspicuous leisure are at man's disposal he can look forward to relegating increasingly the role of *faciens* to machines and utilizing his new-found freedom for enriching his goals and augmenting his more thought-dependent and creative pursuits.

Goals are internal satisfactions

William K. Livingston, my father, and Hadley Cantril perceived that what, in common parlance, we call "mind" originates from internal experiences of feeling.[8,9] Feelings are clear-cut; we can readily identify them subjectively and we automatically express them outwardly. We can quickly decide whether we are depressed or elated, hopeful or without hope, energetic or tired, and whether we have a sense of familiarity with our surroundings or a sense of alienation or foreboding. Feelings stem from the most primordial and most central nervous mechanisms, those lining the central canal and ventricular passages. This matrix, or mother tissue, is composed of neurons that possess relatively short processes, are usually finely myelinated, and provide abundant connections among neurons, yielding many multineuronal transactions. The first person, the "I" in all of us, lives in this part of our brains. Activity in this tissue is directly accessible subjectively; any other experience is secondary; its location is most central and most remote from the outside world. This tissue is the source of our appetites and satisfactions.

Subjective feeling states constitute our original and most intimate experience. Everything else we perceive is adventitious to feeling our existence. Feeling constitutes our initial sensation at birth or, more likely, even before we are born. Feeling provides our last consciousness. Perception of ambiguous signals about our feelings, when we don't know exactly how we feel, is only transient, lasting usually only a few minutes. Ambiguity of a feeling state is prominent, for example, in someone becoming seasick. At the outset he may be uncertain what he is experiencing, but such ambiguity is usually soon followed by conversion to something worse or by return toward normal.

My father and Professor Cantril conceived a phylogenetic origin of what we call mind from states of feeling—a gradual emergence of a body image and awareness of the individual self, of relationships with other objects and beings in space and time, development of a conscious government of sensorimotor skills, perceptual experiences, and behavior. Feeling, they visualized, is the sea within which details of consciousness swim. They perceived the emergence of full consciousness, knowledge, skill, and conscience as built up from an initially crude awareness of feeling. Feeling states provide the prime source for action and self-reference, within which interloop more distantly ranging circuits that penetrate the body wall and provide transactions with the outside world. The elemental circuits involved in feeling are conceived by these authors as fundamental in shaping the essence of the person—his character and emergent personality.

This feeling state is the focal point of the physician's query, "How are you?" His question is serious; a congruent answer is medically meaningful. It may be crucial to the physician's decisions regarding the patient's management and prognosis. The doctor's query is echoed by the commonplace social gambit so much alike in every language: "How are you?"; "Wie geht's?"; Comment ça va?"; "Hur står det till?"; "Nin hao ma?"; "Kak vi poshyvaete?"; I kaga desuka?"; "Ap kaise hai?"; and so forth. This greeting is ordinarily taken so for granted that one responds to it only formally and not informatively. When you are asked the question, "How are you?" the expected answer is, "Fine." If you respond by saying, "I feel unhappy," "I feel depressed," or "I am afraid," the interrogator is at once entrapped, whether he likes it or not. He may advise you to laugh it off, drink it off, or go home and take a nap. But if he perceives you are being congruent, the matter is manifestly more serious; his idle inquiry is likely to lead to elaborate and time-consuming attempts at succor, which he discovers to be a practically obligatory social consequence.

Internal feeling states stem from activity in this innermost compartment of the neuraxis: it includes the lining of the ventricular system, the hypothalamus, central gray matter of the brain stem, and floor of the fourth ventricle. Feeling states are intimately related to the biogenic amine story, which Doctor Kety details in this volume. Excessive production or interference with production of biogenic amines has notable effects on feeling states. The biogenic

amines seem to be formed in and to elicit their main effects by action upon some of these central regions of the neuraxis.

Feelings provide the "go/no go" switch for all behavior. Feeling states govern whether any enterprise can be undertaken. If the feeling state is sufficiently morbid, these same circuits presumably provide the suicide decision. A person may sustain an astonishing degree of misery and deprivation without committing suicide; he must, *ipso facto,* be securing some degree of satisfaction or sustaining some substantial degree of hope for future gratification.

Largely on anatomical grounds, Papez identified the limbic lobe, formerly believed to function exclusively as a "smell brain" (rhinencephalon), with general representation of emotional experience and expression.[10] MacLean, a modern proponent of Papez' conception, differentiates two components within the limbic system: (1) a lateral temporal portion, which he relates to *survival of the individual,* is involved in self-protection and territoriality, the quest for food and water, licking, lapping, mastication, and swallowing; (2) a medial system in the mesio-frontal and septal regions, which he relates to *survival of the species,* is involved in procurement and sharing of food for a mate, grooming, breeding, care of offspring, nursing, and other activities essential for successful reproduction of progeny (Figure 3[11]).

The general outlook and attitude of Western civilization have been greatly influenced by three mutually reinforcing ideas misconstrued from the writings of Darwin, Marx, and Freud. The first involves a notion that evolution depends primarily on conflict, that conflict is the chief fulcrum around which survival is determined. The second maintains that perfection of society depends on social conflict, that society advances largely through the purifying effects of conflict. The third is that each person matures largely as a result of coping with internal conflicts, being perfected as a result of conflicts between his internal appetites and the stipulations of society.

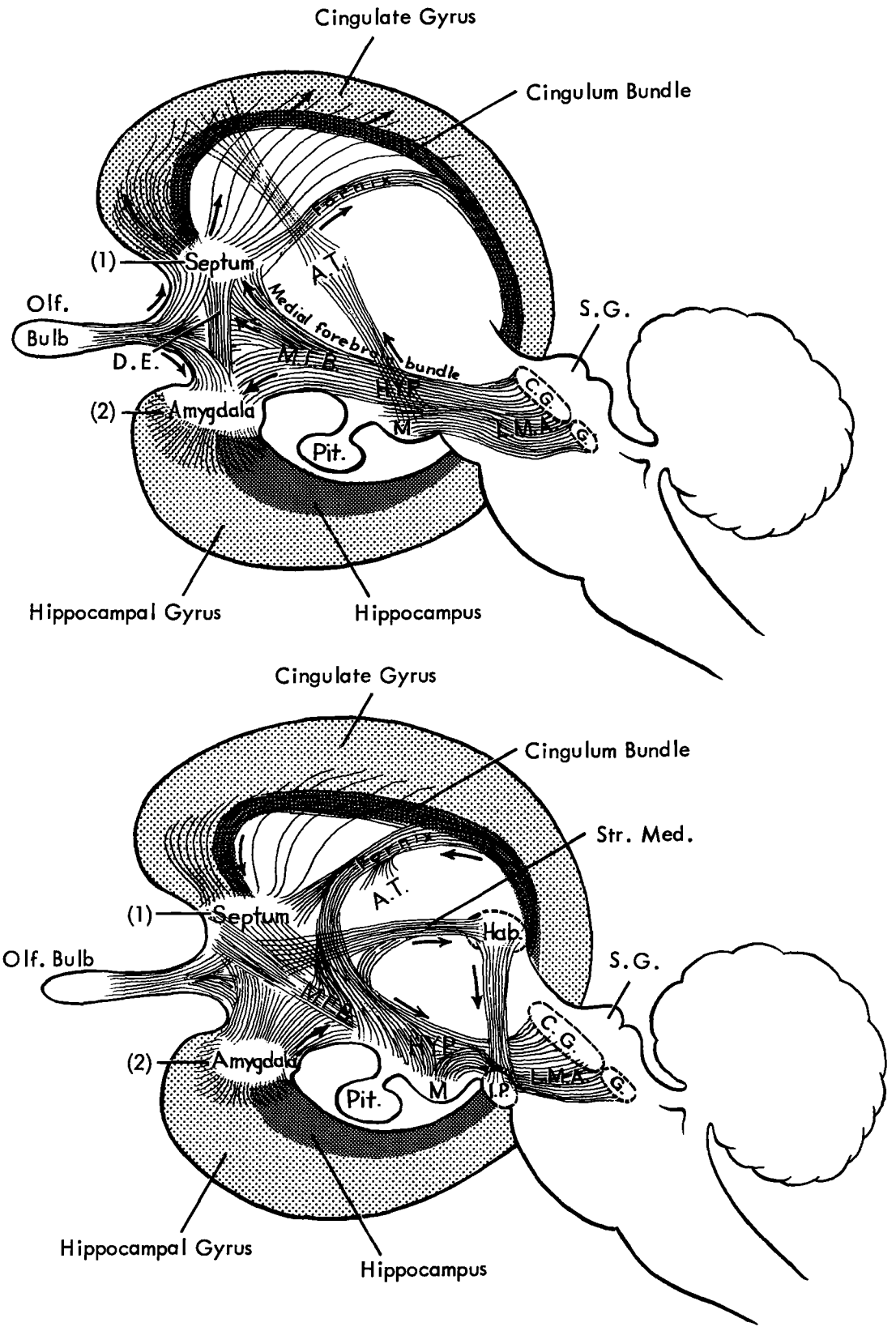
Contemporary biological, social, and pyschological evidence suggest that emphases on conflict reveal only half the story. Conflict does occur, and biological mechanisms for conflict are built into the nervous system; their existence is undoubtedly important for survival.[12,13] But biological mechanisms governing cooperation are likewise built into the nervous system; their existence is also clearly indispensible for survival. Built into the fabric of the nervous system are mechanisms responsible for biological interdependence, obligate and facultative cooperation, faith, mutual trust, and altruism.[13a] Mammals and many other forms of life could not survive a single generation if cooperative behavior were not propelled by internal satisfactions attaching to such activity.

Internal satisfactions include gratifications transcendent to mere reduction of appetites. Gratifications indubitably occur with consummatory behavior, and this may contribute positively as well as reduce discomfort. Gratifications also relate to positive satisfactions springing from buoyant health, vigorous and rested; delight accompanying both genetically endowed and socially acquired values; joys, solitary and shared feelings of pleasant excitement, engendered by exposure to novelty and during the quest for novelty. Gratifications result from satisfaction of curiosity and the pleasures of inquiry, from the acquisition of widening degrees of individual and collective freedom. Positive features of satisfaction enable humans to sustain unbelievable privations and yet to cling to life and, beyond that, to attach importance to beliefs that may surpass the values of life itself.

Olds and Milner,[14] and Delgado, Roberts, and Miller[15] discovered positive and negative reinforcement systems in the brain. Fortunately, positive reinforcement systems are more extensive by a conspicuous amount than are negative reinforcement systems. Implanted electrodes, used to activate regions of positive reinforcement, tend to induce repetition of behavior occurring just before the stimulation. Thus, as Olds and Milner first discovered, a rat can be induced to cross an open field by stimulating positive reinforcement areas each time the animal makes a move in the right direction. The Pied Piper of Hamlin may have activated these regions with the music of his flute. When positive reinforcement areas are stimulated, animals can be attracted and lured toward given objects or actions. Rats will learn to run a maze with no other reward than brief electrical stimulation in positive reinforcement zones. They will cross an electrified grid to obtain such central reinforcement.[16]

Negative reinforcement regions ascend within the brain stem and fan out like a snake's tongue into the posterior hypothalamus and subthalamus. Stimulation by means of an implanted electrode in these regions induces avoidance rather than approach behavior. An animal will perform work and learn complicated activities in order to avoid receiving such a stimulus.[15]

Positive and negative reinforcement systems overlap mechanisms relating to feeling states, appetite satisfaction, and the experiencing and expression of emotion. Stimulation in other vast reaches of the brain, such as the neocortex, yields neither positive nor negative reinforcement of behavior. These indifferent regions can be used as signal transmitting systems, but not as direct systems for motivation and reinforcement.


Cingulate Gyrus
Cingulum Bundle
(1)
Septum
Olf.
Bulb
D.E.
A.T.
Medial forebrain bundle
M.F.B.
H.T.
S.G.
C.G.
L.M.A.
G.
(2)
Amygdala
M
Pit.
Hippocampal Gyrus
Hippocampus
Cingulate Gyrus
Cingulum Bundle
Str. Med.
A.T.
(1)
Septum
Hab.
Olf. Bulb
S.G.
M.F.B.
C.G.
H.T.
(2)
Amygdala
Pit.
M
I.P.
L.M.A.
G.
Hippocampal Gyrus
Hippocampus

Learning is one form of adaptive actions directed toward improved internal satisfactions

What are the main prerequisites for learning? It is obvious that the subject is unlikely to learn if he is not awake, alert, oriented, and motivated. But what do these abstractions mean in terms of nervous system activities?

Figure 4 depicts different levels of organizational complexity, sweeping all the way from molecule to cell, organ, individual, group, community, society. The individual may be perceived as the principal focus of goal-oriented activity at each of these different levels of organization: each of the lower-level systems operates for the survival and integrity of the individual; the individual constitutes the operative unit for each of the more complex levels of organization.

Figure 5 outlines, in a greatly oversimplified manner, the brain-stem reticular formation. The brain stem received its name from German anatomists who used that part of the neuraxis as a practical handle to remove a brain from a jar. The reticular formation is so named because its cells are not assembled into well-defined nuclei and tracts; instead they form a closely packed feltwork of neurons having no readily depictable sources and destinies of connection. In a remarkably prescient article, which predicted on anatomical grounds the generalizations established twenty years and more later by physiological research, William F. Allen referred to the reticular formation as constituting all of the "leftover cells which are not concerned in the formation of motor root nuclei and purely sensory relay nuclei."[17,18]

The reticular formation provides a matrix within which and onto which phylogenetically and ontogenetically recent and readily identifiable structures make their appearance. When neurophysiologists first stimulated this territory electrically in anesthetized or decerebrated preparations, highly variable mixtures of visceral and somatic response were obtained. Responses were so often widespread and ephemeral, and so changeable from one occasion to the next, that the reticular formation was informally dubbed "the manure pile" of the nervous system. Virtually any response could be obtained, although seldom with gratifying consistency. Using techniques for stimulating this region in waking animals, Magoun and his colleagues discovered the pony—that the reticular formation provides generalized control functions.

Magoun and Rhines found that local stimulation in the medial bulbar region induces inhibition of motor responses throughout the body.[19] A more extensive zone, extending upward from the bulb along the brain stem all the way into diencephalic levels, was found to yield an opposite effect—generalized facilitation.[20] As Hess[21,22] and Ranson and Magoun[23-25] had already shown, hypothalamic regions subjacent to the upper end of the reticular formation control two generalized patterns of visceral and somatic activity. Hess identified these as energy expending (ergotropic) and energy restoring (trophotropic).

Figure 6 depicts the ascending influence of the reticular formation. Moruzzi and Magoun discovered that reticular stimulation in the midbrain tegmentum induces generalized activation of the entire forebrain.[26] The reticular formation activates pathways that distribute widely throughout the thalamus and embrace it, continuing by relayed and direct connections to reach all fields of cortex. Ascending impulses induce a generalized shift in activity of cortical cells, which is reflected in an "activation pattern" of low-voltage fast activity as visualized in the electroencephalogram. A parallel downstream readiness for outward behavior occurs at the same time as a result of reticular activation. Incoming sensory pathways destined for cortex send collateral impulses into the reticular formation.[27] It is by access to the reticular formation that sensory signals elicit behavioral and electroencephalographic arousal. The reticular activating system is capable of awakening us from sleep and maintaining arousal; its interference by lesions and anesthetics interferes with arousal and obtunds the waking state.

Figure 7 depicts several cortical areas that project downward into the reticular activating system.[28] Other cortical fields do not possess reticular projections. Thus, the brain-stem reticular formation can activate cortex in an over-all, generalized, diffusely projecting manner, but only limited areas of cortex can exert a reciprocal downward control affecting the reticular formation. These corticifugal path-

FIGURE 3 MacLean's schemata for limbic system organization. The upper figure identifies projections by a variety of divergent pathways from olfactory bulb and brain stem into the limbic system. The lower figure identifies limbic outflow by way of fornix, medial forebrain bundle, and habenulo-peduncular tract to brain stem. The anteriomedial region of this circuit, in the vicinity of the septum (1), is identified by MacLean as relating predominately to *survival of the species.* The anterolateral region of this circuit, in the vicinity of the amygdala (2), is identified by MacLean as relating predominately to *survival of the individual.* (Redrawn from MacLean, Note 13)

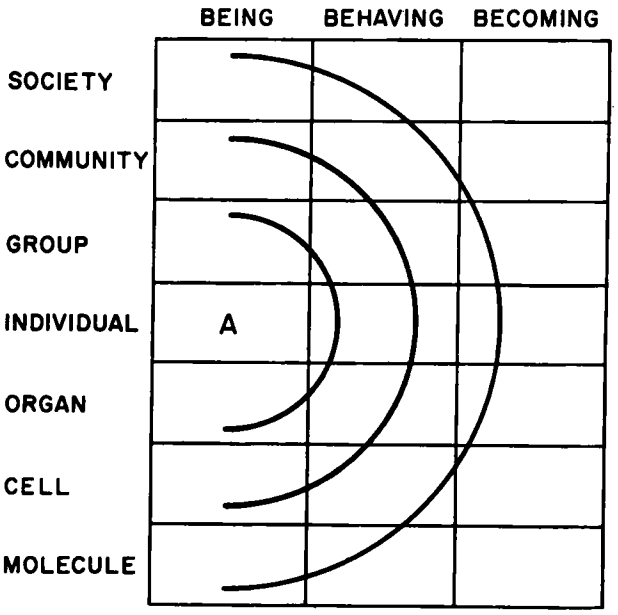


FIGURE 4 Gerard's levels of organization of biological systems. Biology is concerned with all levels of organization: molecule, cell, organ, individual, and beyond, to ethological and ecological systems embracing the whole biosphere. Growth of knowledge has proceeded outward from man's individual sensory experience, dealing initially with structure (being), and extending to function (behaving) and development (becoming) in relation to each level of organization. Growth of knowledge of the nervous system has proceeded from gross to fine structure, with increasing insight into functional and developmental aspects of molecule, cell, organ, and individual and into the mechanisms influencing social structure, behavior and development. (From Gerard, Note 42)

ways acting on the reticular activating system are capable of initiating and maintaining behavioral and electroencephalographic arousal.[29]

Figure 8 displays the limbic system. The temporal region of the limbic system includes the amygdala, hippocampus, and dentate gyrus. A double toroid, originating from the temporal lobes, straddles the upper end of the brain stem. The limbic system receives input mainly from the reticular formation and delivers its output back into the reticular formation; it also receives strategic inputs from the frontal lobes, although this direction of projection is not reciprocated.[30] Elsewhere, Walle H. Nauta describes how this limbic system and the frontal lobes function as a single system. Limbic and frontal areas embrace those central, "where I live" brain parts that are responsible for intrinsic feeling states; parts that are most remote from both input and output relating to the outside world. Limbic and frontal areas embrace feeling states and in turn provide for both the experiencing and the expression of what we call emotion.[31]

Physiologists have long known that motor systems operate according to a descending cascade of controls. Behavior is shaped according to many controls acting all the way along the neuraxis, including some affecting the final neuronal linkage with effectors. During the last decade it has become evident that *sensory mechanisms are likewise controlled by a centrifugal hierarchy of controls.* Perception is thus shaped by a downward cascade of controls, the lowermost of which affect initial sensory receptor units. Hence, the brain controls whatever traffic is permitted to enter and leave the nervous system.[7] The brain controls its own potential contents for perceptual processing. Central con-

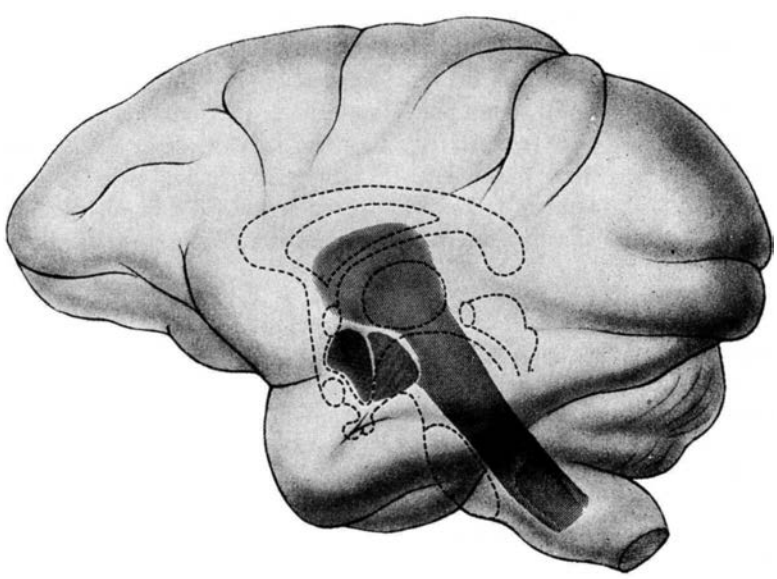


FIGURE 5 A phantom drawing of a human brain, left side view, depicting the brain stem reticular formation. Lowermost part of shaded area represents the bulbar zone from which generalized inhibition of motor responses can be elicited. Major extent of reticular formation constitutes the central core of the neuraxis from bulbar region into medial thalamus; from this region, generalized facilitation of motor response and activation of the cortical EEG is elicited. Hypothalamic extensions of the reticular formation are divided into an anterior *trophotropic* (largely parasympathetic) and a posterior *ergotropic* (largely sympathetic) portion. (From Livingston, Note 43)

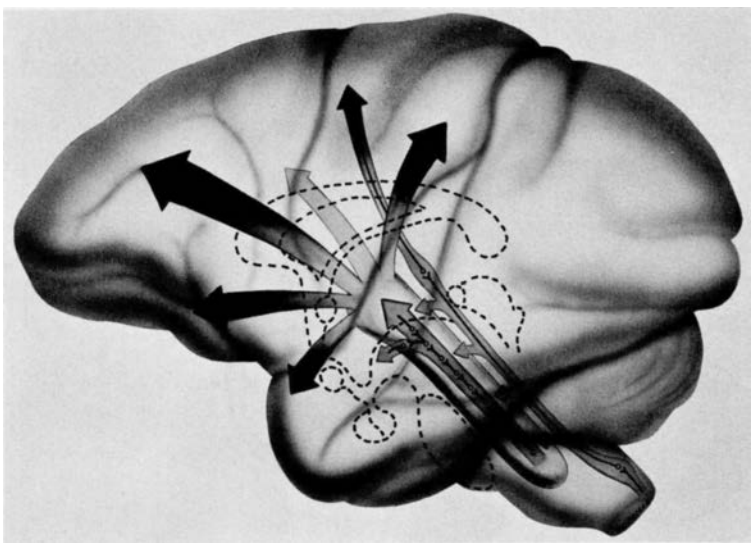


FIGURE 6 Phantom drawing of human brain depicting the reticular activating system. Lemniscal sensory pathways are shown ascending from spinal and brain stem levels to the somesthetic receiving area of cortex. These projections give off collaterals into the brain stem reticular formation. The reticular formation contains extra-lemniscal ascending projections, which influence the hypothalamus and, at the level of the thalamus, diverge to distribute impulses diffusely throughout all areas of cortex. Activation of this ascending reticular system initiates and sustains generalized electrical and behavioral activation of the nervous system. (From Magoun, Note 44)

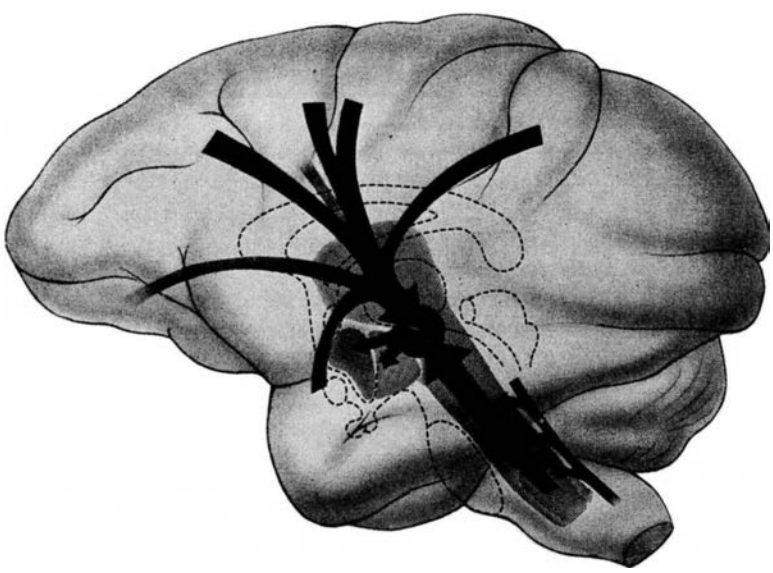


FIGURE 7 Phantom drawing of human brain depicting ascending sensory, descending corticifugal and hypothalamic outflow. The classical (lemniscal) sensory pathways can be seen passing collateral projections into the brain stem reticular formation. These pathways are depicted as though interrupted en route to the somesthetic projection area of cortex. Descending corticifugal projections arise from several cortical areas: frontal eye motor area, sensorimotor cortex, and paraoccipital region, superior gyrus of the temporal lobe, and from more medial regions of orbital surface and cingulate gyrus. Other cortical regions do not possess projections into the brain stem reticular formation. The corticifugal pathways converge in the same general brain stem regions occupied by the extra-lemniscal ascending projections. Convergences of sensory, motor, and visceral projections relate intimately to subthalamic and hypothalamic regions which appear to play a role of switchboard for a wide variety of goal-directed behaviors. (From Livingston, Note 43)

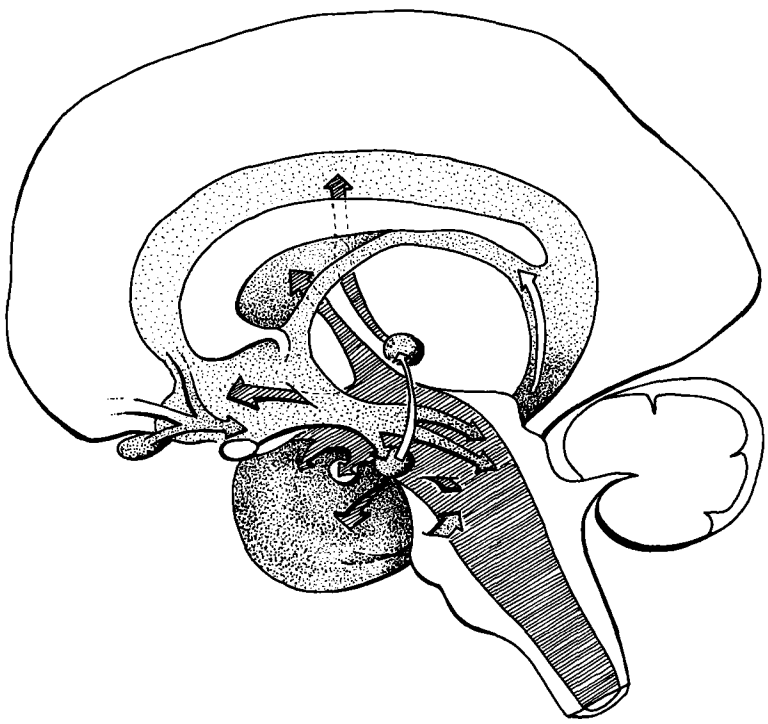


FIGURE 8 Diagrammatic depiction of reticular limbic, and frontal relations. The brain stem reticular formation can be seen projecting into the temporal lobe, directly and via the hypothalamus, through hypothalamus to pituitary, through medial forebrain bundle to the orbitomesial surface of the frontal lobe, and around the fornix into septum. Temporal regions of the limbic lobe project rather directly into the brain stem reticular formation, and also via hippocampus behind the brain stem into fornix, which projects, inter alia, to the mammillary body in the posterior hypothalamus. This, in turn, projects upward to the anterior nucleus of the thalamus and thence to the cingulate gyrus on top of the corpus callosum (unshaded), which cross-connects the two hemispheres. The olfactory bulb, frontal neocortex, and anterior portion of the cingulate gyrus all project into a cortical and subcortical region that interrelates frontal, temporal, and basal forebrain structures. This system, together with the fornix, combines in the medial forebrain bundle to project to hypothalamus and brain stem, terminating in a narrow file of nuclei in the dorsomedial midbrain portion of the brain stem reticular formation.

trol of sensory transmission affects the sensory inputs entering by way of spinal and cranial nerves from all receptors. Sensory receptors and all sensory relays are evidently subject to this centrifugal control.

What nervous system activities seem to be prerequisite for full consciousness? (1) To ensure a wakeful state and alertness, the reticular formation must be active. As Doctors Jouvet and Evarts show (this volume), there are related and perhaps reciprocal brain mechanisms, which, when activated, elicit sleep. (2) For appropriate feeling states and emotional coloration of experience to take place, the most medial lining of the neuraxis and the immediately adjacent limbic and frontal systems must be reasonably intact. Losses from the limbic system are associated with obtunded emotional experience and expression. (3) Neocortex must also be reasonably intact. Cortex is perhaps the least essential of these three entities.

You may ask what is not included among prerequisites for full consciousness? The cerebellum apparently does not modify consciousness except insofar as its loss or defect modifies sensorimotor coordinations. Following cerebellectomy the state of wakeful consciousness at rest is apparently not at all affected. The cerebellum seems to operate as a slave servo-mechanism fairly far downstream from processes involved in consciousness. Spinal transection likewise modifies consciousness only indirectly. Perhaps some of the phylogenetically recent sensory and motor tracts and their cortical projections may be eliminated without directly interfering with consciousness. By contrast, almost any interference with the ventricular lining, brain-stem reticular formation, and fronto-limbic systems seem to interfere directly with the processes, as well as

with the contents of consciousness. Of the three major components, the reticular formation is perhaps the most critically necessary. The inmost ventricular lining and fronto-limbic systems are both more concerned with content than with whether a state of consciousness obtains. The point to be emphasized is that basic mood and motivation, alertness and focus of attention necessary for learning require that several systems—reticular activating system, limbic system, and neocortex—must all be reasonably intact and in a state of coordinated activity.

We become habituated to certain perceptual experiences and to certain predictable outcomes of personal, social, and inanimate object actions. We take familiar events for granted, and our perceptual and motor experiences with them are imperious and automatic. We identify objects to ourselves as chair, lamp, table, and so forth; we walk amidst walls and furniture and through doorways without much error or excitement because such experiences are so familiar. When we are presented something unfamiliar, novel, and unexpected, we become abruptly alerted. This arousal does not require conscious discrimination; a built-in alerting process signals our consciousness whenever a situation appears for which we have no established predictive experience.

Of course, novel objects and novel events present us with undisclosed potentialities for satisfaction or dissatisfaction. Exposure to novelty induces arousal and focused alertness: "What happened?"; "What is it?" Orientation to novelty yields characteristic changes in the EEG, in visceral adjustments preparatory for unknown contingencies as extreme as "fight or flight," and arouses mechanisms involved in motivation, focus of attention, and mobilization of discriminative judgments necessary for purposeful action.

A Maltese cross in bas-relief, Figure 9, appears to extend toward us; but if the same photograph is rotated 180° the cross appears indented. We perceive the same picture in two radically different ways because we "expect" that light on the object is coming from above. The way the figure appears to us reflects a perceptual commitment to customary overhead illumination. Abiding and persuasive sensory commitments are induced in us according to our prior experience. Relatively permanent sensory commitments are not generally considered learning, although they really are greatly overlearned and ingrained by our experiences.

In experiments by Hess (Figure 10), grains of feed were given to newly hatched chicks. The chicks were divided into two groups: one group was fed in light coming from above their cages; another group was fed in light directed from below. After some time, these chicks pecked differentially in response to this photograph, which is made up

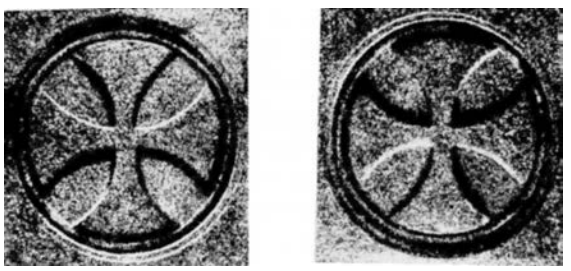


FIGURE 9 Perceptual commitment to position of shadows. Left and right halves of this figure represent the same picture rotated 180°. Because we are generally committed to viewing objects onto which light projects from above, we tend to perceive on the left of this figure a Maltese cross, seen in relief, and on the right, a cross in imprint. If one rotates the book by 180°, this impression changes. (From Kahn, Note 45)

of two identical halves—one rotated 180° so that the images of grains on the two sides are shadowed from opposite directions. Chicks in the group raised with light coming from overhead tended to peck indiscriminately on the two sides. The second group tended to peck predominately on the right half, where the shadows extend above the images of the grain.[32] This chick experiment implies conspicuous readiness for commitment in the newborn, at least the newly hatched.

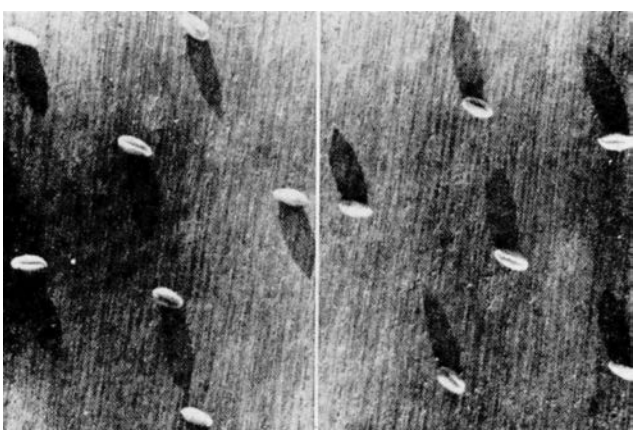


FIGURE 10 Few-day-old chicks are affected according to their experience with the direction of shadows cast by feed grains. The two halves of this figure are similar, but rotated 180°. Chicks fed with light coming down from above tend to peck the left side of this photograph. Chicks fed with light coming from below their cages tend to peck the right side of this picture where shadows project above the grains. The compelling nature of shadowing in chick perception is taken to be similar to that in man. (From Hess, Note 32)

Another familiar type of induced sensory commitment occurs among sailors (Figure 11). When they come ashore after exposure to more than a few hours of ship's motion, they tend to have a rolling gait and to retain a sensation of continuing motion, as if the dock, quayside, and buildings were subject to motions similar to those of their ship at sea. If the voyage has involved a gradually increasing agitation of the sea, passengers and crew may get seasick for the first time on stepping ashore. The French call this *mal de debarquement*. This phenomenon represents an induced commitment of sensed motion that is reversible but may last for several days, gradually diminishing in its compelling perceptual intensity. Such a temporary sensory commitment is not considered learning, although it, too, is acquired and apparently "overlearned." It is thought to result from active functional adjustments, the goal of which is to improve sensorimotor coordinations relative to a moving environment. Such adjustments apparently affect the sensory as well as the motor side of sensorimotor coordination systems.

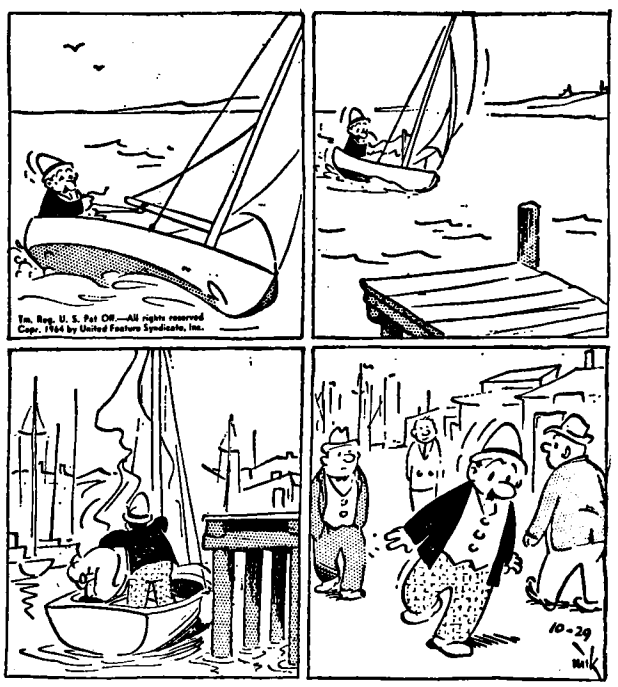


FIGURE 11 Ferdinand, by Mik, illustrates the effects of recent motion on subsequent perceptual experiences. A few hours or days of experiencing motion on a ship have pronounced effects on one's later perceptions ashore. Even a stable environment of docks, streets, buildings, and beds is felt to be in motion simulating that of the ship at sea. This commonplace feeling of persistence of instability of the environment may last for days, and is referred to by the French as *mal de debarquement*. (Courtesy United Features Syndicate)

Transient perceptual commitments also occur following exposure for a minute or more to curved lines (Figure 12). Henceforth, for an appreciable interval, straight-line test patterns appear curved in the direction opposite to that of the original curved lines, and by a predictable amount.[33] Parallel vertical lines looked at for a similarly brief period tend thereafter to distort test circles or squares. A variety of such patterns viewed for a short time yield predictable perceptual deviations. These, again, are not considered learning; they apparently represent temporary modifications in perceptual expectations, induced by immediately preceding experience.

We may infer that the nervous system is continually programming predictively for sensory and behavioral performance on the assumption that conditions presently obtaining will persist. A transiently induced adaptive program that continues after conditions change is likely to be inappropriate and to give rise to predictable perceptual and sensorimotor errors. Perhaps the sailor's sense of false motion ashore results from nervous system adjustments that may have contributed to improvement of his stability and performance despite the ship's roll. Also, the nervous system presumably adjusts to perception of curved lines. In a similar manner, the sense of actual speed in a fast-moving car may be slowed down, so that on a long trip slowing adequately to comply with traffic regulations in a town is perceived as inordinately snail paced. Each of these and other instances imply that the nervous system is continually engaged in sensory adjustments of a goal-seeking nature and that these adjustments have an appreciable hysteresis.

A classical example of transient sensory commitment is the "waterfall effect." If one stares for a minute or longer at a waterfall and then looks away from it toward part of a rocky parapet adjacent to the cataract, a portion of the wall equivalent to the width of the waterfall now appears to be moving upward.[34] Elsewhere the rock wall appears stable, so we must recognize that any altered visual program must be limited to a rectilinear patch of the visual field corresponding to the area occupied by the actual waterfall as seen from that particular vantage point. This effect, therefore, cannot be caused by eye movements, which would presumably affect the whole of the visual field.

We conclude that sensory and sensorimotor commitments may be transient or of long duration. Resultant effects on perception and performance seem to be directed toward improved performance. Sensory goal-seeking is manifested in many examples. Such commitments are both enabling and disabling: the induced changes in perception and sensorimotor coordination perhaps improve performance, but they also exact a toll, because for

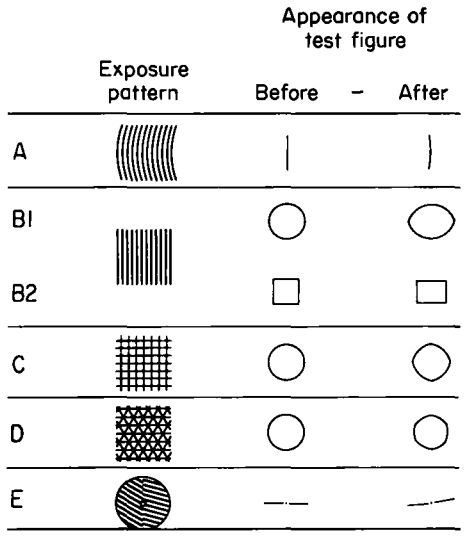


FIGURE 12 Aftereffects induced by active scanning of various patterned fields. A-E: Active scanning of each of these individual patterns for about one minute will alter the appearance of test figures in predictable ways. For example, before the set of curved lines convex to the left is scanned, a straight vertical line appears straight, but afterward, it appears to be curved in an opposite direction, convex to the right. (Adapted from Held, unpublished observations, by Teuber, Note 38)

a while thereafter we cannot correctly detect a straight line or a stable environment, and we perceive the illusion of a patch of motion in the middle of a rock wall. For presumably similar reasons, but with apparently enduring effect, we are mistaken in interpreting a rotating trapezoid; we systematically distort what we perceive through aniseikonic lenses; in other ways we are substantially affected according to our past experiences, expectations, and purposes.[35,36] These perceptual and sensorimotor phenomena must have their neurophysiological correlates.

Significance of sensory and sensorimotor commitments

Even more crucial to the human experiment may be the binding effects of more complex phenomena concerned with acculturation. During the process of growing up, we become committed to certain conventions and expectations regarding space, time, motion, language, music, art, social behavior, and so forth. Our automatic, unconscious reliance on transient and long-lasting commitments in relation to these phenomena may be enabling as long as we confine ourselves to the same physical, cultural, and social circumstances wherein the commitments originated.

Modern man is increasingly driven to confront and to participate in alien physical, cultural, and social systems. He is frequently involved in novel and rapidly changing events for which he has no past experience and no valid foundation for ascertaining the appropriateness of his perceptions, judgments, and actions. Our nervous systems do not prepare us for a world of indefinite variety, but for experiences limited by the past successes and failures of our perceptual and action systems, organized mainly during our infancy and childhood. The world we experience is inevitably circumscribed by our own limited previous experiences.

Our nervous systems are so conservative that we are seldom adequately prepared to recognize and react appropriately to novelty. Yet, perforce, in the variety of our exposure to new phenomena throughout life there is much in what we perceive and in the way we perform that is in gross error. It is rare when we have the benefit of unambiguous corrective evidence, such as is provided by a compass or speedometer. Social groups having similar past experiences share similar distortions and tend to reinforce each other's views, even when confronted by contrasting views, and especially when these latter have been generated by a different set of experiences.

We are oblivious to nearly everything that does not fit into our individually idiosyncratic experiences. Our commitments, including important social commitments, which come into existence automatically and unconsciously as a result of largely fortuitous experiences, are likely to be inappropriate in relation to novel circumstances. Since we have no internal compass to indicate when circumstances have altered, our behavior is frequently inadvertently noncorrespondent. The pity of this is that no one can ascertain whether his confidence in his perceptions, judgments, and actions is warranted or not. He cannot learn this from his cultural surroundings because all societies exist in want of such control, and almost all societies present cultural barriers against reckoning with evidence and events that do not correspond with their own peculiar, culturally bound perceptions and actions.

When we encounter things that do not correspond to our past experiences, there are only two possibilities: the novel experiences are distorted to conform to our past experiences—a process we have shown to be virtually inevitable and certainly not under voluntary control—or, alternatively, noncorrespondence may be detected. The first alternative is the most conservative, and it results in unconscious misperception. This, in turn, tends to entrench our most usually encountered perceptual experiences, those which accord best with previously committed assumptions and expectations. Such entrenchment ensures

us the sense of comfort associated with the familiar.

The second alternative involves recognition of novelty. Whenever that occurs, we encounter what Adelbert Ames, Jr., called a "hitch."[37] We experience an abrupt arousal. We are suddenly jerked alert and oriented toward the novelty. Whenever we encounter a hitch, we apparently inevitably experience learning. Ames and his associates consider that no learning occurs without both (1) exposure to a novel situation and (2) inability of the nervous system to force the novel circumstances into conformity with past experiences, expectations, and purposes.[38] We are almost inevitably victims of our previously committed perceptual and sensorimotor habits, and nearly all of our experiences are caused to conform. Acceptance or rejection of novelty evidently takes place early during the process of perception, prior to awareness and the application of any conscious judgment. It may take place during early input stages of sensory transmission.[7]

The processes of education, and the techniques and attitudes of science, contribute to disabusing us of our prior commitments. Yet there is no royal road to profounder insight. Education and science are both organized to exercise us in the recognition of novelty. They engage us in the deliberate anticipation and pursuit of novelty, employment of novelty in creative ways, and the employment of noncorrespondences for eventually levering mankind as a whole toward novel interpretations. On entry into an education setting or research laboratory, we invade an environment that is deliberately arranged to favor the selection and recognition of novelty. We embrace a socially accepted outlook of excitement and expectation toward the discovery of novelty and engagement with learning. This outlook is deliberately oriented toward things and events that do not conform to our previous experiences, expectations, and purposes; it represents the most fruitful contribution of Western thought. Ancient Greeks conceived the ideals involved in attempting to reconstruct concepts, and Europeans in the Middle Ages provided institutional forms for implementation of these ideals, e.g., constitutional monarchy, trial by jury, parliamentary procedure, and the community of scholars known as universities.[39]

Common sense may be defined as whatever is known before the age of about twelve. What constitutes common sense changes from one generation to another and differs from one culture to another. Recently, by virtue of increased communication and movement, common sense has been challenged to change at a rate more rapid than teachers and educational institutions can consolidate and disseminate. The whole history of education, the mainstream of human intellectual endeavors—including science—has, hitch by hitch, disabused us of much that was previously accepted as common sense. After exposure to the social excitement of experiencing hitches, after realizing improvements in performance following learning, some societies develop what might be termed a societal learning set. They begin to expect increasing benefits through deliberate provision of opportunities for educational and scientific advancement.

"Now print!" mechanisms relating to learning and memory

What happens within the nervous system during recognition of novelty? Only fragments of the process are known sufficiently to formulate a general insight that may be useful to test. The first unusual event is that the reticular formation becomes strongly activated. We have yet to learn how novelty has this effect. Many secrets of memory and learning are bound up in this initial processing of sensory input. We must learn what the mechanisms are and what sets the limits for identification of novelty instead of binding the experience in conformity with past experiences, expectations, and purposes. Reticular activation, in turn, provides a generalized arousal, preparing the forebrain for incoming signals, activating ongoing central integrative processes, and providing visceral and skeletal readiness for action.

Next, the ventricular lining and the fronto-limbic system apparently operate to define the potentialities of the novel event for reward or punishment. This is probably rendered in terms relating both to individual survival and to survival of the species. These appetitive and evaluative systems measure, in effect, that this particular object or event is biologically significant for this individual at this time. Thereupon, limbic outflow discharges into a special region of the reticular formation.

Many people believe that learning occurs as a result of molecular and growth processes taking place at cellular junctions. I suggest that such growth may be induced as a consequence of the waking brain being further aroused through its identification of biologically meaningful novelty.[40] Any novelty has potential biological significance, and is signaled by initial reticular activation. The function of the ventricular lining and fronto-limbic systems is to evaluate significance of the new message. Limbic-induced discharge enters a particular part of the reticular formation and thereby induces a generalized arousal. I have proposed that a consequence of this particular reticular discharge pattern is a generalized order, "Now print!"—an order for all neurons recently activated to undergo growth. This may involve first an alteration of ionic barriers, then a replication of proteins,

and finally a growth of synapses according to particular local configurations that will increase the likelihood of repetition of the patterns of impulse conduction that just transpired.

One advantage of this schema is that any elaborate intricacies of connections will be accounted for: whatever patterns—sensory, motor, associational—are active whenever any biologically important event is repeated, will be printed, reprinted, and overprinted into habit through the exercise of this limbic-reticular "Now print!" discharge consequent to the identification of novelty and its assignment of biological significance.[40] Ongoing activities that are not invariably associated with the biologically significant events will be less regularly printed than any invariant signals. Eventually, only those particular environmental objects and events which are timed so they can be causally related to subsequent internal satisfactions and dissatisfactions will be firmly established as learned behavior.

It should be recalled that not all novelty is learnable. We are exposed to a great many events that involve random concatenations of elements. When we seek to predict in relation to such experiences, we cannot forecast the outcome. A ball with multiple, randomly oriented planes cut into its surface would not be useful in a game of skill; each bounce would yield an unpredictable rebound. No one could learn to bounce and catch such a ball, no matter how long he practiced. Many analogous situations that confront us with unlearnable novelty present themselves in various physical, social, and cultural circumstances. No connections between sensory signals and motor performance can yield more than chance-level success of performance.

However, all novel events that are admitted as novel, are identified as biologically valuable for that individual, and are recurrent in regular enough patterns to be learnable, will be learned. The sensory, sensorimotor, and related signal sequences will become incorporated into circuitry, presumably as a result of new molecular activations and local synaptic growth. This, as we postulate, takes place as the result of reiterated "Now print!" orders originating within that portion of the reticular formation under influence of the limbic system. By this means, any given configuration of stimuli will become learned. Patterns once novel become habituated. The learned performance gradually becomes incorporated as part of the perceptual and sensorimotor repertoire—the enlarged common sense—of that individual.

Summary

Perception, judgment, and action require the nervous system to function in a highly integrated fashion. Some of these complex activities provide clues for neurophysiologists who attempt to find out which parts of the nervous system are indispensible and which are active during given experiences. By inferring from such clues, neurophysiologists have developed notions that certain parts of cells, certain cells, and certain circuits may be essential for local and general systems controls that govern integrative actions. Integrative actions at molecular and cellular levels are discussed by other contributors to this book.

In the following chapters, our goal is to identify *integrative functions of the brain as an organ system involved in sensory intake, storage, discrimination, and adaptive actions, including the complex performances involved in memory and learning.*

Neurophysiological Aspects of Rhythms

FELIX STRUMWASSER

OUR COMPREHENSION OF nervous system mechanisms is quite good for certain fundamental phenomena, such as conduction of the action potential along an axon and the transmission of excitation or inhibition at the synaptic connections between two nerve cells. Admittedly, knowledge is lacking of the particular membrane macromolecules that are involved in electrical excitability of the axon and chemical excitability of the postsynaptic membrane, together with an understanding of their function based on their structure and interrelations with other molecules.

Our comprehension of information processing mechanisms has made a sizeable advance in the last few years, as is evident from Dr. Mountcastle's chapter in this volume. The transformation of signals from peripheral receptors of the somesthetic system to the sensory cortex can be understood currently in terms of predictable linear transforms. The elegant work of Hubel[1] and Wiesel has also taught us that predictable geometric transforms of the receptive fields are performed in the visual cortex of the cat and that certain of the transforms are sensitive to early experience. These approaches obviously tell us a great deal more than the gross, but important, wiring diagrams of the brain provided us by the careful work of the neuroanatomists. But the jump from our present-day knowledge of the fundamentals of conduction, synaptic transmission, information processing, and reflexology to behavior, with its nagging division into instinctive versus learned, is simply too high to take as "the next step." What are the intermediates?

Let us first consider the following behaviors. Every year, during late fall, the gray whales swim several thousand miles from the Arctic Ocean to the warmer waters near Baja California, where they mate and remain for the birth of their young. In late spring both the old and the new generations head back north to the Arctic Ocean. When sockeye salmon are about four years old they begin an irreversible migration that brings them back, in October, from the Gulf of Alaska to their original birth place—the Adams River in British Columbia, where they spawn and then die. When a year old, the young salmon will follow the river down to its junction with the Pacific Ocean, thus starting the outward phase of the cycle again.

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In both of these examples, as well as in the annual migration of certain birds, the entire motor output of the organism is driven by a "state" in the brain that persists for weeks and months until the single goal is accomplished. These examples are dramatic because of the distances covered by the animals to achieve their goal and, of course, because of the long time period of the individual or population cycle. However, shorter-period cycles are an important and well-known part of all animal behavior —sleep (inactivity) and waking (activity), grooming, exploration of territory, searching for food, sexual receptivity, and social relations. In the words of Margaret Mead: "If we look over the whole known human world we may ask: What are the problems that must be solved if a society is to survive? One of these problems is how to set up a rhythm of activity and rest, which in most societies becomes transformed also into the way in which work—activity that is purposeful and directed towards ends that lie outside the activity—and play—activity which is self-rewarding—are alternated."[1a]

All of these behaviors can be classified as *cyclical* because the particular function repeats itself at somewhat periodic time intervals. This chapter contends that the cyclical behaviors, which consume a very large time fraction of all animal behavior, are the intermediates that can be potentially understood in terms of coupled slow oscillations in the state of certain key neurons.

In organization, this paper proceeds downward in level from work with whole organisms to that with isolated parts of the central nervous system and finally to cell-free fractions. A brief review of the laws and current models controlling daily activity cycles in vertebrates is given, followed by a demonstration of a circadian rhythm in the mammalian EEG and a review of our present knowledge and understanding of circadian and higher-frequency rhythms at the level of single neurons in the marine gastropod, *Aplysia californica.*

The free-running (uncoupled) locomotor rhythm is circadian in its period

If one allows any terrestrial mammal to run in an activity wheel under continuous conditions of illumination (no photoperiod), one finds there is but a small probability that it will sustain an exact 24-hour period of activity—

that is, one synchronized with the earth's rotation. This is the first important principle, if you will, of circadian (about 24-hour) rhythms. An example of the most accurate mammalian timer presently known from this sort of measurement is the flying squirrel *Glaucomys volans,* as shown in Figure 1,[2] which records the turning of the activity wheel. This animal was kept in constant darkness; yet, as the figure shows, it advanced its activity schedule in such a systematic way that one could begin predicting the onset time after the first few cycles of activity. The flying squirrel is so accurate that the prediction of the time of onset of activity can be made for any individual animal within a few minutes per day. This squirrel clearly had a free-running activity cycle with a period close to, but significantly less than, 24 hours. That the free-running activity cycle is not exactly 24 (or 24.8) hours is an important phenomenon to establish, because it cannot then result from any of the geophysical forces we know about. From this kind of evidence, the activity cycle must already be intrinsic (or endogenous) to the animal rather than dependent on an exogenous drive. In Figures 2 and 3, the free-running activity rhythms of a chaffinch[3] and an electric sand fish[4] are

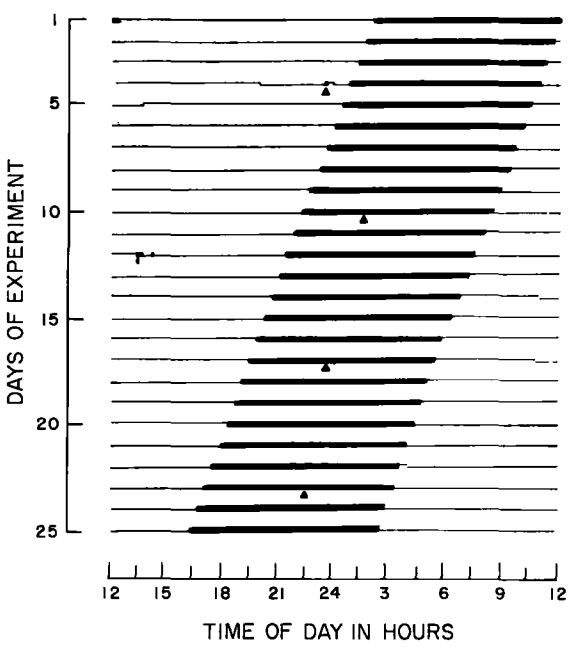


FIGURE 1 Activity record of a flying squirrel (*Glaucomys volans*) in a recording wheel cage in continuous darkness at 20°C. Dark band shows that the squirrel is turning the wheel at a high rate; absence of band indicates that the animal is either asleep or does not choose to exercise. Each line is a solar day, with the succession of experimental days from top to bottom. Solid triangles indicate the time of feeding in darkness. (From DeCoursey, Note 2)

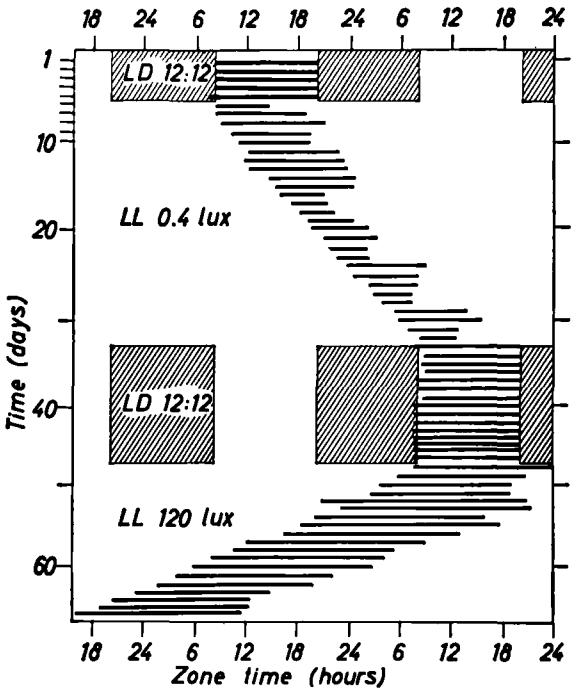


FIGURE 2 Activity record of a chaffinch (*Fringilla coelebs*) in a light-dark cycle with 12 hours of light and 12 hours of darkness (LD 12:12) and in continuous illumination (LL) with an intensity of 0.4 lux and of 120 lux. Black bars, activity time; shaded area, darkness. (From Aschoff, Note 7)

shown, providing further examples of the circadian nature of the endogenous oscillator.

It is well established that under free-running conditions, separated individuals of a species will show a distribution of circadian periods, but the range for certain species can be small: for example, 22 hours and 58 minutes to 24 hours and 21 minutes for 48 test periods using 18 flying squirrels.[2]

The circadian period depends on light intensity

A second principle of activity rhythms is that the free-running circadian period is influenced by light intensity. Aschoff[5] has generalized the findings, many of them his own, by stating that with increasing intensities of light the free-running period is shortened in diurnal animals and lengthened in nocturnal animals. In Figure 2[3] the diurnal chaffinch's free-running period dramatically decreases when light intensity is increased from 0.4 lumens (days 6 to 32) to 120 lumens (days 48 to 66). Work on the chaffinch has also shown that the total amount of activity (perch-hopping) and the ratio of activity (α) to rest time

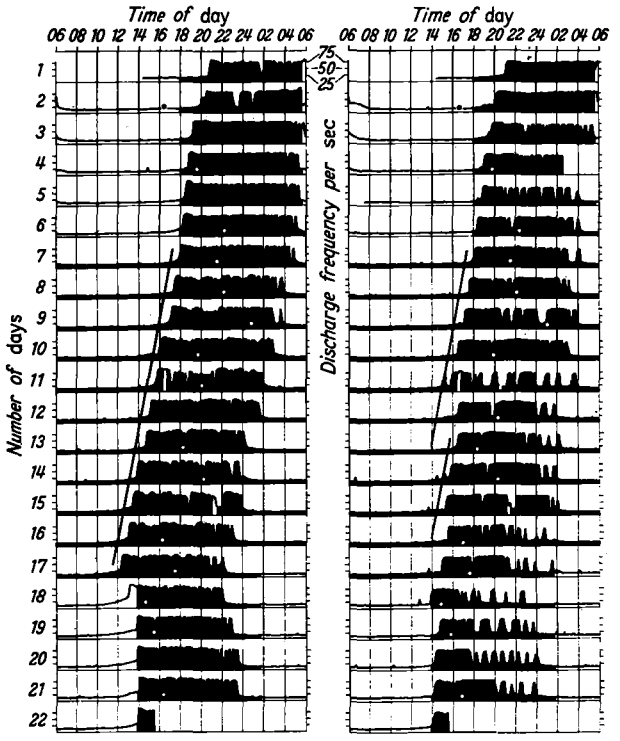


FIGURE 3 The discharge frequency of two electric sandfish (*Gymnorhamphichthys hypostomus*), kept for the first six days in a 12-hour light, 12-hour darkness cycle, followed by 11 days in the dark. Note that the two sandfish show an activity period less than 24 hours when in constant darkness. During days 18 to 22 the dark-light cycle was again imposed, but was advanced by 4 hours. Brief light shocks were applied on day 11 and day 15. 0 = feeding. (Taken from Lissmann and Schwassmann, Note 4)

(ρ), are positive functions of light intensity (compare α:ρ in 0.4 lumens with that in 120 lumens, Figure 2).

The deer mouse, *Peromyscus*, is nocturnal, and lengthens its free-running circadian period when conditions are changed from constant darkness to constant light.[6] Many other examples for diurnal and nocturnal animals and an analysis of certain exceptions to Aschoff's "Circadian Rule" are given by Hoffmann.[7]

Entrainment to a Zeitgeber is mediated by phase control

In nature, the endogenous, free-running circadian rhythm is clearly entrained by the day-night cycle, and there is laboratory evidence for entrainment in birds by sound,[8,9] and perhaps by temperature cycles.[10] Our discussion is limited to light-dark cycles as entraining agents, or Zeitgebers.

The mechanisms by which a Zeitgeber entrains the innate, free-running period are not completely understood. However, certain phenomenological observations are considered relevant to any "final" model of entrainment and there are, of course, several theories. When measurements are made for individuals of a species, the phase-angle difference (ψ) between the entrained locomotor rhythm (onset of activity) and an appropriate phase of the Zeitgeber (dawn, for a diurnal species) bears a definite relationship to the free-running circadian period. It can be seen in Figure 4 that the steady-state phase-angle difference (between onset of locomotor activity and dawn of the Zeitgeber) is a positive function of the free-running circadian period of the chaffinch.[11] This means that a chaffinch with a free-running period of less than 24 hours would anticipate the dawn of the Zeitgeber (i.e., assume a positive phase-angle), whereas one with a free-running period of more than 24 hours would delay the onset of its locomotor activity until after dawn (negative phase-angle). Such a finding implies that the occurrence of dawn *resets* one component of the endogenous circadian oscillator (to its zero state or trough) and the subsequent (steady-state) onset of locomotor activity occurs one "free-running" circadian cycle later (compare with Pittendrigh, Bruce and Kaus[12]). It should be appreciated, however, that the

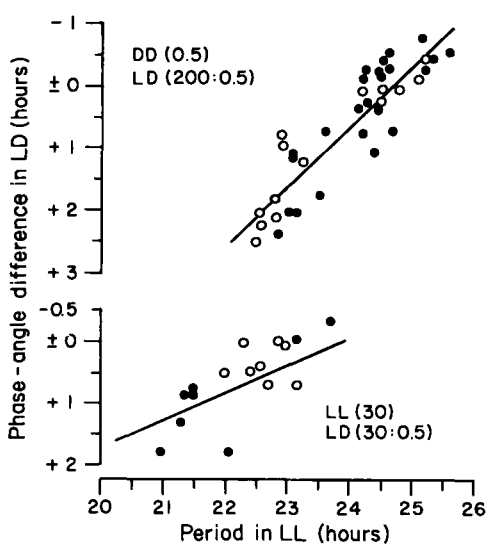


FIGURE 4 Phase-angle difference ψ, measured between onset of activity and 'light-on' of a light-dark cycle, as a function of spontaneous circadian period τ, measured in constant conditions. Results from experiments with 6 male (solid circles) and 4 female chaffinches (open circles). Upper diagram: ψ in LD (200 : 0.5 lux), τ in DD (0.5 lux); lower diagram: ψ in LD (30:0.5), τ in LL (30 lux). (From Aschoff and Wever, Note 11)

endogenous circadian period under light-dark is less than the free-running circadian period measured under dark-dark, as a result of the positive influence of light (during light-dark) on the frequency of the circadian oscillator.

A rhythm of sensitivity to a light pulse has been demonstrated by many workers when applied during different phases of a free-running circadian cycle. By using single "light-shocks" of ten-minute duration, applied at 4- to 25-day intervals during the free-running rhythm measured in darkness, DeCoursey[13] obtained a *light-sensitivity* curve for individual flying squirrels. In Figure 5 the amount of advance or delay of the steady-state onset of activity (in minutes), is shown as a function of the time at which the light-shock is applied in the squirrel's free-running activity cycle. The curves of the two squirrels are both biphasic in form, but show quantitative differences. Near or at the time of spontaneous onset of locomotor activity, the light-shock caused a maximal negative phase response (delay in subsequent steady-state onset of activity), whereas a reversal of response occurred with a light-shock applied near the natural offset of locomotor activity.

The form of these light-sensitivity curves, also called "phase-response curves"[14] is a characteristic of the species, with variations of the amount of phase delay or advance common between individuals. The range of the phase-response curve, under certain conditions, can obviously indicate the range of entrainment by the Zeitgeber. The dynamics of entrainment—that is, the time course of the "transients"[14] on the path to the steady-state—will depend

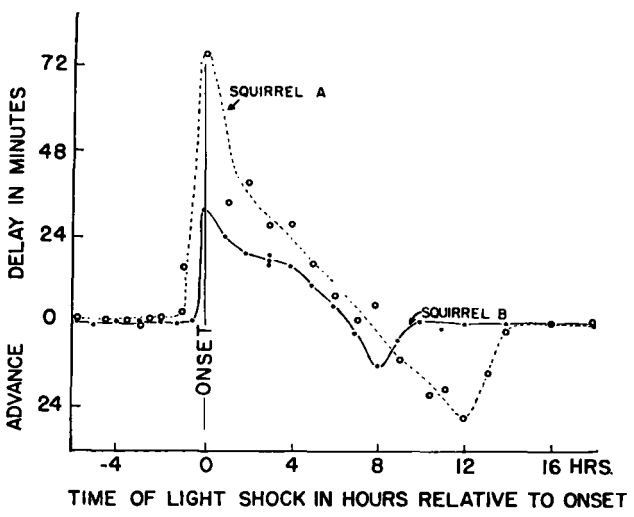


FIGURE 5 Light-sensitivity (or phase-response) curves for two flying squirrels, A and B, showing the phase shift in the rhythm of daily locomotor activity caused by single 10-minute light periods. The squirrels were otherwise maintained in constant darkness. (From DeCoursey, Note 13)

on the slope and range of the response curve. As pointed out by Aschoff,[15] however, there are unfortunately not one but many response curves for even an individual of a species. This is because the response curve is sensitive to a number of parameters of the light-shock (such as duration, intensity, and undoubtedly slope of rising and falling phases and wavelength) and the background illumination. Hence the phase-response curve must be measured under conditions relevant to the entrainment situation before it can be used as a predicter, which makes it of limited use, at the moment, for the construction of general models of entrainment.

Models of circadian rhythms

At least three available models of circadian rhythms exist in the literature.[12,16-19] These models are of very different scope. Wever's model[17-19] is perhaps the broadest, because it accounts for the Circadian Rule, the ratio $\alpha:\rho$, and the phase-response curve. The model of Pittendrigh, Bruce, and Kaus[12] was the first to appear, and was designed around observations made on the eclosion rhythm of *Drosophila;* it attempts to account for the relative temperature independence of circadian rhythms, the mechanism of entrainment by light and the time course of transients during entrainment. Goodwin's model[16] tries to account primarily for the origin of self-sustained oscillations, which is taken for granted in the other two models.

In Figure 6 two factors are considered, and it can be shown that certain predictions emerge. There is a self-sustained oscillation in the organism (of which a single sinusoidal cycle is shown under three different conditions in the Figure). The oscillation has several variables, of which the most important in Wever's model is the *mean value.* There is a fixed threshold. When this threshold is exceeded by some portion of the oscillation, depicted by the shaded area, activity on the part of the organism becomes possible. The activity time (portion of the oscillation above threshold) is called α and rest time (portion of the oscillation below threshold) is called ρ. It can be seen that as the mean value of the oscillation increases, relative to threshold, the ratio $\alpha:\rho$ increases as does the total amount of activity (A)—the area of the oscillation above threshold.

One prediction that emerges is that if light were to increase the mean value of the free-running oscillation, both the ratio $\alpha:\rho$ and the total amount of activity (A) should increase. In Figure 2, it can be seen that $\alpha:\rho$ of the chaffinch increases with a shift in light intensity from 0.4 to 120 lumens. Aschoff[3] has also demonstrated that the total amount of perch-hopping activity of the chaffinch increases with a shift in light intensity from 1.8 to 120 lumens.

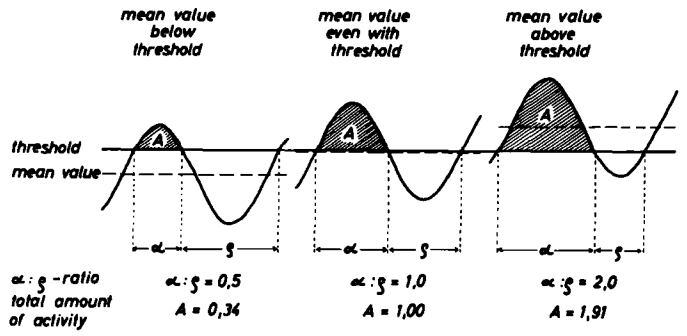


FIGURE 6 Schematic graph of an oscillation whose mean value increases (in three steps) relative to a fixed threshold. The section of the oscillation above threshold is called "activity," the section below threshold "rest." Note that the ratio of activity time to rest time ($\alpha:\rho$) and the total amount of activity (A) increase with the mean value of the oscillation. (From Wever, Note 19)

Wever's[19] quantitative model for circadian periods derives from the well-known Van der Pol equation, which he has modified into the form

$$\ddot{y} + 0.5\,(y^2 + y^{-2} - 3)\,\dot{y} + y + 0.6\,y^2 = \ddot{x} + \dot{x} + x \quad (1)$$

where y is the activity variable, oscillating as a function of time, and x is the controlling variable (light intensity), which may be a function of time (when a Zeitgeber). The first and second derivatives, with respect to time, are shown as ($\dot{x}$, $\dot{y}$) and ($\ddot{x}$, $\ddot{y}$) respectively.

When equation (1) is solved for different values of a time-independent x, the system can be seen to oscillate within a restricted range of x (0.847 to 3.189) as in Figure 7. The most interesting aspect is that the steady-state circadian period shortens as x increases. If x increases with increasing light intensity for diurnal organisms but decreases with increasing light for nocturnal organisms, Wever's modification of the Van der Pol equation can be said to predict the circadian rule mentioned earlier: increasing light intensity shortens the circadian period in diurnal and lengthens the period in nocturnal organisms.

Pittendrigh, Bruce, and Kaus[12] start with a coupled oscillator system. Their oscillator A generates a sawtooth function and is light sensitive but temperature independent; the onset of light immediately resets oscillator A to its zero state, thus allowing entrainment to the Zeitgeber by phase control. Oscillator B generates a square-wave type of function and is temperature-sensitive but light insensitive. These two oscillators are coupled together to permit entrainment of each others' free-running period. The dynamics of entrainment then depend on the strength of coupling between the two oscillators, as oscillator A is immediately reset by a light pulse. An electronic analog of this model can be set up easily with sawtooth and pulse generators (compare with Ehret and Barlow[20]), allowing direct observation of the dynamics of entrainment. Further quantitative refinements of this model have recently appeared utilizing two coupled Rayleigh oscillators with an applied forcing function.[21] The influence of weak interactions amid a population of cells with almost identical periodic functions has been under theoretical study by Winfree.[22]

Goodwin tries to account for the self-sustained oscillations within an individual cell. In his model there are three variables: X_i, which is the quantity of one species of messenger RNA controlling the rate of synthesis of informationally homologous protein Y_i; Y_i enzymatically controls the rate of production of M_i, a metabolite, which

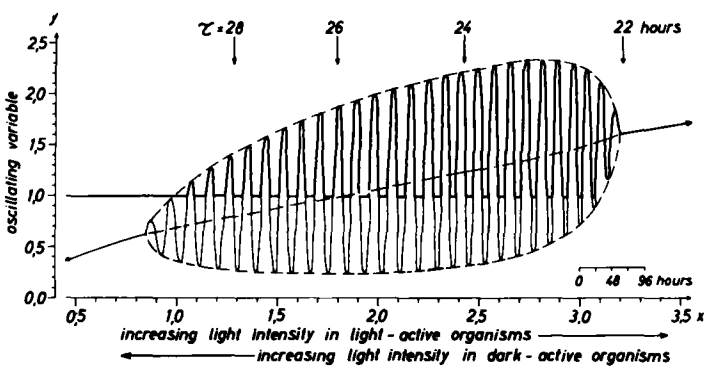


FIGURE 7 Solutions of the model equation (1) in the steady state of the oscillation. The oscillation is schematically drawn as a function of the constant "environmental condition" x. Thick solid lines, "activity"; thin solid lines, "rest"; dotted lines, maximum value, mean value, and minimum value of the oscillation. (From Wever, Note 19)

negatively feeds back on the system controlling messenger RNA production besides participating in metabolic reactions. The motions of X_i and Y_i as functions of time are shown in Figure 8. X_i oscillates around the steady-state value p_i, and Y_i oscillates around the steady-state value q_i; these steady-state values are obtained by setting the left side of the equations in Figure 8 to zero and solving for X_i and Y_i. Depending on the values of the parameters, Goodwin reports that oscillations in the range of 2 to 14 hours can be obtained with amplitudes up to 50 per cent of the steady-state concentration.

Circadian rhythms measured in the vertebrate brain

Locomotor activity is the commonly used indicator of the circadian rhythm in most of the research on animals. However, the recent measurements made on the discharge frequency of the electric organs in sandfish (see Figure 3) come closer to the nervous system, in that each electric organ discharge results directly from impulses in the (electro) motoneurons of the spinal cord. Continuous measurements of brain temperature in the unanaesthetized freely-moving squirrel (*Citellus lateralis*) have demonstrated a strong circadian rhythm, entrainable by photoperiod, as illustrated in Figure 9.[23]

In order to investigate the role of the nervous system in the generation of circadian rhythms, the electrical activity (EEG) of various regions of the squirrel brain has been continuously recorded for periods up to three months. (Techniques are described in Strumwasser, Gilliam, and Smith.[24]) When the EEG of the septum is rectified and integrated over one-hour intervals and autocorrelation performed on the EEG integrals varying with time (Figure 10), a clear circadian rhythm emerges.[23] A similar transform on the continuously recorded EEG of the olfactory bulb shows only a weak circadian rhythm as compared with the septum.

This study of low-frequency oscillations throughout the brain is in its early stages. It is suspected that the circadian period is not common to all brain areas and that oscillators of higher and lower frequency probably exist. Under the special conditions of natural hibernation, the integrated electrical output of the squirrel amygdala shows large oscillations with a 4- to 9-hour period.[25] Arousal from hibernation always occurs when the amplitude of this oscillation becomes critically large, suggesting a functional role for the oscillator.[23]

There are examples of circadian rhythms in the vertebrate brain, at a biochemical level. The 5-HT content of the pineal gland of the rat is maximum at midday and minimal at midnight when studied in animals entrained to a light-dark cycle. This rhythm free-runs when rats are exposed to constant darkness but damps out in constant light.[26,27]

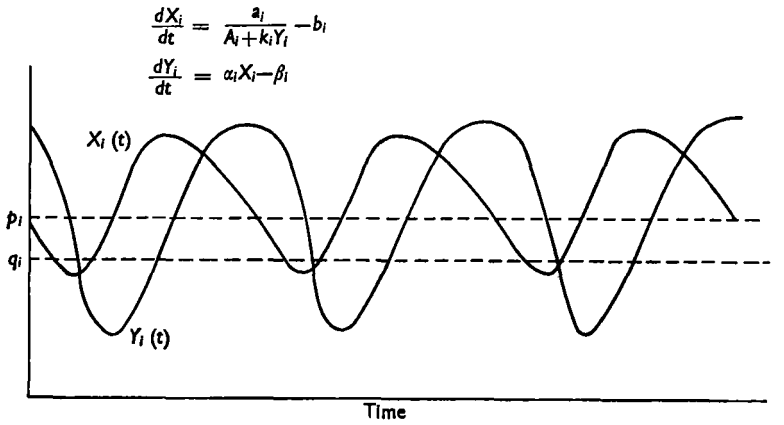


FIGURE 8 The dynamic behavior of the variables X_i (quantity of messenger RNA) and Y_i (quantity of informationally homologous protein) as a function of time. The motion of the variables is governed by the equations shown in the upper left. p_i and q_i are the steady-state values of the variables X_i and Y_i respectively; they are obtained by setting the left hand terms of the equations to zero and solving for X_i and Y_i. α_i, a constant, is a composite parameter containing a rate constant for the template synthesis of protein and concentration terms for activated amino-acids; β_i, a constant, represents the rate of protein degradation; b_i, a constant, represents the rate of messenger RNA degradation; parameters a_i, A_i, and k_i are complicated functions of more elementary constants and are discussed in Goodwin, Note 35a. (From Goodwin, Note 16)

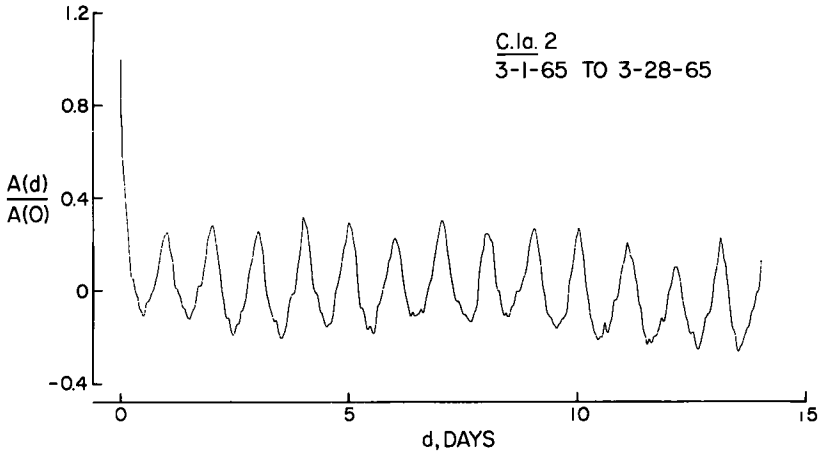


FIGURE 9 Autocorrelation of 28 days of continuously recorded brain temperature of the golden-mantled squirrel (*Citellus lateralis*) while in a 12-hour light, 12-hour dark cycle. Thirty-minute averages of data and 30-minute lag steps were used. (From Strumwasser, Schlechte, and Streeter, Note 23)

Circadian rhythms in isolated invertebrate ganglia

Involvement of the nervous system in the expression of a circadian rhythm does not automatically allow us to conclude that the origin of the rhythm lies totally within a system of neurons. Clearly, at one extreme, the pacemaker could lie outside the nervous system but be sensed by it. Alternatively, the pacemaker property could result from the complex interaction of a system of neurons, hormones, and chemicals of the blood, and feedback from strategically placed receptors.

One step in simplifying the problem of localizing the circadian oscillation is the study of de-afferented parts of the nervous system. Another simplifying step is the study of such a part of the nervous system in vitro, where the bathing fluid is under the control of the experimenter. In this manner, a part of the nervous system can be studied, isolated from peripheral receptors, blood-borne hormones, and the fluctuating levels of other compounds in the natural circulation. These conditions can be imposed, with minimal tissue trauma and requirements, on invertebrate nervous system. The rest of this chapter deals primarily with studies on the isolated parieto-visceral (i.e. abdominal) ganglion of the sea hare *Aplysia californica,* where it is possible to demonstrate a circadian rhythm of spike activity in a single neuron.[28-30]

Time-lapse photography of intact sea hares in a large aquarium showed that these animals were *diurnal,* i.e. they were active during the light portion of a light-dark cycle.[31,32] Locomotor activity starts around dawn and

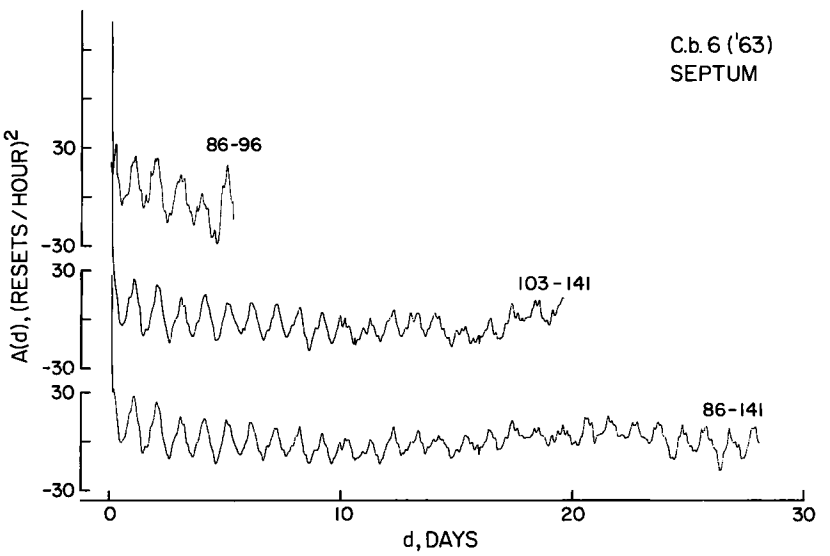


FIGURE 10 Autocorrelation of septum EEG integral for the California ground squirrel (*Citellus beecheyi*) while in a 12-hour light, 12-hour dark cycle. Calendar day numbers during which data was collected are given at end of each curve. The lower curve was obtained by using all the available data. One-hour integrals of rectified data and one-hour lag steps were used. (From Strumwasser, et al., Note 23)

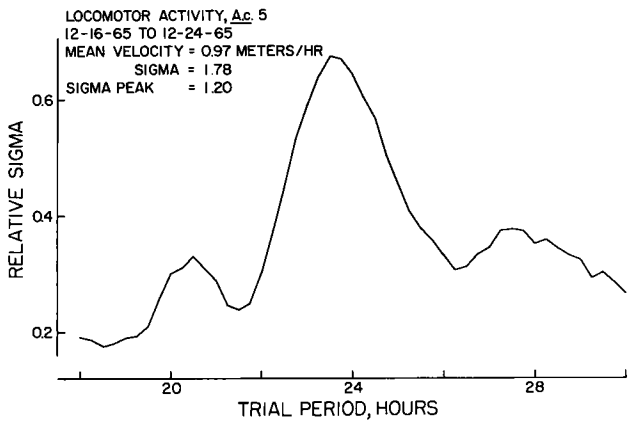


FIGURE 11 Periodogram of nine days of locomotor activity in the sea hare (*Aplysia californica*) while in a 12-hour light, 12-hour dark cycle. Data was obtained by photographing the sea hare, in a 50-gallon tank of flowing sea water, at three-minute intervals on 35 mm. film. The position of the animal was encoded from a numbered grid placed over a film viewer. A computer program (written by Mr. Cary Lu) transformed the grid numbers to velocities. Fifteen-minute averages of data and fifteen-minute lag steps were used.

stops around dusk. The periodogram of Figure 11 shows good entrainment to a light-dark cycle of 12 hours each, for a single sea hare studied, while in isolation, by time-lapse photography.

The dorsal surface of the parieto-visceral ganglion of the Californian sea hare is shown in Figure 12A. On the basis of topography, mapping experiments, and cellular properties, ten to thirty neurons can be reidentified from experiment to experiment.[29,33,34] The branching pattern of the emergent axons of cells 1, 2 and 3 are shown in Figure 12B, as determined by antidromic invasion and orthodromic recording experiments.[33] Many of the neurons in the ganglion that generate intermittent bursts of impulses send their axons into the pericardial nerve (PC, Figure 12B).

When continuous multi-unit recordings are made from the pericardial nerve of the PVG, over a period of several weeks while in organ culture,[35,35a] a circadian rhythm can be demonstrated upon periodogram analysis of the impulse rate (Figure 13). The periodogram in Figure 13 shows the circadian period to be around 26.5 hours (C 55) while the power spectrum of another preparation (C 70) shows the circadian period to be 23.8 hours (Figure 14). This variation in circadian period, from preparation to preparation is to be expected since each preparation is free running and unentrained during the experiment.

The demonstration of a circadian rhythm in the multi-unit impulse rate of the pericardial nerve (representing the output from neurons of the isolated PVG) allows us to conclude that there is built into the ganglion a circadian oscillatory system not dependent on rhythmic input from the environment.

Circadian rhythm in a single nerve cell

The question remains whether single nerve cells in the ganglion are producing the circadian oscillation since a multi-unit counting technique was used in the organ culture experiments described above. By long-term intracellular recording, it has been found that cell 3 (Figure 12) produces a circadian oscillation of impulse activity.[29] Cell 3 is a neuron that intermittently or somewhat rhythmically emits bursts of impulses, as can be seen from the intracellular recording (top trace) of Figure 15. This neuron has emergent axons in the genital and pericardial nerves of the PVG (Figures 12 and 15). Electron microscopic examination of thin sections of the identified and isolated cell have shown that the cell body contains neurosecretory-like vesicles (Bernstein, unpublished observations). Thus, the impulse activity of this cell is probably a record of the release of neurosecretory material at the terminals of this neuron. In the intact animal this activity would affect the genital ganglion and, in addition, neurosecretory material would probably be released into the general circulation through the pericardial nerve.

When the intracellulary recorded impulse activity of cell 3 is studied in isolated ganglia removed from animals previously exposed to a fixed photoperiod, a large increase of impulse rate occurs close to the projected dawn[29] (Figure 16). In about 10 per cent of the preparations the major peak of impulse activity occurs at projected dusk rather than dawn.[33] When sea hares are exposed to constant light for 1 to 2 weeks, cell 3, studied in the isolated PVG, emits an impulse rate that fluctuates with a circadian rhythm, whose form and timing are clearly different from that of light-dark entrained individuals.[29]

The contrast between the performance of a "naive" and an "experienced" cell 3 can be appreciated from the compressed intracellular recordings exhibited in Figures 17 and 18. When in the sea hare, the naive cell 3 (parabolic burster) had information only of one light-dark cycle after one week of constant light. It showed a high background of parabolic bursts, with the major peak of impulse rate occurring around 30 minutes after the projected dawn (line 4, Figure 17). While in the animal, the "experienced" parabolic burster had information of seven light-dark cycles. It showed a low background of parabolic bursts, with the major peak of impulse activity occurring around the projected dawn (lines 4 and 5,

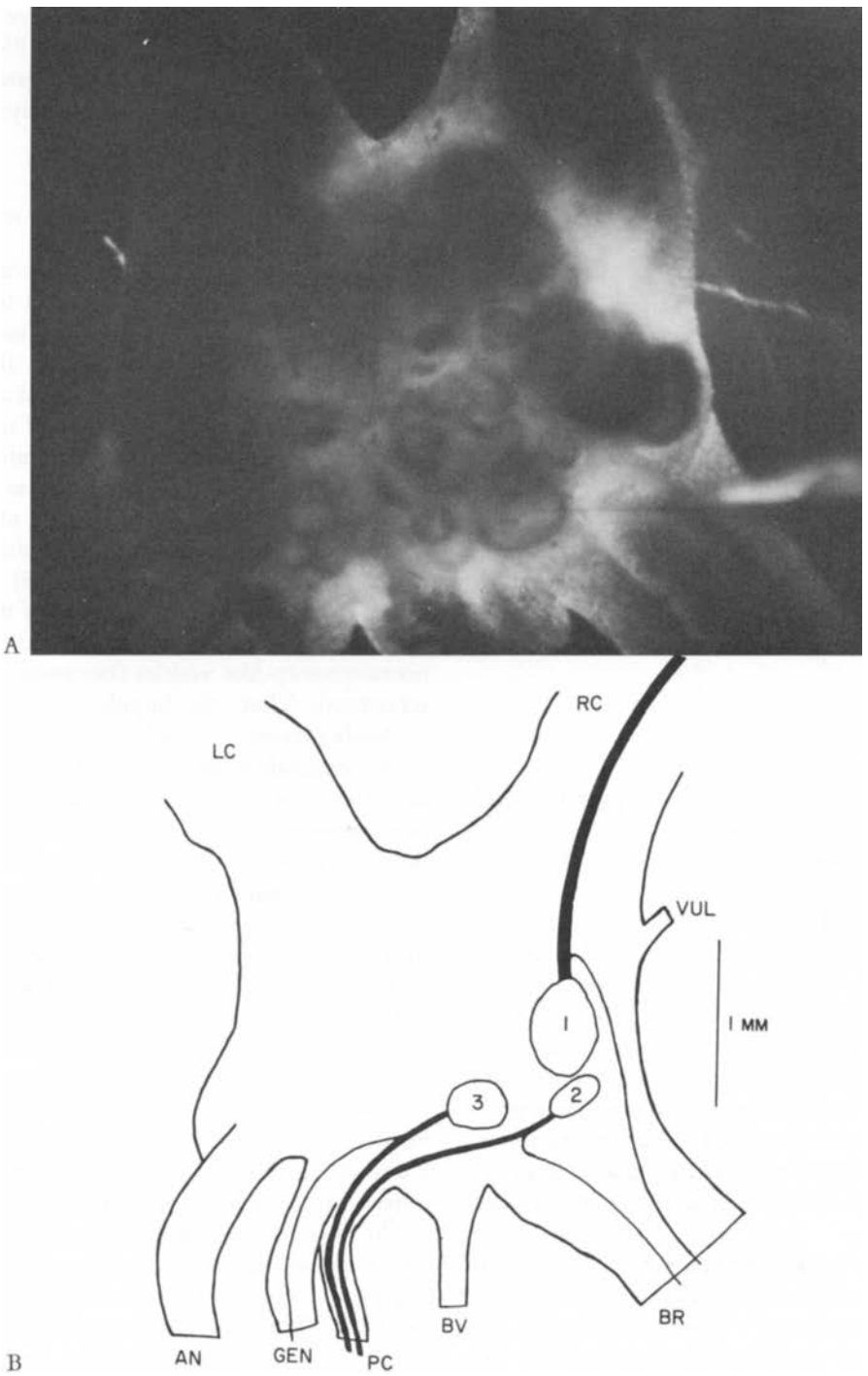


FIGURE 12 A: Photograph of the dorsal surface of an isolated, unstained parieto-visceral ganglion (PVG). A microelectrode can be seen contacting cell 3 (see Fig. 1B[33]) at about 5 o'clock. The "right giant cell" of Hughes and Tauc[36]—cell 1—is immediately above and to the right. Cells 2, 4, 5, 6, 9, and 10 (see Fig. 1B[33]) are readily visible in this preparation. (From Strumwasser, Note 33)

B: The branching patterns emerging from the PVG are shown for the axons of cells 1, 2, and 3. The surface shown is dorsal and the top of the diagram is anterior. The major nerve trunks are (going counterclockwise): RC and LC, right and left pleuro-visceral connectives; AN, anal; GEN, genital; PC, pericardial; BR, branchial; and VUL, vulvar nerves. The terminology is taken from Eales' monograph on *Aplysia*.[37] Additional small nerves around BV (blood vessel) are not shown. Vertical line represents 1 mm. (From Strumwasser, Note 33)

Figure 18). Such effects of prior experience on the form and timing of the activity of a single nerve cell can be used as arguments that a storage of information, concerning the nature and time of past external events, has taken place in a single neuron.[29,33]

Experiments detailed elsewhere[29,38] show that the mechanism of the circadian cycle, as entrained by photoperiod, is not one of counting bursts or impulses. A properly positioned peak will still emerge when, by transmembrane hyperpolarization, background activity is diminished or suppressed. Heat pulses applied to an entrained isolated ganglion during the projected dark period cause a phase advance (earlier expression) of the circadian peak in cell 3.

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis,[39] injected intracellularly during the projected dark period, also causes the circadian peak to advance in phase.[29,38] When actinomycin D is injected into PB, at a time soon after an entrained circadian peak, the subsequent circadian peak is phase delayed with respect to the projected dawn. It appears to be phase locked to the time of actinomycin injection. It has been tentatively proposed[29] that actinomycin D, on binding to DNA, displaces and so releases an available intact nuclear message (presumably in the form of messenger RNA). In this model, the messenger RNA initiates the cytoplasmic production of either a polypeptide (P, Figure 19), which depolarizes the neuron membrane, or an enzyme that controls the production of a depolarizing substance.

An inhibition of the circadian rhythm of luminescence in the marine dinoflagellate, *Gonyaulax polyedra,* by Actinomycin D had been reported earlier by Karakashian and Hastings,[40] leading them to the conclusion that "the clock-like rhythmic mechanism is dependent upon the cell's unimpaired ability to synthesize RNA." Feldman[41] has recently reported that cycloheximide, an inhibitor of protein synthesis, lengthens the period of the circadian rhythm of phototaxis in *Euglena,* in proportion to the dose. The circadian rhythm of photosynthesis in *Acetabularia* is blocked by Actinomycin D in intact[42] but not in anucleate[42,43] preparations. All of these findings imply a DNA-RNA-protein involvement in the control of circadian cycles, but do not as yet shed light on the details of mechanisms.

A model of intracellular control of neuronal rhythms (with macroperiods) is illustrated in Figure 19. The nucleus of the neuron synthesizes and releases messenger (informational) RNA into the cytoplasm. The polypeptide or protein (P) produced on this template either directly or indirectly initiates excitation or inhibition at the surface membrane of the neuron soma by altering its permeability or perhaps by activating or inactivating electrogenic (or

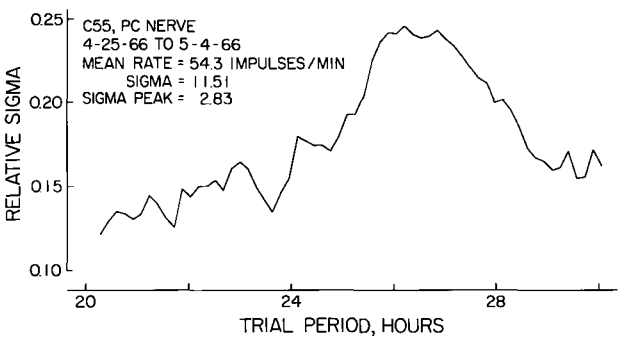


FIGURE 13 Periodogram of ten days of continuously recorded multi-unit impulse activity in the pericardial (PC) nerve while the PVG (C55) was in organ culture.[35] The activity of the PC nerve was recorded by means of a nerve cuff (see Fig. 15). Thirty-minute moving averages of data and ten-minute lag steps were used.

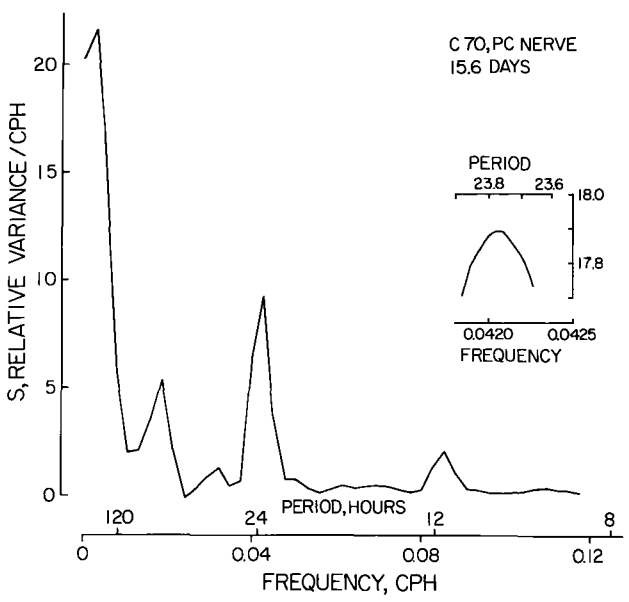


FIGURE 14 Power spectrum of 15.6 days of continuously recorded multi-unit impulse activity in the PC nerve while the PVG (C70) was in organ culture. The activity of the PC nerve was recorded by means of a nerve cuff. The autocorrelation (A) used 30-minute data averages and 30-minute lag steps, and was normalized with respect to the value of A at zero lag. Maximum lag for the spectrum input was 7.8 days. The inset shows, at higher resolution, the peak of the major energy.

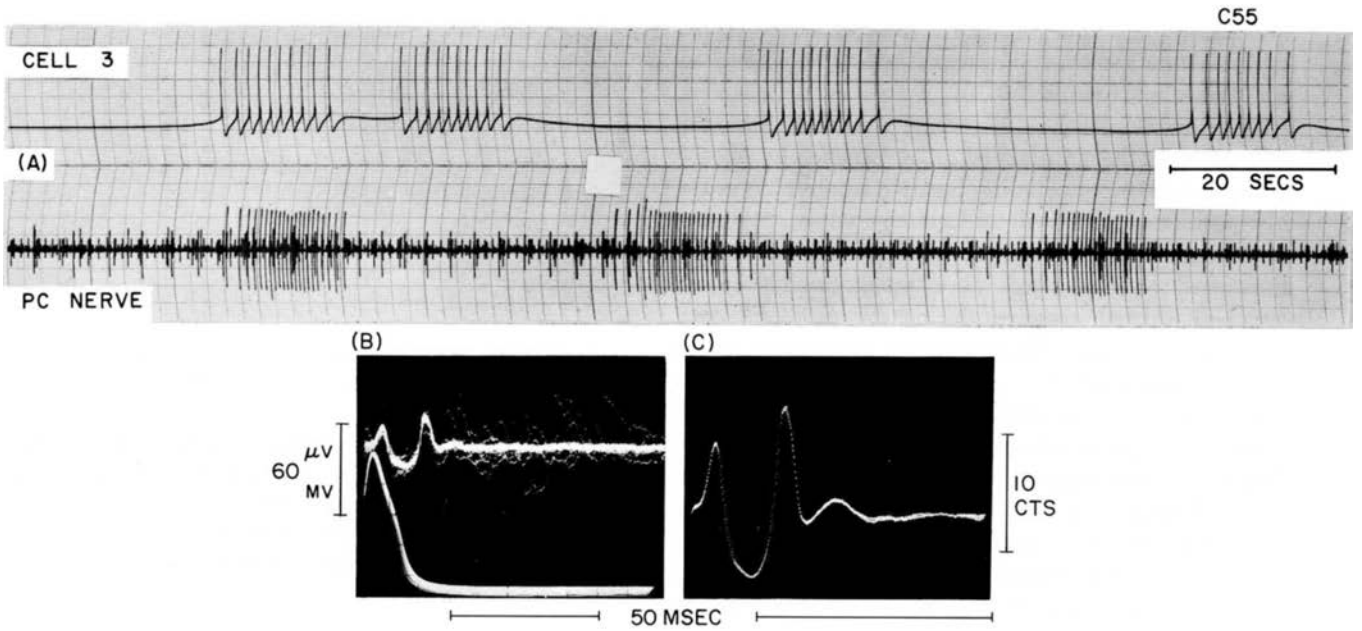


FIGURE 15 A: Simultaneous recordings from cell 3 (*upper trace,* intracellular) and the pericardial nerve (*lower trace,* nerve cuff) from a PVG (C55) in organ culture.

B: The synchrony of a small triphasic axon spike in the pericardial nerve (*upper trace*) with the intracellular spike of cell 3.

C: The orthodromically evoked axon discharge of cell 3 recorded with an on-line averaging computer.

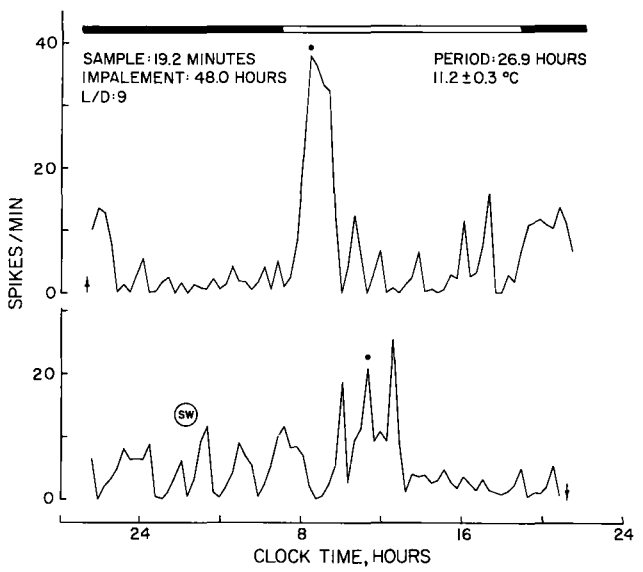


FIGURE 16 Spike output of cell 3 (PB, parabolic burster) as a function of clock time. Organism had been conditioned to nine cycles of light followed by darkness, each of 12 hours' duration. The second day of intracellular recording is plotted below the first. The projected periods of dark and light are indicated as shaded and clear portions, respectively, of the upper rectangular bar. *Sample* refers to the successive lengths of time over which spikes were counted. *Period* refers to the span of time between the two markers (•). At ↑, cell was impaled. Temperature indicated is the average followed by the range. (SW): Sea water changed in main reservoir. (From Strumwasser, Note 29)

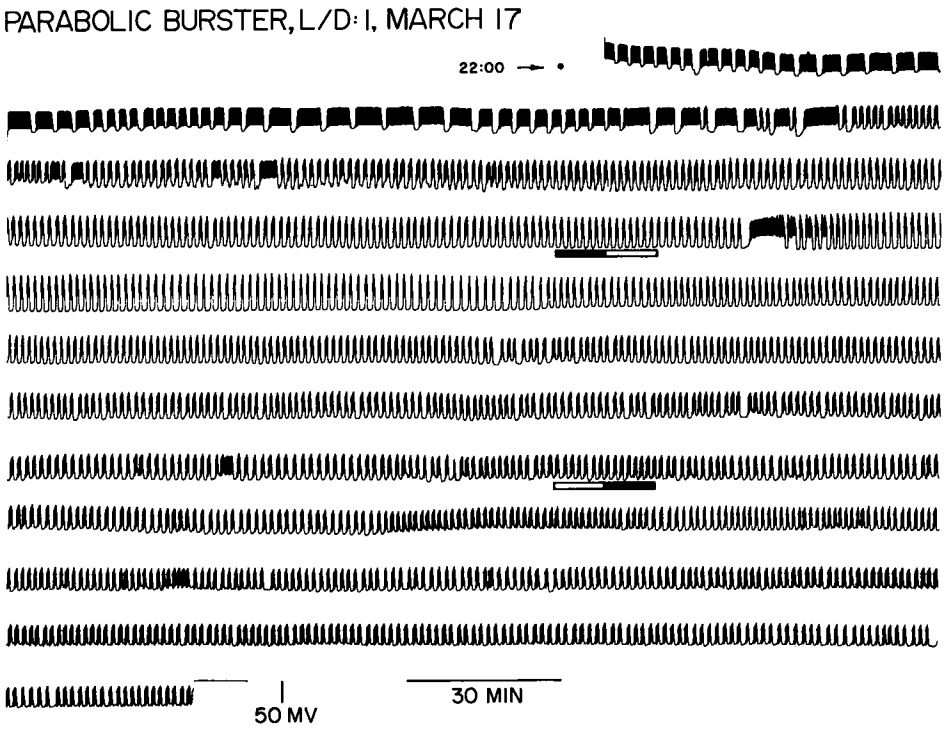


FIGURE 17 Long-term continuous intracellular recording from cell 3 in the PVG. The PVG was obtained from a sea hare exposed first to one week of constant light and then to one cycle of 12 hours light and 12 hours darkness. To compress the time scale, recordings were made on a low speed potentiometric recorder which, however, attenuated the spike amplitude. Each full line represents three hours of cellular output. The projected time of "lights on" and "lights off" are shown by the rectangular bar under lines 4 and 8 respectively.

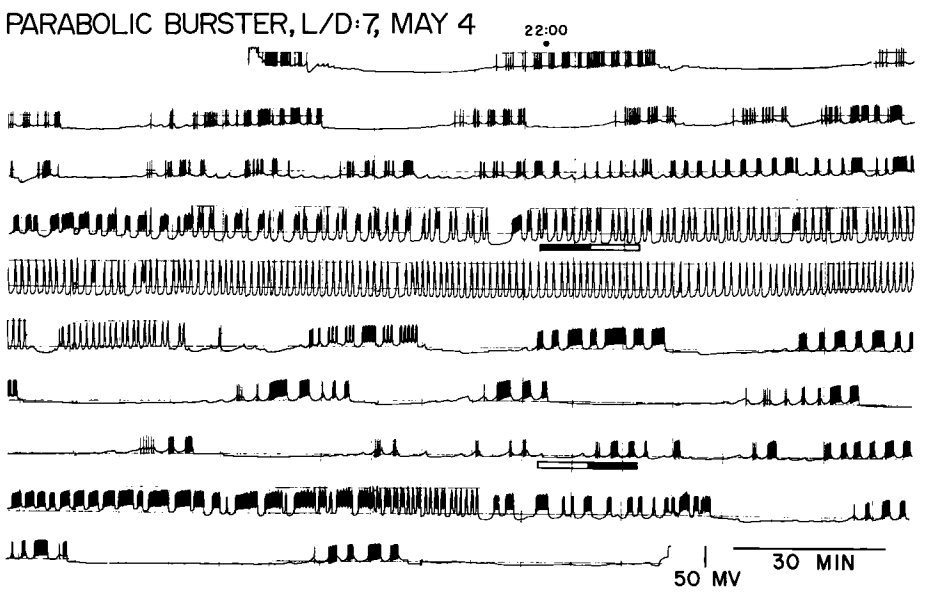
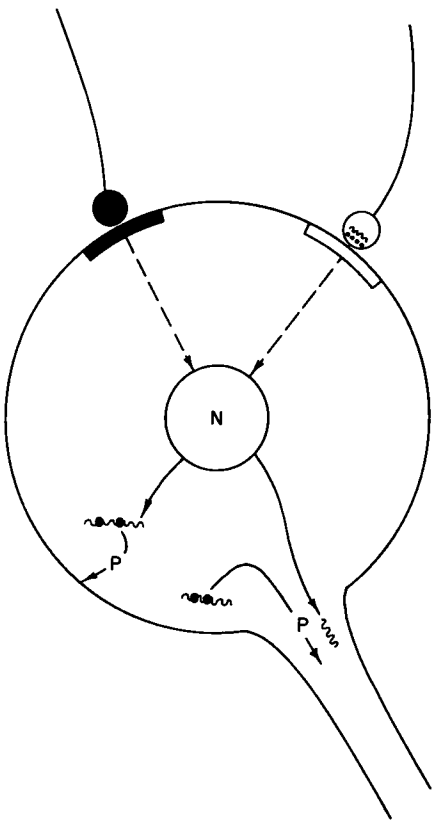


FIGURE 18 Long-term continuous intracellular recording from cell 3 in the PVG. The PVG was obtained from a sea hare exposed to seven light-dark cycles (L/D:12/12 hours). Projected "lights on" and "lights off" on lines 4 and 8 respectively. (See legend of Fig. 17)



neutral) ion pumps in the surface membrane.[29,33] Because the circadian rhythm can be phase controlled, a coupling mechanism is illustrated (dashed line) between the subsynaptic membrane and the nucleus. Presynaptic impulses[44] (or acetylcholine[45]) in the superior cervical ganglion increase the synthesis of phosphatidylinositol, postsynaptically, which implies an excitation-synthesis coupling in these neurons. This coupling might be mediated through the nucleus at the level of transcription, but a coupling at the translational level is, at the moment, just as probable.

FIGURE 19 One model of intracellular control of neuronal rhythms. N is the nucleus and P can be either polypeptide or protein. The wavy line represents messenger (informational) RNA with or without attached ribosomes (●). Coupling between the subsynaptic membrane and the nucleus is shown by dashed arrows. Coupling between the nucleus and soma membrane or axoplasm is shown by solid arrows. Synaptic vesicles (○) shown in nerve terminal on upper right. (See text for additional details.)

Neurophysiology of the States of Sleep

M. JOUVET

SLEEP IS A SUBJECT on which almost every one considers himself an authority because of personal interest and first-hand experience. Yet sleep is still impossible to define properly because we know so little about its mechanisms and function. This review on the neurophysiology of sleep is limited to work that has been done on the subject only during the last six or seven years.

On the one hand, we must assume that our brain, like our kidneys and heart—but unlike the muscular system—does not rest during sleep. On the contrary, it actually undergoes reorganization. In other words, sleep seems to be an active phenomenon. On the other hand, it appears that behavioral sleep does not proceed from a single process but is the manifestation of two closely interconnected, different stages of nervous activity.

The electrical brain activity of a mammal asleep has two recurring patterns from opposite modes. The first mode, which we shall call slow-wave sleep, manifests itself in the presence of a synchronized cortical activity of spindles and/or of high voltage slow waves. The other reveals itself by a low voltage, fast cortical rhythm similar to arousal activity. This is called activated sleep,[1] or paradoxical or rhombencephalic sleep.[2] It has not yet been proved, however, whether these two electrical aspects of sleep are manifestation of a single hypnogenic mechanism or the manifestations of two fundamentally different states.

Phenomenological aspects of sleep

SLOW SLEEP: BEHAVIORAL ASPECTS This section might seem unnecessary because it is apparently so self-evident. Anyone can recognize when a cat is asleep by its posture (by the persistence of a certain muscular tonus), by its slow and steady breathing, and by the stillness of its eyeballs behind closed eyelids. Yet there is no specific behavioral criterion of slow sleep, for the relationship between synchronized, or slow, cortical activity and sleep behavior is not absolute. Indeed, an animal may show spindles or slow cortical waves while standing and apparently awake, while crouching, lying, or curled into a ball.

Thus, in a normal animal, only a few of the behavioral criteria of slow sleep have an absolute value. Ocular signs are useful: the nictitating membranes are relaxed, and there is marked miosis (pupil contraction), which is ascribed to a hypertony of the neurons of the Edinger-Westphal nucleus (part of the oculomotor nucleus in the midbrain). The study of muscular activity does not yield information of diagnostic value. There remains a tonic activity in the neck muscles that is often—but not always—lower than in wakefulness. A deeper analysis of the motor system does not reveal important shifts in the spinal monosynaptic or polysynaptic reflexes, whose amplitude remains the same as in wakefulness.[3,4] The vascular system does not seem to play an important part in the slow sleep state. There is only a small decrease in blood pressure in the cat as compared with the waking state.[5] Changes in cardiac and respiratory activity are not sufficient in themselves to confirm the slow sleep state in the cat.

ELECTROPHYSIOLOGICAL ASPECTS The EEG aspects of slow sleep (Figure 1) have been known for a long time.[6-8] Eleven to 16 cycles-per-second spindles of large amplitude are predominant in the frontal and associated areas; the synchronized activity is less important in the auditory and visual areas, the olfactory bulb, and the pyriform cortex. Spindles are also recorded in bipolar derivation in the mesencephalic reticular formation (RF) and the pyramidal tract. These are often, although not always, synchronous with the cortical spindles. They are usually followed by 1 to 4 cycle-per-second high-voltage slow waves, which can also be recorded in the subcortical structures. The spindles and slow waves are of less amplitude and may be absent from the specific thalamic nuclei, the pulvinar, and the caudal part of the brain stem, where low-voltage, fast activity may prevail.

Some local characteristics of brain activity during sleep should be pointed out. In the dorsal or ventral hippocampus and in structures that are in efferent relationship with the hippocampus, high-voltage spikes of from 500 to 900 microvolts appear. This activity persists in the limbic

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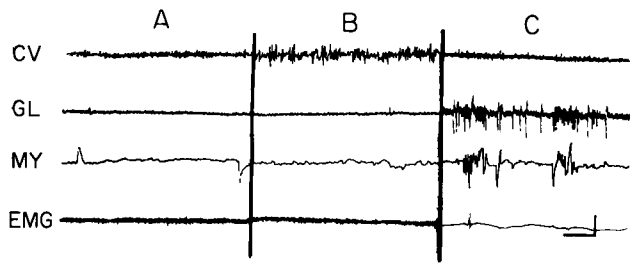

FIGURE 1 The two states of sleep in the cat.

A. Arousal with fast cortical activity (CV), eye movements (MY) and tonic activity of the neck muscle (EMG).

B. "Slow sleep": High voltage slow and synchronized activity at the cortical level. No eye movement. Persistence of tonic muscular activity.

C. Paradoxical sleep: Low voltage fast cortical activity similar to A. Bursts of rapid eye movements accompanied by discharges of lateral geniculate spikes (GL). Total atony of the neck muscle. Cal.: 3 sec, 50 μv.

structures, even when an animal's neocortex has been removed. Thus it is the only electrical index to the onset of slow sleep, and at that time only two consecutive electrocortical aspects—spindles and slow waves—can be recorded in the cat. It appears that when carnivores fall asleep, there is no stage similar to the first stage of sleep in man, during which there is a flattening of alpha activity coupled with a fast activity. A similar short-term stage has been described in primates.[9]

UNIT ACTIVITY DURING SPONTANEOUS SLEEP The unit aspect of sleep will be reviewed in detail in Evarts's paper, so here we shall give only a summary of recent studies.[10-12]

During the manifestation of the surface-positive or surface-negative spindles that typically appear when a cat is falling asleep (the thalamic origin of which seems unquestionable), the unit activity of superficial and deeper layers of the cortex is subject to a phasic enhancement. This increase of discharges is attributed either to a negative feedback that maintains a certain level of cortical tone or to a disinhibition of cortical inhibitory interneurons. Whatever their mechanism, the spindles are accompanied by a phasic increase in background cortical activity in the sensory and motor areas and of the pyramidal tract, and by a decrease of the background activity level in the midbrain reticular formation. This decrease may help us explain a few peripheral signs of sleep: the reduction of muscular tone, despite the amount of pyramidal impulses at every spindle, would then appear as a decrease in the tonic activity of the reticular facilitatory descending system.

During cortical slow waves, when slow sleep gets its most typical EEG manifestation, it is possible to assume that the cortical unit activity decreases (probably through hyperpolarization in the deeper layers). At this stage the reticular activity also seems to decrease.

CORTICAL STEADY POTENTIAL AND OTHER ELECTRICAL PARAMETERS The appearance of spindles and slow waves of physiological sleep, whether they occur spontaneously or are provoked by habituation phenomena, is accompanied by a shift of the cortical steady potential to the positive side,[13,14] whereas waking is accompanied by a negative shift. A reduction in the resistance of superficial layers of the cortex and an increase of the capacitance during slow sleep have been observed by Aladjalova.[15] They are attributed to a "moderating influence of the dendritic potentials over the excitability of the neurons through extracellular currents." The hypothalamic impedance (reflecting, in a way, the blood flow) decreases, whereas cortical and reticular impedance show a tendency to increase at the onset of slow sleep. This is attributed to a relaxation of the vasomotor tone.[16] The temperature of the preoptical area and of the hypothalamus falls by 0.5° during slow sleep.[17]

Sleep with fast cortical activity: paradoxical sleep

All dog owners have seen their sleeping pets suddenly wag their tails, move their lips or legs, or bark. Lucretius, who lived in the century before Christ, attributed these episodes to dream activity, and as long ago as 1765, Fontana[18] called this sleep *sonno profondo* with "convulsions." But 20 years were necessary, following its first electrical description, to integrate this state of sleep into neurophysiology. When EEGs were first used in experiments, fast cortical activity periods were reported[6] during less quiet sleep, "judging by twitching of vibrissae," as being similar to "the alert waking state." This observation was later confirmed.[8] At the same time, the two states of sleep in cats were described and the first EEG recording of the state of *Tiefen Schlaf*, with fast cortical activity and low voltage was published.[7] This work was unfortunately forgotten. Although there were short descriptions of cortical desynchronization[19] or of hippocampal theta rhythm[20] during sleep, until 1958 only the state of slow sleep was known and studied in the cat. Dement and Kleitman,[21] in their studies of man, and Dement in his work with cats[1] deserve the credit for definitely relating the periodic recurrence of activated sleep with rapid eye movements. This state was interpreted as an intermediate phase between slow sleep and arousal. Soon, however, it was shown that this sleep was, in fact, "deeper" than slow sleep, and could be identi-

fied by a specific subcortical electrical activity (pontine spikes) and specific postural criteria (total atony).[22] This, in turn, led to the demonstration of its rhombencephalic origin and to the hypothesis that it was a different *state* of sleep.[22] Since then, numerous correlations have been made on EEGs and behavior during sleep distinguished by fast cortical activity; which has been called, among other things, paradoxical sleep (P.S.).

SOMATIC PHENOMENA Contrary to slow sleep, whose behavioral criteria are imprecise, the onset and the end of P.S. may be fixed and can be assessed within a few seconds from behavioral criteria alone—not only in intact cats, but in decorticated or chronic pontine cats as well. Behavioral phenomena may be divided into two types: tonic and phasic.

The complete disappearance of muscular tone in the antigravity muscles and, above all, in the neck muscles, is the most remarkable manifestation of the inhibition of muscular tone, typical during P.S.[2] Preceding, or following by a few seconds, the cortical desynchronization of P.S., electromyographic (EMG) silence accompanies the sudden fall of the animal's head, when it droops during slow sleep. The end of P.S. is usually shown by a sudden upswing in EMG activity, whether the animal awakes or returns to a slow-sleep state. This typical neck-muscle atonia may also be found in two types of spasticity—gamma (decerebrate animal preparations[23]) or in alpha (decerebrate animal preparations in which the anterior lobe of the cerebellum has been removed). It is also observed after section of the posterior roots from C1 to C7.

Attending this atonia, or even preceding it by a few seconds, a decrease or a diasppearance of heteronymous monosynaptic and plurisynaptic reflexes are seen,[3,4,24] whereas these vary little during slow sleep. The homonymous monosynaptic reflex is not tonically inhibited during P.S. except during bursts of eye movements. Lastly, the posttetanic facilitation of monosynaptic reflex (obtained by stimulating the posterior roots) is abolished during P.S., whereas it persists during slow sleep.[4]

Among phasic phenomena typical of P.S., eye movements are predominant and consequently are discussed separately. They occur with cortical activation, and their pattern is different from that of the eye movements while awake. However, they are not isolated, and other phasic movements accompany P.S. development in a strange and disorderly manner: sudden movements of the ears, the whiskers, the fingers (flexion), the tail, and sometimes genuine clonic jerks of the back muscles. These phasic phenomena are especially developed in the kitten shortly after birth and increase in a striking manner after long P.S. deprivation.

VASCULAR PHENOMENA They are constant in the cat and also express tonic and phasic vascular changes. A drop in blood pressure appears at P.S. onset.[5] It may be interrupted by short, hypertensive phases during bursts of ocular movements, and is accompanied by a great irregularity in heart rate (bradycardia or tachycardia, according to the species of animal). This phenomenon may also be observed when P.S. is induced by brain-stem stimulation. The drops in blood pressure are much larger after bilateral sinoaortic deafferentation. In fact, pressure may be so low during P.S. that episodes of transient cerebral ischemia (electroencephalographic flattening and seizures) sometimes occur.[25] Cerebral blood flow has been studied by methods that utilize the shifts of cerebral temperature or of cerebral impedance. The most striking phenomenon is the large increase of blood flow that occurs during the generalized fall of blood pressure.[26] Several hypotheses have attempted to explain this blood-flow increase—the action of a cerebral vasodilation, or an increase of cerebral metabolism that expresses itself by an augmentation of carbon dioxide, well-known as a vasodilator. An increase of cerebral temperature at the onset of the fast cortical activity of P.S. has also been reported.[27] In addition, respiratory variations are noticeable most of the time; they consist of irregularity and increase of the rhythm, and apnea (shortness of breath) is frequently seen at the end of P.S.

Galvanic skin reflex (GSR), either spontaneous or induced by stimulating the peroneal nerve, has also been studied during P.S.[24] In 70 per cent of the cases there is a noticeable decrease both of spontaneous and induced GSR during paradoxical sleep as compared with slow sleep

If we trust the following criteria, P.S. appears to be deeper than slow sleep: the behavioral arousal threshold by reticular stimulation is much increased (up to 300 per cent) in comparison with slow sleep.[23]

Other behavioral (muscular atonia) and vascular (fall of blood pressure) criteria equally support the idea of a deeper level of sleep during P.S. That is why this state is sometimes called deep sleep as opposed to light sleep (slow sleep). As a matter of fact, the concept of depth or "heaviness" of sleep is ambiguous and depends essentially on the criteria used. The possibility that with conditioning methods some learning could occur during sleep has been extensively studied.[28] It appears that some classical conditioning is possible during slow sleep, whereas it is almost impossible during P.S. On the other hand, judging by the phasic movements of the eyes or legs, P.S. appears to be a restless, less quiet sleep. Moreover, P.S. and slow sleep increasingly appear to be two qualitatively different states, and hence it is, perhaps, misleading to compare them from the quantitative viewpoint of depth or lightness.

Electrophysiological aspects

Paradoxical sleep in an unrestrained, normal, adult cat takes place after a variable period of slow sleep. It then appears periodically during slow sleep. Its mean duration is 6 minutes (but periods of from 15 to 20 minutes can frequently be recorded). Its percentage, in comparison with the duration of behavioral sleep, is from 20 to 25 per cent (about 15 per cent for a period of 24 hours). As in the behavioral aspects, it is also possible to recognize two major components in the electrical cerebral activity during P.S.: tonic (fast cortical activity and regular theta hippocampal activity), and phasic (monophasic ponto-geniculo-occipital spike activity that is associated with rapid eye movements).

Tonic activity is characterized by a neocortical diencephalic and mesencephalic low voltage fast activity (20 to 30 cycles per second), which is similar to the cortical desynchronization that usually accompanies intense arousal or attention states. However, some electrical local cortical and subcortical activity enables us to discriminate between the electrical cerebral activity of P.S. and EEG arousal. The appearance of a continuous theta rhythm in the ventral and dorsal hippocampus is most characteristic. In the dorsal hippocampus, it is steadier and faster (5 to 7 cycles per second) than that observed during complete wakefulness, (4 to 4.5 cycles per second). The olfactory bulb activity also shifts in a characteristic way; the sinusoid rhythm of from 50 to 60 cycles per second, observed during arousal, disappears during P.S.

The close relationship between electrical phasic activity and the visual system makes it necessary to study them together.

RAPID EYE MOVEMENTS Rapid eye movements (REM) appear at the onset of the cortical activation. Their rapidity and frequency—from 60 to 70 movements a minute—and their "pattern" make them distinguishable from observation movements while awake. Most of the time, miosis is maximal and the nictitating membranes are relaxed. Yet, at times, a sudden mydriasis (dilation of the pupil) with retraction of the nictitating membranes may accompany the volleys of ocular movements. This phasic dilation goes on after ablation of the superior cervical ganglia and therefore must be ascribed to an inhibition of the tonic activity of the Edinger-Westphal nucleus. The analysis of the structures responsible for the appearance of eye movements, both isolated and in bursts, has given the following results.[29] The pontine cat whose superior colliculus is destroyed has only isolated lateral and external movements, depending from nerve VI. In the mesencephalic cat whose superior colliculus is intact, more important bursts of ocular movements persist. On the other hand, the electrical coagulation of a zone located at the level of the superior colliculus and the mesencephalic tegmentum in the intact animal suppresses the bursts. These, in turn, are much increased in the decorticated animal. However, the role of the cortex is not unequivocal; the removal of the visual cortex strikingly reduces the isolated eye movements and the bursts, whereas a frontal decortication or a frontal leukotomy produces a marked increase of bursts.

Recently it has been shown that the destruction of the medial and descending vestibular nuclei also suppressed the burst of REM, whereas isolated eye movements were still present during paradoxical sleep. These nuclei, the unit activity of which is increased during P.S., apparently control most of the phasic phenomenon of P.S.[30,31]

PHASIC ELECTRICAL PONTO-GENICULO-OCCIPITAL ACTIVITY Many difficulties and delays necessarily accompany a wide and systematic exploration of the cortical and subcortical structures. This explains why it took several years before a link was found between the "spontaneous" phasic potentials observed during P.S. (Figure 2), which was first described from the pontine reticular formation.[23] One hundred- to 200-microvolt monophasic spikes of a 100-millisecond duration, often occurring in groups of five to six (hence their pseudospindles), were observed from the lateral geniculate nucleus, the occipital cortex, the superior colliculus, the nucleus of III, the pulvinar, and the parietal cortex.

Pontine and geniculate phasic spikes are the first electric signs heralding the appearance of a P.S. episode. They may appear one or two minutes before the cortical activation and the disappearance of the neck EMG, and sometimes occur erratically during slow sleep. Isolated spikes may occur during 5 per cent of slow-sleep time, and during P.S. they usually have a frequency of 60 to 70 per minute.

The latency between the monophasic pontine potentials and the geniculate potentials is very short (5 milliseconds). Geniculate responses with the same pattern as spontaneous spikes can be evoked (gating effect) through stimulation of the pontine reticular formation during paradoxical sleep, when they have a 25- to 35-millisecond latency. On the contrary, it is impossible to evoke geniculate responses through stimulation of the pons during arousal or slow sleep.[32]

The relationship between this phasic activity and REM is not simple: not darkness, destruction of the retina, or even complete removal of the eyes and the extraocular muscles suppress these ponto-geniculo-occipital spikes (at least for two or three days following the operation).

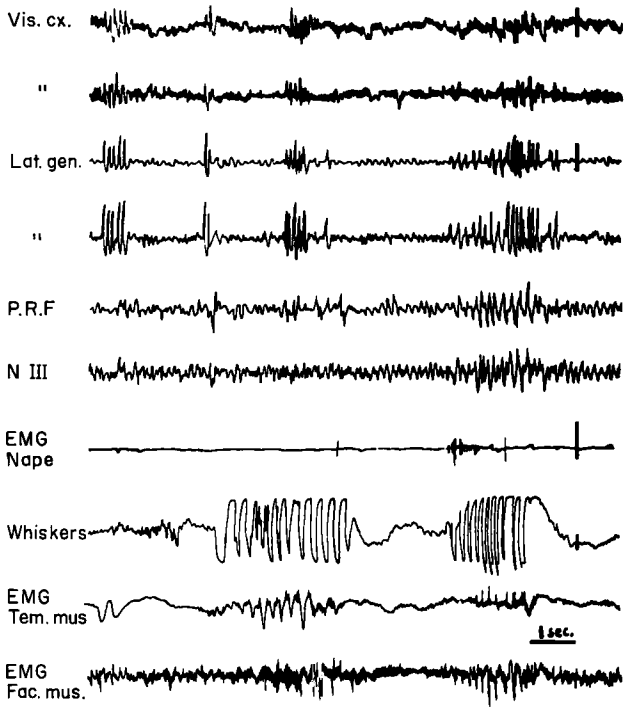

FIGURE 2 Phasic phenomena during P.S. Normal cat: 3 days after enucleation of both eyes. Monophasic peaks grouped in pseudo-spindles in the pontine reticular formation (PRF), the oculomotor nucleus (N.III), the lateral geniculate nucleus (Lat. gen.) and the visual cortex (Vis. cx.). Note phasic twitching of the whiskers, the temporal muscles, and the minor muscles of the face, and absence of nuchal EMG activity. Scale: 1 sec, 50 μv.

Therefore, the activity cannot be a possible feedback of a retinal "on and off" effect, nor can it be of extrinsic muscular origin. In most cases, however, there is a close time relationship between the spikes and the muscular activity of the extrinsic muscles[33]; the latter occurs mainly in phasic bursts, whereas there is a tonic component during arousal.

These data favor an ascending, pontine, extraretinal projection to the lateral geniculate and the occipital cortex. Similar extraretinal input to the lateral geniculate body have been noticed after stimulation of the mesencephalic reticular formation or the labyrinth.[34]

Whatever the complex relationship of the extraretinal input to the lateral body might be, it is likely that the optic tract terminals are involved in the genesis of geniculate monophasic spikes. Indeed, these spikes disappear after the sixth day following enucleation of both orbits[35] or retinal photocoagulation, although REMs and pontine spikes persist.

Structures and mechanisms responsible for slow sleep

It is difficult to study the mechanism of sleep without briefly recalling the classical concept of the wakeful system (Figure 3).[36,37]

Since 1949, the brain-stem reticular formation has been thought to be responsible for cortical "arousal" through the ascending reticular activating system (ARAS), as well as for behavioral arousal. The latter could occur either through the ARAS and so by a secondary corticofugal effect, or by a simultaneous action upon the descending reticular facilitory system. There is, however, a striking difference between the topography of the ARAS when it is delimited by stimulation experiments, which induce arousal, or by lesion, which provokes comatose states. With the first technique, according to the pioneering work of Moruzzi and Magoun,[38] the ARAS extends into the brainstem tegmentum from the medulla to the posterior diencephalon, whereas in the second technique it extends only to the mesencephalon and posterior diencephalon. Thus there is no doubt that the lesion experiment has a better localizing value than does the stimulation technique. (We will meet this fact again in the discussion of hypnogenic structures.)

After the discovery of the waking system, the principal question regarding sleep was: How is the activity of the

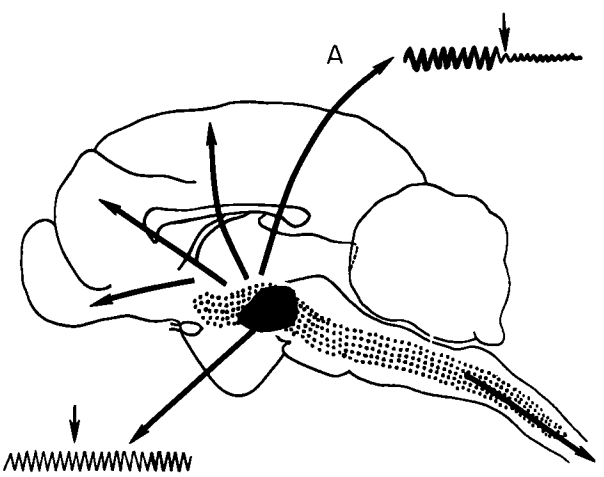

FIGURE 3 The arousal system in the brainstem emphasizing the difference between results obtained by stimulation and by lesion.

In dots: Part of the reticular formation of the brainstem whose stimulation induces arousal (A).

In black: Lesion of the reticular formation, which induces a lasting comatose state during which arousal by sensory stimulation is no longer possible (B). Lesions placed caudally in the reticular formation do not induce coma, but usually increase arousal.

ARAS dampened during sleep? Two theories were offered.

1) If sleep is a *passive* dampening of the activity of the ARAS, there is no need to postulate active sleep-inducing mechanism.

2) If sleep is an *active* process, one must be able to induce sleep by stimulation of some "hypnogenic structures," or, even more convincingly, one should be able to create insomnia by destroying these structures or by disconnecting them from the ARAS.

PASSIVE THEORY OF SLEEP The historical roots of the passive theory of sleep may be traced back many years. Kleitman, who was one of the supporters of the theory, clearly explains its basis: "to fall asleep" or "to be unable to remain awake" do not have the same meaning. The "first term implies an *active* onset of sleep, while the other implies a *cessation of an active condition of wakefulness,*"[39] i.e., a passive mechanism. (The italics are mine.) If so, it is not sleep that must be explained, but wakefulness.

Physiological sleep was then interpreted as the expression of a functional deafferentation of the ARAS "eliminating the waking influence of the ARAS," and thus as the absence of wakefulness. This reticular hypothesis of sleep[40] was founded primarily on the extrapolation of certain experimental results. Coma resulting from the extensive destruction of the mesencephalic tegmentum[41] or from barbiturate narcosis, whose depressive influence over the ARAS had been shown, was attributed to the interruption of an ascending flow of reticular impulse. Physiological sleep, as compared with coma, was then explained as a functional, passive reduction of the tone of the ARAS. So, according to the reticular hypothesis, sleep would be due to the *désactivation en avalanche* of ascending impulses of the ARAS. This deactivation would be initiated by a slowly developing process of neuronal fatigue, precipitated at a given moment by a reduction of sensory inputs.[42] However, the *désactivation en avalanche* neither explains why sleep may be induced by central or peripheral stimulation nor why, if it is a question of "neuronal fatigue," sleep may continue for such a long time (60 per cent of the day in the cat in a cage).

So, the passive theory of sleep does not allow us to explain satisfactorily the processes of falling asleep, and gives way now to *active theories.*

ACTIVE THEORIES OF SLEEP Two types of experiments favor the active hypothesis (Figure 4). They must be considered separately because they have unequal value.

Study of the central triggering of slow sleep was begun, as is well known, by the classic experiments of Hess.[43] In chronic experiments in cats, low-frequency (from 3 to 15 cycles per second) stimulation of numerous structures in the brain may induce spindles or cortical slow waves with or without sleep behavior. As a matter of fact, we know that the presence of recruiting cortical waves by thalamic stimulation may be observed during behavioral arousal.[44] That is why the following have been described or suggested as hypnogenic structures[45]: frontal and somesthetic cortex, anterior and posterior suprasylvian gyrus, visual and motor cortex, head of the caudate nucleus, the internal capsula, the preoptic region, the dorsal or ventral hippocampus, the amygdala, the anterior or posterior hypothalamus, the mamillary bodies, the thalamus, the massa

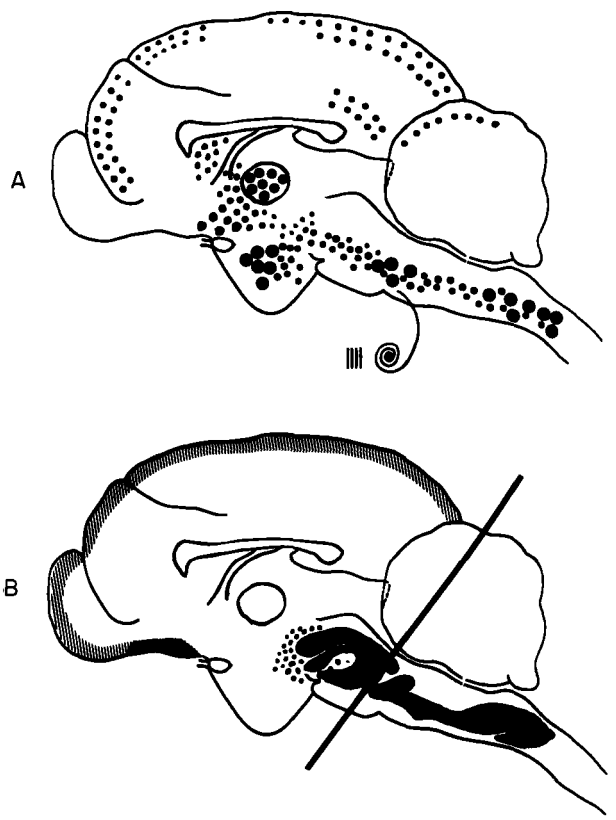

FIGURE 4 A. "Hypnogenic" structures as delimitated by stimulation: dots represent location (upon a schematic sagittal map of the brain) at which stimulation could induce SWS (see text).

B. Hypnogenic structures as delimitated by lesion: the Raphé system, destruction of which led to a permanent insomnia, is in black. The oblique line represents the plane of section of the brainstem in the mediopontine pretrigeminal cat (see text). The neocortex and orbital cortex (vertical line and cross lines) are responsible for the slow EEG activity during sleep (but are not necessarily responsible for behavioral sleep).

As in Figure 3, these diagrams illustrate the difference obtained by two different methods of mapping hypnogenic system.

intermedia, the diffuse thalamic system, the interpeduncularis nucleus, the mesencephalic or pontine reticular formation, the cerebellum, and the region of the solitary nucleus in the medulla. So we see that almost the whole encephalon has been credited with hypnogenic capacities. That is also why there is little justification in claiming a localizing value for hypnogenic electric stimulations.

The interpretation of the results yielded by this method meets numerous difficulties. The stimulation may not act upon neuronal cell bodies, but on their afferent or efferent axons. On the other hand, one stimulation may excite both synchronizing and activating elements, although most stimulations are of low frequency (spindles). Moreover, it is difficult to know whether sleep is induced by the stimulations or has been produced spontaneously, for most investigators agree that the occurrence of sleep is favored by stimulating a "relaxed" animal and that it is extremely difficult to induce sleep during a state of intense alertness except through long, repeated stimulations.

The solution expected from *chemical stimulations* in situ has apparently not yet been obtained. There are serious criticisms as to the specificity of the action of the drugs, which, in addition, have no greater localizing value than do electric stimulations. More and more areas are being discovered in which chemical stimulations induce slow sleep—either EEG or electrical and behavioral. Although adrenalin injected in situ at the brain-stem level has a clear arousal action, the proof that adrenalin or noradrenalin can induce arousal normally has not yet been definitely established[46]; the injection of adrenalin into the carotid has no activating effect.[47] On the other hand, in young birds and kittens, whose blood-brain barrier is permeable, adrenalin injection induces behavioral sleep.[48] In addition, a state "resembling sleep" may be obtained by injecting adrenalin into the ventricles.[49] Acetylcholine or serotonin[50] in microcrystals appear to have a real hypnogenic effect when injected into the caudate nucleus,[50] the preoptic region,[51] the medial thalamus,[50] the pontine reticular formation,[52] and the limbic midbrain circuit.[51]

PERIPHERAL REFLEX INDUCTION OF SLOW SLEEP

Slow sleep may be induced not only by cerebral stimulation, but also by the stimulation of numerous afferent systems. These include auditory stimulations, repetition of insignificant tones during habituation, or the repetition of tones that have acquired an inhibitory signal value (in Pavlovian terminology), intermittent photic stimulations, and stimulation of the cutaneous nerves (Group II) or muscular nerves.[53] The synchronizing cortical action of pressure applied to the skin has also been described. Vascular afferent influences may also induce drowsiness; vagus[54] or laryngeal stimulations, probably acting via vagal afferents, may induce EEG and both ocular and behavioral signs of sleep. Finally, the sleeplike state as observed by Koch[55] through stimulation of the depressor nerves, may induce an EEG sleep pattern in the *encéphale isolé* where there is no change of blood pressure. All these findings show that slow sleep may be actively induced, but they do not give any information about these active mechanisms.

RESULT OF CEREBRAL LESION After a complete midpontine pretrigeminal section (midpontine pretrigeminal preparation: MPP)[56-58] the cortical EEG shows a definite predominance of fast activity—78 per cent instead of 37 per cent (Figure 5). Moreover, the oculo-motor reactions of this preparation (depending from nerves III and IV) undeniably evoke a true alertness; the midpontine cat follows, with vertical eye movements, any object passing across its visual field. There is a mydriatic response to darkness and a pupillary dilation may occur upon the presentation of a significant visual stimulus, such as a mouse. On the contrary, a *cerveau isolé* transection, frontal to (or destroying) the posterior part of the mesencephalic tegmentum, induces a synchronized cortical EEG during the first days, without any ocular sign of wakefulness.[59,60] Because the *encéphale isolé* preparation still presents alternatives of wakefulness and slow sleep,[61] it may be concluded that tonically active EEG synchronizing structures located in the lower brain stem are able to dampen the "arousal" activity of the ARAS.

The lower brain-stem sleep-inducing structures have not yet been localized. There is some evidence that the medulla is involved, because a prebulbar transection induces an increase of the duration of EEG activation produced by reticular stimulation in *encéphale isolé* preparation.[62] Also, local reversible cooling of the bulbar floor of the fourth ventricle in cat *encéphale isolé* produces EEG and behavioral arousal attributed to the inactivation of the bulbar synchronizing structures. Cooling of the pontine floor, on the contrary, produces EEG and behavioral sleep signs.[63]

However, other experiments, seem to support a *posterior pontine localization.* A brain-stem hemisection at midpontine level leads to a desynchronized activity at the level of the homolateral hemisphere; a hemisection made a few millimeters in front induces a synchronized cortical activity. The former is achieved by suppression of the ascending synchronizing influence, the latter by suppression of the ARAS tonic activation. A hemisection situated a few millimeters behind the midpontine section does not cause cortical asymmetry,[64] although logically it should suppress the bulbar ascending synchronizing influences. The chronic experiments of Rossi et al.[65] also demonstrate that retropontine hemisections produce no cortical EEG

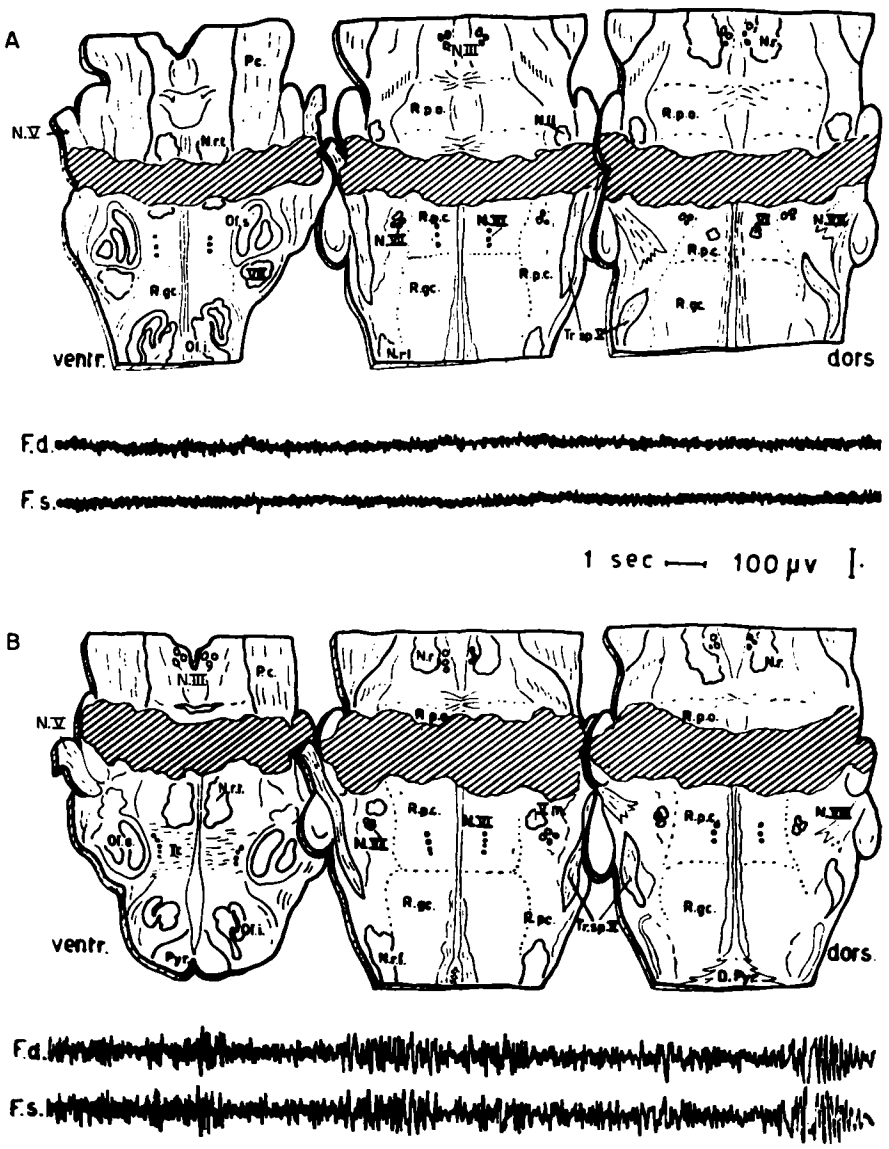

FIGURE 5 A. Mediopontine pretrigeminal section of the brainstem. Desynchronized cortical activity (arousal) obtained by deconnection of caudal synchronizing structures from the arousal system located just in front of the transection.

B. Rostropontine transection. In this case, the EEG is synchronized because the caudal part of the arousal system is destroyed by the lesion. (From Batini, et al., Note 58)

asymmetry, and these workers attribute to the posterior zone of the pons an important part in the triggering of cortical synchronization. All these results could be reconciled with recent data indicating that the Raphé system (serotonergic neurons), which extends from the medulla to the mesencephalon, is involved in sleep mechanism.

Recently, thanks to the new fluorescent techniques of Falk and Hillarp,[66] two systems of monoaminergic neurons in the brain stem have been described by Dahlström and Fuxe.[67,68] (See also chapters in this volume by Kopin and Kety.) The catecholaminergic neurons, most of them noradrenergic and with a green fluorescence, are located primarily in twelve groups (A-1 to A-12) in the lateral part of the medulla, pons, and mesencephalon, whereas serotonergic cell bodies with a yellow fluorescence, which is increased after injection of potent inhibitor of mono-

amino-oxidase (MAO), are almost exclusively located in nine groups in the Raphé nuclei of the brain stem. Serotonergic terminals from these cell bodies have been located in the spinal cord, in the brain stem, and the rostral part of the brain.

Destruction of the Raphé nuclei of the brain stem in chronically recorded cats leads to a state of almost permanent wakefulness,[69] and there is an 80 to 90 per cent decrease of sleep during the first two weeks after the operation. A state of permanent EEG and behavioral wakefulness is obtained during the first four or five days. Destruction of either the anterior or the posterior half of the Raphé system causes slow sleep to decrease by 50 to 60 per cent. Thus there is a correlation between the extent of the lesion of the Raphé nucleus and the amount of sleep. The same kind of result has also been obtained by sagittal section of the brain stem, which destroys most of the Raphé system.[70] These experiments, together with the effects on sleep of neuropharmacological alterations of brain monoamines, have led to the hypothesis that monoaminergic neurons could be involved in sleep mechanisms.[69]

In summary, there are many concordant experimental data in favor of synchronizing and sleep-inducing structures in the lower brain stem. Nevertheless, more experiments are needed to delimit these structures precisely and to determine if they belong to some specific nuclei located in the medulla or the pons, or if they belong to a monoaminergic system, composed of serotonergic neurons that occupy the Raphé nuclei from the medulla to the caudal mesencephalon.

ROSTRAL STRUCTURES INVOLVED IN SLEEP MECHANISM While it appears that the brain-stem hypnogenic structures are involved in the triggering of both behavioral and EEG sleep, there is some evidence that rostral structures are concerned mainly with the EEG aspect of slow sleep. On the one hand, the thalamus appears to be necessary for the occurrence of spindles, because its destruction by coagulation, section, or aspiration abolishes the cortical spindles during the onset of sleep, while cortical slow waves persist.[71,72]

On the other hand, after complete removal of the neocortex,[23] the synchronized or slow activity of the subcortical structures (thalamus or reticular formation) disappears immediately and does not reappear during sleep for several months, even after injection of phenobarbital sodium. On the contrary, if even a small part of the gyrus orbitalis and coronalis anterior is left undamaged, reticular slow waves may persist during sleep in subtotally decorticated chronic cats. This suggests that the basal part of the cortex is essential for *subcortical* synchronization of slow sleep. This hypothesis has found support in the recent experiments of Velasco and Lindsley.[73] In acute conditions, ablation of the entire dorsal convexity and of the medial and cingulate regions of the cortex failed to interfere with the spindle bursts, whereas ablations confined to orbital cortex alone completely abolished these potentials in the cortex and thalamus. Therefore, the orbital cortex appears to be the only region of the neocortex to play a crucial role in the regulation of thalamo-cortical synchronizing function.

Other experiments also support a descending cortical synchronizing influence upon the brain stem, but so far a specific region in the cortex has not been found. After a total mesodiencephalic section of the brain stem, cortical and thalamic spindles persist in front of the section, but none can be recorded caudally to the section site.[23] These data permit us to assume that the synchronized slow activity, observed at the brain-stem level during slow sleep, requires the presence of the cortex—most probably the orbital cortex—and that therefore synchronizing *but not obligatory* sleep-inducing structures exist at a rostral level.

Structures and mechanisms responsible for paradoxical sleep

TRIGGERING STRUCTURES The existence of tonic and phasic behavioral signs, such as the disappearance of EMG activity of the neck, or rapid eye movements, and of subcortical electric signs—monophasic pontine spikes—that are specific to P.S. has enabled us to outline with relative precision the structures necessary and sufficient for the periodic triggering of this state of sleep (Figure 6).[23]

The removal of all neural structures, including the hypothalamus and hypophysis rostral to the pons, does not prevent the periodic appearance of P.S. in the chronic pontine animal. It occurs with the regularity of a "biological clock"; its mean duration is the same as in the intact animal (6 minutes); its circadian percentage is 10 per cent —slightly less than that in the intact animal. Paradoxical sleep is characterized by the sudden disappearance of muscular tone with a complete disappearance of the EMG of the neck, by lateral ocular movements (which depend on activity of the nerve VI), an acceleration of the heart and respiratory rhythms, and the presence of monophasic pontine spikes, whose pattern and regional distribution are the same as in a normal animal during this state.

The experiments of Rossi et al. also support the hypothesis of the localization of the P.S. triggering structures in the pontine tegmentum, as a lateral hemisection located in front of it suppresses or delays the homolateral cortical desynchronization during P.S., while a hemisection at a more caudal level does not involve an asymmetry of the cortical desynchronization.[74,75] The over-all results

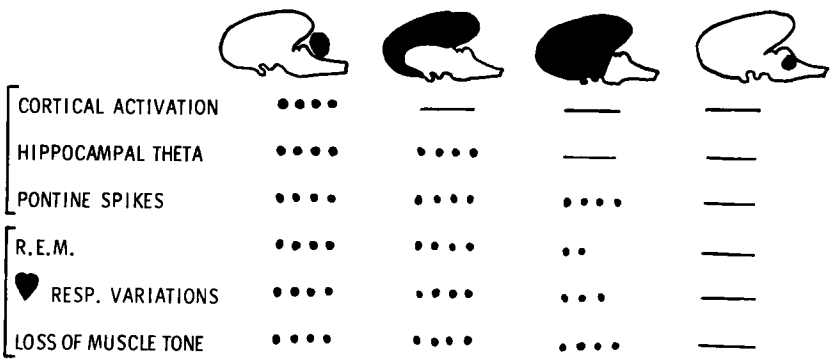

FIGURE 6 Delimitation of the neural structures responsible for the triggering of paradoxical sleep. In black are the lesions of the brain: cerebellectomy, neodecortication, ablation of the cerebrum in front of the pons (pontile cat), lesion of the pontine reticular formation. Dots represent the central and peripheral aspects of P.S., which are still present in pontile cats but absent in cats with destruction of the pontine reticular formation.

of these experiments reveal that the structures sufficient for the periodic appearance of the major behavioral and EEG signs of P.S. are located in the pons.

Another series of experiments shows that some pontine structures are also necessary. The destruction of the nucleus centralis superior de Betcherew and of the medial part of the nucleus reticularis pontis oralis and caudalis has no significant effect upon either state of sleep. But the destruction of the medio-lateral portion of the caudal part of the nucleus reticularis pontis oralis and the rostral part of the nucleus reticularis pontis caudalis suppresses P.S. in chronic cats. Usually, however, there are no changes in slow sleep or arousal.[23,76] Recent experiments favor different pontine structures as responsible for both tonic and phasic components of paradoxical sleep.

TONIC INHIBITION OF MUSCULAR TONUS The bilateral destruction of a limited area situated in the dorsal part of the medio lateral pontine tegmentum suppresses the occurrence of muscular atonia during P.S. (Figure 7),[77] although the phasic geniculo-occipital activity still occurs. This area includes the nucleus locus coeruleus and a zone immediately medial and ventral to it. Behavioral disturbance may occur in animals in which the dorsal part of the medio lateral pontine tegmentum is destroyed. After a period of slow sleep, a sudden increase of lateral geniculate spikes occurs and the cats may suddenly stand up and exhibit typical fear or rage behavior. During these periodic occurrences there is an augmentation of the muscular activity of the neck, but the pupils remain myotic, the nictitating membranes are relaxed, and the animal does not re-

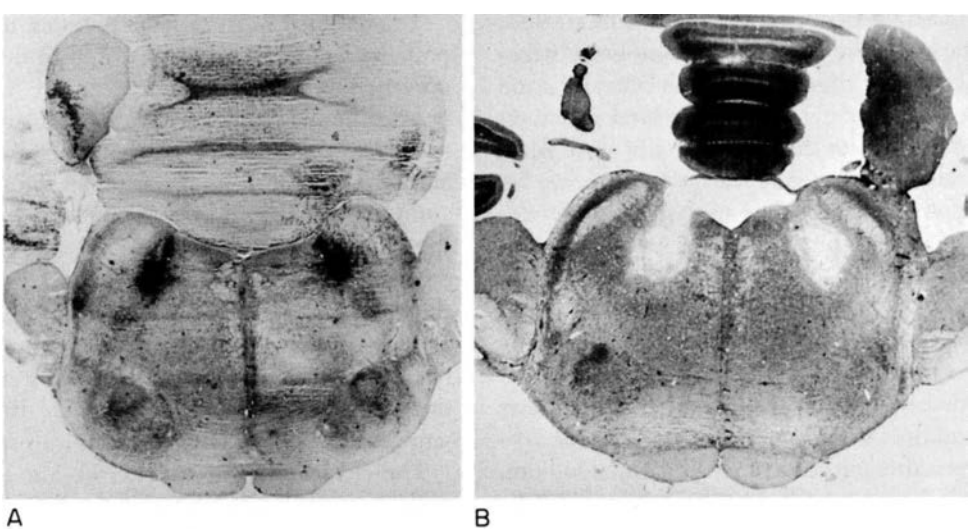

FIGURE 7 A. Concentration of MAO at the level of the locus coeruleus (technique of Glener).

B. Bilateral lesion of the locus coeruleus, which definitely suppresses the tonic aspect of P.S. (Loyez).

act to visual stimuli. The clear-cut dissociation between the ocular aspect of deep sleep and rage behavior has been compared to a "hallucinatorylike state." How the dorsal part of the medio lateral pontine tegmentum controls the supraspinal structures responsible for the tonic inhibition of muscular tonus is still obscure. It must be pointed out, however, that this region is rich in noradrenergic neurons —group A 6 of Dahlström and Fuxe[67]—that are mainly concentrated in the locus coeruleus and immediately ventral to it. This region is also particularly rich in monoamine-oxidase.[78]

PHASIC ASPECTS OF PARADOXICAL SLEEP The medial and descending vestibular nuclei are responsible for the bursts of rapid eye movements, for the clonic jerks of the peripheral muscles, and for some phasic irregularity of the vascular system (sudden pupil dilation or sudden variations in blood pressure) during P.S. Indeed, after bilateral destruction of these nuclei, only a few isolated eye movements without clonic jerks are seen, although there is still a total atony of the neck.[31]

The pontine structures that control the phasic pontogeniculo-occipital activity during P.S. have not yet been described.

The rhombencephalic localization of the structures responsible for triggering P.S. has made it possible to propose the name "rhombencephalic phase of sleep" for this state.[23] The medio lateral zones of the rhombencephalon appear to be triggering zones in relation to some ascending and descending pathways, and are responsible for the electric cortical tonic and phasic phenomena and the behavioral tonic and phasic events specific to P.S.

The complex organization of the neural structures involved in P.S. has been the object of numerous recent investigations.[23,31,76,79] Although a detailed picture has not yet been obtained, a schematic outline may be given. At the level of the pontine reticular formation exist some structures that appear necessary for triggering both the descending and ascending tonic and phasic phenomena.

The medio-lateral-pontine reticular formation is involved in the control of the bulbar inhibitory reticular formation, which acts tonically on the motoneurons through the ventro-lateral-funiculi of the spinal cord. The medial and descending vestibular nuclei are responsible for the burst of rapid eye movements and for most phasic vascular and muscular events of P.S. Phasic influences are transmitted through the dorso-lateral-funiculi of the spinal cord.

The fast cortical activity and regular theta hippocampal rhythm, and the geniculo occipital spikes are also dependent on the medio lateral part of the pontine tegmentum. The ascending pathways responsible for the tonic cortical activation appear to be diffuse in the mesencephalon; the hippocampal theta rhythm is controled by a pathway ascending through the septum. A discrete pathway, independent from the ascending structures mentioned above, is situated in the dorsal part of the brain stem and is responsible for the pontine, ascending, extraretinal input to the lateral geniculate body and the occipital cortex.

SELECTIVE DEPRIVATION OF P.S. The concept that P.S. is a different state of sleep has, of course, raised the question of its function. For this reason, some attempts have been made to suppress it. Although this approach has not yet given a definite answer concerning the role of P.S., it is worth summarizing. The first attempt to suppress P.S. selectively was made in humans by Dement[80] by awakening the subjects immediately after the onset of P.S. A progressive increase in P.S. "attempts" was observed, and an augmentation of P.S. was found during the nights after sleep deprivation.

Similar results have been obtained in animals. In the pontine cat, the suppression of P.S. by an electric shock makes it reappear after intervals that are successively shorter and shorter, so that after a few hours it becomes almost impossible to awake the animal, which immediately collapses into a P.S. state after the shock.[81] Thus, "a need for P.S." is actual, and seems to be the expression of an active mechanism situated in the lower brain stem. The same phenomenon has been obtained in the intact cat, using the same technique.[82]

In the intact cat, the selective deprivation of P.S. is also achieved by placing the animal under conditions in which it cannot completely relax its muscular tone (isolated on a small stand in a bath).[83] Under these conditions, behavioral and EEG slow sleep can persist in a normal way (50 per cent of the day) without P.S. appearing.

Some vascular, electrical, and behavioral changes have been noticed during P.S. deprivation. There is a permanent augmentation of the heart rate,[83] a facilitation of the recovery cycle of acoustic evoked responses at the auditory cortex[84], and a diminution of the seizure threshold for electroshock.[85] Disturbances of sexual behavior, specifically hypersexuality, have also been noticed in male cats or rats.[83,85] These findings suggest that the excitability of the nervous system may be increased when P.S. is selectively suppressed. During recovery sleep, after suppression for several days, an important and durable increase of P.S. percentage is observed—up to 60 per cent of total sleep. This increase may persist for several days and is proportional to the duration of deprivation; that is, it lasts for a time equal to half of the deprivation time. It proceeds mainly from an increase of the frequency of P.S. and not from the increase of its mean duration. At the onset of the

recoveries, P.S. may occur *immediately* after wakefulness, without any intermediary phase of slow sleep. This phenomenon evokes narcoleptic states.

The results of selective P.S. deprivation contrast with the effect of *total* sleep deprivation. In such case the recovery sleep is different. First there is an increase of slow-wave sleep, which is followed by an increase in P.S.[83] Similar results have been found in human subjects.[86]

In summary, these experiments demonstrate that the essential mechanism expressing the "need" for P.S. exists in the lower brain stem. The long-lasting recovery process after P.S. deprivation evokes the eventual accumulation of a neurohumoral or metabolic "byproduct" during deprivation, and meets Piéron's theory of hypnotoxins.[87] The increase of this neurohumoral agent appears to be responsible for a state of increased excitability of the nervous system. Recovery after P.S. deprivation requires a long-lasting increase of P.S. This suggests that the elimination of the specific (and unknown) neurohumoral agent during P.S. is effected through some autoregulating mechanism by which frequency (but not duration) is increased.

Pharmacological influences

The topic of drugs (Figure 8) has been the subject of recent reviews,[69,88,89] and can be summarized briefly as follows.

1) There is no unique and continuous hypnogenic mechanism presiding over the periodic succession of the states of sleep, because it is possible, by altering the level of brain monoamines, to increase slow-wave sleep and to suppress P.S. If there were a single sleep mechanism for both states, P.S., which is considered to be deep sleep, would also have been increased, or at least would not have been suppressed.

2) Increase of brain serotonin, after injection of its precursor, 5-hydroxy-tryptophan, led to an increase of slow-wave sleep and an immediate decrease of P.S.

3) Monoamine-oxidase (MAO) inhibitors suppress

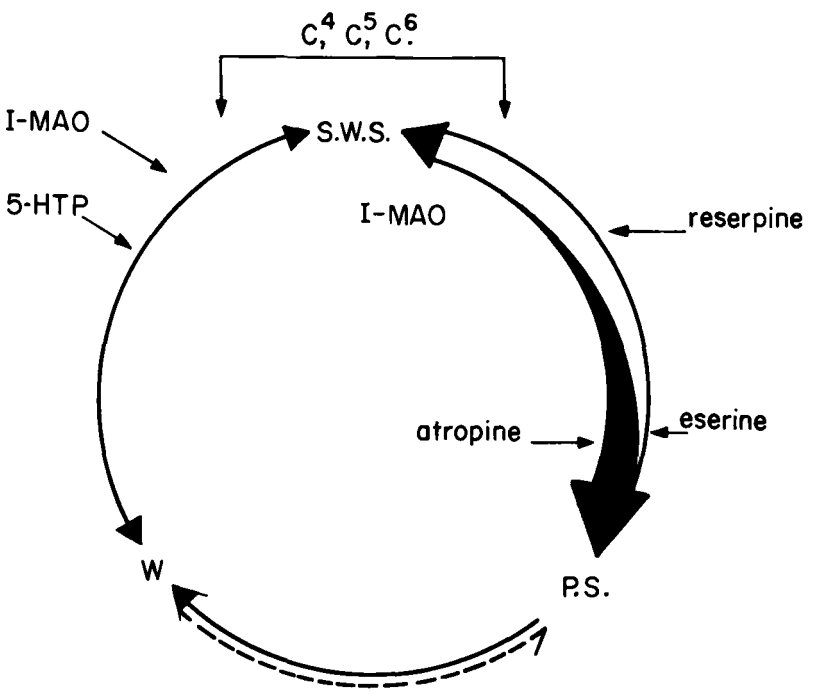

FIGURE 8 Effects of some drugs upon the states of sleep. In 75% of the cases there is a regular cycle from waking (W) to slow-wave sleep (SWS), paradoxical sleep (P.S.), and waking again. But transition from SWS to W or P.S. to SWS may be also frequently seen. In adult cats, one can never see a *direct* transition from W to P.S. However, this can be seen after P.S. deprivation and in man in narcolepsy. Drugs that favor some step during sleep are indicated outside the circle; those inhibiting these steps are inside.

5-HTP and MAO inhibitors (I-MAO) increase SWS but suppress P.S. and its precursory ponto-geniculo-occipital (PGO) activity.

Reserpine electively triggers the PGO activity (white arrow), whereas atropine, which does not suppress the precursory PGO activity, suppresses the final tonic component of P.S., on the contrary eserine may increase it.

Low-chain fatty acids (C4-C5-C6) may facilitate all the steps through a still-unknown mechanism.

P.S. and usually increase slow-wave sleep. Thus it is possible that some metabolic step requiring MAO plays a role in the transition between slow-wave and paradoxical sleep.

4) Reserpine, which is known to release monoamines at monoaminergic terminals, is able to trigger the phasic EEG components of P.S. selectively (ponto-geniculo-occipital spikes).

5) The total atony of P.S. appears to be dependent upon a cholinergic mechanism, because it is suppressed by atropine. It also seems to need a catecholaminergic mechanism, as dopa is able to induce normal P.S. after reserpine has been administered.

6) Finally, it has been shown that short-chain fatty acids are able, in certain conditions, to induce both slow-wave sleep and P.S.[90,91] Their mechanisms are still unknown. Thus, the neuropharmacological approach to sleep, which is still in its early stages, has revealed at least two different mechanisms governing the transition from slow-wave to paradoxical sleep.

The first mechanism, the appearance of the ponto-geniculo-occipital activity that always heralds the occurrence of P.S., is totally suppressed by the MAO inhibitor but not by atropine, whereas the second mechanism, which triggers the total atony of P.S., is dependent upon both cholinergic and catecholaminergic mechanisms.

Relationship to oneiric activity in men

The demonstration of Aserinsky, Kleitman, and Dement[92,93] that dreaming occurs in a specific stage of sleep, called emergent stage I or rapid eye movement sleep, has opened a new area in the objective study of dreaming. The extrapolation of the paradoxical phase of sleep in the cat to the oneiric activity phase (rapid eye movement phase = REMP) in man has raised many arguments. If we compare the paradoxical phase of sleep in cat and REMP in man (in normal subjects)[94,95] or in patients suffering from various cerebral lesions,[97] there is no doubt about the similarity of the phenomena (Table I). There is also obviously a "need" for oneiric activity in man, as has been shown in selective deprivation experiments.[80,96] The demonstration of the relationship between oneiric activity and REM sleep in man leads us to believe that mechanisms and structures, progressively elicited in the cat during P.S., will one day enable us to understand the causes and functions of dreaming. Moreover, subjective data given by subjects awakened from dreams, immediately after REM, seem to reveal a close relationship between the direction of ocular movements and the oneiric scenery.

Numerous questions are still to be answered before the relationship of REM and dreams can be explained. As a matter of fact, P.S. ocular movements persist in subjects suffering from decortication syndrome who are unable to have ocular movements during wakefulness.[97] Moreover, REM occurs during sleep in newborn infants[98,99] and in blind-born adults,[100] who cannot have visual imagery during dreaming. If phasic electrical and oculomotor phenomena both appear to be triggered from the pontine area, the precise determination of a temporal relationship between pontooccipital spikes and REM is essential if we are to understand the integrating mechanisms responsible for dream imagery.

Phylogenesis of the states of sleep

The phylogenetic study of sleep allows us to dissociate[101,102] the appearance of slow sleep from that of P.S. during the course of evolution (Figure 9). In the tortoise, for instance, only the slow-sleep state has been found. In chicks, hens, and pigeons, slow sleep is typical and electrically resembles that of mammals, although there are no spindles and few slow waves, and P.S. is extremely short—about 10 seconds. However, paradoxical sleep is quite

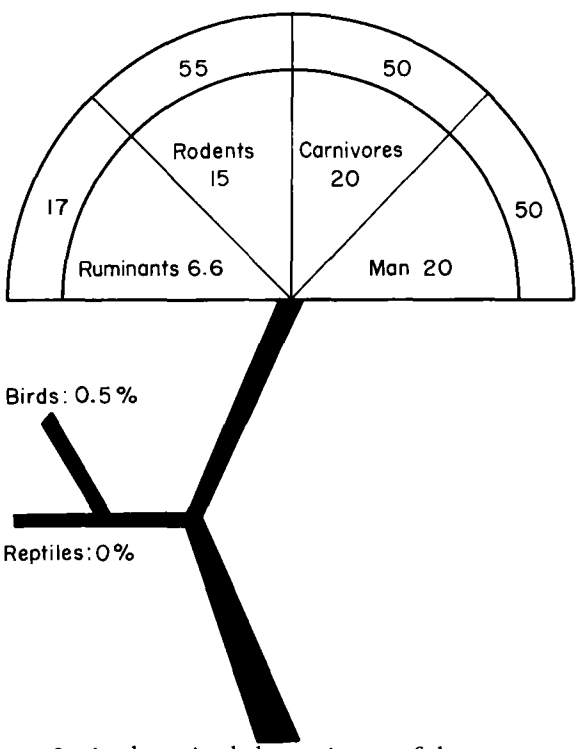

FIGURE 9 A schematic phylogenetic tree of the two states of sleep. Figures indicate the percentage of paradoxical sleep out of total sleep. The outer figures indicate this percentage in newborns, the inner ones, in adults. (From Rechtshaffen, et al., Note 104)

TABLE I

Similarities between paradoxical sleep in the cat and REM sleep in humans

EEG	CAT (Adult)	MAN (Adult)
Cortical activity	Fast, low-voltage (similar to arousal)	— Scalp recording : low-voltage, 6–8 c/s in occipital areas — Cortical recording : low-voltage, fast.
Evoked responses by visual or auditory stimuli	Decreased	Average response decreased
Physiological Behavior.		
EMG activity	EMG activity of the neck totally decreased	Subhyoidian muscle activity totally decreased
Monosynaptic reflexes	Decreased or abolished	Decreased or abolished (H-reflexes)
Ocular movements	Present	Present
Pupillary diameter	Myosis	Myosis
Heart rate	Mostly decreased, irregularity	Mostly increased, irregularity
Blood pressure	Decreased	Increased (but different method)
Respiration	Irregularity	Irregularity
G.S.R.	Decreased	Decreased
Functional Aspects.		
Arousal threshold		
— Auditory stimulation	Increased	Superior or equal to Stage III, equal to Stage IV
— Periodicity	20–28% of total sleep time, always follows slow sleep	20–25% of total sleep time, always follows slow sleep
— Result of selective deprivation	Increased during recovery	Increased during recovery
Structural Aspects.	— Persist after decortication — Suppressed by pontine lesions	— Persist in case of decortication — Suppressed by brainstem lesion involving the pons
Ontogenesis.	— Present immediately after birth, may directly follow waking	— Present immediately after birth, may directly follow waking
Subjective experience.	Unknown	Dreaming.

From Dement, W. C. and Jouvet, M.—General Discussion, *in* Aspects anatomo-fonctionnels de la Physiologie du Sommeil, M. Jouvet, Editor, C.N.R.S., Paris, (in press).

distinct. It is accompanied by a short acceleration of the electrical activity of the hyperstriatum; by an important reduction, but not by a total disappearance, of the neck-muscle tone; by bursts of phasic ocular movements, and by bradycardia. So far, all mammals observed—rat, mouse, rabbit, opossum, cat, dog, sheep, goat, monkey, and chimpanzee—present the two states of sleep we have just considered. In certain species a few special features are known. For example, in the rabbit, P.S. is hormonally dependent, and a long habituation is necessary in order to observe it. On the other hand, while the two states may be easily observed in newborn ruminants, P.S. tends to decrease considerably in the adult animals.

Ontogenesis of the states of sleep

Phylogenesis allows us to dissociate the appearance of the two states of sleep during evolution; such a dissociation may also be observed in the cat during ontogenesis (Figure 10). In kittens and newborn rats,[103] P.S. may be observed immediately after birth, when slow sleep is almost nonexistent. Paradoxical sleep has a peculiar aspect. Phasic phenomena overshadow tonic phenomena, which is why it has been called "sleep with jerks." It takes place for about 50 per cent of the day and 80 to 90 per cent of behavioral sleep, and often occurs immediately after waking, as there is no phase of slow sleep between. Dur-

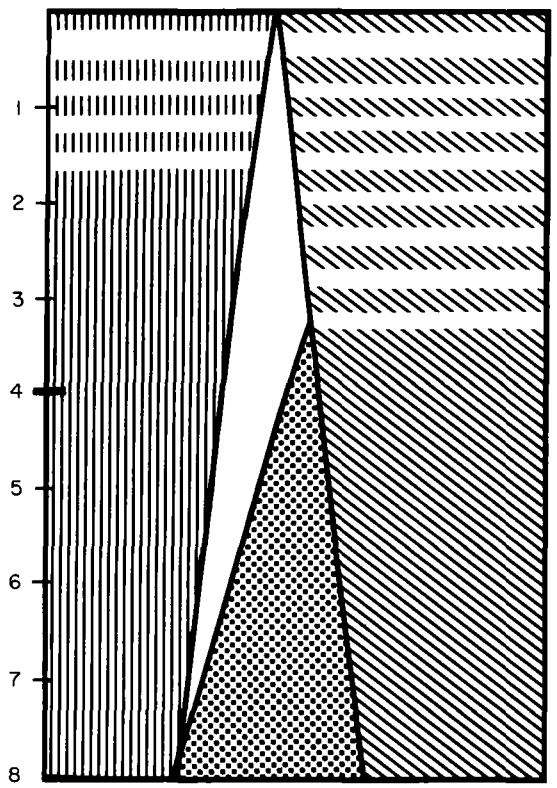

FIGURE 10 Ontogenesis of the two states of sleep (in kittens).
Ordinates: age in weeks.
Abcissa: proportion of the different states calculated on daily recording lasting 3 hours.
Vertical hatching: paradoxical sleep.
Oblique hatching: waking.
White and dots: slow wave sleep.
The white horizontal lines signal the period during which the cortical EEG is different from that of the adult. (Modified from Valatx, et al., Note 103)

ing cortical maturation, slow sleep appears and increases progressively, whereas P.S. decreases, so that at adult age, slow sleep constitutes 70 per cent and P.S. only 20 to 25 per cent of behavioral sleep. Fast cortical activity and hippocampal theta rhythm during P.S. precedes, by a few days, the waking tonic cortical desynchronization, which was suggested as favoring a dissociation of the mechanism responsible both for waking and for P.S. fast cortical activities.

Relationship between slow sleep and P.S.

Numerous results support a *relative* autonomy of the two states of sleep. The possibility of selective suppression of P.S. by pontine lesion, without abolishing slow sleep, shows the duality of the structures in action. To this structural duality, we may add arguments in favor of some duality of mechanisms. A unit theory of sleep necessarily assumes that a period of slow, or light, sleep precedes paradoxical, or deep, sleep. According to this hypothesis, some parallels between the two states must have existed during their phylogenetic and ontogenetic evolution. However, neither ontogenetic nor phylogenetic data support such an idea. Slow sleep exists in all vertebrates so far studied by polygraphic methods, from the tortoise to man; P.S., on the contrary, does not appear to be connected with slow sleep throughout this phylogenetic span. The total lack of P.S. in chelonians and its rudimentary appearance in birds are in contrast with its relatively common and important manifestation in mammals. Although the animal species studied to date are still too few in number to draw permanent conclusions from them, it seems likely that P.S. occurs only from birds upwards, evolutionarily speaking.

The data of ontogenesis also allow us to differentiate between the development of the two states of sleep. In the newborn mammal slow sleep is not necessary for the occurrence of P.S. Consequently, we must assume that the mechanism responsible for P.S. is pre-established at birth, whereas slow sleep is little developed and seems to be governed by mechanisms acquired postnatally.

If, in the adult animal, the differentiation of behavioral sleep into two states appears, thanks to polygraphic study, we can assume that slow sleep must precede paradoxical sleep. However, the selective deprivation technique enables us to dissociate them, because during recovery P.S. may be observed immediately after wakefulness. This has also been noticed in adult man during narcoleptic or cataleptic attacks.[104]

Paradoxical and slow sleep appear, then, to be manifestations of two processes, relatively opposed by virtue of their structures and mechanisms. So we should refer to different *states* rather than to phases of sleep. Behavioral sleep in the adult mammal does not seem to develop cyclicly from light to deep sleep, but seems rather to include two states of qualitatively different activity. It is possible that these two states are induced by different groups of monoaminergic neurons, as is suggested in a possible monoaminergic theory of sleep, which will be summarized.

A possible monoaminergic theory of sleep

Much histochemical[67] and biochemical[105] data support the existence of an ascending serotonergic system whose cell bodies are located mainly in the Raphé system. The intervention of such a system in slow-wave sleep mechanism appears likely, because if the system is almost totally de-

stroyed a state close to permanent wakefulness ensues. Any attempt to increase the serotonin content of the serotonergic neurons, which is determined by the increase of yellow fluorescence after injection of Nialamide, leads to an increase of slow-wave sleep.

While the process governing slow-wave sleep appears to be related to serotonergic neurons, the process governing P.S. appears to be related to catecholaminergic mechanisms. This is strongly suggested by the histochemical structures of the dorsal part of the medio-lateral-pontine tegmentum (noradrenergic neurons group A-6 of Dahlström and Fuxe[67]) which are necessary if P.S. is to take place.

Thus, a discrete sleep "lobby" composed of monoaminergic neurons, the cell bodies of which are located primarily in the Raphé system and the pontine tegmentum, appears to influence all other, far more numerous "classical" neurons of the brain periodically during slow-wave and paradoxical sleep. Neurophysiology must now discover the phylogenetic reasons for the evolution of the monoaminergic neurons responsible for sleep, and eventually for both for sleep and dreaming.

Summary

Sleep can no longer be considered a unique state opposed to wakefulness. It is, in fact, composed of two different states that alternate regularly. The first (slow-wave sleep) is characterized by a synchronized cortical electrical activity and constitutes about 70 to 80 per cent of total sleep; the second (paradoxical sleep) is marked by a fast, low-voltage, desynchronized cortical activity similar to that of waking, by rapid eye movements, and by a total disappearance of muscular tone. In man it is accompanied by dreaming.

These two states cannot be explained by the "passive relaxation" of the arousal system. On the contrary, there is much evidence that they express some active hypnogenic mechanisms. The localization of the hypnogenic structures that inhibit the arousal system has not yet been achieved; most of them appear to be situated in the lower brain stem.

It is possible that structures related to slow-wave sleep belong to some well-defined histochemical system (serotonergic neurons of the Raphé system), whereas noradrenergic neurons located in the pontine tegmentum also appear to be responsible for the triggering of paradoxical sleep.

During ontogeny in mammals, paradoxical sleep is prevalent after birth, whereas slow-wave sleep increases subsequently. Phylogenetically, paradoxical sleep appears to be absent in reptiles, almost nonexistent in birds, and has been found in all mammals studied.

Finally, while the phenomenology of the two states of sleep and some of their mechanisms are beginning to be understood, almost nothing is known about their function.

Unit Activity in Sleep and Wakefulness

EDWARD V. EVARTS

IN WHAT WAY is the nervous system modified by sleep? It would seem safe to assume that the brain that lapses into somnolence at night differs from the brain that awakens next morning. If this assumption is granted, it follows that the key to an understanding of the functional significance of sleep lies in knowledge of the chemical and/or structural aspects in which the waking brain *before* sleep differs from that *after* sleep. Paradoxically, it is possible that some future investigator may discover the functional significance of sleep without studying sleep itself—just as some past nutritionist might have discovered the functional significance of eating by comparing subjects entering a restaurant with those leaving it, without himself having to enter the restaurant at all. Of course, if the nutritionist's knowledge of the physiological role of eating were as rudimentary as the neurophysiologist's knowledge of the role of sleep, he might have gotten valuable clues by actually entering the restaurant to observe the events that took place. Unfortunately, his entry into the restaurant (particularly if he were to enter a three-star restaurant in the company of a gourmet) would have been fraught with danger. Confronted by the elaborate behavioral repertoire of the gourmet and restaurant staff, he might have devoted himself to a study of fascinating gastronomic epiphenomena that would ultimately have provided little insight into the *nutritional* role of intrarestaurant activity. Like this hypothetical nutritionist, the neurophysiologist who records intracerebral activity during sleep and dreams is in the role of an observer confronted by a welter of fascinating epiphenomenal data imbedded in which lie (he hopes!) some hints on the nature of sleep.

In this presentation, I describe some observations on the activity of individual cerebral neurons in sleep and waking, and speculate concerning such small clues as they may provide to the biochemist, anatomist, or molecular biologist who may one day discover what sleep really is for.

Methods

TECHNIQUE OF RECORDING SINGLE UNIT ACTIVITY Little need be said here concerning details of the technique employed to record the activity of single neurons, during natural sleep and waking, by use of microelectrodes. This technique involves attachment of a microdrive (a device for advancing a microelectrode into the brain) to the skull of the experimental animal in such a way that the animal's head movements fail to cause a displacement between the tip of the recording microelectrode and the single cerebral neuron from which it picks up an action potential. The microdrive allows an exploring microelectrode to be lowered into the brain tissue until the action potential of a single cerebral neuron stands out above the action potentials of its neighbors. The amplitude of this extracellularly recorded action potential is largely a function of the proximity of the electrode tip to the cell and provides no useful information beyond the occurrence or nonoccurrence of an action potential. The intracellular events that give rise to action potentials cannot be inferred from the extracellularly recorded action potential. An increase in frequency of action potentials might be caused by an increase in excitatory input to the cell, a decrease in inhibitory input, or some combined change in excitatory and inhibitory input that involves a net increase in excitation.

SAMPLE BIAS Before going on to discuss results, I should like to mention a serious limitation of microelectrode recording techniques as applied to the study of cerebral neurons. This limitation is *sample bias* resulting from the fact that it is easier to record from large neurons than from small neurons. Thus, a given sample of neurons collected by means of microelectrode recording contains a much greater proportion of large neurons than was actually contained in the cerebral tissue that was the locus of recording. This bias may be particularly important in the study of sleep, for it is conceivable that the functional significance of sleep may be different for small as compared to large neurons.

Results

AMOUNT OF ACTIVITY IN VISUAL CORTEX NEURONS Thus far in this chapter, sleep has been spoken of as if it were a homogeneous state; in point of fact, however, we all know that sleep is far from homogeneous. As Dr. Jouvet has pointed out, there are at least two types of sleep that differ strikingly with respect to the behavior of the animal and with respect to the electrical activity of the

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brain. Likewise, when considering activity of individual neurons during waking, one must recognize that waking is even less homogeneous than sleep. For the results on amount of activity in visual cortex neurons to be described in this section, it is advisable to examine the activity of the neurons in the two states of sleep that Dr. Jouvet has already described, and also in two different states of waking.

These four conditions in which the activity of single visual cortex neurons of cat was recorded are summarized in Figure 1. The usual data on electromyogram, eye movement, and electroencephalogram (EEG) were used to identify sleep with EEG slow waves and with low-voltage fast EEG activity. The two states of waking were identified by these same criteria, together with observations of the cat's behavior at the time of recording. The two conditions of waking during which activity of visual cortex neurons was observed were: (1) a waking state when there were no eye movements and when visual stimulation was prevented by covering the cat's pupils with opaque devices, and (2) a waking state when the cat was actively scanning its environment and the contact occluders had been removed, permitting a full range of visual stimulation.

Figure 2 summarizes the results on the amount of activity in visual cortex neurons of the cat under these four conditions. Mean discharge frequencies in groups of neurons recorded during sleep with EEG slow waves (S) and waking (W) in the absence of visual stimulation were approximately the same. On the other hand, the amount of activity in the visual cortex was greatly increased both during sleep with low-voltage fast EEG activity (S-LVF—or, as Dr. Jouvet calls it, P.S.) and during waking with visual stimulation (W-VIS).

The results illustrated in Figure 2 make it clear that S-LVF, a stage of marked unresponsiveness to environmental stimuli, is one in which the brain is far from quiescent. As a matter of fact, activity of cat visual cortex neurons during S-LVF is about as great as during W-VIS, a state of waking associated with patterned stimulation of the retina. Such a high level of activity during S-LVF might seem surprising, particularly if one views sleep as a state of neuronal quiescence.

High levels of neuronal activity during S-LVF seem to be quite general. High discharge frequencies for individual neurons during S-LVF have been described for neurons of the reticular formation,[1] the pyramidal tract,[2] the vestibular nuclei,[3] and the lateral geniculate nucleus,[4] as well as certain other areas. However, the lack of difference in total amount of activity between S and W in this sample of visual cortex neurons cannot be so widely generalized. In a study of pyramidal-tract neurons[2] during waking in the absence of movement and during slow-wave sleep, it was found that many pyramidal-tract neurons are tonically active during waking and become less active during slow-wave sleep, so that mean discharge frequency for pyramidal-tract neurons during S is about half that for W. Activity was about twice as great during sleep with low-voltage fast EEG activity as with slow-wave sleep, however, so that even in this group of neurons the amount of activity during S-LVF was about equal to that for W.

CHANGES IN DISTRIBUTION OF DISCHARGE FREQUENCIES WITHIN GROUPS OF CEREBRAL NEURONS That the mean discharge frequency of a group of neurons is the same in two different states, as was the case for S and W in the visual cortex units, does not mean that individual units

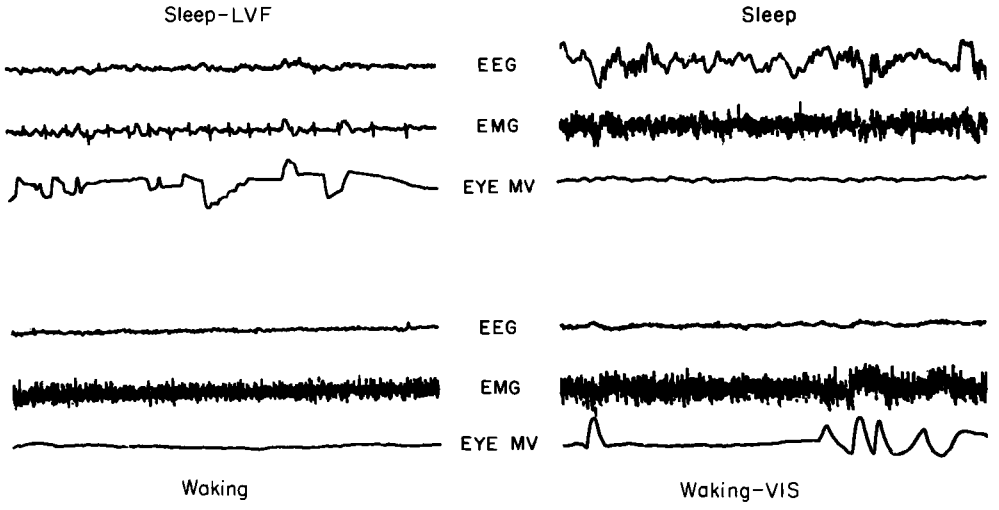

FIGURE 1 *Stages of sleep and waking.* Identification of the four states was based on electroencephalogram (EEG), electromyogram (EMG) and eye movement (Eye Mv).

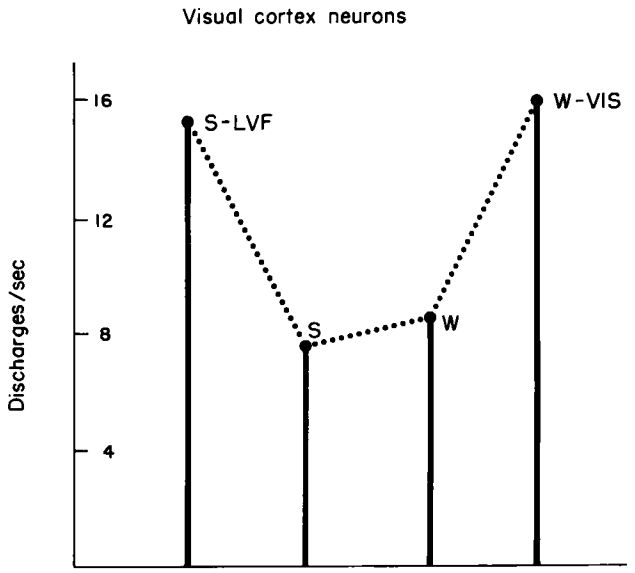

FIGURE 2 *Mean discharge frequency for groups of neurons during S-LVF, S, W, and W-VIS.* Over-all activity of visual cortex neurons is seen to be similar in S-LVF and W-VIS, and to be considerably greater during both of these states than during either S or W. (From *Proc. Internatl. Conf. on Sleep,* 1965, pp. 189–212)

within this group have not changed considerably from S to W. If one compares the variance of discharge frequencies in a group of visual cortex neurons during S and W, one finds that it is much greater during W than during S, even though the mean discharge frequencies are similar in the two conditions. From this, one might suspect that there is a different distribution of discharge frequencies, although there may be no change in mean frequency.

A similar change in distribution of discharge frequencies was also seen for neurons recorded during S and W in the monkey. Figure 3 shows the distribution of discharge frequencies in a group of pyramidal tract neurons recorded from the precentral motor cortex of the monkey during S and W. Each neuron was observed under both conditions. This figure shows that for this group of neurons there was a marked change in the distribution of discharge frequencies during S as compared to W. During W there were a number of neurons with very high discharge frequencies and a number of neurons with rather low discharge frequencies, whereas during S these extreme values disappeared. Neurons which had low frequencies during W tended to speed up with S and neurons which had high frequencies during W tended to slow down with S. Thus, S was associated with a "de-differentiation" of discharge frequencies, such that the sample of neurons became much more homogeneous during S than it had been during W. This elimination of extreme values of discharge frequency with S can be extrapolated to a number of areas throughout the brain. As already mentioned, this reduction in variance was seen in visual cortex neurons. Huttenlocher, in his studies of the activity of neurons in the central brain stem, likewise observed a much greater variance of discharge frequencies during W than during S.[1] Figures 4 and 5 show Huttenlocher's results.

At this point we might look at the possible functional significance of this de-differentiation of discharge frequencies that occurs with S; its occurrence means that in spite of relatively little change in mean frequency for a sample of neurons as a whole, certain *individual* neurons actually have considerably less activity during S than during W. It is also interesting to note that the neurons which are likely to be less active during S than during W are the very ones which were most active during waking.

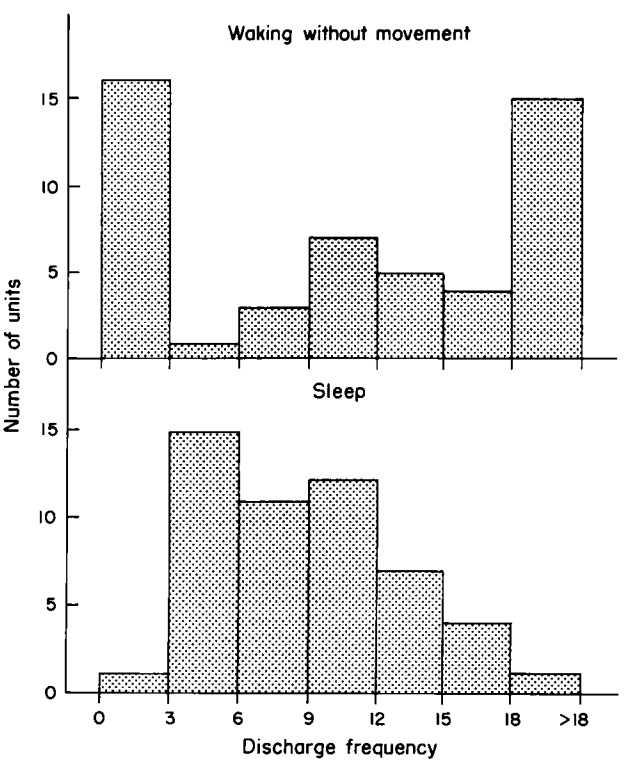

FIGURE 3 Distribution of discharge frequencies in the sample of 51 PTNs during waking in the absence of movement (W) and sleep with EEG slow waves (S). During W, 16 of 51 units had discharge frequencies less than 3/sec., whereas only one of the 51 units had such a low discharge frequency in S. During W there were also many very active units, 15 of 51 units having discharge frequencies over 18/sec. With S, only one of the 51 units had a discharge frequency above 18/sec. (From *J. Neurophysiol.,* 1965, 28:216–228)

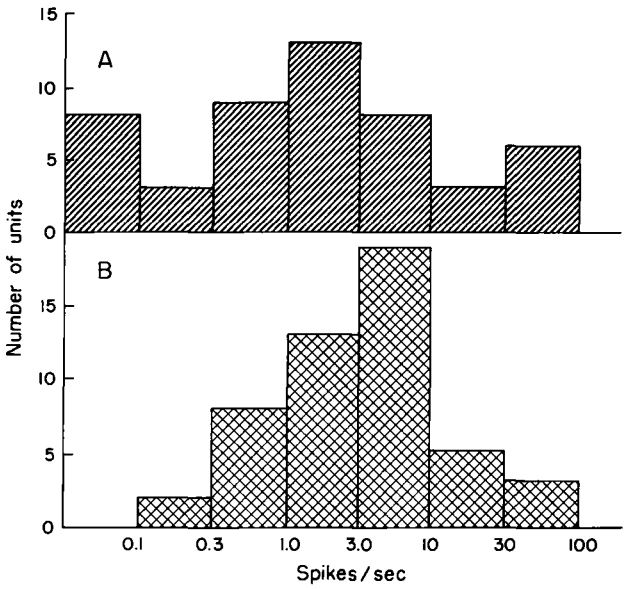

FIGURE 4 Distribution of spontaneous discharge rates of units in brain-stem reticular formation during waking and sleep with slow waves. On the ordinate is shown the number of units in each of the frequency intervals indicated on the abscissa. A = waking; B = sleep. Each of the 50 units was observed under both conditions. (From Huttenlocher, Note 1)

RELATION OF CELL SIZE TO EFFECTS OF SLEEP ON ACTIVITY OF INDIVIDUAL NEURONS The data included so far in this report have dealt with changes in the activity of individual neurons from S to W without considering their functional or anatomical properties. In particular, little attention has been given to the possible differential effects of S on neurons in relation to their size. There are a number of theoretical bases for believing that cell size might be a relevant property of the neuron in differences between S and W.

Figure 6 illustrates the technique used to determine cell size. To be more precise, I should speak of axonal conduction velocity rather than the size of the neuron, since it was that property of the cell that actually was determined. Figure 6 shows the recording microelectrode placed in the precentral motor cortex of the monkey and stimulating electrodes chronically implanted in the ipsilateral medullary pyramid. Stimulation of the pyramid causes antidromic firing of those neurons with cell bodies that lie in the motor cortex and have axons descending through the pyramid. Of course, only a small minority of the cells in the precentral motor cortex send their axons into the pyramid, so that in an experiment such as the present one the microelectrode passes many cells which fail to have antidromic responses before coming to one which does respond with an antidromic spike following electrical stimulation of the medullary pyramid. It is with this minority of antidromically activated cells that the subsequent sections of this report will deal.

The occurrence of an antidromic response following stimulation of the medullary pyramid not only identifies the neuron in question as a pyramidal tract neuron (PTN), but also provides information as to the conduction velocity of this neuron's axon. Thus, units with the highest conduction velocities show a relatively short (0.6 msec) delay between the application of the stimulus to the medullary pyramid and the occurrence of the antidromic response. Neurons which have low conduction velocities show a considerably longer latency from stimulus to response. Since one can infer axonal diameter from axonal conduction velocity, and cell size is generally related to axonal diameter, so the antidromic response latency of a neuron provides indirect evidence as to its size. Large neurons will have short antidromic response latencies and small neurons will have long ones.

Figure 7 shows the relation of antidromic response latency and discharge frequency in pyramidal tract neurons during waking in the absence of movement. The PTNs in this sample have been divided into four groups according to that latency. Those PTNs with the shortest antidromic response latencies (less than 1 millisecond) tend to have low discharge frequencies, whereas the remaining PTNs (with antidromic response latencies greater than 1 millisecond) tend to be tonically active even during the

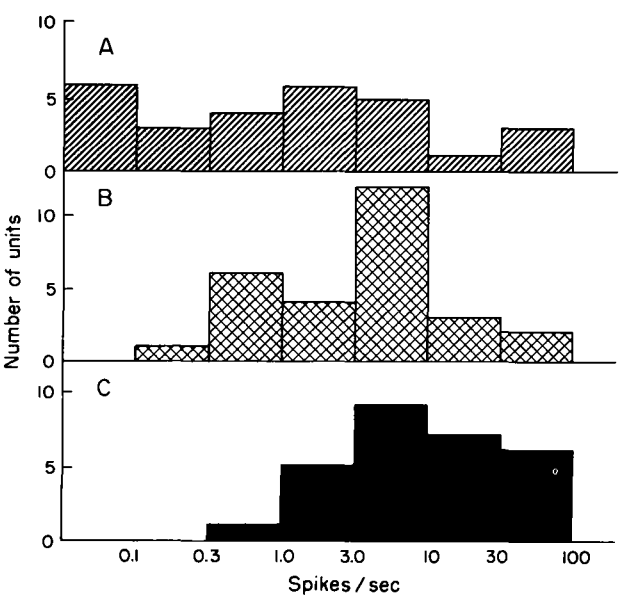

FIGURE 5 Distribution of spontaneous discharge rates of units in brain-stem reticular formation during waking, sleep with slow waves, and paradoxical sleep. A = waking; B = sleep with slow waves; C = paradoxical sleep. Each of 27 units was observed under all three conditions. (From Huttenlocher, Note 1)

absence of movement. During waking there is thus a clear relationship between discharge frequency and axonal diameter.

Although the major purpose of this paper is to discuss sleep, I would like to mention briefly the way in which PTNs behave during *movement.* This departure is to make clear that large PTNs, inactive during waking *without movement,* can discharge briskly during waking *with movement.* Figure 8 shows the changes in discharge frequency associated with monkey contralateral arm movement in the group of neurons whose discharge was shown in Figure 7 for waking without movement. In Figure 8 the increases in discharge frequency with movement are seen to be greatest in the group of PTNs with the shortest antidromic response latencies—that is, with the largest axons. Thus, large PTNs in the arm area of the motor cortex tend to be silent during waking when there is no contralateral arm movement, but show striking increases in activity during such movement. The smaller PTNs are tonically active even with the arm at rest, and although they may show increases in activity with some arm movements, they may show decreases with others. Actually, the group of PTNs with antidromic response latencies greater than 3 milliseconds tend to have a net *decrease* in the amount of activity during movement as compared to nonmovement of the contralateral arm.

Figure 9 illustrates the change in amount of activity from W to S in relation to axonal conduction velocity. The neurons with short antidromic response latencies—that is, those with large axons—tend to be more active during S than during W. Remember that these neurons were inactive during W without movement. At the other end of the axonal size distribution of this group of PTNs, those with the longer antidromic response latencies—that is, those with the smaller axons—were uniformly less active during S than during W.

Perhaps this relationship between axonal conduction velocity and changes in neuronal activity with sleep can be clarified by a sample recording in which the activity of two PTNs was picked up simultaneously by the same microelectrode. This recording is illustrated in Figure 10, which shows the discharge of the two neurons during three conditions: (1) waking in the absence of movement; (2) drowsiness; and (3) sleep with EEG slow waves. During waking, the larger neuron was virtually silent, whereas the other PTN (with the longer antidromic response latency and smaller axon) was tonically active. With drowsiness, the larger of the two neurons had several isolated spikes, even though there was no associated movement. Finally, with the onset of sleep, both neurons showed a considerable change in amount and pattern of activity. Here, then, is a clear example of the de-differentiation of

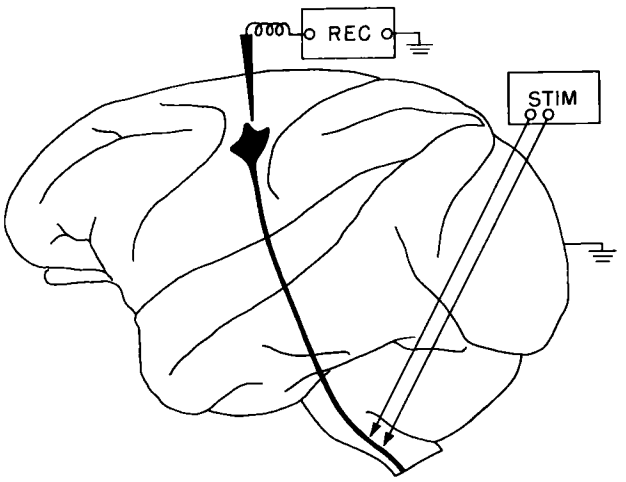

FIGURE 6 Schematic representation of method for identifying pyramidal tract neurons. A pair of electrodes was chronically implanted in the medullary pyramid. Electric shock delivered to a pyramidal tract fiber via these electrodes initiates an impulse which propagates antidromically to the cell body lying in the precentral gyrus, where the resultant action-potential may be recorded by means of an extracellular microelectrode. The latency from medullary stimulus to antidromic response in cortex (antidromic response latency) provides an index of the axonal conduction velocity of the neuron whose activity is picked up by the microelectrode. (From *Sleep and Altered States of Consciousness,* in press)

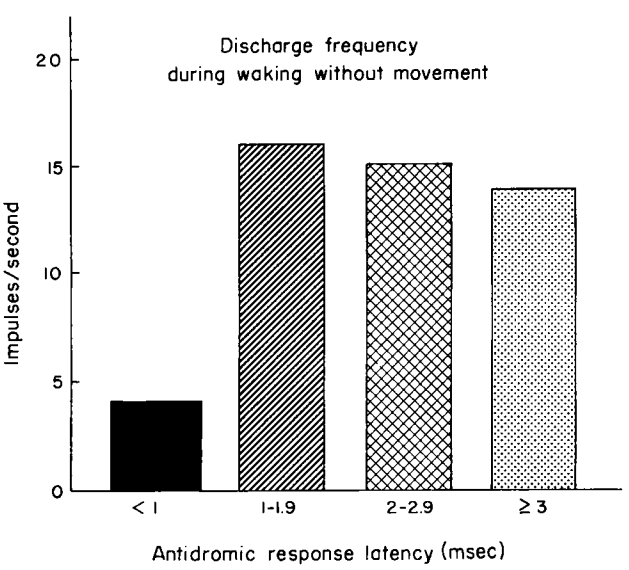

FIGURE 7 Mean discharge frequency for 62 PT units divided into four groups according to antidromic response latency. It may be seen that the mean discharge frequency of the shortest latency group is considerably less than that of any of the three groups with longer antidromic response latencies. (From *J. Neurophysiol.,* 1965, 28:216–228)

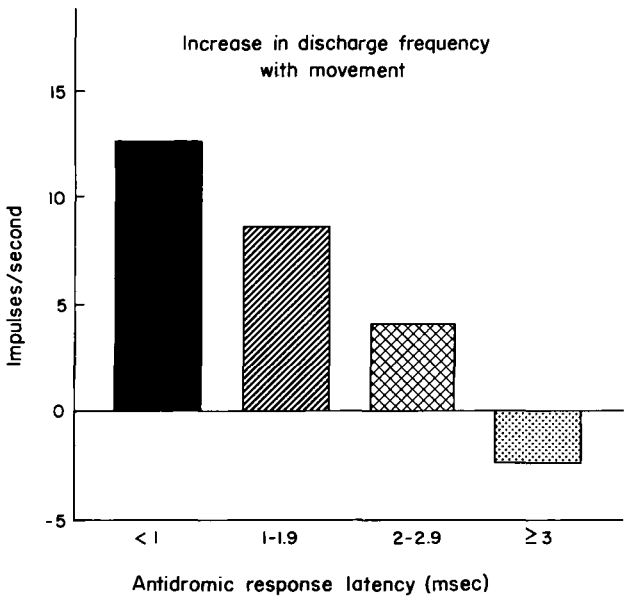

FIGURE 8 As in Fig. 7, the 62 PT units have been divided into four groups according to latency of antidromic response. For each of these four groups, mean discharge frequency during waking without movement has been subtracted from mean discharge frequency during contralateral arm movement. This difference, the increase in discharge frequency with movement, is greatest for the shortest latency units. The longest latency group of units shows a slight average decrease in discharge frequency with movement. (From *J. Neurophysiol.*, 1965, Vol. 28)

discharge patterns associated with S. The two neurons were dramatically different during W but not clearly different in their discharge properties during S. The larger one was more active during S than W without movement, whereas the smaller one was less active during S than during W without movement.

If we look again at Figure 3, a basis for the marked change in distribution of discharge frequencies of PTNs from W to S will be apparent. That figure shows that during W the neurons with large axons have low discharge frequencies and tend to be silent during W in the absence of movement. Figure 3 also shows that a number of tonically active neurons with smaller axons have high discharge frequencies during W. The distribution of discharge frequencies for this same sample of neurons is shown during S in the bottom section of Figure 3. The large neurons, which were discharging at very low frequencies during W, have now become active. At the other end of the distribution the smaller neurons, which were active during W, have become less active with S.

TEMPORAL PATTERNS OF DISCHARGE OF PYRAMIDAL TRACT NEURONS DURING SLEEP AND WAKING It has been pointed out that individual neurons may show marked changes in the rhythmic properties of their activity with the onset of sleep; such changes in the temporal pattern of discharge may or may not give rise to changes in over-all discharge frequency. Figure 11 illustrates typical patterns of neuronal discharge for one PTN during W, S, and S-LVF. During W, discharge of the PTN illustrated in Figure 11 is continual and regular. There are neither bursts of high-frequency discharge nor long periods of inactivity, and short and long interspike intervals are considerably less frequent than they would be for random discharge. During S, discharge is marked by the appearance of short bursts interspersed with short periods of relative silence. As a consequence, the proportion of long and of short interspike intervals is increased. Dur-

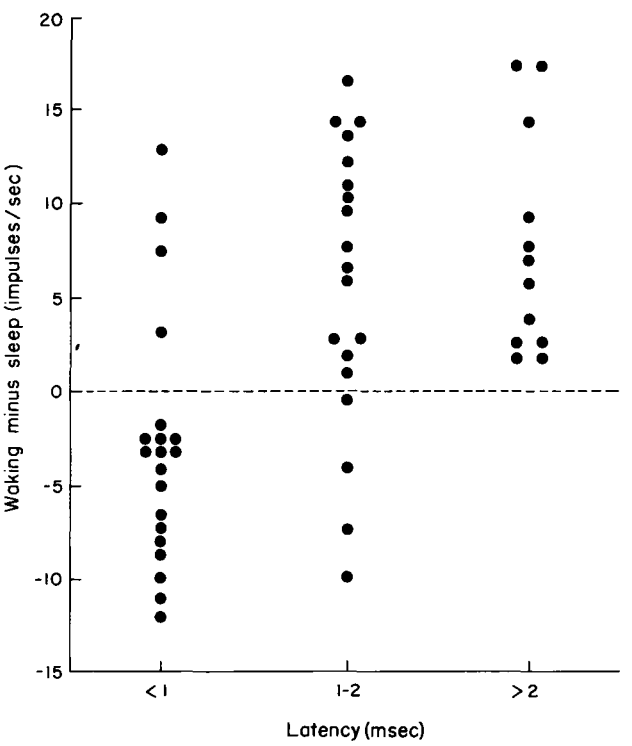

FIGURE 9 Relation of antidromic response latency and change of discharge frequency from W to S. Units have been divided into three groups according to antidromic response latency; each solid circle represents a unit. Change in discharge frequency with S (W minus S) is plotted on the ordinate. For the shortest (< 1 msec) latency group, most units became more active with S, whereas all units in the longest latency (> 2 msec) group became less active with S. (From *Prog. Brain Res.*, 1965, vol. 18)

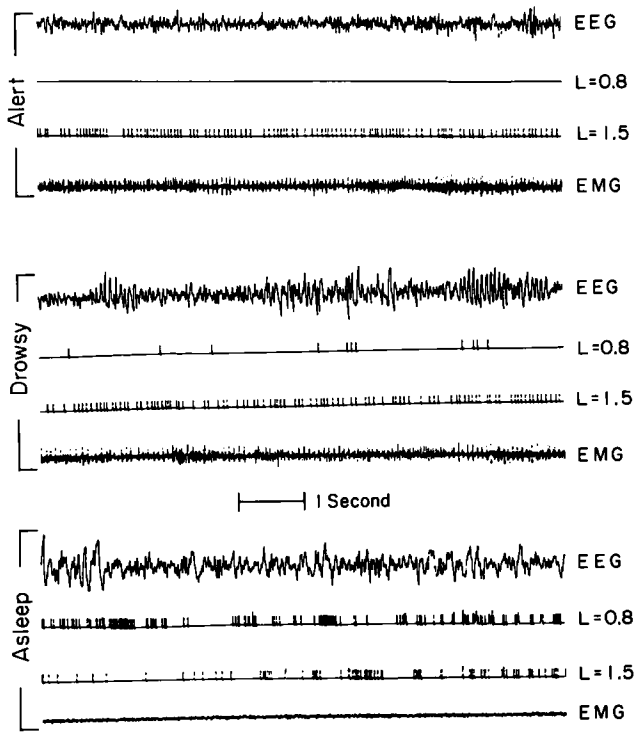

FIGURE 10 Activity of a pair of PTNs recorded simultaneously with the same microelectrode during alertness, drowsiness, and sleep. The unit with antidromic response latency of 0.8 msec. (second line from top) was inactive when the monkey was alert but not moving, showed occasional spikes when the monkey became drowsy, and had high frequency bursts with sleep. The unit with latency of 1.5 msec. (third line from top) discharged regularly during alertness, showed a slight reduction in discharge frequency with drowsiness, and had irregular bursts with sleep. This figure was made by separating the two simultaneously recorded spikes with an "electronic window" and using the separated spikes to trigger pulses which in turn deflected the pens of an ink-writing oscillograph. The deflections on the lines marked L = .8 indicate discharges of the unit with antidromic response latency of 0.8 msec., and the deflections on the lines marked L = 1.5 indicate discharges of the unit with antidromic response latency of 1.5 msec. The electromyograms (marked EMG) were recorded from the muscles of the arm contralateral to the hemisphere from which the unit was recorded. (From *J. Neurophysiol.*, 1965, 28: 216–228)

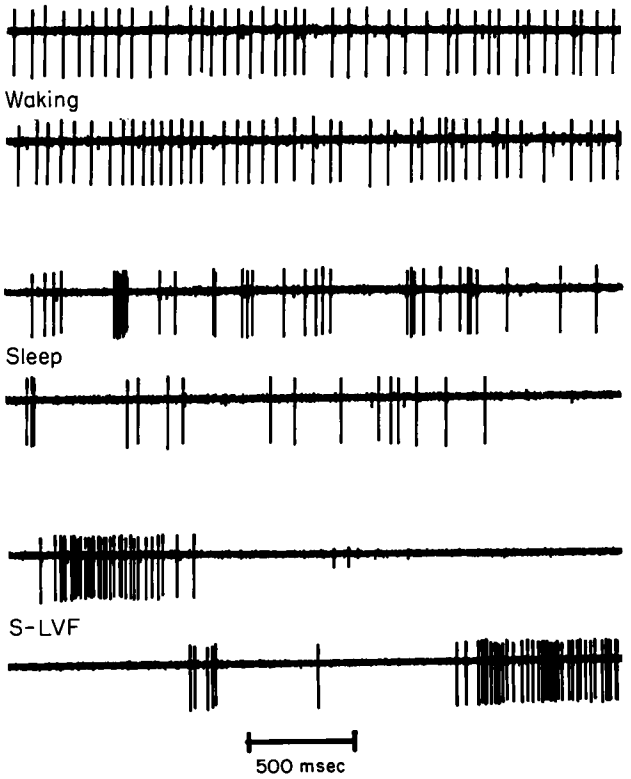

FIGURE 11 Pattern of discharge during W, S, and S-LVF. During W (upper pair of traces) discharge tends to be regular, with an absence both of short and of long interspike intervals. During S (middle pair of traces), there are bursts interspersed with periods of relative inactivity; discharge frequency falls in spite of an increase in the number of short interspike intervals. With S-LVF, burst duration increases, intervening periods of inactivity become longer, and discharge frequency rises. (From Evarts, Note 2)

ing S-LVF, there are also bursts and periods of silence, but the duration of each burst and period of silence increases. The change with S-LVF represents a progression of the modification in the discharge pattern that occurs from W to S. Thus, S-LVF is associated with a further increase in the proportion of short (less than 10 milliseconds) interspike intervals. There is also an increase in the proportion of long (greater than 150 milliseconds) intervals, because the bursts of high frequency discharge during S-LVF are interspersed with longer periods of inactivity.

Distribution of interspike intervals

The grossly apparent alterations of temporal pattern illustrated in Figure 11 must obviously be associated with certain changes of interspike interval distribution. These changes can be expressed numerically and provide a quantitative description of the modifications of temporal pattern. Figure 12 shows the interspike interval distributions for one neuron during W, S, and S-LVF. Among the measurements that may be derived from the data of Fig-

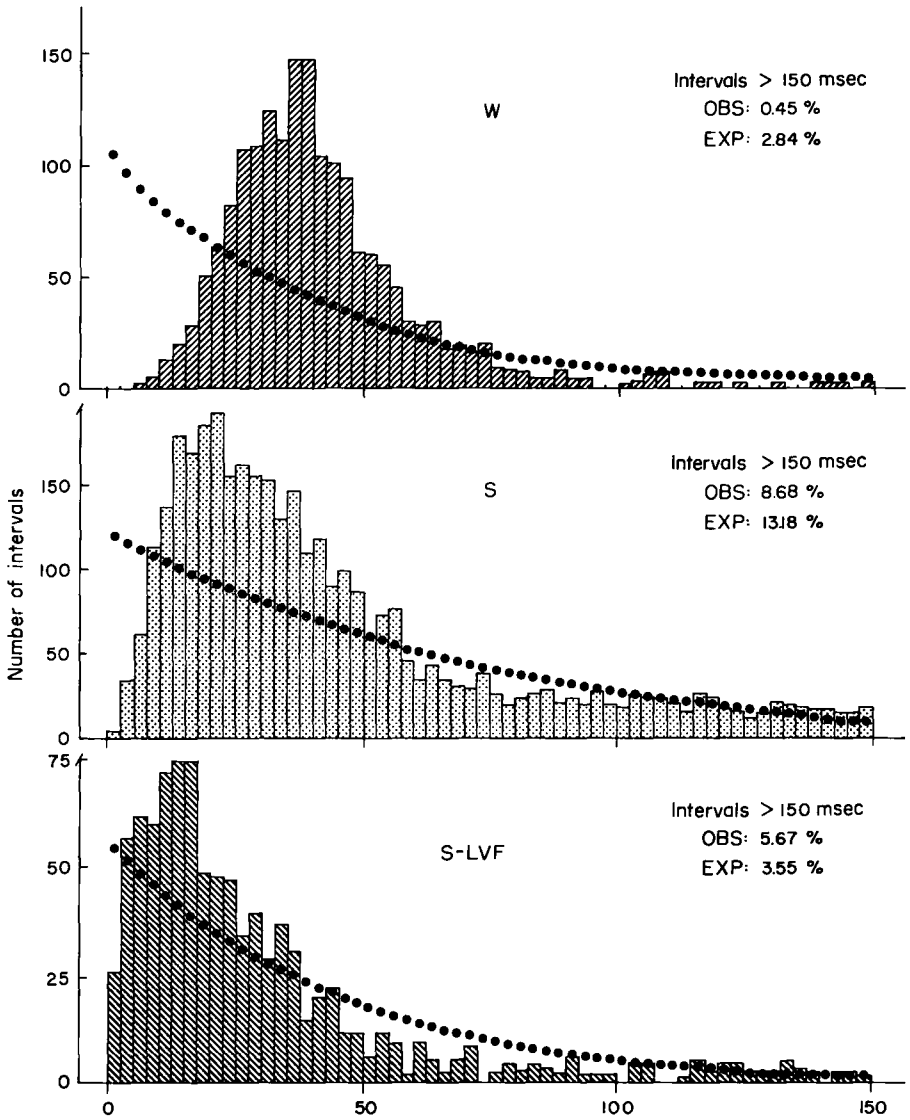

FIGURE 12 Interspike interval distributions during W, S, and S-LVF. The change from W through S to S-LVF is associated with an increase in the proportion of short interspike intervals; there is also an increase in the proportion of long intervals. Thus, during S and S-LVF the number of intervals > 150 msec. approaches or exceeds the number in the Poisson distribution, whereas the number of such intervals during W is considerably less than is the case for the Poisson distribution. The dotted curves represent the Poisson distribution. The histogram of W was based on 1,761 spikes which occurred in 74 sec. For S there were 3,686 spikes in 273 sec. The histogram of S-LVF was based on 987 spikes in 44 sec. Discharge frequencies for the three periods were 23.8/sec. (W), 13.5/sec. (S), and 22.4/sec. (S-LVF). In each of the three histograms, the heights of the successive, vertical bars represent numbers of intervals in consecutive classes of 2.5 msec. width. Thus, during W there were no intervals between 0 and 2.5 msec. or between 2.5 and 5 msec., whereas there were many such intervals during S-LVF. Intervals are divided among 60 classes, each of which is 2.5 msec. wide. Intervals beyond the 60th class (i.e., > 150 msec.) are combined into a single class. Under "intervals > 150 msec." are listed the number of such intervals observed (OBS) and expected on the assumption of random (Poisson) discharge (EXP). (From Evarts, Note 1)

ure 12 are modal interval, mean interval, proportion of long intervals, proportion of short intervals, and frequency of discharge (reciprocal of mean interval). The interval distributions of the figure show a reduction of modal interval from W to S and to S-LVF. The distribution during W differs from a Poisson distribution by a deficiency both of long and of short intervals. The distribution during S-LVF differs from the Poisson distribution in an opposite man-

ner, having an excess both of short and long intervals. These changes from W to S-LVF occur without a significant change in mean interval. Thus, in Figure 12, the mean interval is 42 milliseconds during W and 44 milliseconds during S-LVF. The bursts and periods of silence characteristic of S-LVF tend to balance each other with respect to mean interval, so that the steady discharge of W and the sporadic activity of S-LVF generate about the same over-all discharge frequency. Discharge frequency is less during S than during either W or S-LVF. Inactivity associated with the periods of silence during S is not compensated for by the relatively short bursts. Figures 13 and 14 summarize data on modal interval, proportion of short intervals, and discharge frequency. Modal interval decreases from W to S to S-LVF, the proportion of short intervals rises from W to S and S-LVF, and discharge frequency falls from W to S but rises from S to S-LVF.

Observations on the temporal patterns of activity of PTNs were first made by Adrian and Moruzzi.[5] In that work, it was found that under Dial anesthesia, PTNs tend to discharge in high-frequency bursts of two to three spikes with interspike intervals on the order of 3 milliseconds. These bursts occurred in relation to 10-per-second slow waves, which were recorded from the surface of the cortical motor area. In contrast, units under ether or chloroform anesthesia tended to discharge regularly at approximately 50 per second. In spite of the increase in average frequency of discharge during such anesthesia, there was a decrease in the number of interspike intervals less than 5 milliseconds. It was pointed out by Adrian and Moruzzi that the same neuron may show 10-per-second bursts under Dial and a 50-per-second regular rhythm under ether or chloroform. Bursts of pyramidal tract cell discharge during strychninization or electrical stimulation of the cortex, and the absence of such bursts during light $MgSO_4$ anesthesia, made it appear unlikely that high-frequency bursts occur in the normal animal.

Only recently has it become possible to obtain recordings of single-unit activity in normal (unanesthetized, intact) animals. Among the first results of the technique for obtaining such recordings was Hubel's[6] finding that, during natural sleep and waking, neurons in the lateral geniculate nucleus discharge with brief bursts of two to eight spikes at frequencies up to 500 per second. These bursts did not occur during arousal, and the geniculate neurons discharged at "more or less random intervals." Hubel's observations made it clear that clustered firing can occur in the central nervous system as a natural event. He has reported similar findings for certain neurons of the striate cortex.[7] Creutzfeldt and Jung[8] observed that arousal eliminated clustered firing of motor cortex units in the cat *encéphale isolé*. In our laboratory, the tendency of neurons

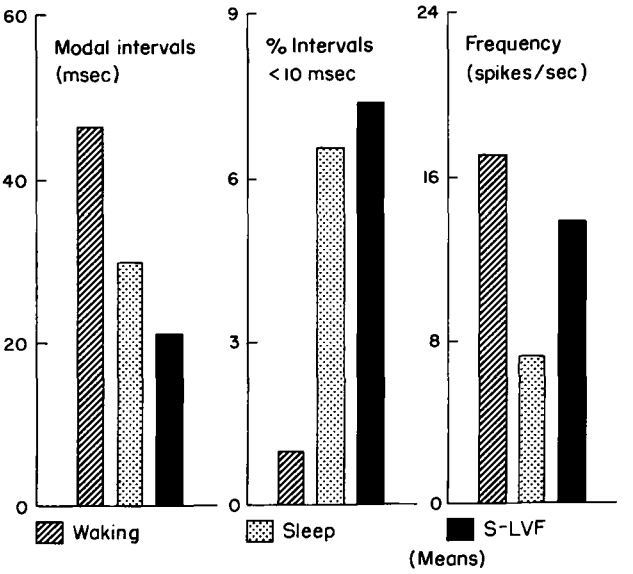

FIGURE 13 Mean values of discharge measures for a group of PTNs. Modal interspike interval was determined for each PTN during W, S, and S-LVF. The average for each one of these three sets of modal intervals is shown in the left-hand chart. It may be seen that modal interval drops from W to S, and drops further from S to S-LVF. W differed significantly from S ($t = 3.47$, $P < .001$) and from S-LVF ($t = 5.08$, $P < .001$). The change in modal interval from S to S-LVF did not reach statistical significance. The proportion of intervals less than 10 msec. (shown in center chart) exhibited a corresponding change from W to S and S-LVF. W differed significantly from S ($t = 4.44$, $P < .001$) and from S-LVF ($t = 3.76$, $P < .01$). Discharge frequency was greater during W than during S ($t = 3.36$, $P < .01$), and greater during S-LVF than during S ($t = 3.34$, $P < .01$). The difference between mean discharge frequencies during W and S-LVF was not statistically significant. (From Evarts, Note 1)

to fire in bursts during sleep has been seen in units of brain stem,[1] striate cortex,[9-11] and suprasylvian association cortex.[12]

Mechanisms underlying change in temporal pattern

The regular discharge pattern of the PTN during W resembles that of the spinal motoneuron.[13] This similarity, together with certain functional analogies between the two kinds of neurons, makes it appear probable that mechanisms which play a role in stabilizing the discharge of the motoneuron[14-18] may be important in maintaining the regular discharge pattern of PTNs during W. Afferent inhibition, recurrent inhibition, and postspike hyperpolarization are among the processes that may operate to limit the motoneuron's frequency of discharge; the interaction

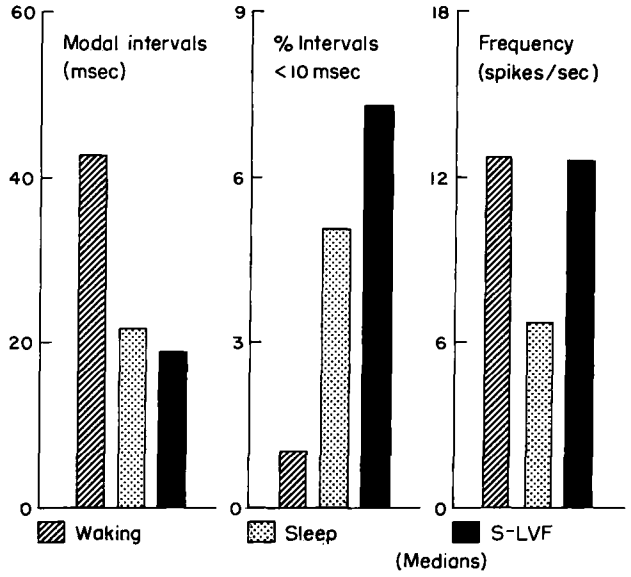

FIGURE 14 Median values of discharge measures for a group of PTNs. This figure shows the medians of those measures whose means are shown in Fig. 13. Medians are presented because means may be greatly influenced by extreme values, whereas medians are not so influenced. The median value for the modal interval was greatest during W and least during S-LVF, S being intermediate. The proportion of short (< 10 msec.) intervals showed a corresponding rise from W through S to S-LVF. Discharge frequency dropped to one-half its waking value during S, but returned to near the waking value with S-LVF. It is apparent that the pattern of change in the means of Fig. 13 is equally clear in the medians of Fig. 14. (From Evarts, Note 1)

of excitatory drive with these (plus certain other) mechanisms determines the motoneuron's pattern of discharge.[17,18] A complex relationship of this sort makes it impossible to ascertain the basis of a particular change in activity solely from analysis of the associated modification of temporal pattern. The change of discharge pattern of the PTN from W to S-LVF could result from marked fluctuations of excitatory drive, from a failure of some frequency-limiting mechanism, or from some combination of the two.

It seems unlikely that an alteration of excitatory drive is the entire basis for changes of PTN activity from W to S or S-LVF. Discharge frequency during the intense bursts of S-LVF reaches values greater than those occurring at any other time—greater even than the peak frequencies associated with movement. It is likely that there is considerable excitatory drive associated with the maximum discharge frequencies during movement. Thus, one may suppose that S and S-LVF are associated with a reduction in the effectiveness of one or more of the frequency-limiting mechanisms. Recurrent inhibition, one such mechanism in PTNs, has been studied by Phillips through intracellular recording.[19,20] The results led him to suggest that recurrent inhibition might act to prevent high frequency bursts. The regular discharge pattern and the absence of short interspike intervals, which characterize pyramidal tract cell discharge during W, may depend upon the system comprised of the recurrent axon collateral and the proposed cortical analog of the Renshaw cell. Postspike afterhyperpolarization and afferent inhibition might also stabilize the discharge pattern during W. Further experimentation may discover the combination of changes of excitatory and inhibitory inputs to PTNs that may underlie the striking differences between sleep and waking.

Thus far we have dealt with differences between the discharge patterns of S and W and have given relatively little attention to the differences between sleep with EEG slow waves (S) and sleep with low-voltage fast EEG activity (S-LVF). S and S-LVF were found to differ both in frequency of discharge and in temporal pattern. Frequency of discharge was, on the average, twice as great during S-LVF as during S and, in fact, was only slightly less during S-LVF than during W. These observations confirm the recent findings of Arduini et al.[21] Although similar in average discharge frequency, W and S-LVF are at opposite extremes in the temporal pattern of activity in the individual neuron. In fact, the change from the regular discharge of W to the sporadic bursts of S becomes extreme with S-LVF. Bursts are even more intense, and intervening periods of silence are even longer, during S-LVF than during S. The method of recording used by Arduini and his colleagues[21] provides an index of integrated activity in large populations of neurons but does not reflect modifications in patterns of discharge of the individual neuron. Arduini and coworkers did not detect this difference between W and S-LVF, but this is in no way incompatible with the present findings. They found that, during S-LVF, pyramidal activity is characterized by a more steady course than during S or W. This indicates that the intense bursts occurring in individual PTNs during S-LVF do not occur synchronously in large groups of PTNs whereas the less intense bursts of S occur synchronously.

The change of discharge pattern of PTNs associated with the onset of sleep suggests an underlying reduction of both inhibitory and excitatory inputs. Observations in acute experiments by previous investigators[22,23] indicate that stimulation of the thalamic reticular formation, or arousal by other means, causes transient inhibition of PTN discharge. It seems probable that these subcortical structures exert their inhibitory actions via interneurons and not directly on PTNs. As Eccles[16] has emphasized for the spinal cord, ". . . there is no recorded instance of central

inhibitory action directly exerted by primary afferent fibres or by volleys in descending tracts. In every case an interneuron with a short axon is interpolated." It seems reasonable that inhibition within the cortex should also depend on interneurons, and that the excitability of these interneurons should be to some extent under the control of subcortical structures. Subcortical control of spinal cord interneurons is now a well-demonstrated fact, and it would seem likely that there are analogous subcortical controls for cortical interneurons.

Sleep may involve a progressive reduction in the excitability of these interneurons. Such a reduction would tend to eliminate the temporospatial patterning that must underlie the coordinated activity of the waking state and would give rise to progressive changes in the organization of neuronal discharge.

Speculations concerning the nature of sleep

The results and comments presented here have been designed to make four basic points about the activity of single neurons during sleep. The first concerns the considerable amount of activity that takes place during sleep with EEG slow waves and that during S-LVF many areas of the brain are actually more active than during waking. It may be concluded that *sleep cannot involve any generalized inhibition of the activity of cerebral neurons.* The second point concerns the change in distribution of discharge frequencies from W to S, which makes it clear that although the brain as a whole remains relatively active during sleep, many individual neurons in the brain are less active during S than during W. Although sleep cannot represent a period of *generalized* rest for the brain, it could represent a period of *relative* rest for certain specific elements. The third point is the relation between cell size and the effects of sleep. This will now be discussed in some detail and from two points of view, for it may have some implications for the nature of sleep.

We have said that the largest PTNs are relatively inactive during W and speed up from W to S and that the smallest PTNs are relatively active during W and slow down from W to S. This relationship may be viewed as a reflection of the differing functional properties of the large and small neurons during waking. Alternatively, it may be of heuristic value to speculate upon the possible teleological significance of the observed relationship between neuronal size and change in activity from waking to sleep.

One may begin by considering findings of previous investigators on the relation of axonal diameter to the electrochemical consequences of the action potential. Ritchie and Straub[24] have calculated that a short period of repetitive discharge (150 impulses in 10 seconds) would cause practically no increase in the concentration of sodium ions inside the giant squid axon, whereas a similar train of impulses would increase the sodium concentration of a 1-micron-diameter C fiber by 24 millimoles per liter, and of a 0.2-micron-diameter fiber by 120 millimoles per liter. As nerve fibers become smaller and the ratio of their membrane surface area to axoplasmic volume increases, there is a corresponding change in the metabolic and electrochemical results of their activity. A myelin sheath, which greatly limits the surface area through which ion movements take place, reduces the extent of sodium influx and potassium efflux during activity. Hodgkin[25] has calculated that for a given number of impulses a myelinated fiber must exchange only 1/300 as much sodium as an unmyelinated fiber of equal diameter. Even for myelinated fibers, however, a given amount of activity causes a relatively greater increase of sodium concentration in the small neuron than in the large one. The reduction of ionic movement resulting from the myelin sheath does not extend to the neuronal soma, and for the membrane of the cell body movement of ions and the consequent requirement for sodium-potassium exchange may approximate that of the unmyelinated C fiber. In addition, during W the smaller neurons tend to engage in relatively continuous tonic activity, whereas the large neurons have less over-all discharge although they are active during movement. It is impossible to say whether discharge frequencies of the order of 20 per second (a value seen in a number of tonic PTNs) maintained over a number of days and nights of waking might build up a significant metabolic and/or ionic debt. However, it does not seem unreasonable to suppose that if any PTNs might benefit from a period of reduced discharge frequency it should be the small ones, because their tonic discharge during W and their structure make them inherently less capable of long-sustained, continuous discharge.

This teleological analysis took as its point of departure the relation between cell size and the electrochemical consequences of the action potential. An alternative and nonteleological explanation for the relation of cell size to changes with sleep may be proposed if one considers the relation between cell size and the cell's role in the over-all neural system. Commonly, large neurons are phasically active and small neurons are tonically active. Eccles et al.[26] have found that the average conduction velocity of soleus motoneuron axons is 72 per cent of that of gastrocnemius motoneurons. The α-motoneuron fibers innervating the gastrocnemius (a pale, phasically active muscle) have a correspondingly larger diameter (Eccles and Sherrington[27]) than do the fibers to the soleus (a red, tonically active muscle). Granit et al.[28] have emphasized the relationship between structural and functional properties in α-motoneu-

rons and have presented several lines of evidence that tonically active α-motoneurons are smaller than those that are phasically active. To cite another example, the large, Ia afferents of the nuclear bag intrafusal muscle fibers of the muscle spindle carry information that is particularly related to transients, whereas the smaller, group II afferents of the nuclear-chain intrafusal muscle fibers tend to carry steady-state information (cf. Matthews[29]). The efferent supply to the intrafusal fibers also shows a relation between function and structure. The larger γ_1 fibers with high conduction velocities innervate the phasically active, nuclear-bag, intrafusal fibers, and carry transient information, while the smaller γ_2 fibers with low conduction velocities innervate the more slowly contracting nuclear-chain fibers, carry steady-state information, and are tonically active.[30,31] Differences in discharge pattern as a function of cell size, seen so clearly for PTNs during W, disappear with S, during which sporadic bursts of impulses occur in small and large cells alike. Large PTNs become more active with S and small ones become less active, but in spite of this, the mean discharge frequency of the small cells during S is actually somewhat greater than that of the large cells. During S the group of 13 relatively small PTNs (latencies greater than 2 milliseconds) had a mean discharge frequency of 9 per second. For the 19 relatively large PTNs (latencies less than 1 millisecond), the mean discharge frequency during S was 6.5 per second.

Another point against the teleological argument is the behavior of PTNs during S-LVF, at which time they have discharge frequencies that approximate the value of W, and are, on the average, twice as great as those of S. Discharge frequency increases from S to S-LVF regardless of antidromic response latency, and both large and small PTNs discharge in intense bursts. That the smaller PTNs discharge almost as rapidly during S-LVF as during W provides additional grounds for questioning the teleological explanation for the discharge frequency reduction of small PTNs from W to S.

Here then, we come to the crux of the problem of the possible functional significance of sleep. Is it reasonable to suppose that sleep is primarily concerned with allowing rest for neurons, in the sense of providing them with a period of freedom from activity during which they may replenish those substrates necessary for the generation of action potentials? The results of microelectrode recording cast some doubts on this hypothesis. It is, of course, true that many neurons are less active during sleep than during waking. If the purpose of sleep were to restore certain substrates, however, that aim is not being achieved efficiently, because large numbers of neurons continue to have such intense activity during sleep. The time course of sleep is another point against sleep being involved in replenishment of some substrate necessary for the occurrence of the action potential. Studies on isolated neural tissue[32] indicate that neurons can be driven by electrical stimulation at frequencies that lead to their virtual exhaustion, and yet, given adequate external metabolic substrates, they can recover within periods of minutes—periods that are extremely short in contrast to the rather long period the subjects spend asleep. It is hard to imagine a state of neuronal fatigue occurring under physiological circumstances that would need "eight hours of sleep" for its elimination.

Alternatively, it may be proposed that the functional significance of sleep *does not* lie in the restoration of substrates for action potential generation. This alternative has recently been discussed in a most eloquent paper by Moruzzi,[33] who proposed that sleep has a very different role in the function of the nervous system. Pointing out that sleep may not be primarily concerned with short-term recovery processes associated with action potential generation, Professor Moruzzi suggests that it may be related to recovery processes concerned with those relatively long-term, plastic, structural changes that the nervous system must undergo to make memory and learning possible.

A related hypothesis has been advanced by Feinberg[34] on the basis of studies of sleep patterns in normal aged subjects and in aged subjects with diffuse organic disease of the brain. He observed that organic brain disease associated with failure of memory formation was associated with a reduction in sleep time and reasoned that the brain whose structure is no longer compatible with normal learning and memory shows a reduced sleep requirement.

In conclusion, I would like to suggest that the hypotheses of Feinberg and Moruzzi be carefully examined by the molecular biologist interested in the higher function of the central nervous system, for they point toward some highly promising experiments that may help to solve the problems of learning and memory, as well as those of sleep and waking.

Summary

During sleep, cerebral neurons show modifications in the organization of activity, but do not show a generalized reduction in the total amount of discharge. With rapid eye movement sleep in particular, many neurons actually show marked increases of activity. These results introduce the consideration that the biological function of sleep is not to allow restoration of those chemical substrates necessary for the generation of action potentials, and suggest the need for new hypotheses on the nature of sleep.

The Biology of Drive

PHILIP TEITELBAUM

Analysis and synthesis in the study of motivated behavior

ONE HUNDRED and one years ago, Claude Bernard wrote in his *Introduction to the Study of Experimental Medicine,* while discussing the nature of scientific reasoning:

> Reasoning is always the same whether in the sciences that study living beings or in those concerned with inorganic bodies. But each kind of science presents different kinds of phenomena and complexities and difficulties of investigation peculiarly its own.[1]

This was particularly borne in upon me by the chapter presented elsewhere in this volume by Dr. Edelman on the nature of the immune response. In Figure 2 of his paper, he described a program of thought and work based on the processes of analysis and synthesis. Thus, to make an experimental approach to the problem of understanding the structure and action of the immunoglobulin molecule, one tries various ways of breaking it down and putting it back together. Edelman devised ways of chopping the molecule into half molecules or even smaller fractions. He then studied the action of the simpler parts. He also punched holes in the immunoglobulin molecule by protein digestion techniques to produce aberrations which might shed light on its normal action. Furthermore, he studied the synthetic reconstitution of such crippled molecules, with great success in revealing the importance of normal structure in the normal action of the immunoglobulin molecule.

There is a close parallel in the physiological analysis of motivated behavior. As in the study of the immunoglobulin molecule, we seek ways of cleaving behavior into simpler units and of recombining such units to reconstitute the behavioral phenomena in which we are interested. The nervous system controls behavior, so we must take it apart. Chop it into smaller parts and our motivated behavior should decompose into simple fragments. Recombine the levels of nervous function and we should synthesize the complex phenomena of motivation.

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Levels of central nervous function

About 150 years ago, Pierre Flourens, one of the great early French physiologists, used this approach to the study of the nervous system.[2] By cutting at various levels, and by removing entire parts, he completely separated the lower portions of the nervous system from the influence of higher parts. He then studied the behavior that remained. Sherrington similarly analyzed the integration of reflexes in the spinal cord.[3] Later, Philip Bard used the same approach in the study of sexual behavior in the cat.[4] Although it may be quite elementary for some, I would like to describe Bard's work in detail, because it illustrates clearly some of the levels of behavior I wish to distinguish between.

The female cat becomes sexually receptive about twice a year. If its back or genital areas are touched or stroked, the cat in heat will crouch by bending its forelimbs, and tread in place by alternately flexing one hind limb and extending the other. If a glass rod is inserted into its vagina, it will move its tail to one side and tread very vigorously, while giving the low throaty estrual call. If stimulated sufficiently, the cat will reach a violent climax signaled by a loud cry and followed by an after-reaction of rolling and squirming on its back while purring. When not in heat, or if its ovaries have been removed, such stimulation of a cat's genitals causes it to move away or display anger.

Bard studied this sexual behavior pattern in castrated female cats whose nervous systems were transected at various levels. In a spinal cat, even with its gonads removed, stimulation of the genitals elicited the normal pattern of crouching, tail deflection, and hind-limb treading. However, injection with estrogen had no effect on the threshold of sexual response to genital stimulation. The same was true of a decerebrate cat, in which the transection was made at the level of the midbrain. Only if the transection was above the hypothalamus, allowing it to remain connected to the lower part of the nervous system, did this behavior pattern show a sensitivity to hormonal influence.

Adding only a little bit of tissue (that contained in the hypothalamus) restores hormonal control of sexual behavior. Therefore, tissue responsive to hormones exists in the hypothalamus, and such tissue without any of the brain above is sufficient to exert control over spinal reflexes.

Where such a demonstration has been made, one can speak of neural centers of integration. By extrapolation, although such rigorous proof is still largely lacking, one may think of hypothalamic integration of feeding, drinking, temperature regulation, and so forth.

By this classic approach, we can clearly distinguish at least two levels of complexity of behavior and nervous function: (1) simple reflexes, which are not subject to specific hormonal control; and (2) hormonally controlled reflexes. I call the latter instinctive, because as far as I can determine, hormonal control is common to all instinctive behavior.

The comparative approach: simplify by phylogeny

It is clear, then, that simply by transecting the nervous system at different levels we immediately discover varying levels of behavioral complexity. However, such operations, although they reveal some simple types of integration, often prevent us from observing more complex forms of behavior. As soon as we cut through the nervous system, we can no longer study any behavior that is dependent upon a nervous connection that goes across the transection. Therefore we must look for an alternative way of simplifying the nervous system.

One way is the comparative approach, which has been used most fruitfully in recent years by ethologists.[5] Phylogenetically lower animals, such as fishes and birds, have simple nervous systems, so their behavior patterns are stereotyped, easily observable, and amenable to study.

Consider the instinctive mating pattern of a stickleback fish.[6] In the appropriate season, a male stickleback, after having set up a mating territory and built a nest, encounters a female. As the male approaches, the receptive female turns upward in front of him, displaying her belly, swollen with eggs. The male then turns and leads toward the nest; if the female is fully receptive she follows; he shows the nest and she enters; he rubs against her, stimulating her to deposit eggs; then she swims out of the nest and he swims through and fertilizes the eggs. This complicated behavior pattern is characterized by a chain of stimuli and responses. The stimuli themselves are complex —they are patterned configurations that the ethologists call social releasers, or sign stimuli. A sign stimulus is another differentiating characteristic of a higher level of behavior. One way of demonstrating the existence of a sign stimulus is to use a dummy model. If the dummy elicits the behavior, we are dealing with a fixed instinctive response to that sign stimulus. Like reflexive behavior, the more appropriate the stimulus, the more easily the response is elicited. But, in addition, the higher the concentration of the appropriate hormones circulating in the animal's blood, the more receptive it is to sign stimuli—even to crude ones, which are ordinarily ineffective. Indeed, if the hormone level is very high and the animal has not engaged in the act for some time, the instinctive behavior pattern may occur spontaneously as a "vacuum" activity in the absence of any observable releasing stimulus. Thus, the comparative approach agrees with the analysis by levels of function and shows that instinctive behavior, as distinguished from simple reflex action, is doubly quantified.

The development of behavior

Still another way of simplifying behavior is by studying its development, which often proceeds from simple to more complex levels of integration. Consider, for instance, the feeding behavior of young thrushes.[7] When the parent alights on the nest, thus shaking it, the newborn thrush gapes vertically. A few days later in its development one can elicit gaping by touching the side of the thrush's mouth, but the gaping is still vertically oriented. Later, after the eyes open, the sight of the parent bird or even the sight of a finger will elicit gaping, but it is still vertically oriented; whereas, still later in development, gaping is directed toward the visual stimulus. We see here the development of increasingly complex forms of stimulus control over an important element of feeding behavior—the gaping response. At first, the response is elicited and controlled by very proximal stimuli—vestibular or kinesthetic receptors detect the shaking of the nest—and gaping is vertical (controlled by gravity). Stimulus elicitation gradually becomes more distal (from vestibular, to touch on the skin of the mouth, to visual) and the direction of the gape becomes subject to guidance by visual stimuli.

The comparative approach has a major limitation for a psychologist who is primarily interested in human behavior. Although the approach is fruitful, it is certain that some of the phenomena of human behavior are not to be found in the repertoire of lower animals. Furthermore, the application to human behavior of principles derived from animal studies is often not obvious, although it will no doubt become so eventually.

REFLEXES IN THE NORMAL HUMAN INFANT Ideally, what we would like is a human preparation that is as simple as a Sherringtonian decerebrate cat or dog, but one that will slowly become capable of all the complex motivated behavior characteristics of the adult human. Such a preparation is very easily found. The newborn infant possesses many of the reflexes seen in spinal or decerebrate preparations.[8] These reflexes can be understood in terms of their function: they increase or decrease the infant's contact with the stimuli evoking them. In general, reflexes of

approach are elicited by stimuli of weak intensity and reflexes of withdrawal are evoked by stimuli of great intensity.[8,9] As shown in Figure 1, touching a rubber nipple to the cheek or chin of a newborn infant will immediately elicit the rooting reflex, a head movement that serves to bring the mouth closer to the nipple. At the same time, the mouth opens, and if it contacts the nipple, the baby begins to suck. These reflexes are completely automatic and can occur when the infant is asleep or in a coma. They are approach reflexes of the head and mouth, which serve to bring the infant into contact with the mother's breast and enable the infant to feed.

Touching an infant's palm causes its fingers to flex and close around the stimulating object. This is the grasp reflex (Figure 2). A grasp reflex of the foot (plantar reflex) is also seen. These reflexes are extremely powerful. If the infant is caused to grasp a rope reflexively with its hands, as in Figure 3, it will cling so firmly that it is entirely supported when suspended in mid-air. This is forced, involuntary grasping—the child cannot let go although it screams with discomfort in the air.

If a bright light is flashed into an infant's eyes (Figure 4) it will shut them instantly, thus cutting off the blinding stimulus. Similarly, as in Figure 5, a bitter taste causes immediate rejection and firm closure of the mouth, with downturned corners. Painful stimulation of the hand elicits a withdrawal reflex, characterized by spreading extension of the fingers. A similar withdrawal reflex is elicited by painful stimulation of the sole of the foot. This is the Babinski reflex—a spreading of the toes.

The preceding figures also show one principle of elaboration of reflexes into more complex acts. Reflexes spread in response to increased intensity of stimulation.[8] Allied reflexes, those that have in common the functions of approach or withdrawal, occur together to cooperate in approach or avoidance of the stimulus. For instance, the rooting reflex can occur alone. If, however, the infant has been long without food, or if its cheek is stroked repeatedly with the nipple, then vigorous head-turning will occur in combination with mouth-opening and sucking.

Thus, simple reflexes, in response to strong stimuli, can combine into chains, which make the response more effective in approach or withdrawal. The nervous mechanism of such a spread of allied reflexes must be basic to the integration of chains of behavior.

Some reflex chains, which form part of adult behavior, are already present at birth.[8] A newborn infant cannot stand upright, but if its body is supported so that its legs are placed in contact with the surface of the floor below and its body is moved forward, smoothly synchronized walking movements occur. The infant cannot walk voluntarily; therefore, the presence at birth of such reflex chains shows that they are inborn nervous patterns that await the development of mechanisms of voluntary control.

REFLEXES IN THE ANENCEPHALIC HUMAN INFANT It is true that a human infant's behavior is simpler than it will be when it is adult, but how can we say that it is essentially a decerebrate preparation? Natural aberrations in human development furnish the proof we need. Some infants develop abnormally in utero and are born with incompletely formed nervous systems. Such anencephalic infants usually die quickly, but in rare instances they live for days or months, long enough for a relatively complete assessment of their behavioral capacities. One such anencephalic child was found at autopsy to be devoid of any nervous tissue above the medulla, although its spinal cord and medulla appeared to be normally developed.[10] Yet its behavior was amazingly similar in many respects to that of a normal newborn child. It rooted, sucked, and nursed at its mother's breast. It grasped with hand and foot. In response to a pin prick, the foot withdrew quite readily. Another anencephalic infant developed midbrain structures in addition to the spinal cord and medulla.[11] It, too, displayed normal rooting, sucking, grasp, and withdrawal reflexes. However, in contrast to the rhombencephalic infant described above, this mesencephalic child breathed without any difficulty and showed clear patterns of waking and sleeping similar to those of a normal newborn. It nursed, but not sufficiently to gain or even to maintain its weight. It steadily lost weight and would have died within a few days if it had not been tube-fed. However, with the help of tube-feeding, it lived 57 days.

Thus, at birth, the normal child is in many ways comparable to an animal without cerebral hemispheres, incapable of voluntary action. Yet it differs from a decerebrate anencephalic infant; it displays spontaneous cyclic activity —screaming and struggling when wet, cold, hungry, or in pain. Like a hypothalamic cat, its behavior is more instinctive than reflexive; the thresholds of its reflexes and its spontaneous activities are strongly influenced, not merely by external stimuli, but by its internal state as well.

THE DISAPPEARANCE OF REFLEXES The simple involuntary reflexes displayed by the infant at birth disappear as development progresses.[8] By the age of four to six months, the automatic grasp reflex of hand and foot, the rooting and sucking reflexes of the mouth, the coordinated stepping patterns seen earlier, all become increasingly difficult to elicit as the capacity for voluntary reaching, grasping, eating, and walking develop. Eventually, voluntary purposeful acts dominate the behavior of the child; reflexes seem to disappear. Does this mean they are no longer a part of his behavior? Can we neglect these simpler units in

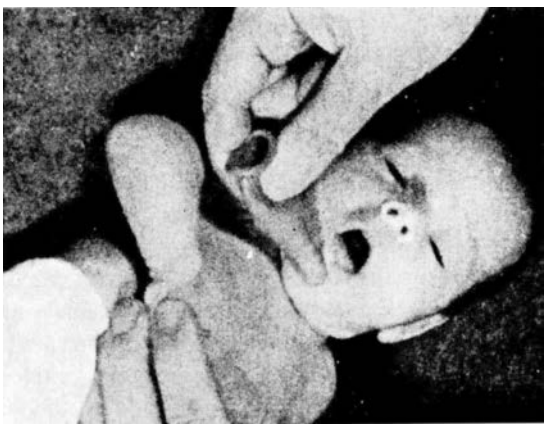

FIGURE 1 Reflexive rooting and mouth-opening in newborn infant on being touched with a nipple. (From Peiper[8])

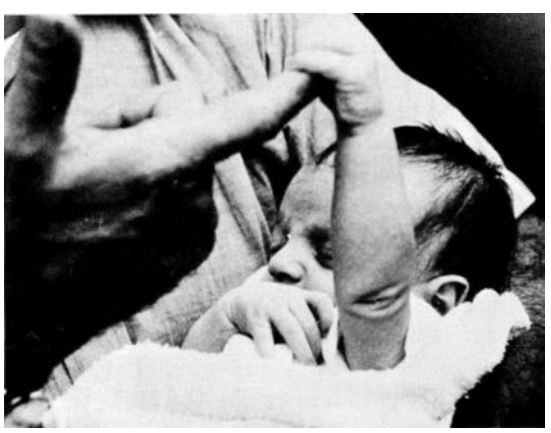

FIGURE 2 Palmar grasp reflex of newborn. Touching the palm causes flexion of the fingers and grasping of the touching object. (This infant, the author's son, was born at the NRP conference in Boulder, Colo., a few hours after this paper was delivered.)

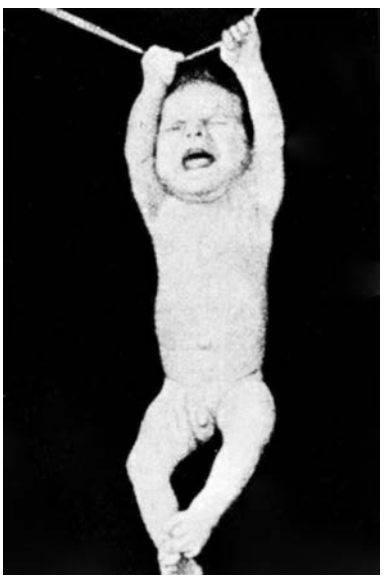

FIGURE 3 Forced grasping strongly enough to suspend a prematurely born 4-week-old infant. (From Peiper, Note 8)

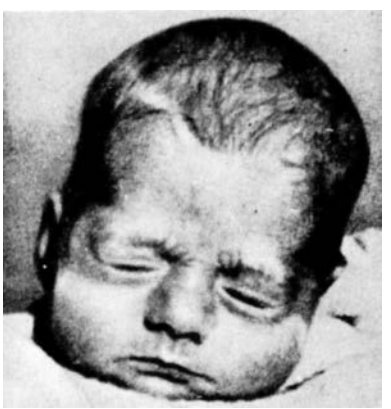

FIGURE 4 Eye and mouth closure of a 7½-week-old girl on exposure to blinding photic stimuli. (From Peiper, ibid.)

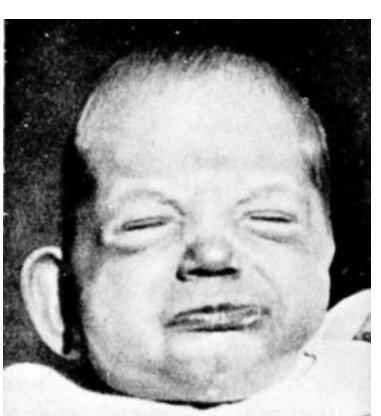

FIGURE 5 Facial reaction, spreading from mouth to eyes, caused by quinine in a female infant 2½ months old. (From Peiper, ibid.)

an attempt to understand his motivated behavior? Has he used these simple mechanisms merely as crutches during his early development only to cast them away when he becomes capable of the amazingly complex behavior of the adult human being?

REAPPEARANCE OF INFANTILE REFLEXES IN BRAIN-DAMAGED ADULTS Clearly, the simple reflex mechanisms are still present in the nervous system. From the work of Denny-Brown[12-14] on brain-damaged adult patients, it is evident that many of the infantile reflexive patterns of approach and withdrawal reappear after damage has been done. For instance, after damage to the frontal lobes, we observe release of automatisms of approach. Touching or stroking the side of the patient's cheek near his mouth often elicits mouth-opening, head-turning toward the stimulus, and, if the mouth makes contact with the stimulating finger, sucking. If the patient is asked why he is doing this, he may show surprise and embarrassment, replying that he was not aware of his action. He may even deny it, yet he may be unable to stop.

Similar approach automatisms can be demonstrated in the hand and foot. If, while the frontal patient's attention is distracted, the palm of his hand is lightly touched and stroked by the examiner's index finger in a movement from the center of the patient's palm outward between the thumb and forefinger, grasping will be elicited. Depending on the amount of damage to the frontal lobes, and also on how much paralysis has been caused by damage simultaneously occurring in the motor areas of the brain, various components of grasping can be evoked. Deep pressure in the palm elicits strong maintained flexion of the fingers (grasping). Light touch in a stroking movement of the skin elicits closure of the fingers around the stimulating object (trap reaction) in such a way as to prevent it from leaving the palm. Rhythmic flexion and extension of the fingers (forced groping) then occur in a manner that acts to bring the object back to the center of the palm, where it can be firmly grasped. If the object is moved out of the palm, the hand and arm follow after it (pursuit reaction), and if the object is moved slowly enough, the hand and arm follow while just clinging to it (magnet reaction). In some cases, the mere sight of an approaching object or person will elicit involuntary reaching and grasping. The same phenomena can be seen in the foot.

Thus, the approach reflexes of mouth, hand, and foot, which disappear during infant development, reappear in the adult after damage to the frontal cortex. One may speculate that in the course of normal development the frontal cortex exerts over these reflexes an inhibitory influence which, in some manner, integrates them into voluntary behavior. It is interesting to note, in this respect, that recovery of voluntary control after frontal-lobe damage parallels the development of voluntary control in infancy. The same progression occurs in both—from control by local contact reflexes (forced grasping in response to deep pressure) to control by more tenuous and distant stimuli with projection into space (forced groping, magnet reaction, and visual control over grasping) to eventual voluntary control of reaching and grasping. A similar progression has been described with respect to the development of stimulus control of gaping in the thrush.

Aberrations in eating following hypothalamic damage

In principle, therefore, we should be able to learn a great deal about normal adult motivated behavior by studying simplified preparations broken into various levels of function by phylogeny, by surgery, or by immaturity in development. Still another approach is possible—the study of aberrations in motivation. Just as Edelman chemically punched holes in the immunoglobulin molecule, we can use a stereotaxic instrument to damage the hypothalamus, where a great deal of motivated behavior is integrated. We can then compare the behavior of hypothalamic-damaged animals with normal ones, to see whether the abnormal may reveal important elements of normal behavior.

Consider the behavior involved in eating—a motivated behavior that has been frequently studied. Work on the hypothalamic control of food intake has rapidly accelerated since the demonstration by Hetherington and Ranson in 1939 that obesity in people with pituitary tumors (Fröhlich's syndrome) can be produced in animals by discrete lesions in the ventromedial nuclei of the hypothalamus.[15] In 1943, Brobeck, Tepperman, and Long showed that such obesity was caused by overeating, not by a disturbance in the metabolism or utilization of food.[16] In 1951, Anand and Brobeck discovered that small lesions in the lateral hypothalamus would produce the opposite effect—refusal to eat and death from starvation.[17] They therefore suggested that these two antagonistic feeding mechanisms—a lateral, excitatory, "feeding" system held in check by a medial, inhibitory, "satiety" system—are sufficient to achieve the homeostatic regulation of food intake. Hypothalamic stimulation confirms this view: lateral stimulation elicits feeding in satiated animals, whereas medial stimulation inhibits the intake of hungry animals.[18,19]

Reflexes involved in feeding are also integrated in these hypothalamic regions. Thus, bodily states associated with lack of food, such as increased gastric motility, decreased blood sugar utilization, and decreased body temperature all apparently act to increase the activity of the lateral

"feeding center."[20-22] Changes associated with repletion, such as gastric distension, increased blood sugar utilization, and a rise in body temperature, probably cause an increase in the activity of the ventromedial "satiety center," which inhibits the "feeding center" and stops the behavior involved in eating.[21,23,24] Although there are psychological correlates of these states—the urges and pleasures of hunger and satiety—for purposes of parsimony, they usually are considered epiphenomena of the homeostatic regulation of food intake; they are believed to accompany regulation, but not to cause it.[25]

However, work on the aberrations in feeding that result from hypothalamic damage has forced a drastic reinterpretation of the nature of such regulations. Consider, for instance, a hyperphagic animal. Shortly after medial hypothalamic damage, the animal eats two to three times as much as normal and gains weight rapidly. This is the dynamic phase of hypothalamic hyperphagia.[16] As shown in Figure 6, such animals become quite fat. Once obese, a static phase ensues, in which the weight levels off at a high plateau and the animal's food intake drops back to only slightly more than normal. When their diet is diluted with nonnutritive cellulose, normal animals increase their intake to maintain caloric regulation (Figure 7). Even at fifty and seventy-five per cent dilution, when tremendous bulk must be eaten to get an adequate amount of food, normal animals still eat appreciable quantities. In contrast, hyperphagic animals do not increase their intake when the diet is mixed with cellulose.[26] Although they eat two to three times as much of the ordinary diet, they refuse to overeat

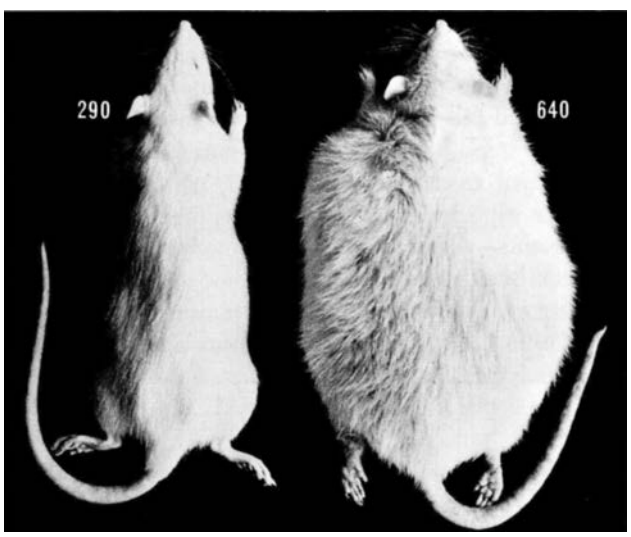

FIGURE 6 Hypothalamic obese female rat (right) compared with its normal control. (From Teitelbaum, Note 25)

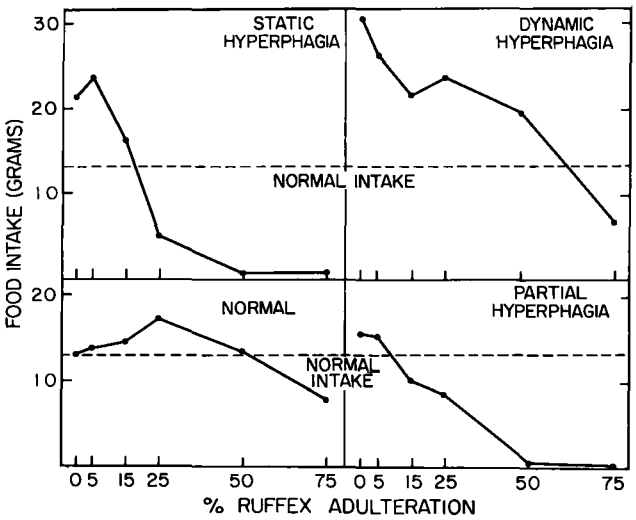

FIGURE 7 Food intake of normal and hyperphagic animals when offered a diet mixed with nonnutritive cellulose. (From Teitelbaum, Note 26)

when the food contains as little as twenty-five per cent cellulose. This finicky eating is particularly marked in obese hyperphagic animals, which eat little or nothing for a week at a time and lose a great deal of weight. Making the diet slightly bitter by adding quinine will also completely prevent them from eating, although normal animals remain unaffected. However, if one sweetens the diet, making it more palatable, static hyperphagic animals that had ceased overeating and had leveled off in weight show dynamic hyperphagia all over again. They overeat vigorously and become more obese than they had been before.[26]

A similar phenomenon can be seen in animals with lateral hypothalamic lesions. Such animals are typically active and alert and are capable of chewing and swallowing, but they do not eat or drink, and will starve to death in the face of food and water. Nevertheless, if they are kept alive by tube-feeding, they eventually recover,[27] and their recovery yields a great deal of information about the nature of the deficits produced by the lesions.[28] Long before they accept ordinary food and water, they can be induced to eat appreciable quantities by offering them wet and palatable foods, such as milk chocolate, wet cookies, and egg-nog liquid diets. However, like the anencephalic decerebrate, they do not eat enough to stay alive.[11,29] It is as though they are drawn to the food by its taste and smell, not driven to it by hunger. Even after they have recovered their capacity to regulate their food intake (as demonstrated by their increased intake of a liquid diet when it is diluted with water), when they are subsequently offered only their

ordinary diet and water, they refuse to eat or drink and starve to death.[30] Once again, palatability controls regulation.

TASTE AND REGULATION OF FOOD INTAKE Is taste essential to the regulation of food intake? Can an animal regulate his caloric intake in the absence of taste? It is difficult to remove the sense of taste surgically, so we used a gastric tube to bypass it. A slender, polyethylene tube is slipped into a rat's nostril, through the nasopharynx, into the esophagus, and down into the stomach. The outer end of the tube is brought under the skin of the snout and scalp to the top of the skull, where it is anchored firmly by screws and cement. Water or liquid food can now be pumped directly into the animal's stomach, completely bypassing the nasal and oropharyngeal receptors for taste and smell. By training the rat to press a lever to inject a liquid diet directly into its stomach, we can study the regulation of food intake in the absence of taste and smell and without the consummatory acts of chewing and swallowing. This arrangement is shown in Figure 8.[31]

The rat was first taught to press a bar for delivery of fluid into a cup in its cage. It ingested the fluid by mouth. Then the pump was connected to the animal's gastric tube so that when it pressed the bar, food was delivered into its stomach. A watertight swivel joint coupled between the animal's gastric tube and the pump allowed him to spend day and night in the cage. The joint swiveled with the animal and prevented its movement around the cage from kinking the delivery tube. The rat could therefore feed itself whenever it wished for months at a time, without tasting or even eating the food.

A normal rat switched from oral to intragastric feeding maintains normal intake and weight. It regulates its caloric intake precisely when the diet is diluted with water or when the size of each injection into the stomach is changed. If the animal is forced to work harder to obtain food, by increasing the number of presses required for each gastric injection, it does so and achieves normal intake.[31]

The hyperphagic animal, however, is severely affected by the absence of taste and smell.[32] As shown in Figure 9, a dynamic hyperphagic animal pressing a bar to obtain a liquid food delivered directly into its food cup ate the diet avidly, pressing the bar many times to obtain over 100 milliliters daily, which is about three times the normal intake. But when the diet was delivered directly into the stomach, the hyperphagic animal pressed the bar less frequently each day, until by the seventh day it was eating nothing at all. Although we have not permitted it, it is likely that such animals will starve to death when deprived of the taste and smell of their food. However, as is also shown in Figure 9, if we merely provide a sweet taste, by delivering a few drops of sodium saccharin solution into the animal's food cup at the same time that the liquid diet is delivered intragastrically, the animal works vigorously, regulates its intake, and gains weight rapidly. How does taste transform starvation into hyperphagia?

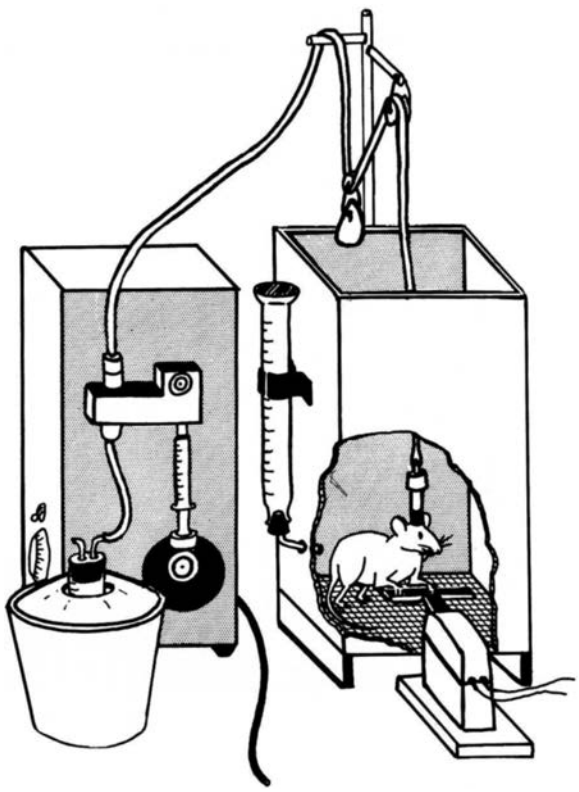

FIGURE 8 Schematic drawing of the apparatus for intragastric self-injection by the rat. The rat presses the bar in order to activate the pipetting machine (center), thus delivering a liquid diet from the reservoir (left foreground) through the chronic gastric tube directly into its own stomach. (From Epstein and Teitelbaum, Note 31)

MOTIVATION AND THE REGULATION OF FOOD INTAKE When an animal uses a learned arbitrary act to obtain food, psychologists say that the food is a reinforcing stimulus. They say that the reinforcement maintains the learned (operant) performance, because without it the animal quits working. As shown very clearly by the intragastric feeding experiment I have described, the taste of food is a powerful reinforcement.[33,34] It maintains the operant behavior necessary to achieve caloric regulation of food intake. Without taste, the hyperphagic animal fails to regulate his food intake. The normal animal, with unimpaired hunger motivation, is able to ignore taste, and will work hard to

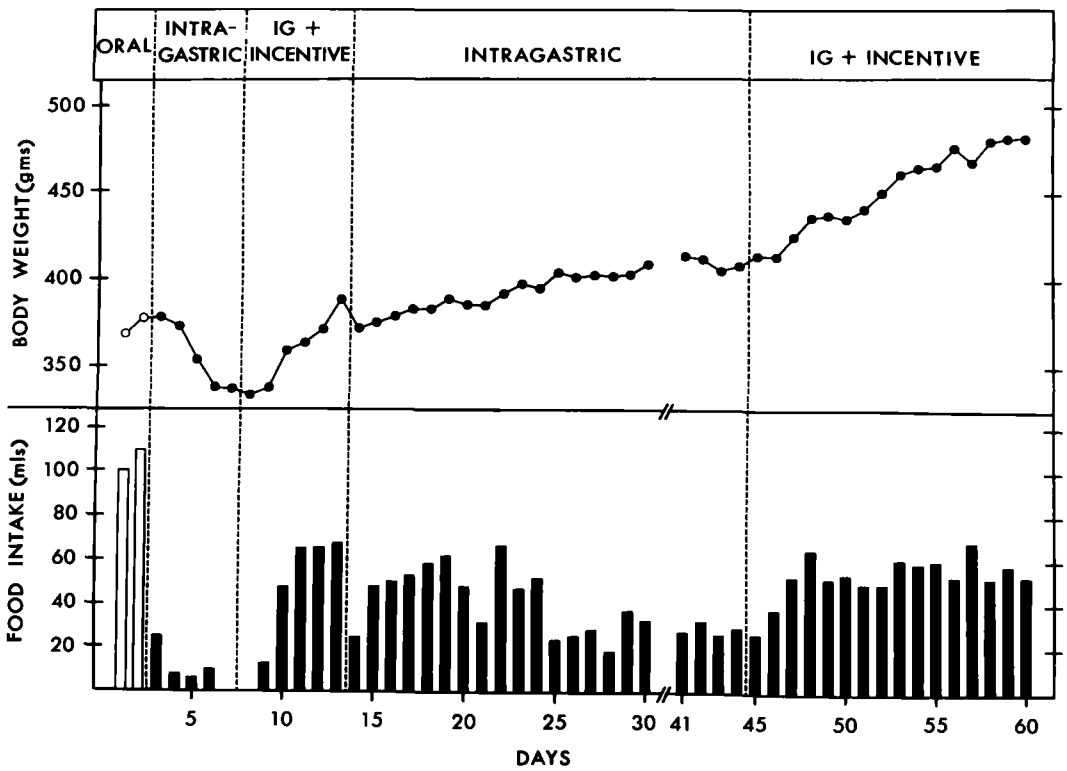

FIGURE 9 Body weight and daily food intake of a dynamic hyperphagic rat under conditions of oral intake, intragastric self-injection, and self-injection with a saccharine taste incentive. (From McGinty, et al., Note 32)

obtain sufficient food, whether that food is palatable, unpalatable, or completely tasteless. However, normal feeding also depends on reinforcement; if the amount of food injected into the stomach is made very small, the normal animal acts just like the hyperphagic—it stops working for food. But, also like the hyperphagic, its performance is restored if a sweet taste is added. Thus, taste is a powerful reinforcing and motivating stimulus. It is a psychic energizer that contributes to the animal's hunger motivation and is magnified in importance whenever the urge to eat is impaired.[34]

Taste controls oral feeding in the same way. When a normal animal eats, he is highly motivated. In a bar-pressing situation, he more clearly displays his motivation by the way he works for the reinforcements (taste, stomach fullness) provided by food. Therefore, the same variables that influence learned motivated behavior control the normal regulation of food intake.

Clearly, this does not fit a concept of reflexive regulation of food intake. A reflex, such as leg withdrawal from pin prick, is a built-in, fixed response to a particular kind of stimulus. Such an act can occur in an animal that is asleep or unconscious, or in a spinal animal in which no impulses reach or descend from the brain. The act is completely involuntary and can occur without the animal being aware of it or being able to emit it voluntarily.

If we think about it for a moment, we will realize that there is no such thing as reflexive feeding in any adult mammal. All such acts of feeding are voluntary, motivated acts, subject to the same laws that control operant behavior.[35] Of course, reflexes do exist in feeding. They are seen in decerebrate animals,[29] and also in the sucking displayed by newborn infants.[8] But with increasing encephalization, involuntary reflexes disappear. They are replaced by voluntary acts that are used to obtain the reinforcement provided by food. The voluntary act is completely arbitrary—once taught that a bar press (or any other operant act) will produce food, the animal can use the arbitrary response to obtain food just as readily and can regulate just as adequately as if he were using the ordinary instinctive acts of approach, chewing, and swallowing. The acts that completely control approach to and ingestion of food are voluntary and motivated, not reflexive.[35]

We see, then, that there are at least two kinds of homeo-

static regulatory mechanisms: reflexes and behavioral regulations. Reflexes, such as those involved in control of heart rate, blood pressure, and respiration, go on automatically, without awareness or purposiveness, whether the animal is unconscious or awake. Behavioral regulations, on the other hand, can occur only when the animal is awake; they require motivated, directed behavior to achieve the desired end. Feeding, drinking, and many aspects of temperature regulation are such behavioral regulations. They are governed by the same variables of past experience and future expectation, of sensations, urges, and pleasures, as are all motivated acts.[25,35]

DRIVE VERSUS MOTIVATION Does motivation exist in animals low on the phylogenetic scale of evolutionary development? Consider an insect such as the common blowfly. Is it hungrily searching for food as it buzzes around a heap of garbage? In the laboratory, flies eat and thrive on pure sugar solutions.[36] The longer a blowfly has been without food, the lower the sugar concentration it accepts and the more of a given concentration it will eat. Thus, food deprivation facilitates its approach to and ingestion of food. This looks like motivated behavior, but if we demand independent proof of motivation as a central state existing independently of built-in nervous reflexes, we find that we are not yet able to say that the fly is hungry—that is, that it wants to obtain food. The blowfly's act of eating is completely reflexive. It is a stereotyped chain of acts: when the fly happens to step into a droplet of a sweet substance, taste receptors on its legs are stimulated, the fly reflexively extends its proboscis, and it sucks the fluid. Whether the response occurs depends entirely on the state of sensory adaptation of the taste receptors and on signals transmitted from the foregut. When the sensory threshold is low, the response occurs, unless inhibited by signals from the foregut. If the recurrent nerve from the foregut is sectioned, inhibition no longer occurs, and feeding is continued until the fly bursts from overeating.[37] Therefore, such eating is a fixed response over which the fly apparently has no control. Although it has been tried many times, with many methods, it has not yet been possible to prove that a fly can learn anything, not even to use some arbitrary response to obtain food.

However, the reflexes involved in feeding are clearly easier to elicit when the fly has been deprived and are more difficult to evoke when its foregut is full. Its general level of activity is higher when deprived, and therefore it is more likely to encounter food and more easily stimulated when it does so. The internal state of the fly does influence the excitability of its nervous system and does control its feeding reflexes. Such a state, which is characteristic of all instinctive activities, can be called a drive state. A hunger drive exists: the fly's approach to and acceptance of food are increased automatically in contrast to motivated behavior, in which a central state apart from reflexes can be inferred each time the animal uses an arbitrary act to obtain food. Perhaps by using insects such as the honey bee, which are clearly capable of learning, it may be possible to demonstrate and study various motivations in insects.[38]

It is quite probable, therefore, that all instinctive behavior is accompanied by a corresponding drive. When each sign stimulus elicits the next act in the instinctive behavior chain, and when these responses are triggered in a highly stereotyped way by sign stimuli, it is difficult to determine the degree of motivation that accompanies the act unless an arbitrary act such as pressing a bar can be interpolated between the members of the chain. When that is possible, we have a definition and a measure of the motivation accompanying the act. If, for instance, the male stickleback can be taught to press a bar to obtain the opportunity of mating with a female, we have proof that, even though its mating behavior is stereotyped, the male fish wants to mate with the female. And if its rate of bar pressing increases as the final act of fertilization approaches, we interpret this as evidence that the fish is more strongly motivated to mate as the sexual act nears completion. Such an assessment of the motivation accompanying stereotyped reflexive and instinctive behavior is already being carried out.[35]

The same methods can be used to study the motivation that accompanies all instinctive behavior that is elicited by stimulation of various levels of the nervous system, such as the medulla, hypothalamus, rhinencephalon, and neocortex. Wherever they have been studied, the results of both stimulation and ablation of the hypothalamus show that hypothalamic activity produces motivated instinctive behavior. Thus, lateral hypothalamic stimulation not only elicits eating but also the previously learned act of pressing a bar for food.[39] But when lower levels of the brain, such as the midbrain and the medulla, are separated from the integrating action of the hypothalamus, their functions have been shown to be fragmentary and uncoordinated. Cats decerebrated below the hypothalamus show isolated fragments of emotional or sexual behavior, but never their integrated expression.[4,40] Similarly, the eating evoked during stimulation of the dorsal motor nucleus of the vagus in the medulla[41] may well be different from the feeding elicited by hypothalamic stimulation, in that merely a fragment of the instinctive act of feeding is elicited. If so, an animal should not work for food during medullary stimulation, whereas it will do so when receiving lateral hypothalamic stimulation.

Not only does hypothalamic stimulation elicit motivated instinctive behavior patterns, it can also be used to

provide immediate positive or negative reinforcement.[42,43] Animals will bar-press many thousands of times hour after hour to obtain electrical stimulation of the brain. People, when stimulated in specific subcortical regions, report pleasure or pain.[44] Olds, Travis, and Schwing[45] have shown that the system of brain structures for which stimulation is reinforcing seems to follow the course of the medial forebrain bundle. This runs from the olfactory bulbs through the septal area and the lateral hypothalamus into the midbrain, making connections with and receiving fibers from many of the structures it passes along the way. In the more medial periventricular system that runs through the ventromedial areas of the hypothalamus into the midbrain, stimulation is aversive: rats press a bar to escape stimulation in this medial system.[46]

The tissue of the lateral hypothalamus that excites feeding lies within the system in which stimulation is reinforcing, whereas the inhibitory "satiety center" lies within the aversive region.[47,48] They are interwoven functionally as well as anatomically. Thus, lateral hypothalamic self-stimulation is more reinforcing when an animal is hungry than when it is satiated. Furthermore, as shown in Figure 10, stimulation of the ventromedial area or satiety induced by excessive feeding inhibits lateral hypothalamic self-

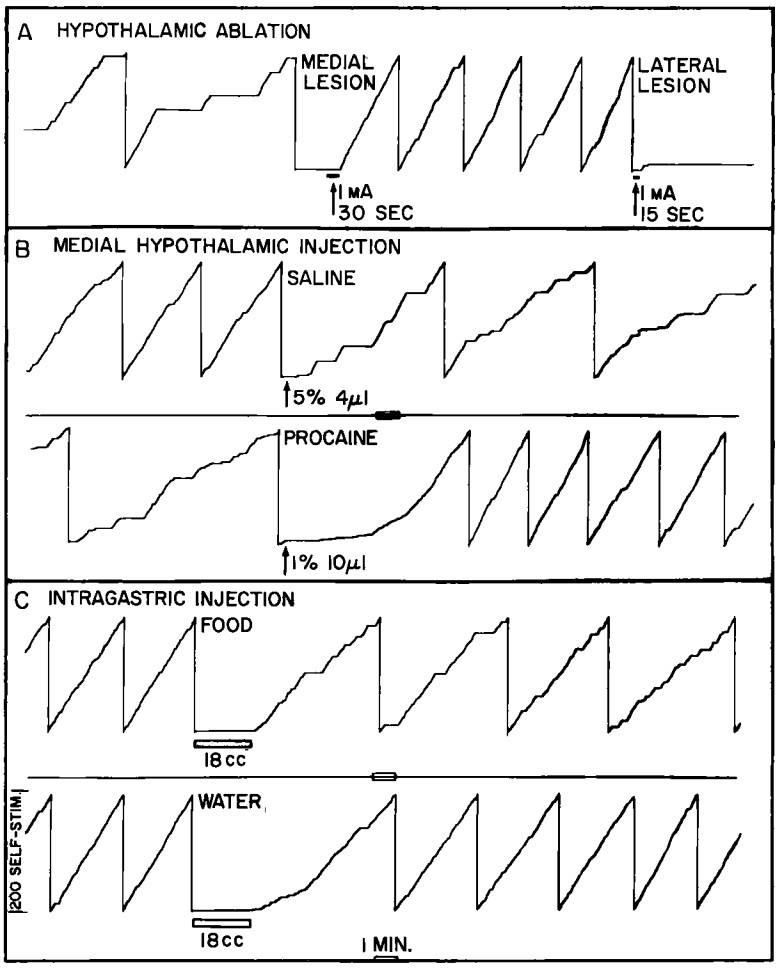

FIGURE 10 Representative cumulative recorder records showing the changes in lateral hypothalamic self-stimulation rate produced by experimental influence of the hypothalamus or by feeding.

A. Acceleration of self-stimulation caused by destruction of both ventromedial regions.

B. Inhibition of self-stimulation by chemical excitation of both ventromedial regions, and subsequent disinhibition of self-stimulation by their anesthetization.

C. Prolonged inhibition of self-stimulation by tube-feeding a liquid diet but only transient inhibition by tube-feeding an equal volume of water. (From Hoebel and Teitelbaum, Note 48)

stimulation; ventromedial destruction disinhibits it. This suggests that the feeding systems are involved not only in the regulation of food intake, but also in the urge to eat and in the pleasure that results from eating.[48]

The technique of self-stimulation may reveal the affective correlates of motivated behavior. Just as the rate of bar-pressing for food or water is a sensitive measure of the hunger and thirst drive, so too the rate of bar-pressing for brain stimulation in the feeding systems is indicative of changes in hunger or satiety. Perhaps self-stimulation of the brain will prove ultimately to be an even more sensitive measure of motivation than is bar-pressing, which is reinforced in the usual way.[35] Self-stimulation has the added advantage of identifying the parts of the brain involved in each type of motivation.

Parallel between development and recovery of hypothalamic function

Finally, in a last parallel to the work on the immunoglobulin molecule, the reconstitution of the molecule has its counterpart in the synthetic processes of ontogeny and recovery of nervous function. With increasing encephalization, the infant's behavioral capacities develop through transitional stages of increasing complexity—reflexive, instinctive, and voluntary. By using surgical thyroidectomy to slow development in newborn rats, Teitelbaum, Cheng, and Rozin[49] have shown that elements of the regulation of food intake appear one at a time in exactly the same sequence in which the adult rat recovers them after lateral hypothalamic damage. When tested at 21 days of age (the normal weaning age), thyroidectomized rats display every symptom of the lateral hypothalamic syndrome. If greatly retarded in development (as measured by their body weight at weaning), the weanling is completely aphagic and adipsic when offered ordinary food and water. It nurses reflexively (recall that aphagic adult lateral hypothalamic rats lick and swallow milk reflexively from a dropper), but does not voluntarily ingest food or water, no matter how palatable. Others, more fully developed, accept wet, palatable foods. The smell and sight of food elicits feeding (feeding is projected into space), but as in the decerebrate rat or anencephalic child, caloric regulation is not present: the animal does not eat enough to maintain its weight (anorexia) and must be tube-fed to stay alive.

With further development, motivational components are added; voluntary feeding manifests the urge to eat and the appearance of internal regulatory controls (thermostatic and glucostatic regulation). But subtle transition states in this process are revealed. The thyroidectomized rats begin to regulate their caloric intake of a liquid diet (they double their intake when the caloric content is halved), but are still adipsic and will die if offered only dry food and water. Later, when they accept dry food and water, they drink only when they eat; they are prandial drinkers and do not drink in response to bodily dehydration. They are very finicky, and although they eat more in the cold, such rats do not eat more in response to hypoglycemia. These subtle defects are also seen in adult rats, which are eating and drinking again after lateral hypothalamic damage, but actually are only incompletely recovered.[28] Thus, the parallel is perfect—hypothalamic recovery of feeding recapitulates its encephalization in ontogeny. Indeed, this parallel may be true of many types of recovery of function.

Summary

We have used the processes of analysis and synthesis to dissect and reconstitute the structure of motivated behavior. By means of the simplifying techniques of surgery, phylogeny, ontogeny, and recovery we reveal some of the stages in the transition from reflexes through instincts to voluntary motivated behavior. We must systematize the common laws that operate at each stage and must distinguish clearly each emergent control that adds complexity. As behavioral scientists, we must—like every human infant—work through the behavioral transition states that exist between the views of Sherrington[3] and Skinner.[50] Only then will we understand the biology of drive.

Reinforcement

ROBERT B. LIVINGSTON

DR. LEHNINGER has illustrated the potentialities of molecular systems in biology by giving an example relating to a single protein. If we take a modest-sized protein, he has said, involving only 100 residues, and use all twenty amino acids to synthesize one each of all potential isomers of that single protein, we would accumulate a mass exceeding the weight of the universe. It is perhaps appropriate to indicate the potentialities of neuronal circuits by a parallel analogy. An example appears in the writings of C. Judson Herrick:

"Every neuron of the cerebral cortex is enmeshed in a tangle of very fine fibers of great complexity, some of which come from very remote parts. It is probably safe to say that the majority of cortical neurons are directly or indirectly connected with every cortical field. This is the anatomical basis of cortical associational processes. The interconnections of these associational fibers form an anatomical mechanism which permits, during a train of cortical associations, numbers of different functional combinations of cortical neurons that far surpass any figures ever suggested by the astronomers in measuring the distances of stars. . . . It is the capacity for making this sort of combination and recombination of the nervous elements that determines the practical value of the system. . . . If a million cortical nerve cells were connected one with another in groups of only two neurons each in all possible combinations, the number of different patterns of interneuronic connection thus provided would be expressed by $10^{2,783,000}$. . . .

"On the basis of the known structure of the cortex, the following computation may be regarded as a conservative statement of the number of intercellular connections that are anatomically present and available for use in a short series of cortical associational processes. Starting with a million cortical neurons of the visual area simultaneously excited by some retinal image . . . the total number of such connections would far exceed the $10^{2,783,000}$ already mentioned as the theoretically possible combinations in groups of two only."[1] For comparative purposes, recall that the number of atoms in the universe is estimated to be about 10^{66}.

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Lehninger's and Herrick's examples illustrate that biological systems have acquired adequate potentialities, at both molecular and cellular levels, to account for even the most complicated phenomenological processes this volume addresses. Furthermore, with known structural complexities, even subtle effects, such as weak interactions at the molecular level and slight shifts in the bias of cellular excitability in feedback loops, can account for highly complex transactions. By transactions we mean multiple, mutually interdependent systems in simultaneous action, a many-bodied problem in which each body is itself a complex, dynamic system.

The nature of reinforcement

TOWARD A DEFINITION OF REINFORCEMENT Reinforcement subsumes processes involved in what are popularly called *reward and punishment, experiences liked and disliked, and behaviors impelled toward (approach) and away from objects and experiences (avoidance)*. The term reinforcement has been adapted by psychologists from physiology (but the nervous system operates without regard to distinctions in the minds of those who study it). The original physiological definition of reinforcement expresses strengthening of a response to one stimulus by the concurrent action of another stimulus. In psychology the term is generally used to identify such strengthening of interactions between stimuli, specifically relating to the learning process. Miller has defined reinforcement in the following terms: If, by doing something in conjunction with a stimulus (perhaps a whole stimulus situation), you obtain a response that is thereafter more likely to occur in relation to that stimulus, you may say that learning has occurred. . . . The 'something' you do in conjunction with the stimulus to be learned is called reinforcement.[2]

For example, in classical Pavlovian conditioning, the experimenter rings a bell in the presence of a hungry dog; afterward, and overlapping with that stimulus, he presents food to the dog, while measuring the production of saliva. At first the bell alone elicits no salivation; food presented alone evokes salivation; after a few trials of bell preceding and overlapping with food presentation, the bell elicits salivation *prior to* presentation of food, and *in the absence of* presentation of food. The bell is considered a conditional stimulus. The food presentation is considered

an unconditional stimulus; the food is also acting as a reinforcement. The animal is prepared by being accustomed to the apparatus and by being kept hungry, so that he becomes a virtually automatic instrumentality in classical conditioning. In this same experimental tradition—in the example given, involving a somatic sensory signal (bell) and a visceral response (salivation)—experiments have been performed to obtain conditioning between somatic sensory and somatic motor, visceral sensory and somatic motor, and visceral sensory and visceral motor; in short, all combinations are exemplified.

A distinctly different kind of conditioning, called *instrumental, operant,* or *type II* conditioning (as contrasted with *Pavlovian, classical,* or *type I* conditioning described above) depends on the animal emitting a certain response, which is then reinforced. In instrumental conditioning, the animal's own actions lead to the presentation of reinforcement. In Konorski and Miller's original experiment, the dog placed its paw on the cover of a food dish and was reinforced for that gesture by being given food.[3] In instrumental conditioning, reinforcement is linked to the emitted response instead of being bound to a given stimulus. Thus, a rat presses a bar to receive food. Control lies with the animal; the animal can pace the rate of reinforcement; the experimenter then interprets the animal's behavior as an index of the strength of the reinforcement. The experimenter acts only indirectly, through his control over the environment; thus he maintains the animal in a slightly deprived (e.g., hungry) state and provides reinforcement (e.g., food) for any emitted behavior he may select within the animal's natural repertoire. The experimenter can, of course, further shape the animal's behavior by gradually refining and making more difficult the animal's selected response, contingent upon reinforcement. Nevertheless, the animal's act, by whatever way the animal performs it and whenever the animal elects to perform it, becomes instrumental in providing reward or escape from punishment.

It is relatively easy to anthropomorphize external stimuli: food for a hungry animal, shock to skin, etc. External reinforcements can usually be appreciated in accordance with commonplace experiences and it can be anticipated that certain external stimuli will be liked or disliked, will constitute rewards or punishments, and will therefore provide positive or negative reinforcement. In 1954, two radically different experiments, conceived and pursued entirely independently, yielded an exciting new dimension to conceptions about reinforcements: these experiments revealed for the first time primary (central) reinforcement —negative or positive according to directly stimulated brain regions. Reinforcement by brain stimulation cannot readily be anthropomorphized nor appreciated in terms of commonplace experience; we must depend entirely on empirical findings, except perhaps for those individuals with electrodes implanted in their heads.

NEGATIVE REINFORCEMENT Delgado, Roberts and Miller found that central stimulation applied to appropriate areas in the brain of the cat, can be used to establish both classical and operant conditional responses.[4] Cats learned to turn a wheel to avoid central stimulation; they were motivated to perform trial and error learning, including escape from a compartment in which stimulation had previously been given, into a compartment in which no stimulation had been given; hungry animals were taught to avoid food; animals previously trained to perform specific acts to avoid pain (from cutaneous shock) and conditional fear (to a buzzer) quickly responded or promptly learned to respond in a similar way following central stimulation. In short, Delgado, Roberts and Miller demonstrated that *brain stimulation in certain areas yields negative reinforcement.* Delgado had earlier shown that pain pathways invade parts of this same territory.[5-7] Presumably negative external and internal reinforcements are confluent.

POSITIVE REINFORCEMENT In the same year, in Montreal, Olds and Milner disclosed an opposite effect of central brain stimulation. Instead of aversive behavior, they elicited approach behavior.[8] Working in an open field experiment with rats carrying implanted electrodes, Olds and Milner discovered that mild electrical current in the septum induced the animals to stop whatever they were doing. This was perhaps similar to the "arrest response" that had been seen by Knott and Jasper some time earlier as a result of intralaminar thalamic stimulation. Olds and Milner asked themselves: Does the rat stop because of an automatic arrest reaction or does it seek more stimulation? By deciding arbitrarily that "we want the rat to go to that corner of the table," then stimulating only when the animal made a step in the preferred direction, and withholding stimulation when the animal stood still or went in another direction, the investigators discovered that they could attract animals to any selected location in an open field.

Exploiting this new evidence for positive central reinforcement, Olds showed that rats can learn a maze when the only object is access to a lever by which they can stimulate their own brains; they will cross an electrified grid for the same privilege, showing greater willingness to sustain painful shock in the process than will hungry animals to obtain food, and sex-deprived animals to be with a receptive mate. Thus, *brain stimulation in certain areas yields positive reinforcement.*

BRAIN LOCI FOR CENTRAL REINFORCEMENT Areas for positive and negative reinforcement are extensive and generally separate, but they possess overlapping and perhaps interpenetrating boundaries. Most neocortex is essentially neutral as regards reinforcement.The most distinctive sites for negative reinforcement lie in the dorsolateral part of the mesencephalon and along a bifurcating zone of the diencephalic brain stem. The most distinctive areas of positive reinforcement are the limbic system and ventromedial hypothalamus. Regions of positive reinforcement are several times as extensive as those for negative reinforcement. In an early survey of loci, Olds estimated that approximately 60 per cent of his placements were neutral, 5 per cent were negative, and 35 per cent were positive.[9]

Demonstrations that both positive and negative reinforcement can be obtained by central stimulation proved revolutionary. Philosophy had more or less abandoned hedonism at the turn of the century: now it was forced to accept direct evidence to the contrary. Another body of theory held that all behavior is based on satisfactions obtained by *drive reduction*. That is, that all organisms are subject to drives for air, water, food, and so forth, and that all satisfaction (and shaping of behavior thereto) is provided by drive reduction—in effect, that all behavior results from pressure for reducing negative drives. The Olds and Milner findings implied otherwise—that the brain is also organized for positive satisfactions. Considering lines of evidence relating to both positive and negative central reinforcements, now extended to many animal forms including man, we are obliged to reckon with central nervous mechanisms responsible for primary positive as well as primary negative reinforcement.

The substantial contributions of Delgado, Roberts, and Miller, and Olds and Milner indicated that organisms possess internal systems of punishment and reward that can shape behavior. Intentional activity by an organism, and its primary relationship to its environment, is dictated by internal states predicated on impulses generated within the organism. Before such lines of evidence were available, it could be maintained that behavior is shaped on the basis of pleasant and unpleasant *sensations* impinging from the outside. What makes certain sensations pleasant and others unpleasant would depend primarily on the nature of the sensory ending. It is now appreciated that behavior stems largely from activity in central systems, including central reward and punishment systems—all built into the chassis. These central systems for reinforcement can themselves generate approach and avoidance behavior, motivate learning, and, as we shall see presently, modulate as well as respond to sensory input.

CONSCIOUSNESS AND MOTIVATION Thus far, we have taken an entirely external viewpoint in interpreting the apparatus of behavior. If we now consider these events from the viewpoint of the organism itself, we shall come to grips with the foundations of experience. We all possess some degree of self-awareness whenever we are awake. Apparently all nervous systems possess this capacity for self-awareness, at least to some degree. Insects, for example, with less than 100,000 nerve cells, show appetites, satiety, moods, and approach and avoidance behavior. Indeed, a whole industry is based on controlling insects by anticipating their approach and avoidance activities. As Dethier concluded after describing the brain of the fly in relation to various elements of its behavior: "The idea of a dichotomy that sets insects so far apart from vertebrates as to be qualitatively different is founded as much on a fear of anthropomorphism, however well disciplined, as on a paucity of data. . . . Perhaps these insects are little machines in a deep sleep, but looking at their rigidly armored bodies, their staring eyes, and their mute performances, one cannot help at times wondering if there is anyone inside."[10] Jellyfish and sea anemones show analogous behavior even without possessing central nervous systems; and the lowly hydra, which has only a primitive system of intercellular communication, shows voraciousness of appetite, directedness of behavior, and even postprandial lassitude.

What may be the basis for emergent self-awareness? Coghill and Herrick suggested that mechanisms associated with motor activity and the sources of spontaneous rhythmic activities that we now refer to as "biological clocks" may be responsible for the conative sense of effort and duration. The most primitive sensibilities relating to what is taking place in the outside world provide foundations for intelligence. Such emergent sensibilities, in their most elementary forms, are looked upon as largely automatic and reflexive. In more elaborate brains, these operations develop expanded central resources and greater options for experience and action. As options improve, there is an obvious evolutionary advantage to increasing the organism's own control over any options exercised.

Somewhere in the process of evolution, and probably long before the development of ganglionic brains, there must have emerged some sense of satisfaction and dissatisfaction. Behavior may be successful or unsuccessful. A behavioral pattern may be terminated or switched to another pattern in accordance with changes effected in the *milieu interne* as a consequence of the original behavior. Coghill and Herrick visualized a combination of this sense of effort, this simplest and most primitive sensibility and the most primitive sense of effect associated with elements of satisfaction and dissatisfaction, together to provide con-

sciousness.[11] Aristotle defined appetite as the "tendency toward something we do not have" and "which we need." Spinoza conceived will to consist of "the conscious effort of desire." As nervous systems become more elaborate there arise increasing potentialities for visceration, expression, and effectuation in motoric behavior and for parallel pursuit of satisfactions deliberately sought for the sake of an increasing panoply of internal experiences.

As expanded potentialities for memory and learning arise, there appear enlarged potentialities for rehearsal of past sensory and motor experiences and a beginning rehearsal and forecast of the consequences of anticipated sensations and behaviors. In mammals, such operations are extensive, and they evidently depend on the combination of limbic and frontal systems. Frontal and limbic circuits forecast the outcome of behavior, based on past experiences and expectations, making a pre-vision of movements and of the consequences of self-actions, the actions of others, and the actions of other animate and inanimate objects.

REINFORCEMENTS RELATING TO COOPERATIVE BEHAVIOR Let us now take a departure to consider reinforcement in more naturalistic conditions. We shall make an incursion into ethology to review the results of a long series of experiments on canaries carried out by Hinde.[12-18] In the springtime, when the sun begins to expand the day and warm the countryside, the hypothalamic-pituitary axis of the canary is affected; in both male and female canaries this change in sunlight induces an increase in sex hormones and gonadal size. In the male, such neurohormonal exaltation elicits singing and display behavior and search for a mate. The male's singing and display, in turn, constitutes a positive stimulus appreciably affecting the physiology of the female. This further augments her neurohormonal tides and starts her collecting material for a nest. As she collects, she begins to lose feathers from the front of her breast, forming a brood patch that becomes increasingly sensitive and suffused with blood. As she continues constructing the nest, this brood patch becomes a stimulus that both attracts her to the nest and contributes to the ripening of her endocrine and reproductive systems. She undergoes conspicuous enlargement of eggs and oviduct.

About the time the nest is completed, the female canary begins to pluck her own soft feathers to line the nest, evidently to reduce discomfort of the brood patch when snuggling down into the nest. The canaries then copulate several times and the female begins to lay eggs, one a day for several days. The combination of the irritant brood patch and protective nest, her internal urgency to lay the eggs and the physical presence of eggs in the nest, all constitute stimuli that encourage her incubation of the eggs. She becomes "broody." The male, in witness of these processes, begins to stand guard over her and the nest.

Specific external and internal stimuli become newly rewarding. A shift in setting of central neuroendocrine controls in both male and female canaries alters their behavior and changes the scale of values of things and events that interest and satisfy them. Each major step in this entire sequence, and the exact order of the sequence, is indispensible for survival of the species. The birds continue, during all stages, "doing what comes naturally." This unfolding of changing conditions, goals, and satisfactions depends on physiological alterations, which are sometimes bound to the environment and sometimes to activities in the partner. The whole reproductive cycle has a compelling "logic line," like the force of characters in a great novel and themes in a great symphony.

Hinde dissected canary behavior to establish the role of the brood patch and of the male's singing and display, alone and in combination, and the disruptive influences on both partners when the sequence is broken or mismatched in specific ways. The interlocking events include inanimate as well as animate stimuli, reciprocal behaviors and differential internal maturations, swellings and hormonal tides. This provides a single example, but by no means an extraordinary one, from which can be visualized the important survival value for many families, genera, and species, of biological processes underlying cooperation, mutual trust, and altruism.

REINFORCEMENTS RELATING TO CONFLICTFUL BEHAVIOR By way of contrast, we should illustrate linked behaviors relating to conflict and aggression, also indubitably affected by hormones, mutually reinforcing stimuli, and conditions of the environment. I have selected a story "Safety First! But Where Is It?" from Charles Russell's *Trails Plowed Under*.[19]

"Old Bedrock Jim tells me one time about him and his pardner. They're prospecting in the Big Horns. One morning they're out of meat. They ain't gone far till they jump an elk. It's a bull. Bedrock gets the first shot—that's all he needs. The bull goes to his knees and rolls over. They both walk up, laying their guns agin a log. The bull's laying with his head under him. Bedrock notices the blood on the bull's neck and thinks his neck's broke, but when he grabs a horn and starts to straighten him out to stick him, the bull gets up. And he ain't friendly and goes to war with Bedrock and his pardner. He's between the hunters and their guns. There's nothing to do but give the bull the fight.

"Bedrock makes a scrub pine that's agin a rock ledge. This tree won't hold two, so his pardner finds a hole under

the ledge. It's late in the winter; there's plenty of snow and the wind's in the north. There ain't much comfort up this jack pine. When Bedrock looks around, he notices that Jack Williams (that's his pardner's name) keeps coming out of the hole. Then the bull will charge them. Jack goes back but he don't stay long. The bull ain't only creased, and he's mighty nasty. His hair's all turned the wrong way and the way he rattles his horns agin the rocks around that hole tells he ain't jokin'. But Bedrock can't savvy why, when the bull steps back, Jack comes out of the hole.

"Bedrock's getting cold and plumb out of patience, and he finally hollers down from his perch, 'If you'd stay in that hole, you damn fool, that bull would leave and give us a chance to get away!'

"Jack is taking his turn outside. The bull charges. Jack ducks in as the bull scrapes his horns on the rocks. The bull backs away, shakin' his head. This time when Jack shows, he yells up to Bedrock, 'Stay in the hole, hell! There's a bear in the hole.' "

Hinde's work illustrated an elaborate sequence of essentially positive reinforcements arising both from within and outside the birds. Cooperative, mutually interdependent approach behaviors proceeded to yield mutual internal satisfactions. Russell's story portrayed an idiosyncratic sequence of essentially negative reinforcements stemming from both internal and external stimuli (testicular, pain, and mutually reinforcing responses to threat) tending toward aggression and defensive activities that mutually reinforce one another. In both examples, there is probably a confluence among innate and learned behaviors.

Central mechanisms underlying drive

RESPIRATORY DRIVE—LOWER LEVELS The lower levels of respiratory control organization are centered in that portion of the brain stem where the spinal cord widens as it passes up beneath the cerebellum. In this bulbar region, an extensive territory within the reticular formation is organized into inspiratory and expiratory centers governing pulmonary ventilation. Certain local neurons are critically sensitive to oxygen lack, carbon dioxide excess and alterations in *p*H. These centers receive inputs from sense organs located at the bifurcation of the carotid arteries whose receptors are sensitive to blood pressure and oxygen concentration.

The brain stem respiratory centers send and receive impulses via the vagus nerves, which are concerned on the motor side with trachial and bronchiolar dilatation and secretory activity, and hence with pulmonary resistance. On the sensory side, the vagi respond according to the degree of expansion of the lungs. This feedback loop prevents overdistention of the lungs by inhibiting inspiration and exciting active expiration, and when the lungs are underinflated, it activates inspiration and inhibits the expiratory center. As output, the brain stem respiratory centers project both upward and downward. Their downstream projections deliver impulses for diaphragm control to nuclei of the phrenic nerve in the cervical region, and for forced expansion and contraction of the chest wall to those nuclei that serve the intercostals and the other muscles.

These elements of a respiratory system are all an animal needs for pulmonary exchange at rest. They are not adequate, however, to meet vigorous ventilatory demands. An immediate adjunct for respiratory regulation is provided by additional reticular tissue lying further up the brain stem in the pontine region. At midbrain level, as shown by Magoun and his colleagues,[20] are located brain stem mechanisms for gesture vocalization, representing automatic involuntary grunting, vocal ejaculations, laughing, crying, and other activities constituting vocal expression of emotions. This part of the respiratory system exerts its own limited priority in the control of ventilation, as do bulbar, pontine, and midbrain mechanisms controlling coughing, vomiting, etc., during which exertions pulmonary ventilation may be embarrassed. Many other activities require deliberate control of thoracic and abdominal muscles and other axial musculatures affecting respiration: for purposes of lifting and throwing, establishing axial stability so that limbs can be moved, and in the humble processes of urination and defecation.

Extending from midbrain into hypothalamus are additional systems controlling panting, shivering, and other aspects of ventilation and temperature regulation. Magoun and others have shown that when you heat localized regions of the hypothalamus, you may induce not only panting but a whole constellation of heat-releasing activities involving somatic, smooth and cardiac musculature, secretory activity, including activity expressed in bodily posture, trial and error cool-seeking behavior, and drinking. Processes involving higher, as well as lower, executive functions, are thrown into action simultaneously by hypothalamic heating.[21] Hypothalamic cooling has a contrary, but equally involved, pattern of behavioral adjustments. Hypothalamic controls affecting respiration and temperature regulation interact with temperature controls initiated by cutaneous as well as central receptors.

Hess and his associates have shown that hypothalamic regions may be described as essentially *trophotropic*, that is, energy-conserving and consolidating, rest-giving, restorative types of activities, including the regulation of respiration, represented anteriorly and ventrally in the hypothalamus; and *ergotropic*, energy expending systems,

including the expenditure of respiratory reserves, located more posteriorly and dorsally in the hypothalamus.[22,23]

RESPIRATORY DRIVE—HIGHER LEVELS To complete the main features of respiratory control, we must include extensive areas of the limbic system, a great ring of cortical and subcortical structures influencing respiration and located in medial and inferior temporal lobe regions, and medial hemispheric and orbitomesial surfaces. In one illustrative fragment of data, removal of a small portion of the orbital surface of the frontal lobe renders monkeys less sensitive to high altitude anoxia. They maintain useful consciousness at higher altitudes and for longer periods at a given altitude.[42] This advantage may relate to their respiratory control of CO_2. After ablation of this patch of orbital cortex, the animals may not bail out CO_2 so effectively; a slight retention of CO_2 would contribute to sustaining consciousness in the face of altitude hyperventilation.

In examining respiration, we can visualize, level by level, the acquisition of increasing finesse in systems of control, and at the same time increasing degrees of freedom. The singer and wind instrument player, particularly, but even the ordinary speaker, must learn to develop controls he can exercise from somewhere upstream, in ways that will invade lower level reflexive centers sufficiently to obtain breath control essential for communication. At midbrain levels, where gesture vocalization is represented, we account for practically all the vocalizations that dogs, cats and monkeys exhibit. It is presently assumed that dolphins and whales may have some higher levels of brain organization representing language. Man certainly possesses a higher level of representation of language, usually ascribed to areas of the temporal and parietal cortex in the dominant hemisphere.[25,26]

Using the respiratory system as one example, we find that goal-seeking control begins with cellular mechanisms, which in turn indubitably reflect subcellular, molecular control mechanisms. Respiratory cells in the inspiratory and expiratory centers of the brain stem have apparently been specially differentiated and suborned through millenia to the control of ventilation on behalf of gigantic multicelled organisms, which require regulation of ventilatory responses to oxygen deprivation, carbon dioxide excess and alterations in *p*H. Further evolutionary changes have organized and accommodated and enriched the repertoire of still other cells in various parts of the brain to provide increasingly elaborate options relating to respiration. As the analysis proceeds upward, we first witness the most primitive kinds of organization on behalf of homeostasis, in continuity with higher level organizations engaged in elaborate intellectual transactions. The respiratory system cuts vertically through the supposed boundaries between brain and mind. All integrative processes are directive: higher functions are not simply additive, but they provide emergent qualities expressed nowhere within simpler systems.

OTHER DRIVES A similar account might be rendered for cardiovascular organization and regulation, including the important psychosomatic controls that are organized in the hypothalamus, limbic lobe and forebrain, and affect blood pressure, cardiac rhythm, intracardiac conduction systems, coronary blood flow, and even the pattern and amount of cholesterol deposition in the walls of arteries.[27,28] Bear in mind, moreover, that there is conspicuous functional overlap and mutual interdependence between cardiovascular and respiratory systems. Indeed, the respiratory controls discussed are but preliminary steps toward fulfillment of tissue needs for imbibing oxygen and bailing out carbon dioxide—needs that are finally fulfilled by diffusion to and from the capillary bed. Similar disclosures might be reviewed concerning the regulation of food and water intake, temperature regulation, sexual behavior, and so on. Beyond these, there are still more complex drives exhibited by many animals—and quite elaborately by man—relating to freedom, curiosity, creativity, and aesthetics.

It is now established beyond peradventure of doubt that various levels of nervous system organization are interdependently interrelated with one another. Somehow, by means that are still mysterious, purposive behavior organized at each of these different levels of integrative function becomes expressed by a linked sequence of over-all purposes representing some kind of final judicious reckoning among contending functions. The purposes of the whole organism are clearly manifested and continuously served according to some integrated internal point of view.

We are unable to define a level at which purely reflexive behavior occurs, nor to point to another level at which purely learned behavior occurs. The boundaries between these activities probably fluctuate up and down from time to time according to the state of alertness or excitement of the individual and also to some extent according to the individual's habits, training and experience. It is likely that through training and practice we can reach down to modulate and interfere with some relatively lowly reflex levels. Conversely, at other times even the most practiced voluntary controls can undoubtedly be overwhelmed according to the demands of lower reflex systems.

RELATIONSHIPS BETWEEN DRIVE AND REINFORCEMENT One important relation between drive and reinforcement

was identified by Pavlov forty years ago: "In the hungry animal, food naturally brings about a powerful unconditioned reflex, and the conditioned reflex develops quickly. But in a dog which has not long been fed, the unconditioned stimulus has only a small effect, and alimentary conditioned reflexes either are not formed at all or are established very slowly."[29] Intensity of food reinforcement by the "unconditioned stimulus" is directly linked to the intensity of the drive. Stimulation of an area in the lateral hypothalamus that induces feeding behavior in an already satiated cat, hence presumably elevates the level of hunger drive, can be conditioned in the classical paradigm by association with an auditory conditional stimulus. Even in the absence of electrical stimulation of a hypothalamic "feeding center" there is augmentation of "spontaneous" electrical activity in that center whenever the appropriate auditory cue is presented and not when an indifferent auditory cue is presented.[30] It is generally accepted that a conditioned stimulus is itself reinforcing, hence we have evidence that a reinforcement can modify drive, apart from whatever changes it may induce in the milieu interne. Reciprocally, arousal of aggression by hypothalamic stimulation will motivate cats to learn a maze, the only object being to obtain a rat they could attack directly.[31-35] Aversive properties of foot shock can be attenuated by self-stimulation of the hypothalamus.[36] Rats will seek out aversive stimulation of the tegmental area if it is paired with positive hypothalamic stimulation. Heath reports that patients receiving electrical stimulation of the septal area describe immediate relief from intractable pain.[37]

It is evident that central stimulation in areas of positive or negative reinforcement can be used to modify drive states. The close relationship—anatomical, physiological, and subjective—between drive and reinforcement has been firmly established, but by no means entirely clarified. This indicates that we are on the threshold of many fascinating experimental opportunities.

SENSORY CONTRIBUTIONS TO REINFORCEMENT In our discussion thus far, we have concentrated on the motor side of performance and experience. It is equally necessary to illustrate in parallel fashion the sensory side. "Bug detectors" in the retina of the frog, as described by Lettvin, Maturana, Pitts, and McCulloch, for example, give rise to intense impulse discharge centralward.[38,39] Do these central projections alter the drive states of the frog? Had the drive state of the frog already affected retinal circuitry to increase the responsivity of the bug detectors? Do retinal bug detectors project centrally in ways that will directly alert or release into action bug catching motor systems? Retinal detection of massive shadow movement and blue color detectors in the frog are known to contribute to the release of escape (jumping) behavior directed toward that part of the visual field corresponding to retinal elements excited by blue light.[40]

Wenzel has succeeded in recording electrical activity in the olfactory bulb of conscious birds during carefully controlled olfactory experiments.[41,42] Grain-feeding birds, like the pheasant, show maximum activity of the olfactory bulb when presented with odors associated with feed grains, but do not show such activation when presented with meat odors. Meat- and carrion-eating birds, like the vulture, do not show such activity when presented with odors of feed grains, but exhibit marked olfactory bulb excitation with very low levels of concentration of meat fragrances. Omnivorous birds, like the pigeon, show some response to both kinds of odors.

In short, there is strong evidence, at least in some animals, that there are specially differentiated and susceptible systems, *in the peripheral parts of sensory systems*, which may contribute directly to approach and avoidance behaviors. A sensory short-cutting of decision-making seems to occur in relation to sensory input just as it does for sensorimotor reflex patterns where, for example, pain stimuli give rise to immediate flexor withdrawal or stretch stimuli give rise to appropriate reflex stepping. It is known that nociceptive pathways (those pertaining to pain) directly invade negative reinforcement centers.[43-46] Perhaps other systems of sensory input have more or less direct access to positive and negative reinforcement centers in the brain. Perhaps they also have access to hypothalamic centers which are known to be important in the release of approach and avoidance behavior. Such short-cutting input pathways would contribute to conservation of the life of the individual and to survival of the species. The degree to which such short-cutting input pathways may nevertheless be subject to modulation as a result of past experiences, expectations and purposes would insure the possibility of constructive adaptation.

REINFORCEMENT AND BEHAVIOR To survive, an organism must be able to modify its behavior in such ways that its basic needs are met despite changes in circumstances. The nervous system is organized so that behavior is not only goal directed; it is guided by the consequences of the behavior it has generated. Actions are initiated by internal feeling states and drives. These actions are maintained or discontinued or diverted according to the animal's success or failure in fulfilling satisfactions sought. Central reinforcement and drive mechanisms, affected by the consequence of behavior, further modify behavior. As indicated in my earlier chapter: the nervous system is built for actions; the actions are goal-directed; the goals are internal satisfactions; systems for satisfaction and dissatis-

faction are built into the chassis of the nervous system and need not be consciously experienced for behavior to be directed; perceptual processes, memory, and learning, including the processes of consciousness, are all biological actions directed toward improved satisfactions.

The question arises as to the relations of drive and reinforcement systems to emotional experience and expression. Locke claimed that "Pleasure and pain are the hinges on which our passions turn." Emotion (or passion), the outward expression of our internal state, was the concern of medieval philosophers merely in regard to: How is passion used in oratory? How is it employed in moral leadership? How is it made effective in politics? They assumed the role of emotion in social signaling and social integration and asked only how it might be used for good or ill. Descartes, in his *Passions of the Soul,* was the first to take departure from this tradition by treating emotion solely in terms of mechanism. He considered that passions are elaborated in automatic fashion, as are other bodily activities in accordance with his scheme. It is in this same tradition that Darwin wrote *The Expression of Emotion in Man and Animals.*

When Olds first began mapping the central representation of positive reinforcement, according to the relative effectiveness of his electrode placements, he had difficulty interpreting any organizing principle. When he heard a lecture on the functional organization of the limbic system, it immediately became apparent to him that his positive reinforcement loci were predominately concentrated along the course of the median forebrain bundle, with other, weaker positive reinforcement sites located throughout the remainder of the limbic system. It was already generally accepted that the limbic lobe and associated frontal, hypothalamic, and brain stem mechanisms are directly involved in the experiencing and expression of emotion.

Evidence is now accumulating for a general scheme as follows: in the centralmost portions of the brain, lining the ventricular passages, lie systems involved in feeling tone, approach and avoidance, appetite and satiety. At least in respect to feeding, lesions in appetitive centers lead to a loss of drive; lesions in aversive centers lead to inadequacy of the check on drive exerted by satiety. A much thicker and more complicated brain system surrounds this central appetitive core. This seems to be concerned with discriminative evaluation of and, to some degree, choice of sensations and actions in terms of goal directedness. The limbic system is less pronounced in either positive or negative reinforcement, in guiding aversive or approach behavior. This system is less imperious, less compelling, and more plastic and modifiable through experience than are the underlying core systems. In this territory there is flexibility for the estimation of judgment as regards: Is this event biologically significant? If it is biologically significant, is it to be approached or avoided?

Out beyond the limbic system lies neocortex. Neocortex is neutral as regards approach or avoidance. It is evidently not essential for the exercise of judgment regarding biological significance. Stimulation of neocortex does not give rise to bar-pressing for the satisfaction derived from that stimulation. Only a secondarily learned response will involve neocortex in discriminative activity.

Reward and punishment can exist without obvious biological advantage beyond the actuation of particular brain parts. Reward and punishment can be involved in connection with actual biological gain, such as food for a hungry animal. But it is not essential that there be an obvious biological gain for reward or punishment to be manifested by approach or avoidance of a lever for self-stimulation. Behavior associated with reward and punishment also can exist without any obvious drive reduction.

REINFORCEMENT AND NEUROCHEMISTRY Carbamylcholine introduced intraperitoneally causes an augmentation of self-stimulation. Physostigmine causes a reduction in self-stimulation. The anticholinesterases can go either way, predominantly to augment self-stimulation. Chelation of calcium causes changes in self-stimulation that are counteracted by a whole combination of neural transmitters, including epinephrine, norepinephrine, serotonin, and gamma-aminobutyric acid (GABA).[47]

Stein has shown that amphetamine will augment self-stimulation. Imipramine, chlorpromazine, cocaine, and monamine oxidase inhibitors will also increase self-stimulation. The behavioral stimulating action of amphetamine appears to be mediated by the local brain release of a naturally occurring amine. It is suggestive that this amine may be a phenethylamine derivative such a catecholamine.[48]

SIGNIFICANCE TO PHYSIOLOGY The tissues involved in the mechanisms thus far discussed are obviously also involved in the central control of arousal. They are intimately involved with the mechanisms that have been discussed by Jouvet and Evarts, and with the biological rhythms described by Strumwasser (this volume). These mechanisms somehow have a capacity to distinguish stimulus novelty. We do not know the mechanisms that govern how novelty is recognized. We do know that drives can modify sensory input signals. Under the influence of modified drives, sensory signals are caused to vary in distribution and amplitude. These processes are probably also related to the DC potential shifts discussed by Row-

land in this book, and which Rusinov[49,50] and Morrell[51,52] and Rowland[53] have exploited experimentally to produce at least transient conditioning in cortical units, as further reported here by Morrell.

We now can visualize how these systems are implicated in what were referred to in our initial article as the "Now print!" mechanisms. The steps are postulated to occur as follows: (1) reticular recognition of novelty; (2) limbic discrimination of biological meaning for that individual at that moment; (3) limbic discharge into the reticular formation; (4) a diffusely projecting reticular formation discharge distributed throughout both hemispheres, a discharge conceived to be a "Now print!" order for memory[54,54a]; and, finally, (5) all recent brain events, all recent conduction activities will be "printed" to facilitate repetition of similar conduction patterns. We know that all sorts of stimuli and events, central and peripheral, have repercussions throughout the brain, without leading to remembrance. Only those occurrences that are biologically meaningful, only those that receive *reinforcement* by a sensory reinforcing signal, like pain, or a sensory approach signal like a "bug detector," or by a central reinforcement signal, can contribute to the third, fourth and fifth stages of this postulated "Now print!" sequence. Let me give an example of an occasion which you probably all stamped with a "Now print!" order yourselves.

I suggest that almost all of you will remember exactly where you were on November 22, 1963, when you heard the news that President Kennedy had been assassinated. You can probably tell us where you were, with whom, and very likely whether you were sitting, standing, or walking—almost which foot was forward when your awareness became manifest. Some people can even tell the details of the dials of a radio they happened to hear the news from, and so forth.

It is suggestive, from a wide variety of evidences in psychological literature, that whenever something that is biologically highly meaningful to the individual and very significant for the individual, the associated remembrance includes even aspects which themselves are not pertinent to the meaningfulness of the occasion. The brain "prints" remembrance of all events immediately preceding, regardless of whether the events have any real significance for the central matter involved. The brain records these incidental events in startling detail.

The advantage of having a generalized "Now print!" order is that any concatenation of sensory, motor and central events taking place throughout any field of cortex will be affected by a "Now print!" order regardless of causal relations.[54a] Following a generalized "Now print!" order, everything that has been ongoing in the recent past will receive a "Now print!" contribution in the form of a growth stimulus or a neurohormonal influence that will favor future repetitions of the same neural activities. The effect may be by cutting off a protein to make a facilitated pathway, or something else fairly specific and fairly local in relation to any territory throughout the hemisphere wherever there was immediately preceding activity.

Whenever you experience an abrupt and highly meaningful event, everything will get printed, and printed elaborately on the basis of one experience. If the event experienced is only mildly significant biologically, but is recurrent, the printing will take some time to sharpen up a specific recollection or sensory or motor skill. There will be a modest amount of printing on one event, and on subsequent occasions further repetitions of printing. On every occasion, there are many more stimuli, many more aspects of the environment, than will ultimately be discriminated. It is repetition of a biologically meaningful signal within this wide concatenation of elements of experience that selects out those changes which are significant, those parts of the stimulus, those aspects of the environment which are regularly associated with time immediately in conjunction with and preceding reinforcement.

It is timing and biological meaning that contribute to learning and memory. Of course, from the point of view of the organism, any event that regularly immediately precedes a biologically rewarding or punishing event can be taken to be (a) either causative or (b) a reliable index to the important (to be remembered or learned) event. Satisfaction is the touchstone. The reticular formation that is known to project diffusely throughout the hemisphere is recipient to limbic discharge. It seems we may have the important elements to put together to account for learning and memory. At least these ideas are suggestive for experiments that may lead us toward further understanding.

Summary

The primitive attitude of the organism toward the environment lies within the organism itself, and this is conditioned by sensory input. Actions emitted by the organism are goal-directed, and the goals are internal satisfactions. These satisfactions need not be consciously experienced, nor the behavior consciously directed. There is an interface between reflexive and innate behavioral patterns and learned patterns, between non-conscious and conscious patterns, which we cannot differentiate and which may actually involve a moving boundary. For behavior to be learned, for improvement in biological directedness and goal satisfaction, these steps do not need to be ex-

perienced consciously. And as William James emphasized, even a consciously learned response can become so habituated that it submerges to the non-conscious level and thereafter is taken care of quite automatically by the nervous system, more or less on the same plane as genetically endowed behavior.

Perception, memory, learning, and consciousness are all biological activities of the nervous system. As Herrick remarked: "The problem with which science is properly concerned is not a search for liaison between brain as a physical instrument and some other entity which is mind. What we must do is to discover those characteristics of brain as living tissue which enable it to have as one of its own intrinsic physicalistic properties the awareness that we call mind. . . . What we need here is more knowledge of the actual structure and operation of the nervous mechanisms that do the thinking."[55]

Anatomical Basis of Attention Mechanisms in Vertebrate Brains

M. E. SCHEIBEL and A. B. SCHEIBEL

A COMPLEX OF neurons and fibers that constitute the most primitive part of the brain wanders through the substance of the brain stem. This archaic system shows little evidence of rigid structure, and approaches, in some respects, the communication engineer's ideal of the statistical net. We cannot call this *reticular formation* truly isotropic, but here one can find no hint of the columnar arrays of cortical pyramids, the file-on-file of bidimensional Purkinje cells of cerebellum, nor even the whorled clusters of sensory field cells, such as characterize analogically mapping fields of sensory relays.

But perhaps as a result of this muted statement of its structural theme, there resides within the brain-stem core a remarkable pluripotentium of functional roles that have become increasingly obvious over the past quarter century and that probably mark it as the most critical integrative center of the brain—determiner of operational modes, gating mechanism for all sensory influx, modulator and monitor of cortical function, readout mechanism for the cortical differentiative and comparative processes and gain manipulator for motor output. A brief, chronologically oriented survey of the development of our thoughts about this multi-faceted system may prove useful in gaining insight into its present stature.

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Historical résumé

"It is known from embryology that most of the leftover cells of the brain stem and spinal cord which are not concerned in the formation of motor root nuclei and purely sensory relay nuclei are utilized in the production of the formatio reticularis." In this curiously negative manner commences a short and prescient article by W. F. Allen,[1] published in 1932, which sketched out an envelope of functional roles for the brain-stem reticular core that was to be followed by investigators for the next three decades. But the tenor of the sentence also reflects almost a century of neurological prejudice, which conceived the reticular core of the brain stem largely as filler—a kind of neuroectodermally derived excelsior, in which were cushioned the more functionally attractive cranial nerve nuclei.

During the past quarter century, there has been a dramatic change in value judgments. Compared to the spectrum of presently recognized reticular functions, ranging from homeostat operations practiced on the internal milieu to modulation and control of highest psychic functions, the workings of cranial nerve nuclei seem almost humdrum. If our appreciation of the astonishing range of physiological possibilities inherent in the core seems a strictly contemporary phenomenon, it was not necessary that it be so, for the anatomical and physiological literature has been replete with clues pointing in these directions since the turn of the century.

Anatomical studies of Kohnstamm and Quensel,[2,3]

which suggested pooling of a number of afferent and efferent systems upon the reticular core, led them to propose this area as a "centrum receptorium" or "sensorium commune"—a common sensory pool for the neuraxis. Although their attempts to interpret these data within a clinical frame were not entirely convincing, implications of their thesis included the integration of heterogeneous convergent stimuli and an alternative centripetal path lashed in parallel with the classical long sensory tracts—both astonishingly contemporary conceptions.

At about the same time, Golgi chrome–silver studies by Held[4] and Cajal[5] emphasized characteristics of input–output systems and the possibilities for a spectrum of interactions between reticular cells and intrinsic axonal systems. Cajal called attention to the characteristic bifurcating axonal output of a number of reticular neurons and their projection toward more rostral and caudal areas of the neuraxis. The caudal-flowing system was established as projecting upon spinal cord by retrograde studies of Kohnstam[6] and van Gehuchten,[7] and by anterograde (Marchi) studies in the hands of Lewandowsky.[8] However, the classic Marchi study was that of Papez,[9] who described three major descending (reticulo-spinal) tracts, their apparent origins in the brain stem, and their approximate terminal locations in the cord. This work, later to be amplified by anatomical studies of Niemer and Magoun,[10] Torvik and Brodal,[11] and Nyberg-Hansen,[12] provides the hodological (fiber bundle or tract) substrate for reticular modulation of spinal mechanisms.

The studies of Allen[1] offered a highly intuitive preview of the physiological role of the core in modulating rostral and caudal structures. Allen was primarily an anatomist, so his conclusions were largely speculative. Furthermore, the time was not ripe for such ideas, and his paper was to be forgotten for over a decade.

Equally pregnant was the discovery of Bremer,[13,14] made only three years later, regarding the relation of brain stem to cortex. He noticed that after decerebration the electrocorticogram of the resultant "*cerveau isolé*" preparation swung into and maintained a high-voltage, slow-wave rhythm that was maintained as long as the animal survived. Such rhythms had just been identified by

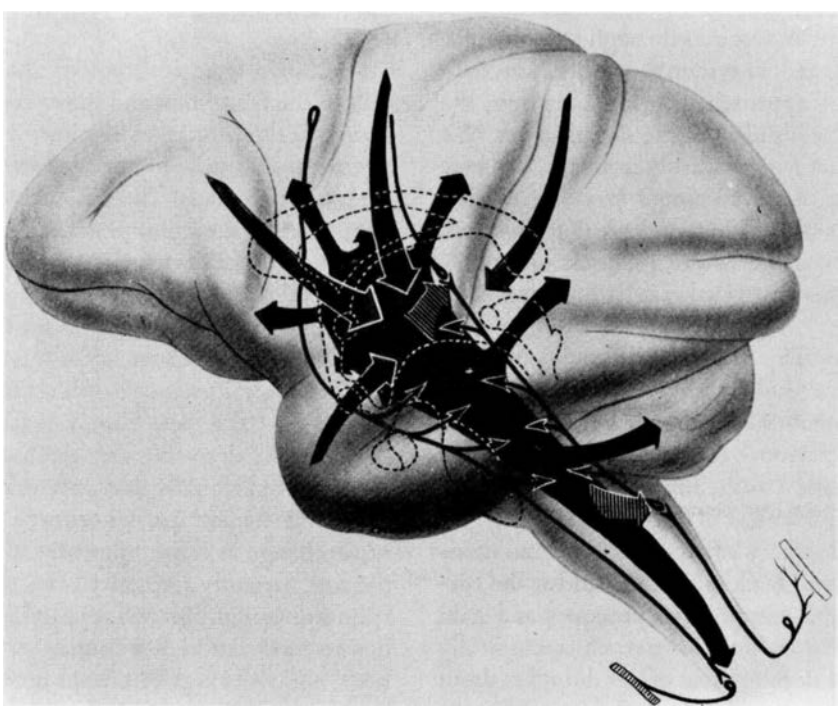

FIGURE 1 Schematic representation of the relations of the reticular core of the brain stem (black) with other systems of the brain. Collaterals pour in from long sensory and motor tracts (thin lines) and from the cerebral hemispheres (arrows directed downward into reticular core). The core acts in turn on cerebral and cerebellar cortices (upward-directed arrows) and associated structures, on spinal cord (long, downward-directed arrow), and on central sensory relays (striped arrows). (From F. Worden, and R. Livingstone, 1961. Brain stem reticular formation, in Electrical Stimulation of the Brain [D. Sheer, editor], Univ. of Texas Press, Austin, pp. 263–276.)

Loomis, Harvey, and Hobart[15] and by Gibbs, Davis, and Lennox[16] as consonant with sleep; so Bremer was able to reach a conclusion of signal importance to experimental neurology. The cerebral cortex needs tonic sensory influx to maintain the wakeful state and, by implication, one of the important roles of the long sensory tracts was to maintain, via continuous stimulus bombardment, a state of cortical—and accordingly, organismic—vigilance. The brilliance of this interpretation was scarcely marred by Bremer's error in identifying the agency involved.

The group around Alexander Forbes was the first to establish some of the physiological characteristics of the rostral end of the extra-lemniscal (reticular) system. A significant series of papers spanning the period 1936–1943 delimited its probable location within the thalamus, the widespread nature of its projection upon cortex, and the characteristic pattern of recruitment waves that followed its stimulation at low frequency.[17,18] Following the war, these studies were expanded by Jasper and his associates.[19-21] who defined in greater detail the probable anatomical paths and electrophysiological characteristics of this system. An interpretative capstone was placed on this growing body of data with the suggestion by Penfield[22] that the diencephalic intralaminar system might well serve as a "centrencephalon" for all neural activity. The classical teachings of Hughlings Jackson[23] as to the ultimate status of the cerebral cortex in the neuraxial hierarchy was thus supplanted. It had now become penultimate to the upper end of the reticular core, and the principle of thalamo-cortico-thalamic circulation already proposed by Cajal,[5] Campion,[24] and by Dusser de Barenne and McCulloch[25] gained support and experimental verification.

In the meantime, the range of autonomic control exerted by brain-stem reticular mechanisms had been investigated by Ranson and his colleagues,[26,27] thereby extending the work of early investigators in this area, and culminating in concepts of overlapping medullo-pontine fields subserving respiratory and circulatory patterns. Out of this developed the observations of Magoun and Rhines[28-30] on brain-stem reticular override (inhibition and facilitation) of ongoing spinal motor activity, with subsequent conceptual modifications by Sprague and Chambers.[31] Shortly thereafter followed the epochal report of Moruzzi and Magoun,[32] describing reticular control over cortical activity and its relation to the spectrum of conscious states from attentive awareness to deep sleep. These workers attributed such effects to ascending polysynaptic conduction over successive chains of short-axoned cells. That these phenomena, in reality, depended importantly on high-priority conduction in oligosynaptic and, in some cases, monosynaptic channels to cortex was suggested by our Golgi studies[33] and more recently by intracellular analyses of Magni and Willis.[34]

Results of selective destruction of lemniscal and extra-lemniscal (reticular) systems by Magoun and a group of collaborators[35,36] now placed the original findings of Bremer[13,14] in correct perspective, as it became increasingly clear that extralemniscal, rather than direct sensory-tract volleys, were crucial to maintenance of cortical tonus. By implication, sleep remained a passive process consequent to decrementing levels of reticular activity, despite earlier studies by Hess,[37,38] which had shown that it could be obtained by stimulation of appropriate brain-stem sites. It remained for Jouvet[39,40] and Rossi[41,42] to document a complex of interrelated states and to focus attention on an anatomical interface in the rostral pons separating areas apparently productive of rhombencephalic (activated) sleep from classical slow-wave sleep (see Jouvet, this volume).

Still another significant facet of reticular-core activities has been revealed by Galambos[43,44] and Hernández-Peón,[45,46] who have been able to relate the size of sensory evoked potentials to the focus of active interest momentarily expressed by implanted, freely mobile animal. Our own Golgi investigations[33,47] have revealed a suitable substrate in the penetration of most first- and second-order sensory stations by reticular collaterals. Notwithstanding a recent disclaimer by Worden and Marsh,[48] it is generally conceded that, aside from modulation of tonic and phasic components that comprise the activated state,[49] the reticular core mediates specific delimitation of the focus of consciousness with concordant suppression of those sensory inputs that have been temporarily relegated to a secondary role.

Knowledge of the intimate physiology of the core has been gleaned from extracellular microelectrode studies by a number of investigators,[50-52] and most recently by a limited number of intracellular analyses[34,53,54] that have examined in some detail the problems of convergence of sensory signals, habituation (response attenuation) of individual units to iterative stimuli,[55,56] and a cyclical alternation of unit sensitivity to the stimulus array.[57] At the same time, a group of Golgi studies has revealed much about the relevant substrate,[33,58-61] and in providing circuit paradigms for a system with this order of functional complexity, has also suggested program modes applicable to computer design.[62]

The core as a mosaic

In the tradition of the great cortical cytoarchitectonicists (i.e., Brodmann,[63] the Vogts,[64] von Economo,[65] etc.), a number of attempts have been made to subdivide the reticular core of the brain stem into component nuclei on

the basis of its appearance after aniline staining. The most detailed essay in this direction is that of Olszewski and his collaborators,[66,67] who have identified more than 40 nuclear entities in man, and almost as many in rabbit, in the interval between the spino-medullary junction and the rostral mesencephalon. The most obvious advantage of such a system is the moderate degree of uniformity provided in an invariant vocabulary for descriptions of various portions of the core. Thus, all who are acquainted with Olszewski's atlas will visualize approximately the same sector when asked to consider, for example, the nucleus reticularis pontis caudalis. However, the original bases for cytoarchitectonic identifications are often arbitrary, and individual judgments vary as to where a pattern of somal morphology or distribution becomes sufficiently different to merit recognition as another nucleus. The problem is particularly vexing in the reticular core, where obvious transitions are the exception rather than the rule, and cells of many sizes are intermingled.

The significance of somal size and distribution can be questioned as adequate criteria for isolation of a nuclear field. In a complex territory like the brain stem, it is at best an assumption that these factors will mirror differences in input arrangements, efferent destinations, local specialization of the microchemical environment, presence of specific synaptic mediators, etc. A more likely disclaimer to the validity of such parceling is the appearance of these areas when impregnated by one of the Golgi modifications. The long, relatively unramified dendrites characteristic of cells of the reticular core stream in all directions, especially when studied in transverse section. Such processes transcend the cytoarchitectonically determined nuclear limits, projecting into neighboring and even remote nuclear subdivisions. Examination of a number of species indicates that the total extent of the dendritic domain of a single cell may exceed one-third of the total cross-sectional area of the stem.[68] The processes of such a cell can easily invade as many as half a dozen extraneous nuclear pools.

We may conclude that the apparent isolation of each core nucleus from its neighbor is entirely an artifact or, alternatively, that the relative segregation of cell bodies and the mingling of dendrite shafts express fundamental differences in the physiology of these entities (Purpura, this volume). As is usual in cases such as this, truth will probably be found to lie somewhere between.

Dendritic apparatus

The morphology and patterning of dendrites has been a subject of interest since the initial studies of Golgi.[69] With the exception of the lateral reticular nucleus (*noyau du cordon lateral*[5]), dendrites of reticular core cells appear relatively straight, long, and unramified. The uniqueness of this pattern has been remarked by a number of investigators,[5,70,71] and referred to as isodendritic by one group,[72] who see in its similarity to archaic patterns a relation to integrative rather than differentiative or comparative function. Such dendrites are characteristically thick (3 to 8 microns) at the somal junction, and may taper over a course of several hundred microns or more to terminal segments only a fraction of a micron in diameter. Golgi sections suggest, and electron micrographs show, that the dendrite tips may be quite as densely covered with presynaptic structures as more proximal portions of the shafts. The functional significance of terminal structures committed to sections of postsynaptic membrane so distant from the presumed spike-trigger area around the axon hillock remains a question of more than theoretical interest (Purpura, this volume, and Note 78).

The available dendritic (postsynaptic) membrane is large, often constituting 85 to 95 per cent of the total receptive surface of the neuron.[73] Such a figure agrees with estimates of Sholl[74] and others for the fraction of postsynaptic membrane represented by the dendritic segment of cortical neurons. The surface of these processes is irregular, often nodular, and unevenly covered by hairy protuberances, or "spines." The reality of the spiny apparatus has been attested by electron micrographs by Gray[75] and others, with the suggestion of elaborate intraspinous structures—at least in cortical spines—and a special population of presynaptic junctions effected at the apex or base of the spine. Although these structures are not so numerous (0.1 to 0.3 per linear micron) as in cortex (0.4 to 0.7 per linear micron),[76] their presence is virtually invariant and indicates an as-yet-unknown specialization of postsynaptic membrane.

If the dendrites radiate widely, as seen in cross section, their appearance is very different in sagittal planes. The majority of reticular neurons over the medial two-thirds of the core continue to show impressive degrees of spread in the transverse dimension (dorso-ventral and latero-lateral), but little or no projection in the rostro-caudal dimension. The appearance is one of marked compression along the long axis of the stem, resulting in a series of flattened dendritic domains, piled one on another like a stack of poker chips. We originally suggested[33] that this configuration introduced a significant dimension of localization of input, because each cell thereby "looked" only at a limited series of inputs from a single level along the input continuum. This seemed especially likely, as presynaptic components invariably parallel the postsynaptic (dendritic) surface, and virtually all terminating axonal elements pour into the core at right angles to the long axis.

The modular nature of this "stack of chips" organiza-

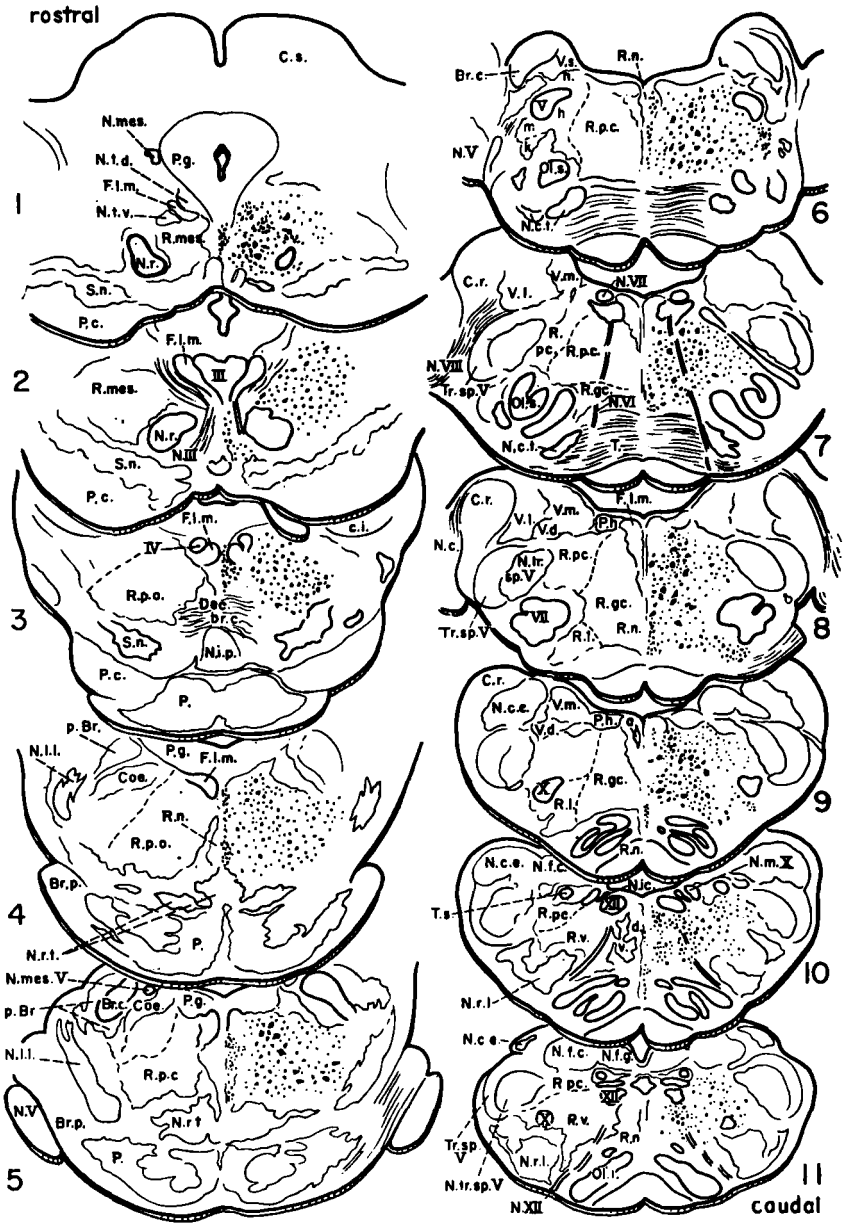

FIGURE 2 A series of equally spaced drawings of transverse Nissl-stained sections through the brain stem of the cat to show the grouping of cells in the reticular core. Various-sized dots on the right indicate specific cell bodies, while dotted lines on the left indicate approximate boundaries of major reticular nuclei. Terminology and nuclear areas are based on the studies of Olszewski in the rabbit, with minor modifications. The following list of abbreviations refer only to structures making up the reticular core: a, Accessory group of paramedian reticular nucleus: d, Dorsal group of paramedian reticular nucleus; h, region poor in cells of Meesen and Olszewski surrounding motor trigeminal nucleus; k, cell group k of Meesen and Olszewski; m, cell group m of Meesen and Olszewski; N. ic., Nucleus intercalatus; N. in., Nucleus interpositus; N. r. l., Lateral reticular nucleus; N. r. p., Nucleus reticularis paramedianus; N. r. t., Nucleus reticularis tegmenti pontis; N. t. d., Dorsal tegmental nucleus; N. t. v., Ventral tegmental nucleus; P. g. Periaqueductal gray; P. h., Nucleus prepositus hypoglossi; R. gc., Nucleus reticularis giganto-cellularis; R.l., Nucleus reticularis lateralis (of Meesen and Olszewski); R. mes, Reticular formation of mesencephalon; R.n., Nucleus of the raphe; R.p.c., Nucleus reticularis parvocellularis; R.p.o., Nucleus reticularis pontis oralis; R.v., Nucleus reticularis ventralis; v, Ventral group of paramedian reticular nucleus. (From Brodal, Note 80)

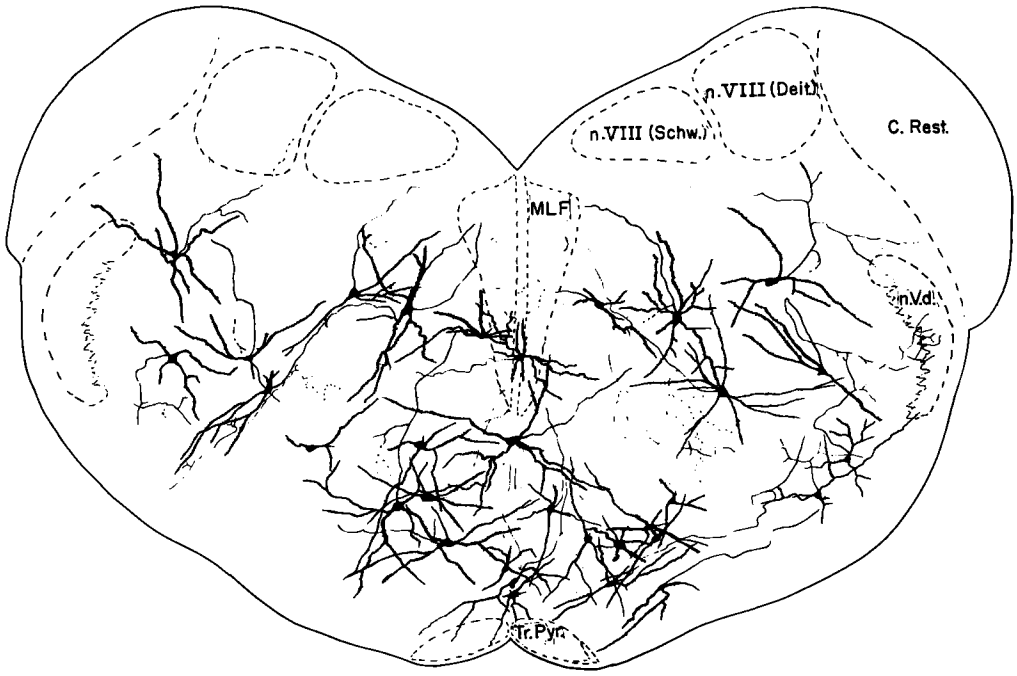

FIGURE 3 Cross section through upper medulla of new-born kitten, stained by the Golgi method, showing the general appearance of reticular cell bodies and dendrites at this level. There is marked variation in size and shape of both dendrites and cell bodies. The dendrite systems tend to radiate in all directions in this plane of section and sometimes to invade a fiber bundle from which they receive excitation. Abbreviations: C. Rest., restiform body; n VIII (Deit.), Deiters nucleus of the vestibular complex; n.VIII (Schw.), Schwalbe's nulceus of the vestibular complex; MLF, medial longitudinal fasciculus; n.V.d., descending nucleus of the trigeminal nerve; Tr. Pyr., pyramidal tract.

tional pattern has recently been exploited by Kilmer and McCulloch,[62] who have used it as a paradigm for a new generation of computers, which will show increased degrees of flexibility in dealing with data influx, will habituate over time to iterative stimuli, etc. Although the concept of a reticular core made up of a subinfinite number of modules as numerous as its cellular complement may seem overly divisive, it shows a rather good conceptual fit with ideas emerging from single-unit analysis of the structure.[50-52]

As we will stress again, most neurons appear to receive upon their soma–dendrite surfaces an appreciable, but not unlimited, convergent sampling of the many inputs afferent to the core. Each neuron receives its own idiosyncratic mix—in terms of afferent selection and loading—from the total influx, so it has seemed appropriate to some of us[52] to consider each neuron as an integrating subcenter, operating upon its own peculiar combination of afferents. The sum of these neuronal subcenters would, in turn, make up the total mosaic of the operating reticular core, in which each element performed operations upon one segment of the total envelope.

If we accept each reticular element as one of a family that samples a limited and idiosyncratic fraction of the total reticular afferent supply, we might pause to consider the relative significance of neuronal soma and dendrite, respectively, in servicing the presynaptic array. The widely ranging dendritic apparatus has already been seen to penetrate areas rather remote from that of the parent cell body, in quest of presynaptic excitation. We have suggested elsewhere that the dendrites appear to point toward, and reach for, potent sources of influx, which suggests that trophic or neurobiotactic forces are operant during the developmental phase. We have previously shown evidence of clear-cut segregation of afferent terminals on various portions of dendrite shafts in cortex[77] and on spinal interneurons.[78] Although we do not have evidence of concentrated afferent terminal populations on various portions of single reticular dendrites, there seems little doubt that many reticular cell dendrites will show marked predominance of inputs, depending on the location of that shaft relative to the spectrum of afferent sources (Figure 5). Thus, for a cell located in the ventral-lateral quadrant of medulla, it might be appropriate to refer to shafts that are predominantly cortico-spinal loaded, spino-thalamic load-

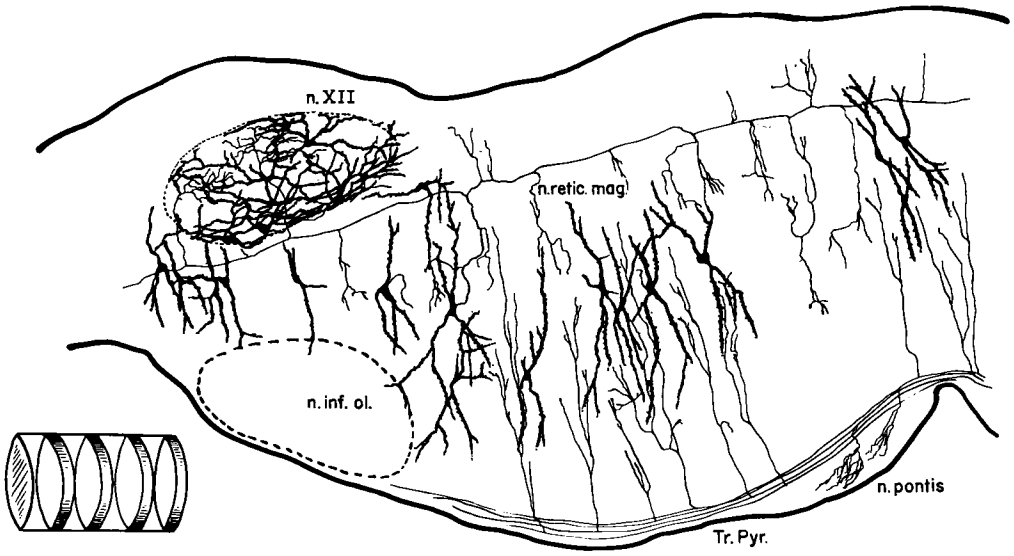

FIGURE 4 Sagittal section through the lower half of the brain stem of a 10-day-old rat. Most of the dendrite mass of reticular core cells is organized along the dorso-ventral axis as seen in this type of section, with marked compression along the rostro-caudal axis. This orientation places the dendrites parallel to the terminal presynaptic components which in this case arise from pyramidal tract (Tr. Pyr.) and from a single axon of a magnocellular reticular neuron (n. retic. mag.). This type of dendrite organization, which is especially characteristic of reticular cells of the medial 2/3 of the core, produces sets of essentially two-dimensional modular neuropil fields leading to the stack of chips analogy (see text and inset diagram at lower left). This is contrasted with dendritic patterns in the adjacent hypoglossal nucleus (n. XII); n. inf. ol., inferior olive; n. pontis, the pons. (From Scheibel and Scheibel, Note 33)

ed, descending trigeminal loaded, etc., thereby emphasizing the predominant population of afferents to be found on that process.

However, this concept suffers some dilution because each reticular cell lies in a matrix of fibers, largely (although not entirely) core derived and projecting for varying distances in the rostro-caudal (and occasionally transverse) direction. It is a characteristic property of such axons to give off collaterals at right angles more or less continuously along their path.[33,60] These collaterals make up a spectrum of lengths; some constitute little more than minimal specializations of the fiber in transit (*boutons-en-passage*). Each reticular dendrite must thread among the paths of tens of thousands of such axons, so a considerable number of axodendritic contacts must be expected from this source, even if mere contiguity of nonspecialized axon conductors with dendritic membrane is not in itself sufficient for information transfer. It seems logical to expand the hypothesis of sensory-annotated dendritic monads to include in each case a sprinkling of core-derived afferents. The moment-to-moment dendritic membrane loading would thereby represent an integrate of specific and nonspecific inputs. In turn, the algebraic summation of such states for each major dendrite shaft of a specific reticular-core neuron would represent (with additions from the somal synaptic scale) the level of synaptic drive being applied to the soma–initial segment at a specific point in time.

It seems beyond question that the output of each reticular element represents a vector of this type; it therefore follows that *specific informational content, intrinsic to each of the afferent sources, is lost in the integrative process.* The output of each unit must represent intensity only. It may not be out of place to suggest the analogy that a major function of the core is to describe not the pageantry and color of the passing parade, but the loudness of the shouting that accompanies it.

The presypnatic influx

The reticular core of the brain stem may be thought of as sitting athwart all incoming and outgoing information-carrying systems. The collaterals and terminals that pour in at every level receive a continuous stream of samples of the activity ongoing in these tracts. In general, archaic afferent and efferent systems, like the spino-thalamics and

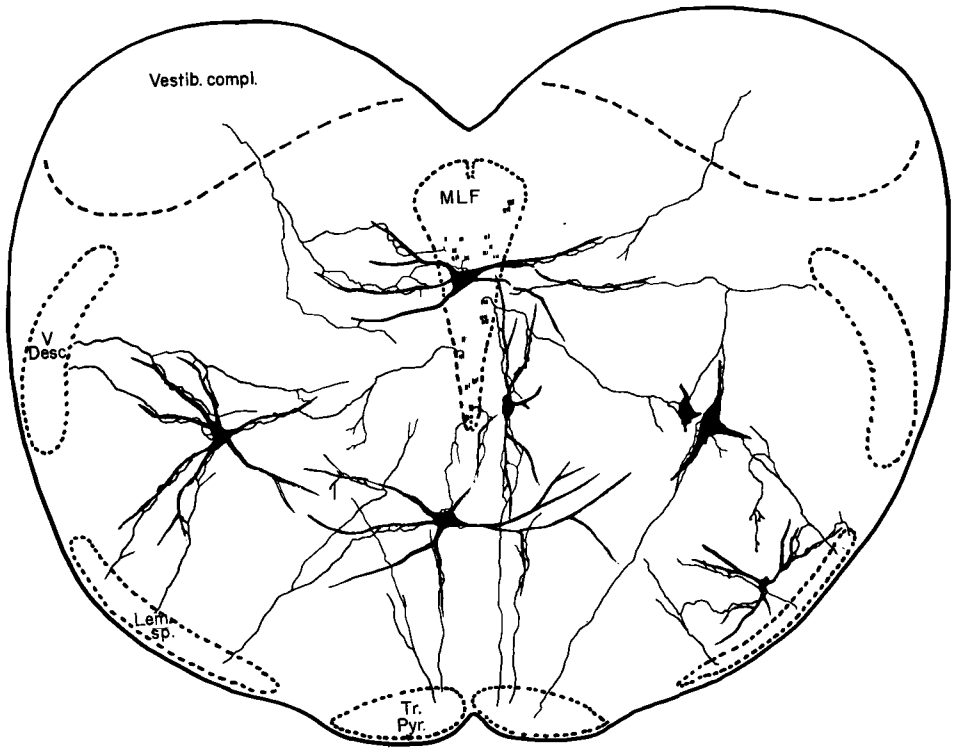

FIGURE 5 Semi-schematic cross section through upper third of medulla, showing relations of a few presynaptic components to dendrites and somata of reticular neurons. Some dendrites appear to point toward the source of their most significant presynaptic supply, individual fibers of which may effect multiple terminations along a single dendrite shaft. The nature of synaptic loading along each of a group of dendrites radiating from a single soma may be unique. Although the apparent segregation of presynaptic supply is very obvious in this sketch, the density of presynaptic terminals in the core actually results in appreciable degrees of convergence of heterogenous afferents upon each shaft. Vestib. compl., vestibular nuclear complex; V. Desc., descending trigeminal; Lem. sp., lemniscus spinalis (spinothalamic tract); Tr. Pyr., pyramidal tract; MLF, medial longitudinal fasciculus.

extrapyramidals, are more thoroughly represented than the phylogenetically newer tracts, such as the dorsal column—medial lemniscus and pyramid. The former (medial lemniscus), in fact, probably contributes no collaterals to the core, thus suggesting that information with a high degree of locus and mode specificity is not crucial to the operation of the reticular mosaic.

A fairly massive spino-reticular system introduces afferent activity into medullo-pontine levels, along the long axis of the tract, as do descending components in the central tegmental fasciculus and the brachia efferent from cerebellum. The most consistent afferent source, however, appears to be the collaterals that pour in from sagittally coursing fillets along the ventral and lateral aspects of the stem and on each side of midline.[5,33] In general, the aggregate of reticulopetal fibers (fibers afferent to the reticular core) may be divided into several categories, which include: (1) fibers from more rostrally placed centers (cerebral cortex, basal ganglia, diencephalon, and limbic or allocortex); (2) fibers ascending from the spinal cord; (3) fibers originating in the cerebellum and other brain-stem structures such as the geniculate bodies, colliculi, etc. These data are discussed in comprehensive form in several places.[79,80] Accordingly, only a few summarizing statements will be made here.

THE CORTICO-RETICULAR PROJECTION This makes up the most dramatic, if not the largest, descending system. The fibers originate in an area centering on the sensorimotor strip, but extend rather widely into adjacent cortical areas, and terminate in "nucleus reticularis giganto-cellularis, more rostally than caudally, while the pontine terminal region is found in the nucleus reticularis pontis oralis and the nucleus reticularis pontis caudalis, chiefly in its

rostral part."[80] Some terminations are also found in nucleus reticularis tegmenti pontis.

Of particular interest are the components descending indirectly from the limbic system upon midline tegmental structures, a projection studied in some detail by Nauta.[82] It seems clear that several routes are taken through thalamus and hypothalamus, with the eventual focus upon the mesencephalic nuclear fields of Bechterew and Gudden, from which arise an ascending system to complete a massive limbic-midline circuit. Informational by-products of activity in this circuit may also be disseminated laterally throughout the tegmentum and central tectum by the somewhat enigmatic radiation first described by Weisschedel.[83] We are as yet unable to decide from our material whether this represents the fine-fibered lateral projection of large numbers of small neurons situated in the periaqueductal gray, or, in whole or in part, a projection of neuroglial stalks, running from periependymal neuroglia that line the Aqueduct of Sylvius. If the latter possibility should be proved, it might introduce interesting possibilities for neurohumoral interactions at this level of the core.

DESCENDING TECTAL COMPONENTS Originating in the colliculi and pretectal area, these components terminate in the same general areas as do cortico-reticular components, probably as part of the tecto–bulbo–spinal correlation system. The terminal patterns of reticulo-petal components from basal ganglionar and diencephalic sites must still be worked out completely, although Golgi material indicates a hierarchy of fiber lengths and terminal stations throughout the length of the core.[33,73]

SPINO-RETICULAR FIBERS These fibers originate from all levels of the cord,[80,84] ascend in the ventro-lateral funiculus of the cord and terminate along an extensive core of medullo-pontine tissue, roughly congruent with areas of termination of the cortico-reticular projection. In addition, an appreciable fraction of spino-reticular elements project through, or terminate in, the lateral reticular nucleus, which also receives many of the lower brain-stem terminals of the spino–bulbo–thalamic system. This nucleus projects, in turn, upon cerebellum, while the majority of more centrally placed reticular fields (excluding paramedian nucleus and nucleus reticularis tegmenti pontis) relate back only to upstream or downstream centers.[80]

THE CEREBELLO-RETICULAR CONTINGENT The contingent enters the stem directly via the superior and inferior cerebellar peduncles. (The former is found primarily in the hooked bundle of Russell.) As summarized by Brodal,[80] major terminal stations in the reticular formation include nucleus reticularis tegmenti pontis and nucleus reticularis pontis caudalis, the paramedian nucleus, and portions of giganto-cellularis.

AFFERENT PROJECTION UPON THE RETICULAR CORE Details can be studied in Golgi sections through the upper medulla, which will serve as a paradigm. Figure 6 shows that collaterals from long ascending and descending tracts (i.e., spino-thalamic, descending trigeminal, vestibulospinal, tecto–bulbo–spinal, cortico-spinal, etc.) turn into the plane of section and terminate in overlapping patterns within the reticular core. At a higher level of resolution (Figure 7, right side), these terminals can be seen to cover the soma and dendrites of most neurons within the field, thereby making up the total roster of afferent supply or *synaptic scale*[85] upon the neuron.

The convergence of such a heterogeneous afferent array can also be demonstrated physiologically by means of occlusion techniques with macroelectrodes and by single-unit recording methods with the extracellular or intracellular microelectrode. The left side of Figure 7 shows strips from a series of records illustrating the response of a single unit to a group of disparate inputs, as well as its lack of response to others. Data of this sort underline the extensive but not unlimited convergence of inputs upon each reticular element. The unique quality of the synaptic "mix" upon each neuron emphasizes the mosaic-like nature of the core, with each neuron acting as a tiny integrating subcenter within the complex.

Cyclic phenomena

Anatomical analysis of an input array spread over the soma–dendrite complex of the average reticular neuron is sharply and unequivocally stated when subjected to successful Golgi staining or electron micrography. Furthermore, mechanical techniques that attempt the separation of synaptic boutons from the subsynaptic membrane bear witness to the tenacity with which presynaptic endings cling to the postsynaptic ensemble. Such procedures, in the hands of de Robertis,[86,87] who has differentially centrifuged the various fractions of crude separata down through a series of sucrose interfaces, reveal numerous elements in the bouton fraction still clinging to fragments of postsynaptic membrane. Clearly, this is no relationship that can be reversibly severed by the swing of an astrocyte tail, as was once hypothesized by Cajal.[5]

Yet, electrophysiological data that we have gathered from long-term, extracellular recording from a number of medullo-pontine reticular elements suggest that no matter how anatomically stable the synaptic relationships appear to be, their functional interactions are dynamic and cyclic.[57] In about 80 per cent of the reticular elements from

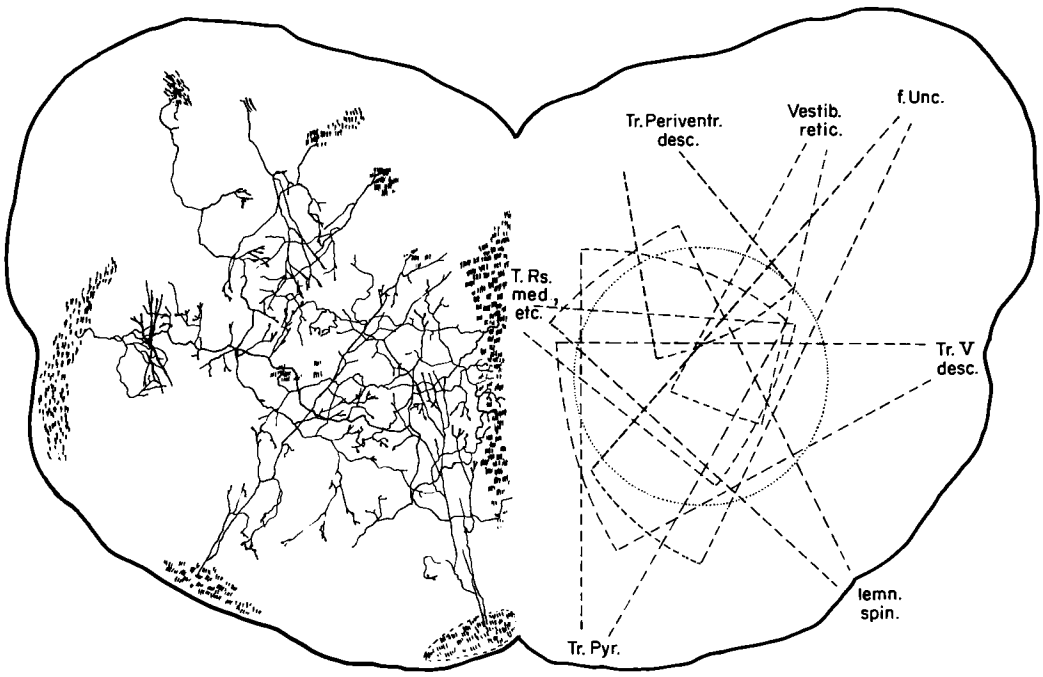

FIGURE 6 Transverse section through the upper third of the medulla of a 10-day-old kitten, showing the convergence and overlapping of terminating afferent fibers in the reticular core. On the left, a small group of fibers are drawn directly from the microscope; on the right, as a group of overlapping sectors. Reading clockwise from the top, the afferents include the descending periventricular system (Tr. Periventr. desc.); vestibulo-reticular fibers (Vestib. retic.); the uncinate fasciculus from cerebellum (f. Unc.); the tract of the descending fifth nerve (Tr. V desc.); lemniscus spinalis (spinothalamic tract) (lemn. spin.); pyramidal tract (Tr. Pyr.); and midline white matter, including medial reticulospinal fibers (T. Rs. med., etc.). (From Scheibel and Scheibel, Note 33)

which we were able to record for periods of from 10 to 12 hours each, there was a succession of reactive phases during which the cells appeared alternately sensitive to sensory bombardment from the external milieu (mild sciatic shocks of 1 to 2 volts of ½ millisecond, administered at 1-per-second frequency) and from the internal milieu, as exemplified by rhythms following the respiratory cycle. During these respiration-sensitive periods, the reticular elements were completely refractory to exteroceptive stimuli (Figure 8). Similarly, during sciatic shock sensitive phases, no traces of interoceptive-modeled activity were ever seen. Each cell seemed to follow a unique temporal pattern within a general order of magnitude of ½ to 3 hours (Figure 9). There was no discernible relation of these swings to sleep–wakefulness cycles nor were there indications of general physiological changes serving as substrate to the alterations.

Speculation on the mechanisms underlying this phenomenon include hypothecated endocellular rhythms, which periodically change the receptive sensitivities of the subsynaptic mosaic (see notes 88 and 89 for empirical data of possible relevance to this problem). An alternative suggestion regarding mechanisms is the presence of a population of pacemaker neurons that controls postsynaptic response either through presynaptic manipulation[90,91] or via interaction of satellite oligoglia, which we have shown receive terminating collaterals from the same preterminals that innervate the neuron.[92] Each suggestion carries with it its own intrinsic problems, the most intriguing of which endow our speculations on the pacemaker.

In a cellular matrix as complex as the reticular formation, if one hypothesizes a pacemaker elite that comprises a fraction of the mass of followers, the quest for order suggests a hyper-elite to pace the pacemakers, and so on to the ultimate absurdity of one supreme, pontifical neuron. A more attractive variant of this hypothesis involves mobile and redundant command, which temporarily vests pacing activities on those local arrays whose information loading is of a more biologically urgent nature than that of adjacent, more indecisive domains. As this content-mosaic

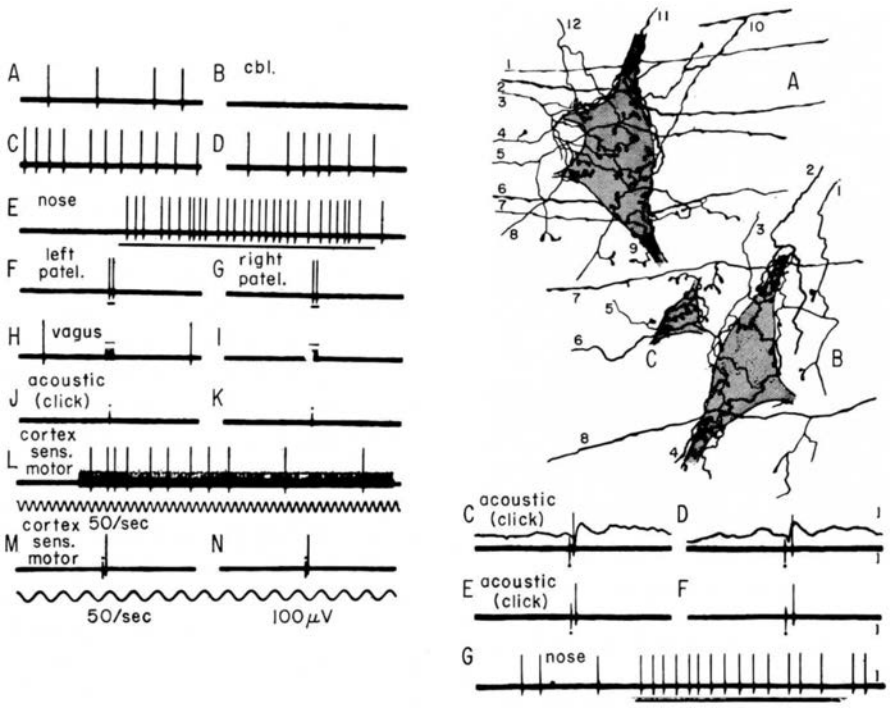

FIGURE 7 Convergence of heterogenous afferents upon single elements of the brain stem reticular core demonstrated physiologically and histologically. Strips A through N and C through G illustrate patterns of extracellularly derived spike discharges from two elements of bulbar reticular formation. A: spontaneous discharges; B: inhibition by cerebellar polarization (anterior lobe, surface positive); C: rebound following cessation of polarization; D: return toward normal spontaneous pattern; E: driven by nose pressure; F and G: driven by patellar tendon taps bilaterally; H and I: unaffected by short bursts of vagal stimuli; J and K: unaffected by clicks; L: driven by repetitive cortical stimulation; M and N: driven after a brief latency by single shocks to cortex (note expanded time base beneath these last two records). Strips C, D, E, and F show another unit that is sensitive to nose pressure and can also be driven by clicks.

Cells A, B, and C are bulbar reticular elements in 10-day-old kitten whose synaptic scale of terminating afferents is partially shown. Horizontal-running fibers such as A, 1 through 7, and B, 6 through 8, may belong to long spino-reticular and reticulo-reticular components while B, 1 through 4, represent sensory collaterals and cerebelloreticular terminals. (From Scheibel and Scheibel, Note 33)

varies through each fraction of time, it might be resonable to expect that centers of pacemaking activity would shift widely through the core—a suggestion not out of context with our own data already mentioned, which indicates multi-modal operations of reticular elements.

If we assume that the relatively small fraction of reticular elements from which we have led potentials is representative of the entire array, and that periodically the majority of brain-stem-core neurons lower their threshold to inputs, first of one sort and then of another, the significance of such behavior becomes a matter of some concern. We can only speculate that the cyclic activity which seems to couple and uncouple neural elements from one circuit or another may help preserve functional isotropicity within the reticular net. It may well be that without periodic input gating of this sort, masses of reticular elements would gradually be drawn into the full-time service of that system, sensory or motor, within whose geographic domain they most nearly fall. It is conceivable that by such a process of gradual acquisition and entrapment, increasing numbers of reticular elements would be lost to that range of multi-potent functions that characterize core operations. A neural ensemble, previously free to engage in multi-modal integrative operations, could then conceivably become a crazy quilt of satraps, each one only an extension of the specific system demanding its fealty. Make-break swings of the sort we have described might effectively counter such a trend.

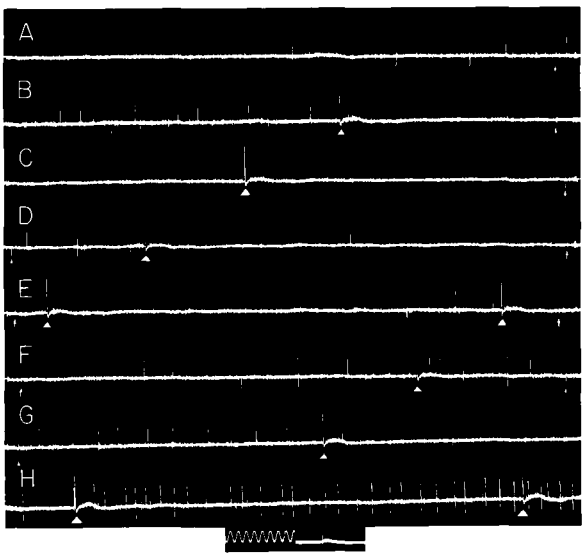

FIGURE 8 Bulbar reticular unit showing responsiveness first to endogenous, then to exogenous stimuli. Strips A through G show a bulbar unit driven by respiratory activity but totally nonresponsive to sciatic stimulation (1/sec, 2 volts, 1/2 msec). Strip H shows activity of the same unit 1/2 hour later. It is now highly responsive to the same type of sciatic stimulation but shows no evidence of being driven by respiratory activity. (From Scheibel and Scheibel, Note 57)

The axonal outflow

The axonal outflow of the reticular core consists of a spectrum of conducting systems projecting in three main directions—caudally upon spinal cord, rostrally upon subcortical and cortical centers, and dorsally to cerebellum. In addition, a vast and complex pattern is generated by the output of the core operating upon itself through collaterals *en passage*. Axonal collaterals of greater length also penetrate sensory and motor nuclei of cranial nerves en route. The first three projections can be demonstrated with some degree of rigor by the usual hodological methods employing retrograde or anterograde degeneration. Using modifications of the latter, Brodal[80,93] has shown that a system of reticulo-cerebellar projections originates from the lateral and paramedian nuclei and from the nucleus reticularis tegmenti pontis, which differ appreciably in their terminal stations. Use of the same technique has shown that the reticulo-spinal projection arises from cells spread along the medial two-thirds of medulla and pons, with particular reference to nuclei pontis oralis et caudalis, nucleus reticularis giganto-cellularis, and reticularis ventralis. Similarly, ascending reticular axons identified by lesions at the meso–diencephalic junction (Figure 10) arise from the same general nuclear fields, suggesting either an in-

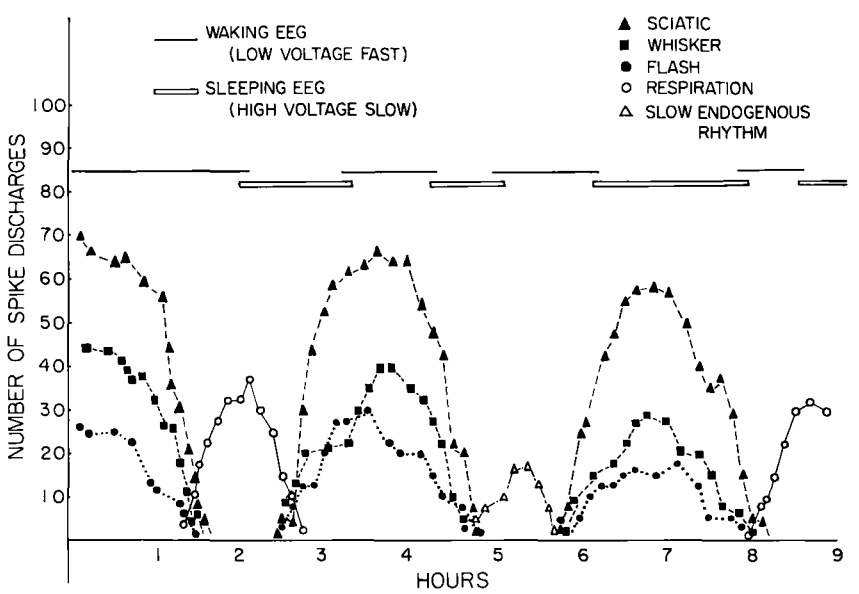

FIGURE 9 Cyclic response pattern of a single bulbar reticular unit followed for 9 hours. Almost three complete cycles of exogenous-driven and endogenous-driven activity are charted here. It will be noted that periods of sensitivity to exogenous sensory inputs are almost twice as long as those during which the unit is apparently responsive only to slow endogenous rhythms. There is no obvious relation between these swings and the state of consciousness of the preparation as indicated by the line-bar notation above the curves. (From Scheibel and Scheibel, Note 57)

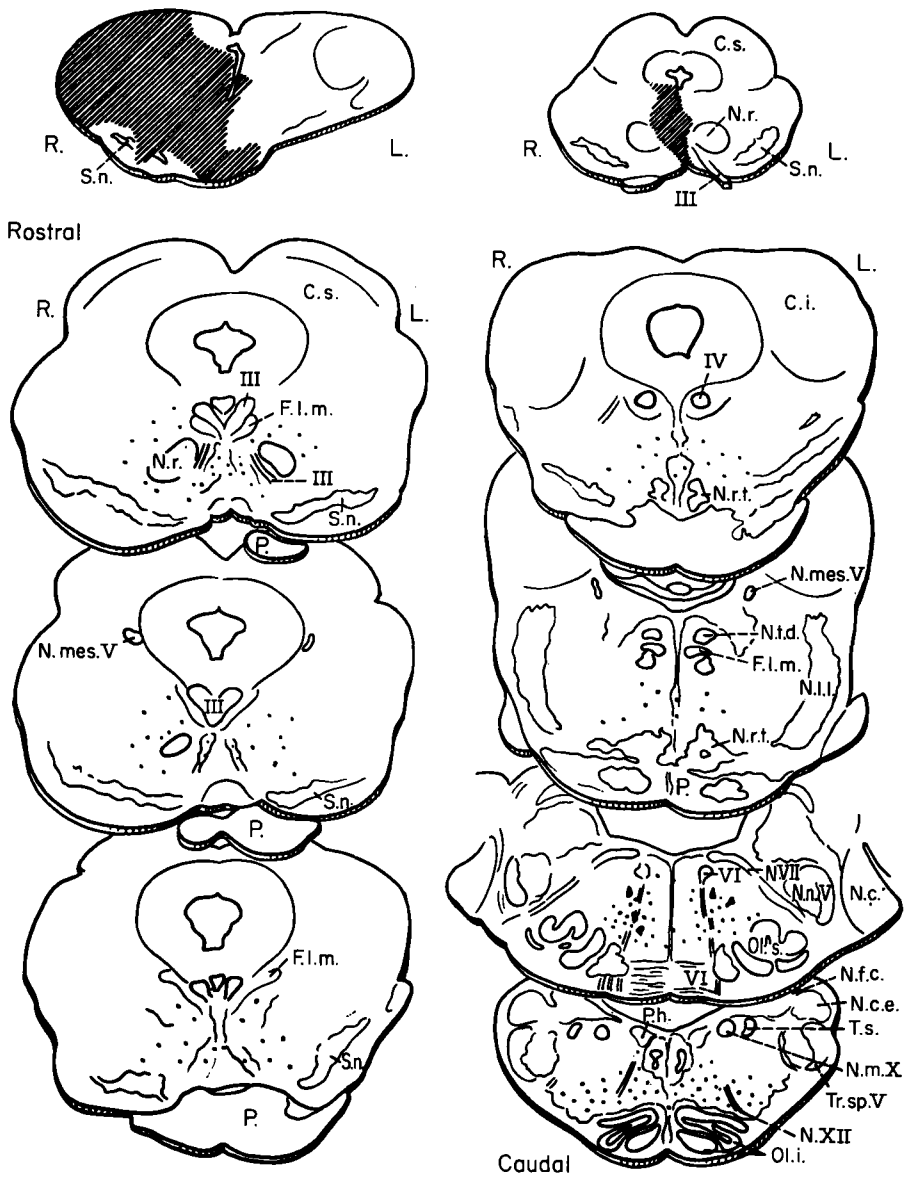

FIGURE 10 A lesion at the meso-diencephalic junction of cat brain stem and the position of cell bodies originally sending rostral-coursing axons through the site as determined by retrograde techniques (see text). Notice that a predominantly unilateral lesion results in degenerating cell bodies (dots) on both sides of the stem more caudally, and as far posteriorly as the middle third of the medulla. S.n., substantia nigra; III, third nerve nucleus and nerve; F.l.m., medial longitudinal fasciculus; N.r., nucleus ruber; P, pons; C. s., superior colliculus; IV, fourth nerve nucleus; N.r.t., nucleus reticularis tegmenti; C.i., inferior colliculus; N. mes. V, mesencephalic root of the fifth nerve; N.t.d., dorsal tegmental nucleus; N.l.l., nucleus of lateral lemniscus; VI, sixth nerve nucleus; N VII, seventh nerve; N.n.V, sensory trigeminal nucleus; N.c., cochlear nucleus; Ol.s., superior olive; P.h., perihypoglossal nucleus; N.f.c., cuneate nucleus; N.c.e., external cuneate nucleus; T.s., solitary tract and nucleus; N.m.X, motor tenth nerve nucleus (Vagus); Tr. sp. V., descending trigeminal tract and nucleus; N. XII, twelfth nerve; Ol.i., inferior olive. (From Brodal, Note 80)

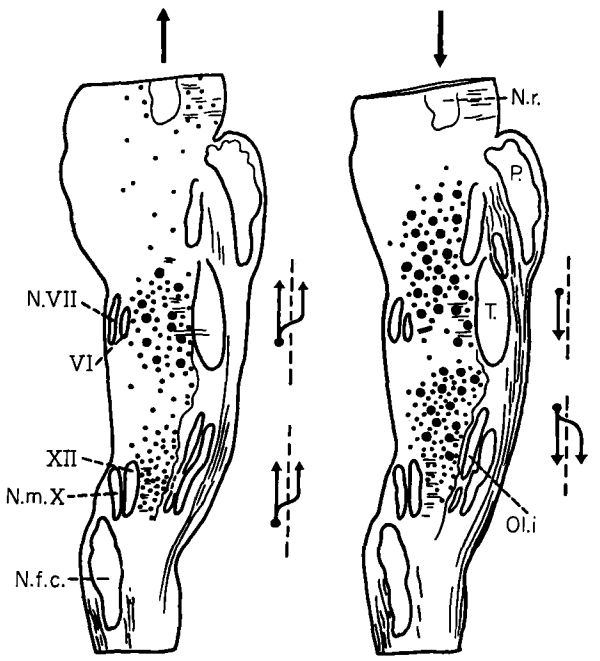

FIGURE 11 Distribution of reticular cells sending axons rostrad (left) and caudad (right) is indicated in two semi-schematic sagittal sections of brain stem of cat. Although there is a good deal of overlapping, caudally directed axons appear to arise somewhat more rostral than rostrally directed fibers. The arrows at the side of the figures indicate that all axon systems are both crossed and uncrossed, except fibers descending from pons, which are uncrossed. Abbreviations as in previous figures. (From Brodal, Note 80)

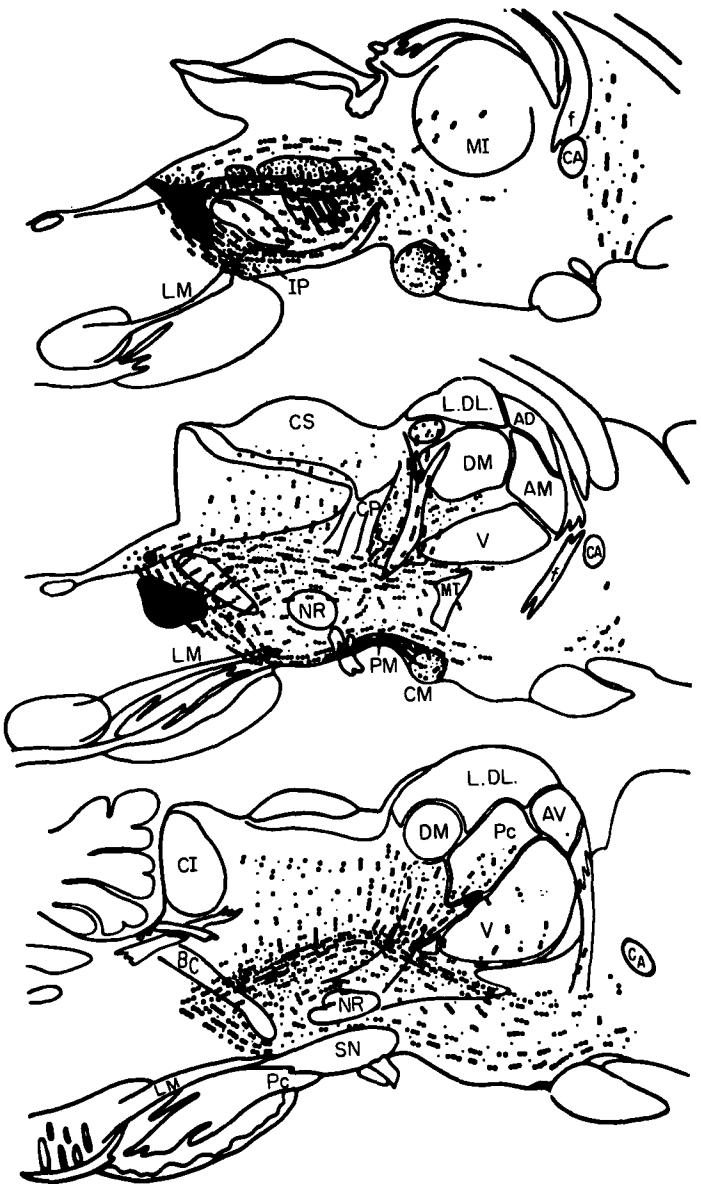

FIGURE 12 Degeneration of ascending fiber systems, as seen in sagittal section following a lesion in the caudal midbrain tegmentum, visualized by means of the Nauta method of anterograde degeneration. Large dots represent fibers of passage; fine stipple represents probable preterminal axons. LM, medial lemniscus; IP, interpeduncular nucleus; MI, massa intermedia; CS, superior colliculus; NR, nucleus ruber; MT, mamillothalamic tract; V, ventral nuclear complex; L.DL., lateral dorsal nucleus; DM, dorsomedial nucleus; BC, brachium conjunctivum; SN, substantia nigra; AD, anterior drosal nucleus; AM, anterior medial nucleus; f, fornix; CA, anterior commissure; CP, posterior commissure; PM, mamillary peduncle; CM, mamillary body; Pc, paracentral nucleus; CI, inferior colliculus. (From Nauta and Kuypers, Note 95)

discriminate mingling together of rostral- and caudal-projecting cells, or a source in common from many of the same neurons (Figure 11). Our Golgi studies indicate that both of these alternatives exist.

Anterograde studies, employing modifications of the Bielschowsky reduced-silver method, allow certain statements to be made as to terminal stations of ascending and descending projections. Reticulo-spinal fibers have been followed in ventral and lateral funiculi to termination in spinal laminae 7 and 8.[12] In some cases, Golgi preparations of spinal cord show such fibers terminating along dendrites and somata of internuncials and occasionally along the outer segments of motoneuron dendrites. In the latter, the anatomical material is not sufficiently precise to differentiate alpha from gamma motoneurons.[73]

Rostral projections have been followed by a number of workers using Marchi, Glees-Bielschowsky, and Nauta methods (Figure 12). There is general consensus that the ascending system projects dorsally into the thalamic intra-

laminar system, especially to the paracentral and central lateral nuclei and possibly as far rostrally as the nucleus reticularis thalami. Although there are some reasons—both anatomical and physiological—for expecting terminations in centre median, it has also been suggested that degeneration granules found in this area are purely of *en passage* variety and do not represent functional preterminal elements.[94] The ventral prolongation of this system can be traced into the zona incerta of hypothalamus and the fields of Forel. Lesions placed progressively more rostrally and ventrally in mesencephalic tegmentum reveal axonal systems that reach preoptic, septal, basal-forebrain, and basal-ganglionar stations. The vexing problem of whether direct monosynaptic connections are established with cortex by any of these elements has not yet received a satisfying answer from these techniques.[95]

As useful as these tracking methods have proved to be, it remains for the Golgi methods to demonstrate the richness and complexity of reticular projection systems—preferably viewed in sagittal sections of postnatal mice and rats (Figure 13). Although admittedly "simpler" than carnivores and primates, the rodent brain stem can well serve as a paradigm for higher forms, while escaping much of the circuit redundancy that makes Golgi analysis of larger brains so difficult.

The axons of reticular neurons form a spectrum of conductors of many lengths. Some neurons appear to project rostrally only; and others only caudally, but a significant number of medium-sized and large reticular elements generate the familiar bifurcating axon (Figure 14) that may project to distant stations both upstream and down. Virtually all of these are characterized by collaterals of varying lengths that leave the main stem perpendicularly and penetrate into adjacent reticular areas. Longer collaterals may reach cranial nerve nuclei and/or sensory relay and extrapyramidal motor fields. A number of fiber counts

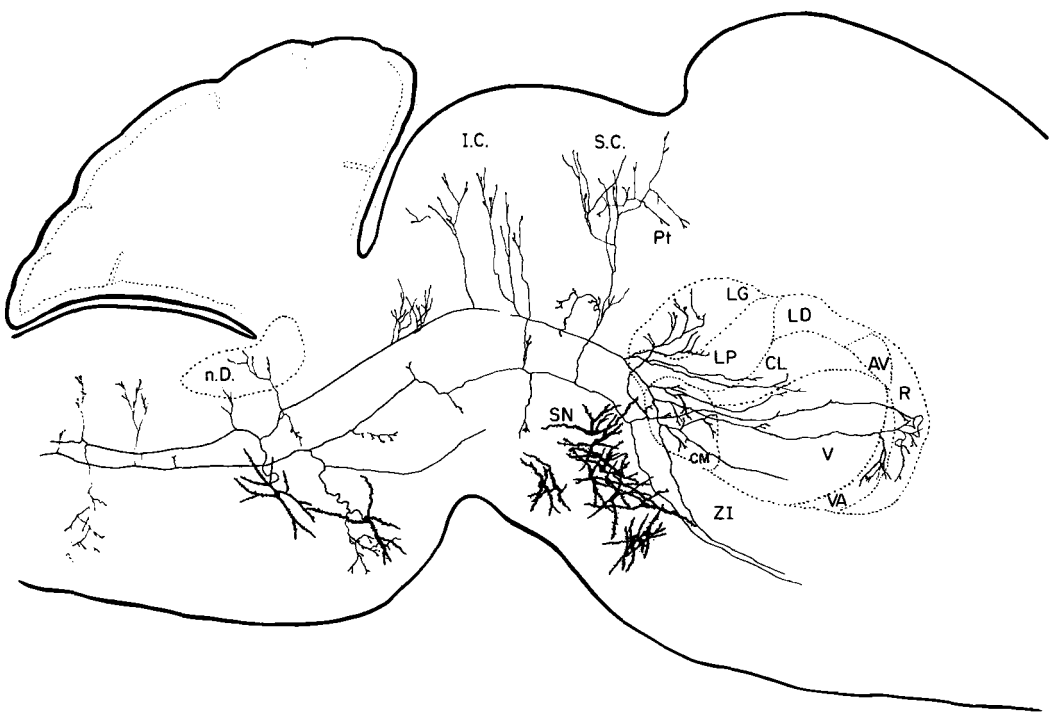

FIGURE 13 Sagittal section of an entire mouse brain (7 days old) showing 2 reticular cells in the magnocellular nucleus of rostral medulla. Both cells emit axons that bifurcate and course rostrad and caudad. A number of collaterals are given off by each axon, some of which reach cranial nerve nuclei, such as Deiter's component of the vestibular complex, n.D.; both inferior and superior colliculi, I.C. and S.C.; and pretectum, Pt. Other abbreviations include CM, centre median; LP, lateral posterior; LG, lateral geniculate; LD, lateral dorsal; CL, central lateral; AV, anterior ventral; V, ventral complex; VA, ventral anterior; R, nucleus reticularis thalami; ZI, zona incerta; and SN, substantia nigra. (From Scheibel and Scheibel, Note 33)

FIGURE 14 Sagittal section through the brain of a young rat showing the axonal trajectory of a single neuron of the nucleus reticularis giganto-cellularis, R. The rostral-coursing axonal component supplies collaterals to inferior colliculus, j; the region of the III and IV nerve nuclei, i; mesencephalic tegmentum, h; posterior nuclear complex of thalamus, f; dorsal, intralaminar and ventral thalamic nuclei respectively, e, d, and c; zona incerta of hypothalamus, g; nucleus reticularis thalami, b; and basal forebrain area, a. The posterior-directed component sends collaterals into the substance of the reticular core, m; the hypoglossal nucleus (XII), k; the nucleus gracilis, l; and the intermediate gray matter of the spinal cord, n. (This illustration was originally prepared for publication in Brazier, M. A. B., "The Electrical Activity of the Brain"; Third Edition, New York, The Macmillan Company; in press, and is reproduced here with permission of the author.)

have suggested to us that, on the average, each reticular axon releases one collateral of approximately 100-micron length for each 100 microns of trajectory.[33] This does not include large numbers of smaller, bulb-like enlargements along the course of the axon, called by Cajal *boutons-en-passage*[5] and representing, according to electron-micrographic analysis, centers especially rich in mitochondria and other intra-axonal organelles.

The picture that emerges is one of continuous, intensive interaction between large numbers of conductors and the surrounding matrix of core neurons. The nature of these contacts must vary, depending on the presence or absence of a myelin sheath and the frequency of axonal specializations. No definite statement can be made about the functional significance of the untold numbers of contacts established among structures without discernible axonal (or postsynaptic) specialization. It is currently fashionable to discount the significance of such membrane appositions as seen in electron micrographs if there is no evidence of membrane thickening, increased opacity to the electron beam, or presence of synaptic vesicles behind one of the apposed membranes. However, the presence of transmembranal electrogenic effects has been shown to exist at least in invertebrates,[96,97] and Nelson and Frank have mapped a considerable field effect around spinal motoneurons in the cat.[98] Pending more rigorous analysis with recording techniques of higher resolution, no definitive statements can be made about the presence or absence of a spectrum of field interactions and threshold manipulatory effects in a dense complex of long conductors and axodendritic neuropil such as the reticular core.

However, the patterns of connectivity that can be traced out through the core suggest a circuit scheme so richly redundant that convincing arguments could be advanced for almost any conceivable loop or chain. Figure 15 summarizes three alternatives. The first depends largely on polysynaptic chains of short-axon elements similar to the scheme originally suggested by Moruzzi and Magoun.[32] The second illustrates the collateral-rich, long-projecting, high-priority axon now known to characterize many reticular neurons. The third combines a group of these, geographically staggered, so that each is activated somewhat

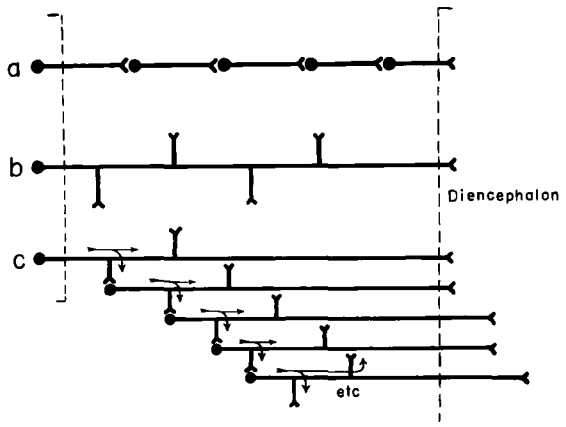

FIGURE 15 Schematic showing three possible circuit paradigms through the reticular core of the brain stem. a: the chaining of short-axoned cells as suggested by Moruzzi and Magoun. b: a single long-axoned, high-priority conductor reaching from lower brain stem (left) at least as far as diencephalon (right) without synaptic interruption. This resembles a type of reticular axon actually found in large numbers in the core. c: an ensemble of such long axons, whose collateral systems, playing upon adjacent elements similar in nature, but geographically staggered, can result in a spectrum of conduction latencies and divergence of information. (From Scheibel and Scheibel, Note 33)

later in time, resulting in appreciable degrees of spatial and temporal dispersion. Such an array could also account for the appearance of a family of response latencies over intrareticular conducting paths, as demonstrated by Adey, Segundo, and Livingston.[99]

The rostral course of axonal elements of medullo-pontine and mesencephalic tegmental neurons has already been mentioned in part. While the dorsal thalamic leaf almost certainly projects no farther than the intralaminar fields and nucleus reticularis thalami, the ventral leaf, extending through ventrolateral hypothalamus, in some cases may be traced to, and through, the basal forebrain area and ventral caudate-putamen. By using very thick sections (200 micron) of perinatal mouse, we have been able to trace a limited number of axons from brain-stem reticular formation to the base of the anterior third to half of cortex. We estimate that the number of such monosynaptic corticopetal conductors is relatively modest—perhaps of the order of 5 to 10 per cent. Although we are still working on this problem, it is of interest that Magni and Willis[34] have recently succeeded in antidromically activating small numbers of micropipette-impaled, brain-stem reticular neurons via cortical stimulation. They consider their data consistent with the presence of some reticular elements that project monsynaptically upon cortex.

The thalamic system

The nonspecific system of the thalamus differs in organizational pattern and functional role from the remainder of the brain-stem reticular core. It is also partially isolated from reticular structures of the lower two thirds of the stem, so it seems appropriate to deal with it separately. According to Jasper[100] "the thalamic reticular system is the dorsal limb of the cephalic end of the brain stem reticular formation. . . . [It] is sometimes identified with the unspecific thalamocortical projection system. This may not be accurate, since unspecific projections may be only one important property of the thalamic reticular system." Generally included within its domain is the calyx of medial and intralaminar nuclei that run forward from the centre median–parafascicular field to surround the dorso-medial complex and separate it from the ventro-lateral fields just outside. More rostrally, the system includes—at least at a functional level—portions of antero-medial and ventral-anterior nuclei and the nucleus reticularis thalami (Figure 16). The group of small cell masses making up the internal medullary lamina itself includes central-medial, paracentral, central-lateral, and a group of more variable midline-bridging nuclei, i.e. reuniens, rhomboidalis, interantero-medialis. Previous descriptions of these areas may be found in various studies,[5,101-104] while adequate summarizing statements of their functional capabilities have been offered by Jasper.[100,105] More recent discussions of problems inherent in the experimental analyses of the system have also been provided.[94,106]

The present discussion is limited to data that have emerged from our analysis of thalamic nonspecific systems, using Golgi methods. It is worth passing comment that problems in visualization of the intricate neuropil of this system were noted by Cajal 60 years ago[5] and probably have been largely responsible for the dearth of information on structural substrates of this intriguing area. Our own material is presently based on analysis of approximately 2000 rodents, cats, and a few primates, and can be considered introductory.

Expressed in terms of its constituent neuropil as revealed by the Golgi chrome-silver, Golgi-Cox, and ancillary techniques, the thalamic nonspecific system consists of a group of fields stacked along the rostro-caudal axis, and connected by a dense feltwork of axons of varying lengths and diverse projections. We shall consider the centre median–parafascicular complex as the common caudal component, despite rather compelling evidence that the centre median is a relatively late phylogenetic acquisition and is virtually absent in rodents. From this complex, axons pour rostrally (Figure 17), fanning out both laterally and medially; the latter elements bridge midline somewhat more

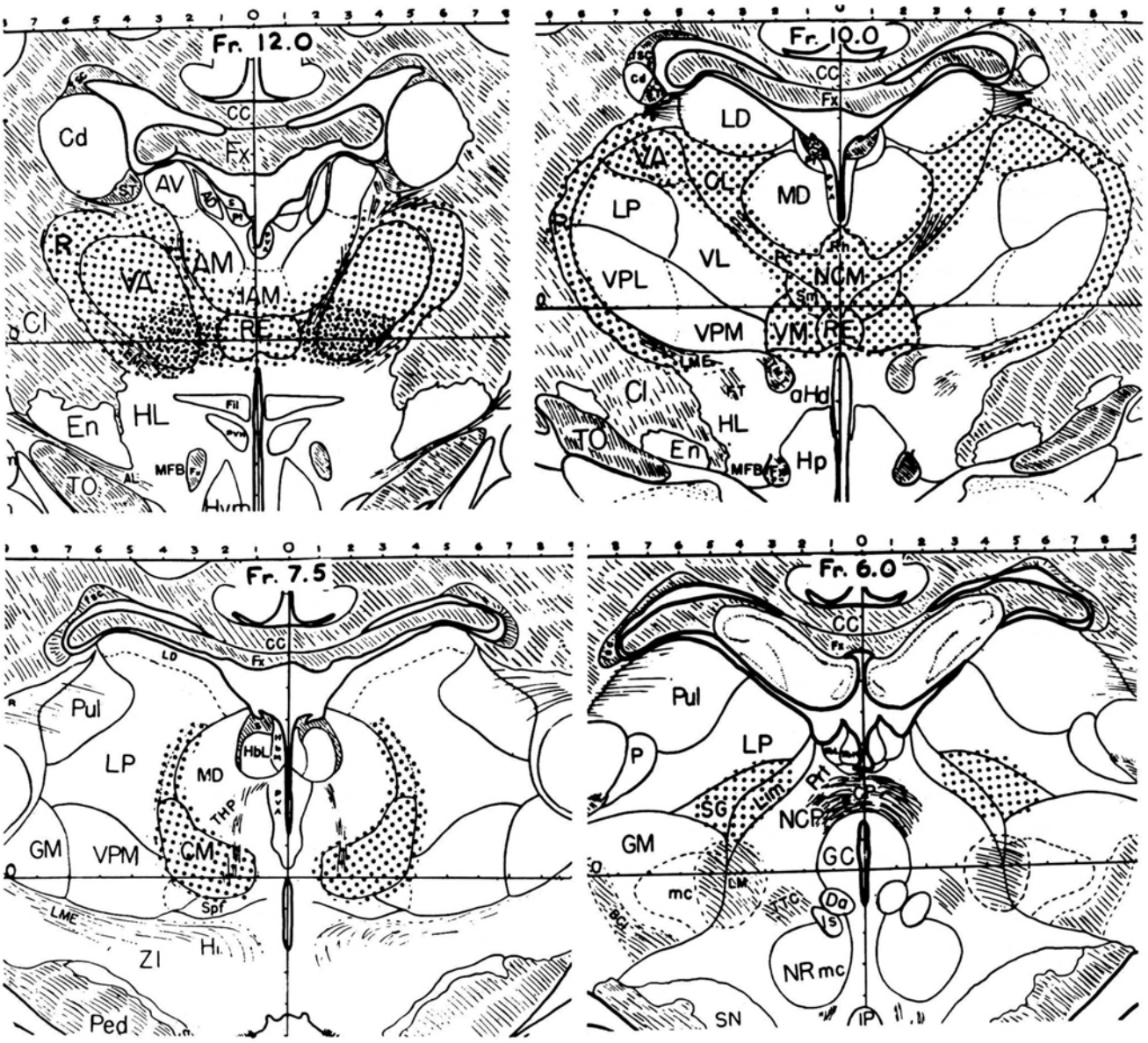

FIGURE 16 The electrophysiologically determined limits of the nonspecific (reticular) system of the thalamus. AD, antero-dorsal nucleus; aHd, dorsal hypothalamic area; AL, ansa lenticularis; AM, antero-medial nucleus; AV, antero-ventral nucleus; BCl, brachium of the inferior colliculus; CC, corpus callosum; Cd, caudate nucleus; Cl, claustrum; CL, central lateral nucleus; CM, centre median nucleus; CP, posterior commissure; Da, nucleus of Darkschewitsch; En, entopeduncular nucleus; Fil, filiform nucleus; fsc, subcallosal fasciculus; FT, thalamic fasciculus; Fx, fornix; GC, central gray matter; GM, medial geniculate body; HbL, lateral habenular nucleus; HbM, medial habenular nucleus; Hi, field of Forel; HL, lateral hypothalamus; Hp, posterior hypothalamus; IAM, interanteromedial nucleus; IP, interpeduncular nucleus; Is, interstitial nucleus; LD, lateral dorsal nucleus; Lim, nucleus limitans; LM, medial lemniscus; LME, external medullary lamina; LP, lateral posterior nucleus; mc, pars magnocellularis; MD, medial dorsal nucleus; MFB, medial forebrain bundle; NCM, central medial nucleus; NCP, posterior commissural nucleus; NR, red nucleus; P, posterior nucleus; Pc, paracentral nucleus; Ped, cerebral peduncle; Prt, praetectum; Pt, parataenial nucleus; Pul, pulvinar; PVA, anterior periventricular nucleus; PVH, periventricular hypothalamic nucleus; R, reticular nucleus; RE, nucleus reuniens; S, medullary stria; SG, supragenicular nucleus; Sm, submedian nucleus; SN, substantia nigra; Spf, subparafascicular nucleus; ST, terminal stria; THP, habenulopeduncular tract; TMT, mammillothalamic tract; TO, optic tract; TTC, central tegmental tract; VA, ventral anterior nucleus; VL, ventral lateral nucleus; VM, ventral medial nucleus; VPL, ventral posterolateral nucleus; VPM, ventral posteromedial nucleus; ZI, zona incerta. (Figure and caption from Jasper, Note 100)

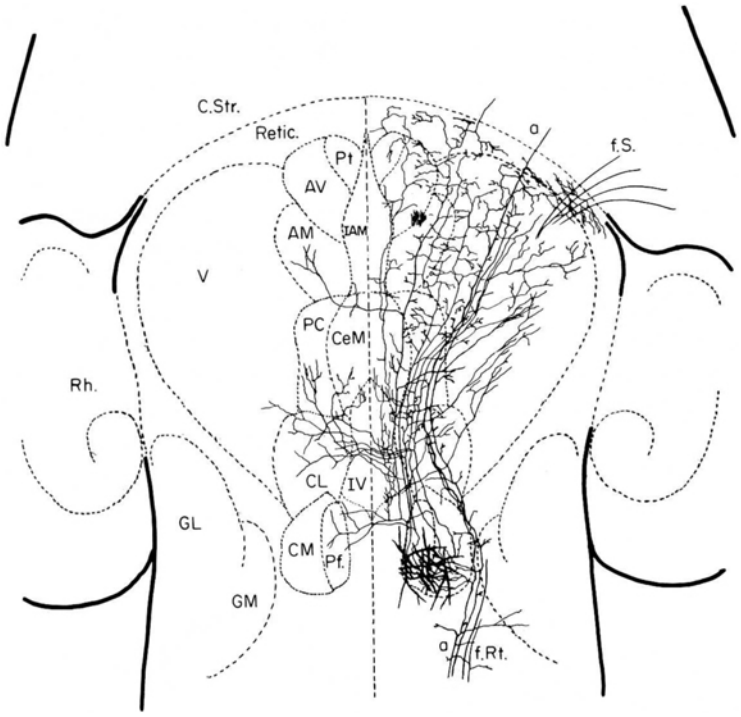

FIGURE 17 Horizontal sagittal section through diencephalon of 12-day-old mouse, showing certain aspects of rostral-coursing axonal projections from the thalamic nonspecific system and the brain stem reticular core. The latter, f. Rt., runs through the lateral aspect of the intralaminar nuclei, giving off collaterals to, and lying in parallel with, the projection of the thalamic system that here is limited to a small number of fibers pictured issuing from the centre median-parafascicular, CM-Pf complex, although the amount of centre median tissue present in the rodent is uncertain (see text). Collateralization of these systems appears to be both ipsilateral and contralateral, extending into specific and nonspecific nuclear masses. Most of these fibers are shown terminating no farther rostral than the nucleus reticularis thalami (Retic.), although there is some evidence that elements among them may reach striatum and even cortex (see text). One such fiber is shown at a, while f.S. represent a small group of specific thalamo-cortical elements. Other abbreviations include: Pt, parataenial nucleus; AV, anterior ventral nucleus; AM, anterior medial nucleus; IAM, interanteromedial nucleus; PC, paracentral nucleus; CeM, central medial nucleus; CL, central lateral nucleus; IV, interventricular nucleus; GL, lateral geniculate; GM, medial geniculate; Rh., rhinencephalon. (From Scheibel and Scheibel, Note 33)

rostrally in the reuniens and rhomboidalis components of the massa intermedia. Rostro-lateral components peel off in arcs, flowing through or around more lateral nuclear masses to reach putamen and caudate.

Although it has generally been agreed that no axons of the posterior half of the thalamic nonspecific system reach cortex, more sensitive tracking methods in the hands of some investigators[106] now suggest that delicate cortical projections may, in fact, exist. Golgi methods have not enabled us to trace such axons to their destination, although there seems no question about cortical terminations for more rostrally situated elements. The Golgi methods do, however, show the intensive collateralization of these caudal and rostral components, which provide a rich substrate for interaction among the various fields of the nonspecific system and with adjacent specific and associational nuclei. As suggested by Figure 17, the ascending axonal system from the posterior two-thirds of the brain stem runs roughly parallel to the thalamic nonspecific system and provides one of its most important sources of afferent excitation.

This relationship is shown more clearly in Figure 18, where a dense collateral mass infiltrates the intralaminar system from ascending brain-stem reticular fibers as well as from the adjacent, much attenuated spino-thalamic tract. Individual elements of this influx penetrate not only the entire ipsilateral intralaminar system, but reach some of the contralateral fields—at least in the rodent (specifical-

ly rats and mice). We have not yet been able to identify the entire category of afferents to this system, nor is such information available from degeneration and/or evoked-potential studies. However, we have identified axonal elements from basal ganglia; the fields of Forel; colliculi and pretectum; from adjacent associational nuclei such as lateral posterior and pulvinar; hypothalamus; the anterior nuclear complex; the stria medullaris–habenula–fasciculus–retroflexus complex; and a massive, fine-fibered contingent from (at least) orbito-frontal cortex. The nature of this afferent array suggests that the thalamic nonspecific fields are more sheltered from the onslaught of externally derived data than is the reticular mosaic of the lower brain stem.

This presynaptic influx reaches the thalamic fields along two predominant axes, rostro-caudal and transverse. The majority of dendritic elements of this system appear to be organized transversely (see Figure 19); so we must assume that those conductors with terminal elaborations in the rostro-caudal axis either develop less potent synaptic drive along the dendrites or terminate preferentially on somata. Although the Golgi evidence is suggestive in this regard, the data cannot yet be considered definitive.

Intensive pentration of adjacent, specific thalamic fields is a common feature of this system. Figure 20 shows a number of such elements entering the neuropil mass of the ventral–lateral (VL) nucleus, whose main afferent supply comes from cerebellum via brachium conjunctivum. Physiological support of this relationship is supplied by Purpura, et al., who have found a frequency-specific type

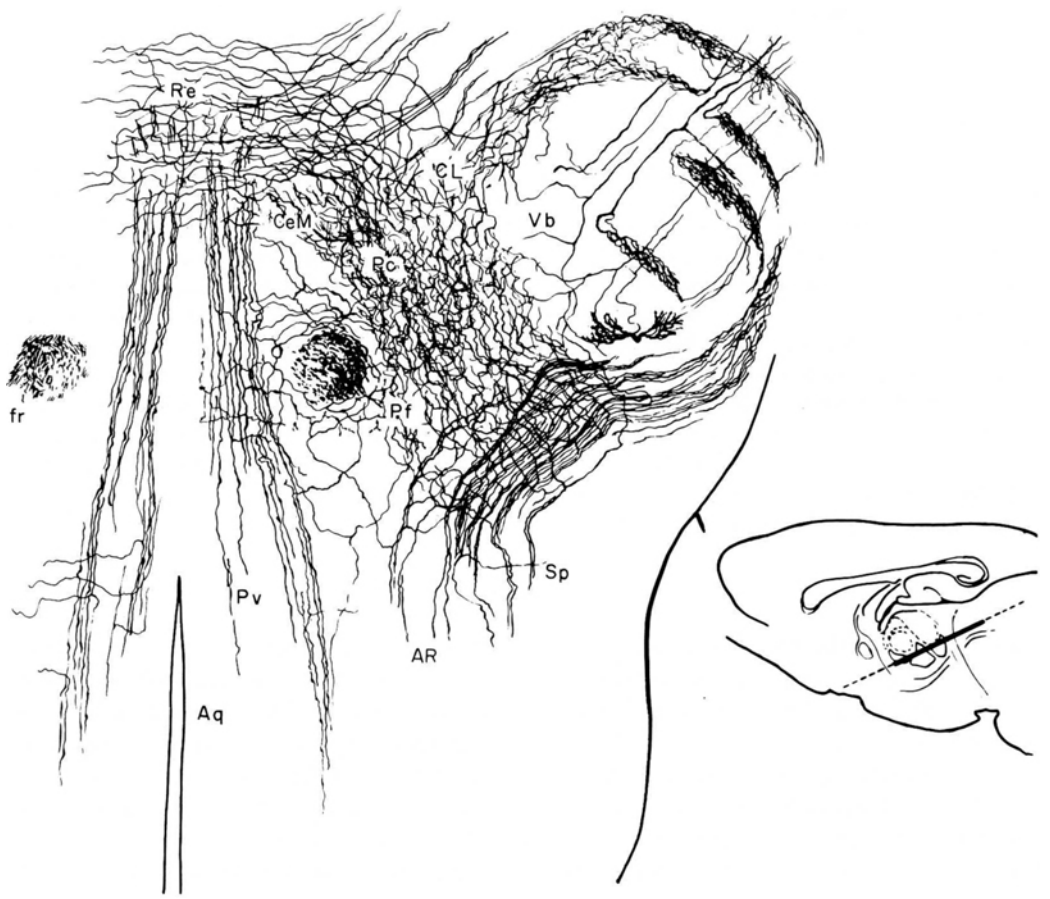

FIGURE 18 Mass of collateral and terminal fibers from ascending reticular components (AR) and spino-thalamic tract (Sp) terminate in the posterior half of the intralaminar fields, including parafascicular (Pf); paracentral (Pc); central lateral (CL); central medial (CeM), and project contralaterally via the reuniens nucleus (Re). Other abbreviations include Aq, aqueduct of Sylvius; Pv, periventricular fibers; and fr, fasciculus retroflexus. Ten-day-old rat. Rapid Golgi modification.

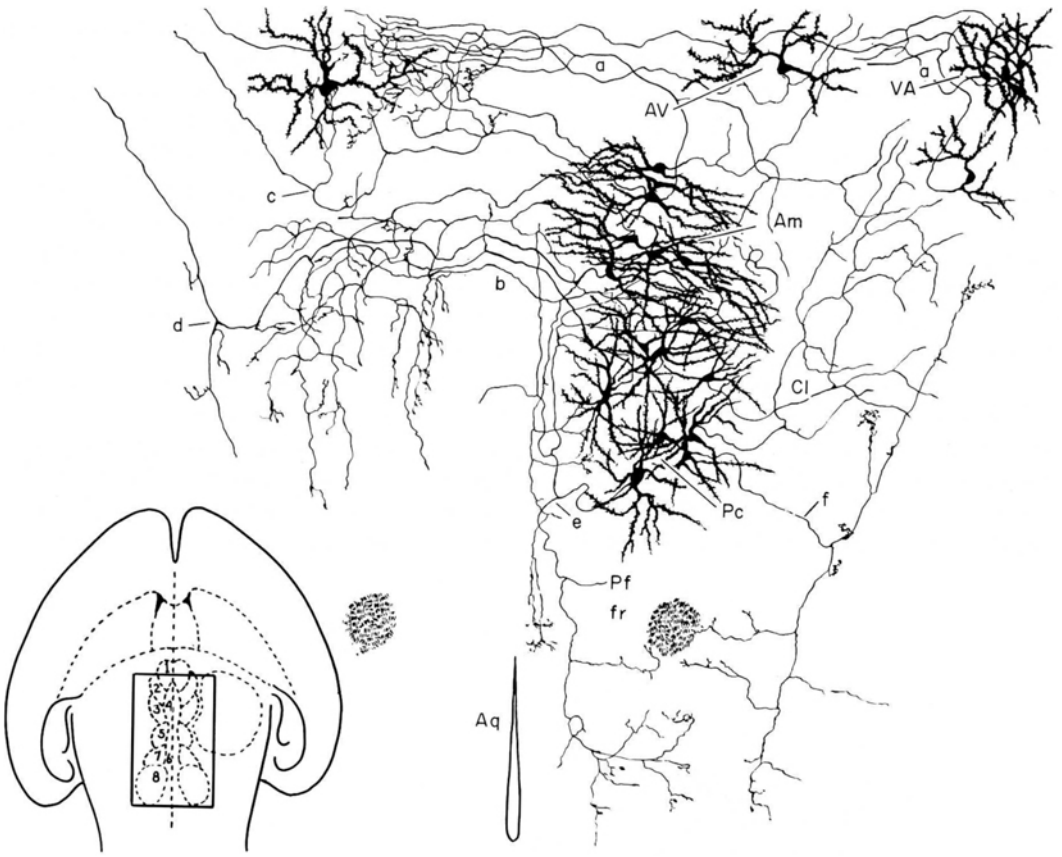

FIGURE 19 Axonal and dendritic organization of portions of the thalamic nonspecific system. Orientation of dendrite masses of neurons in paracentral (Pc), central lateral (Cl), and anterior medial (Am) appears largely transverse or oblique, thus paralleling contralateral axonal inputs, b, and collaterals from descending centrifugal fibers, d. Some intralaminar axons, like e, seem to remain largely ipsilateral, connecting adjacent nuclei, while others, such as f, project laterally into adjacent fields of the ventral nuclear complex (see figure 20). A number of intralaminar-derived axons such as c and d collateralize contralaterally and then project rostrad toward striatum and/or cortex. Similar patterns are found in axons from cells of anterior ventral (AV) and ventral anterior (VA), which are not included in the intralaminar complex. The two axons marked a follow trajectories of this type. Other abbreviations include Aq, aqueduct of Sylvius; and fr, fasciculus retroflexus. Twenty-day-old mouse. Rapid Golgi modification.

of modulatory control exerted by intralaminar nuclei upon a background of ongoing, brachium-derived excitation in VL neurons.[107]

In the rodent, massive commissural patterns are formed by intralaminar axons. A series of fibro-nuclear masses that enable intensive bilateral communication have been described, including the nuclei reuniens, rhomboidalis, and interantero-medialis. This allows for a massive system of re-entrant loops of varying lengths connecting both sides of the thalamus, and also facilitates communication of nuclear fields with both cortical hemispheres. The intensity of such crosscommunication decreases as the phylogenetic scale is ascended and midline-bridging tissue shrinks in size and importance. One might wonder whether this feature of rodent thalamo-cortical anatomy might not be substantially implicated in the phenomena of cortical equipotentiality described by Lashley[108] in rats (Chow, this volume). In a similar vein, with the progressive decrease of commissural mass to the point at which 30 per cent of the human thalami may be totally devoid of massa intermedia bridging,[109] there may reside substrate for the rise of laterality in primate and man.

It appears highly likely that a cortically directed projection arises from the anterior third of thalamic nonspecific fields. In Figure 21, part of this system can be seen leaving the critical antero-medial angle of the field. Such axons

may project rostrally, unilaterally, or bilaterally, and as they emerge from this pool, send rich collateral masses into adjacent nuclei such as ventral–anterior and nucleus reticularis thalami.[110,111]

The latter is of special interest to us. It continues as a nuclear plate or shell surrounding anterior, superior, and inferior surfaces of the thalamus, and all thalamo-cortical projections and the greater part of the cortico-thalamic reflux must penetrate through it. It is, accordingly, in a critical position to monitor and modulate most thalamo–cortico–thalamic interactions. Several investigators[111,112] have, in fact, likened it to the screen grid in a vacuum tube. Our Golgi studies indicate that the dendritic ensemble of this field is densely intertwined and covered with long, hairlike spines, forming a net or sievelike structure through which most axons of these thalamo–cortico–thalamic systems perforate, either directly or with production of collaterals.[111] Opportunities for intensive axodendritic interactions are obvious (Figure 22).

The axonal projection of the nucleus reticularis thalami is clearly of great physiological importance. Cajal reported that these axons seemed to run caudally,[5] but a long series of retrograde degeneration studies following massive, selective, cortical ablation appeared to favor a cortical termination.[113,114] As a result of evoked-potential studies, it was asserted by some investigators that the nucleus reticularis thalami actually constituted the final common pathway along an intralaminar polysynaptic chain to cortex.[21] The importance of such data was somewhat weakened by the inability of the stimulating electrode to differentiate between nucleus reticularis cells and fibers of passage. Recent Golgi studies have shown clearly that at least 95 per cent of the axons of nucleus reticularis thalami cells are, in fact, distributed caudally to thalamus and mesencephalic

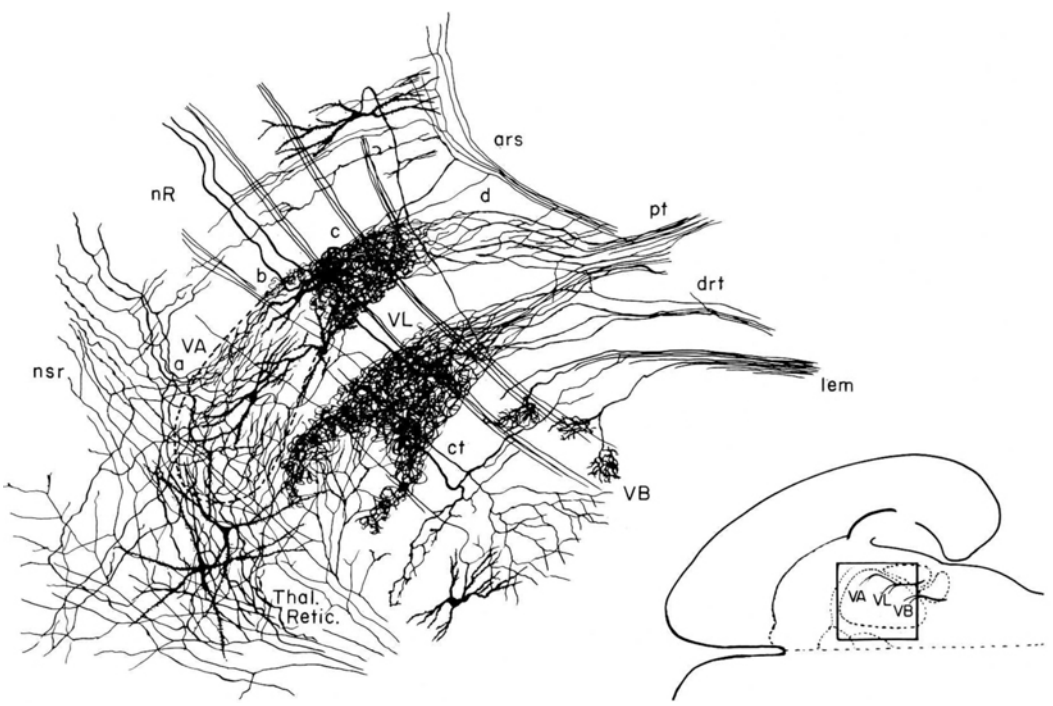

FIGURE 20 Vignette taken from a horizontal sagittal section through the thalamus of a 7-day-old rat showing some interrelations between thalamic nonspecific system fibers and the ventral lateral and ventral anterior fields. Cells and fibers of the thalamic nonspecific system (Thal. Retic.) culminating in the rostral-running projection or nonspecific radiation (nsr) send terminals and collaterals into the neuropil fields of ventral lateral (VL) and ventral anterior (VA) nuclei, whose primary afferent sources are the dentato-rubro-thalamic, (drt) and pallido-thalamic (pt) bundles respectively. Also visible are sectors a, b, c, and d, of VA, the nucleus reticularis thalami (nR) more anteriorly, and the ventrobasal complex (VB) more posteriorly, receiving the terminating medial lemniscus (lem). One centrifugal fiber (ct) is seen ending in VB, while the ascending reticular system (ars) sends collaterals toward the VA field. Rapid Golgi modification. (From Scheibel and Scheibel, Note 110)

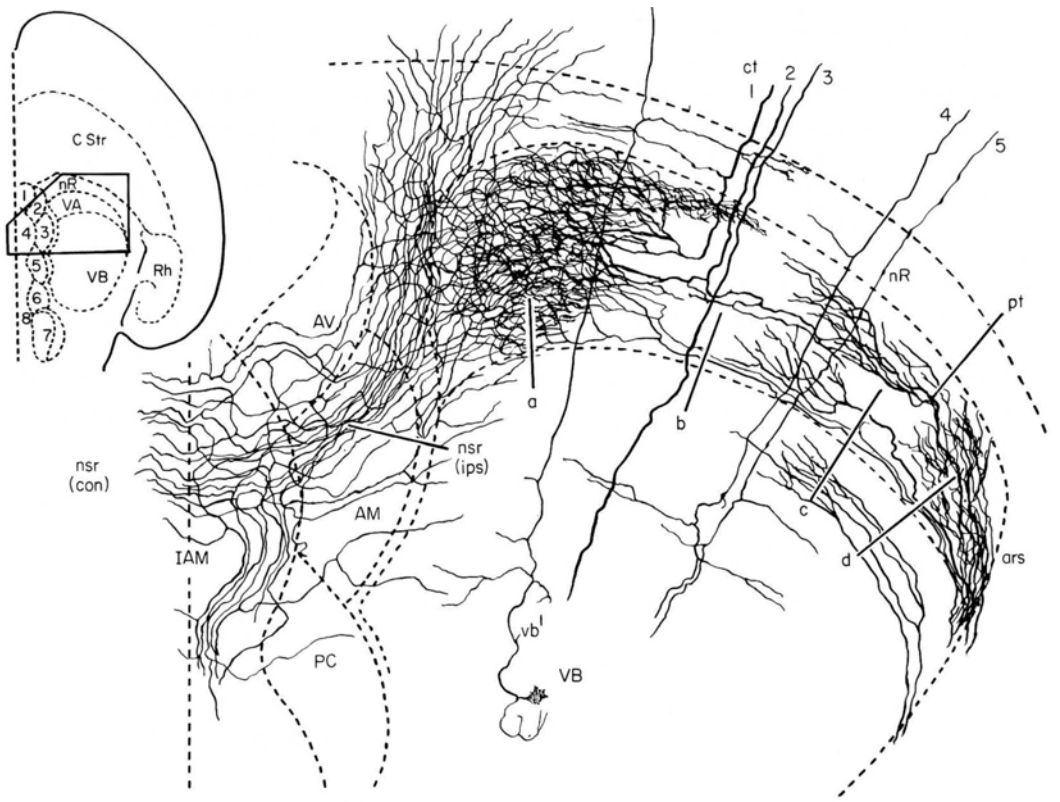

FIGURE 21 Horizontal section through the anterior end of the thalamus showing the area of origination of the thalamic nonspecific projection upon cortex. Collaterals and terminals from the ipsilateral nonspecific radiation, nsr (ips), and some elements from the contralateral system, nsr (con) fill the medial portion—sector a of VA. This region is also reached by individual elements of the pallido-thalamic bundle (pt), which arborizes maximally in sector c, while shorter collaterals from the ascending reticular system (ars) terminate in sector d. One rostrally-directed axon (vb^1) from a ventrobasal cell (VB) is seen, as are 3 heavy caliber and 2 fine cortico-thalamic fibers (ct 1–5), the first two of which terminate in VA. Other abbreviations including those in the inset diagram include: 1, parataenial; 2, anterior ventral, AV; 3, anterior medial, AM; 4, interantero-medial, IAM; 5, paracentral, PC; 6, central lateral nucleus, CL; 7, centre median-parafascicular complex; 8, interventricular; C Str, corpus striatum; Rh, Rhinencephalon. Three-day-old kitten. Rapid Golgi modification. (From Scheibel and Scheibel, Note 110)

tegmentum (Figure 23), thereby providing a hierarchy of re-entrant circuits.[111] Such feedback loops appear to play on neurons of both specific and nonspecific thalamic systems and may serve as substrate for recruitment potentials.[18,19] They may also provide circuitry essential to the alternating excitatory postsynaptic potential (EPSP) and inhibitory postsynaptic potential (IPSP) swings described in specific thalamic cells secondary to afferent barrage.[115]

THALAMO-CORTICAL PROJECTION The projection of thalamic nonspecific systems upon cortex poses a problem that requires further investigation. Investigators do not fully agree on how recruitment potentials are distributed over cortex following intralaminar stimulation,[19,20] although there is a consensus that the distribution transcends that produced by stimulation of specific thalamic nuclei. Our own investigation of this projection is still incomplete, but the data are suggestive. In rats and mice, the axonal outflow from the anterior third of the intralaminar fields appears to project rostrally and ventrally via the inferior thalamic radiation, just lateral to the mass of the septal nuclei. The fibers can be followed forward to apparent simple ascending (axodendrite?) cortical terminations in orbito-frontal cortex. Each fiber may subdivide into many branches while still in the subgriseal white matter, and the most caudal branches may reach almost to the frontal motor fields.

Such data appear to complement the findings of Velasco

and Lindsley,[116] who observed that orbito-frontal ablation in the cat effectively blocks recruitment phenomena over the remainder of cortex, thereby identifying orbito-frontal fields as primary cortical distribution stations for ascending intralaminar control. On the other hand, Purpura has removed large blocks of frontal cortex and underlying subcortical tissue without disturbing recruitment phenomena.[117] If further structural and functional studies should bear out the importance of the orbito-frontal field as a distribution center, it would be logical to suppose that the further backward spread of recruitment waves over cortex would depend on a complex series of cortical chains of varying lengths.

Conclusions

Disparate but converging bodies of evidence now enable us to define certain characteristics of the reticular core of the brain. It is an archaic, centrally located mosaic of subcenters almost completely shielded from direct contact with the self–world interface, yet continuously apprised of all transactions crossing that interface. Its outputs, continuously variable along an intensity continuum, do not reflect *stimulus mode* so much as *stimulus quantity*. This output is projected simultaneously upon downstream effector centers, upon rostral mechanisms devoted to differentiation and comparation of information, upon primary

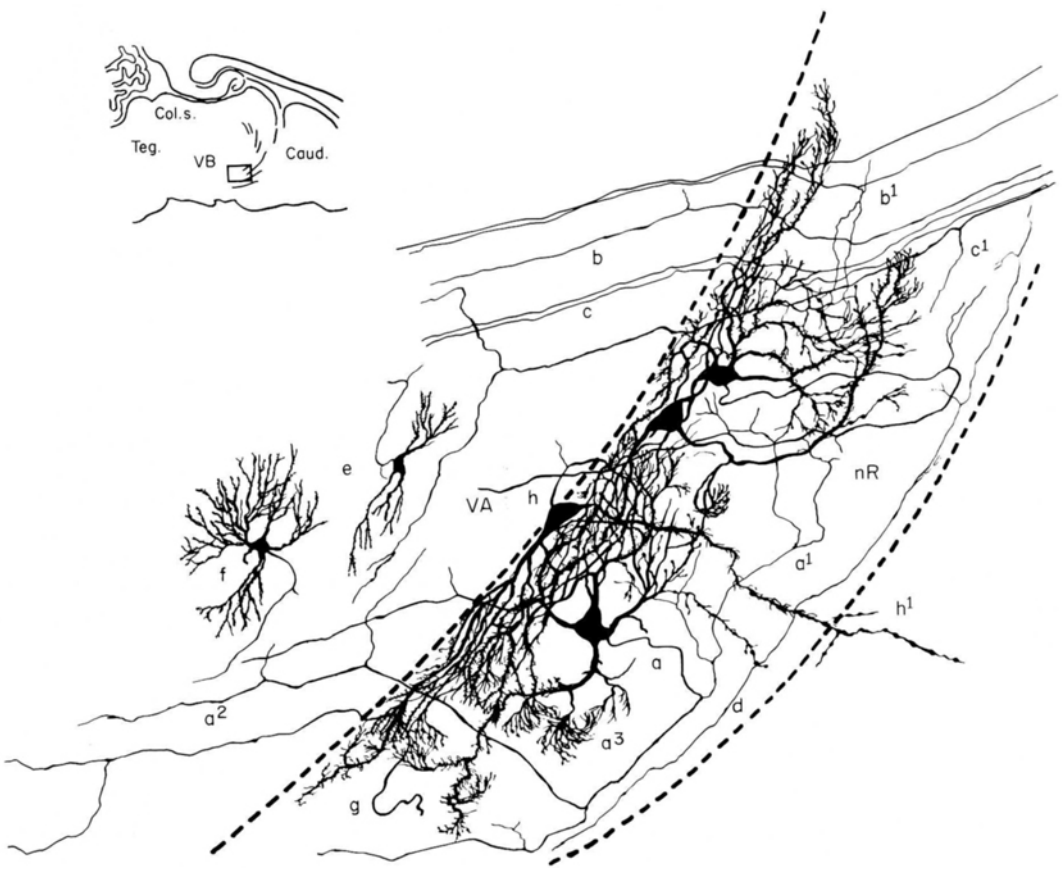

FIGURE 22 Portion of the anterior ventral sector of the nucleus reticularis thalami in 12-day cat as seen in sagittal section. The dense matting of the dendrite mass is seen paritcularly along the inner border of the nucleus, nR; a, initial intranuclear path of an axon with its primary collateral system limited to the n. reticularis; a^1, its caudal path; a^2, penetrating the ventral anterior nucleus, VA; and a typical bushy terminal dendrite structure, a^3. Fibers b and c, are centrifugal and centripetal elements, respectively, penetrating the nucleus and emitting collaterals b^1 and c^1 en passage. The entire observed course of a fiber of unknown origin, d, lies within the nucleus. Two cells, e and f, of the ventral anterior nucleus, show dendritic configuration typical of this field. Axon g is emitted from a n. reticularis cell whose soma remains unstained. Cell h of the n. reticularis emits dendrite h^1, projects into more rostral white matter (cerebral peduncle), and loses its filamentous spines as it does so. Other abbreviations include VB, ventrobasal complex; Col. s., superior colliculus; Teg., Tegmentum; Caud., caudate. (From Scheibel and Scheibel, Note 111)

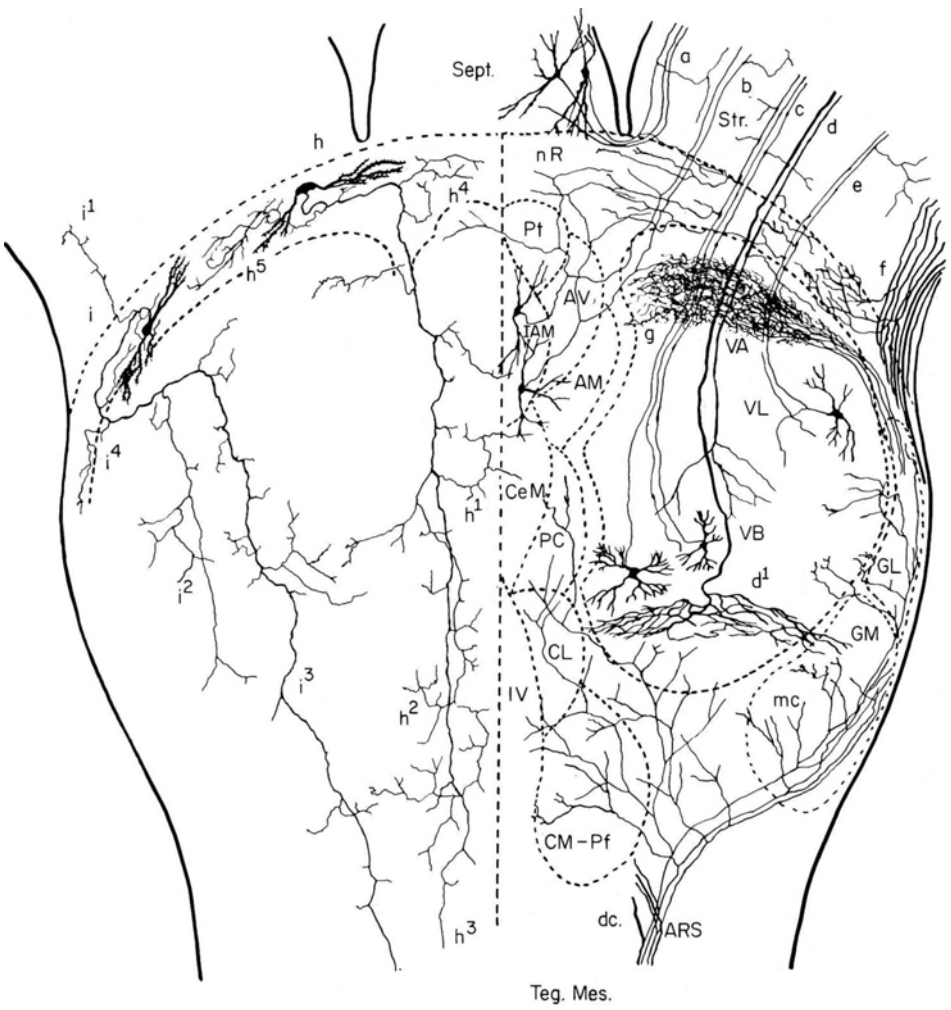

FIGURE 23 Slightly schematized horizontal sagittal section through the thalamus of the mouse, showing some thalamic afferent and efferent constituents on the right, and the course of two nucleus reticularis thalami axons on the left, elements h and i and two n. reticularis neurons. At h^4 and h^5 the axon of h collateralizes within n. reticularis, while $h^{1,2,3}$ represent collaterals generated along the length of the axon in its caudal trajectory and projecting into specific and nonspecific thalamic fields. Similarly, in the case of neuron i, structures marked $i^{2,3,4}$ represent collaterals infiltrating the thalamic ventral and lateral nuclear masses while i^1 represents a short collateral projecting rostrad into the striatum (Str). All other abbreviations as in previous figures. (From Scheibel and Scheibel, Note 111)

and secondary fields that relay raw information rostrally, and upon the remainder of the reticular core itself. Its volume is small in comparison with cortex and cerebellum, and economy probably demands repeated use of its relatively limited amount of modular logic.[62]

It seems likely that stimulus patterns continuously recirculate through the millions of re-entrant loops as decisional modes are reached. At the same time, competition for the interest of the reticular arrays must be high, and neural supremacy is gained for that moment in time only by those data that are most "exotic"—or most compelling biologically. Like some stern, harried father figure, the core has limited patience and limited time-binding resources. Its logic is wide but superficial, and its decisionary apparatus does not permit the luxury of hesitation.

Summary

Modern structural and operational concepts of the reticular core of the brain stem have emerged over the past 60

years, although a number of current ideas were already implicit in studies published at the turn of the century. The complex may be thought of as a mosaic of subcenters placed athwart all input and output systems of the neuraxis, receiving a constant sampling of information from activity ongoing in these systems. This mass of heterogeneous, convergent data is integrated along the postsynaptic membrane of individual reticular neurons whose output represents an algebraic summation of these inputs. The output of these reticular-core elements, representing intensity rather than mode, is then projected rostrally and caudally to modulate the degree of synaptic drive on neurons spread widely throughout cortex, brain stem, spinal cord and, in some cases, as far peripherally as the first-order sensory cell.

An impressive group of physiological mechanisms are subserved by this structural substrate and include gating of sensory inputs; modulation of transmission along major rostral-coursing and caudal-coursing tracts; modulation and control of spinal effector mechanisms; multileveled control over most visceral functions; and the active manipulation of a spectrum of states of consciousness from deep coma through a series of sleeping states to maximal vigilance.

Subcortical and Cortical Mechanisms in Arousal and Emotional Behavior

ALBERTO ZANCHETTI

THE DIFFERENT EMPHASIS that has been placed at different times upon neural activity at either the cortical or the subcortical level can be well exemplified by the history of recent theories of emotion. Psychological thinking on this subject was dominated at the end of the last century and throughout the first three decades of the present one by the so-called James–Lange theory.[1,2] The neural basis for this theory is schematized in the drawing of Figure 1 (left), taken from a paper by Cannon.[3] As Cannon says, "according to the James–Lange theory an object stimulates one or more receptors (R, in Figure 1), afferent impulses pass to the cortex (path 1) and the object is perceived; thereupon currents run down to muscles and viscera (path 2) and alter them in complex ways; afferent impulses course back to the cortex (paths 3 and 4), and when there perceived transform the 'object-simply-apprehended' to the 'object-emotionally-felt'; 'the feeling of the bodily changes as they occur *is* the emotion—the common sensational, associational and motor elements explain all,' to quote James's expression." Obviously, then, in the James–Lange theory the cerebral cortex plays a predominant role in emotion, first in simply apprehending the object, then in feeling it emotionally. If we want to state this somewhat differently, the cerebral cortex enjoys predominant attention in the James–Lange theory because it refers exclusively to emotional *feeling*.

Cannon[3,4] strongly objected to James's and Lange's opinions and formulated the so-called thalamic theory of emotion. The neural basis for this theory was schematized by Cannon[3] himself in the drawing reproduced in Figure 1 (right), and has been conveniently summarized by Lindsley[5] as follows: "An external emotion-provoking stimulus excites receptors (R) and starts impulses toward the thalamus (path 1). . . . Thus efferent discharges are set up in path 2, either through direct activation of the thalamus over path 1 or after impulses have passed to cortex (path 1′), where they inactivate inhibition over path 3, which allows patterned motor responses in the diencephalon to find expression in effectors via path 2. At the same time an upward discharge in path 4 carries to the cortex an appreciation of the pattern just released. . . . The difference between this view and that of James and Lange is that for Cannon 'the peculiar quality of the emotion is added to simple sensation when the thalamic processes are roused.' "

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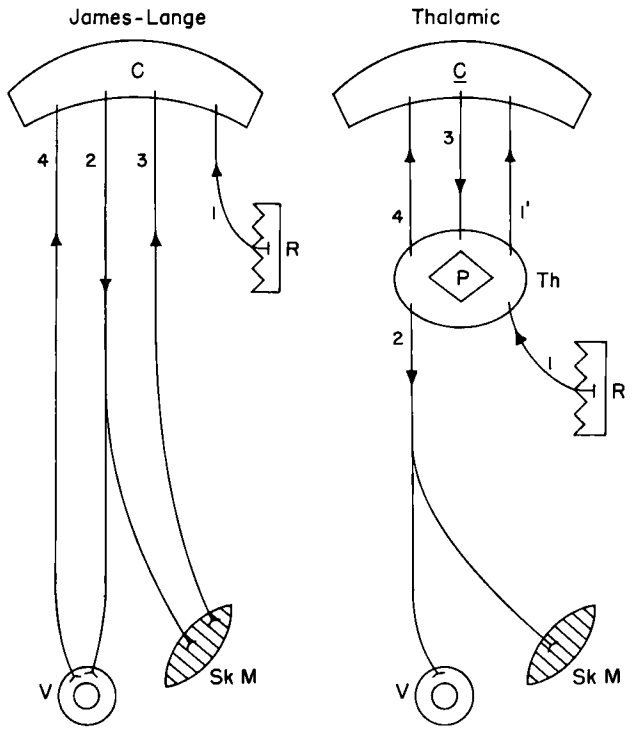

FIGURE 1 Diagramatic representation of the James-Lange (left) and of the thalamic (right) theories of emotion. R: receptors, C: cerebral cortex, V: viscera; Sk M: skeletal muscles; Th: thalamus; P: pattern. The connecting lines represent nervous pathways; direction of impulses is indicated by arrows. (From Cannon, Note 3)

Cannon's theory results from several considerations that run counter to the views of James, as well as from experiments that suggest that the diencephalon plays a dominant role in emotional behavior. Among the former considerations, it has been found that almost complete separation of the viscera from the central nervous system does not alter emotional behavior in animals.[6,7] Among the latter is the fundamental observation, provided by Bard[8] in Cannon's laboratory, that "after removal in lower animals of all of the cerebrum anterior to the thalamus the behavior commonly designated as 'rage' is exhibited; and when in addition the thalamus is removed the reaction disappears."[3]

One should recognize that both kinds of arguments do not really dispose of the James–Lange theory. As Hebb[9] correctly pointed out, James did not claim that abolition of sensory reverberation from muscles and viscera would suppress emotional *behavior,* but rather emotional *feeling.* The fact is that James was concerned only with feelings, and Cannon, although he mentioned the peculiar quality of the emotion, was actually interested only in behavior.

The history of the dispute on the James's and Cannon's theories of emotion is instructive, in that it points to modern psychology's shift in emphasis from feelings to behavior. The strength of the James–Lange theory, which placed it beyond the reach of Cannon's criticism, is the reason for its failure. As has been recently written,[10] "the criterion of a scientific theory is its falsifiability or refutability or testability. . . . A theory which is not refutable by any conceivable event is non-scientific."

More than Cannon, Bard was well aware of what could be learned from a physiological study of emotion when he wrote:[11] "The word emotion implies both a mode of acting and a subjective experience. In experimental work on animals emotions can be studied only as behavior patterns; to consider the subjective aspects of emotion in an animal is to proceed on the basis of an inference which, however plausible, has no place in objective physiological work." This lucid sentence well exemplifies the foundations and the limits of the behavioristic approach to studying emotions. There is no doubt that this is the only means available when the location or the functioning of the neural mechanisms of emotions are to be studied with lesion or stimulation experiments, which obviously cannot be performed on man. Little progress, moreover, can be expected in this field if introspective methods are applied to the study of human emotion.

Before leaving this introductory topic, it should be remembered that in his classical treatise "The Expression of Emotions in Man and Animals" Charles Darwin[12] became the founding father of the behaviorist approach to the study of emotion. While his descriptive techniques may seem rather naive today, his concept that "behavior patterns are just as conservatively and reliably characters of species as are the forms of bones, teeth, or any other bodily structures"[13] paved the way for modern investigation in the field.

It should be clear, therefore, that this presentation deals with emotions only as *patterns of response* and with neural mechanisms as emotional *expression.* Instead of discussing separately the mechanisms of arousal and emotional behavior, I will concentrate on their relationships; that is to say, on the relationships of the mechanisms for emotional expression and the reticular activating system. The role of this brain stem system in mediating arousal and maintaining wakefulness, first described by Moruzzi and Magoun,[14,15] has already been discussed in this volume by Jouvet[16] and by the Scheibels,[17] who have also outlined its anatomy. Therefore I would like to ask the following questions:

1) Are the basic mechanisms for emotional expression a part, although a specialized one, of the reticular activating system?

2) Does the excitability of these mechanisms depend on an ascending activating influence exerted by the reticular system?

3) Are the motor pathways mediating emotional expression a part of the descending reticular system?

Central mechanisms for emotional expression

The location of the centers of emotional behavior has been studied in animals by ablation and stimulation techniques. The first important step was accomplished when Goltz,[18] at the end of last century, and Dusser de Barenne[19] and Rothmann[20] later, showed that dogs and cats deprived of their cerebral hemispheres were nonetheless capable of motor reactions that closely resembled the rage of a normal animal. As I have already pointed out, this observation was such as to displace the neocortex from its high rank as "the seat of emotions," and Cannon[21] stressed the concept that because emotional behavior was a primitive, urgent reaction directed toward the preservation of the individual and common to the various members of the vertebrate series, it was more reasonable to think that this behavior depends on phylogenetically older divisions of the nervous system.

A more precise location of the subcortical mechanisms responsible for emotional expression came from the classical ablation experiments of Bard,[8] who showed that the rage activity of the decorticate cat "is conditioned by central mechanisms which lie within an area comprising the caudal half of the hypothalamus and the most ventral and most caudal fractions of the corresponding segment of the thalamus." However, it could not be inferred from Bard's data whether these mechanisms were diffusely represented in the relatively large area delimited by his research, or instead were restricted to one or more of the different anatomical structures included in the ventroposterior pole of the diencephalon: lateral hypothalamus, posterior hypothalamus, mammillary bodies, subthalamus, and the thalamic nuclei parafascicularis and centrum medianum.

The current opinion that the basic mechanisms of emotional behavior are restricted to the lateral and posterior hypothalamus results from the stimulation experiments of Hess,[22,23] and of Ranson and Magoun.[24] As a matter of fact, when Hess electrically stimulated these areas in an unanesthetized cat, he could induce the motor patterns of rage, and Ranson and Magoun[24] elicited several of the visceral concomitants of rage in an anaesthetized animal. However, electrical stimulation of other subcortical structures, and particularly of the mesencephalic periaqueductal gray substance,[22,23,25,26] can evoke rage activity. Furthermore, it has been shown by several authors[27-31] that rage behavior, although relatively incomplete in pattern, can also be obtained with strong peripheral stimulation in animals with a transection at the mesencephalic-diencephalic border, which has left only the midbrain intact (hereafter called a high mesencephalic animal).

Figure 2 summarizes a more systematic study of the location of the basic mechanisms for emotional expression that we have recently performed in our laboratory. In this study, the intensity of rage patterns and the threshold for their reflex induction have been assessed in animals subjected first to diencephalic sections performed at various levels, and then to selective electrolytic lesions of some of the spared portions of the diencephalon.[32] The anatomical drawings show the residual portions of the diencephalon and mesencephalon in a cat subjected to a brain transection. The operation was similar to the one by Bard[8] that was followed by full rage behavior. In acute thalamic animals such as this, the excitability of rage mechanisms is at its highest, as shown by the frequent appearance of "spontaneous" outbursts of intense rage, with struggling movements, hyperpnea, mydriasis, and rise in blood pressure. When either the lateral and tuberal hypothalamus or the posterior hypothalamic area is further destroyed electrolytically, intensity and excitability of rage activity are not appreciably decreased. Simultaneous destruction of both lateral and posterior hypothalamus, although still permitting strong outbursts of rage, abolishes all spontaneous activity. Rage can be precipitated only by peripheral stimulation. However, very light tactile stimuli are still capable of eliciting rage outbursts, which demonstrates that, even after ablation of the whole hypothalamus, the excitability of the rage mechanisms is still remarkably high, although undoubtedly decreased. Additional destruction of the remaining parts of the diencephalon—the subthalamus and the thalamic nuclei parafascicularis and centrum medianum—further diminishes the excitability of the rage mechanisms. However, fully integrated outbursts of rage can also be evoked in the high mesencephalic animal by strong, noxious stimuli. As this activity is observed a few minutes after the transection at the mesencephalic-diencephalic junction, the rage behavior of the high mesencephalic animal is likely to represent the residual activity of the central mechanisms for rage, whose excitability is simply decreased, but not suppressed, by ablation of the hypothalamus and subthalamus.

It has been contended by Hunsperger[25,33] that destruction of the periaqueductal gray substance suppresses for long periods naturally occurring, affective reactions, as well as those elicited by electrical stimulation of the hypothalamus. Our experience with the sham rage of the acute thalamic cat has been quite different:[34] Figure 3 shows a wide electrolytic destruction of the central gray substance, extending from the periventricular gray sub-

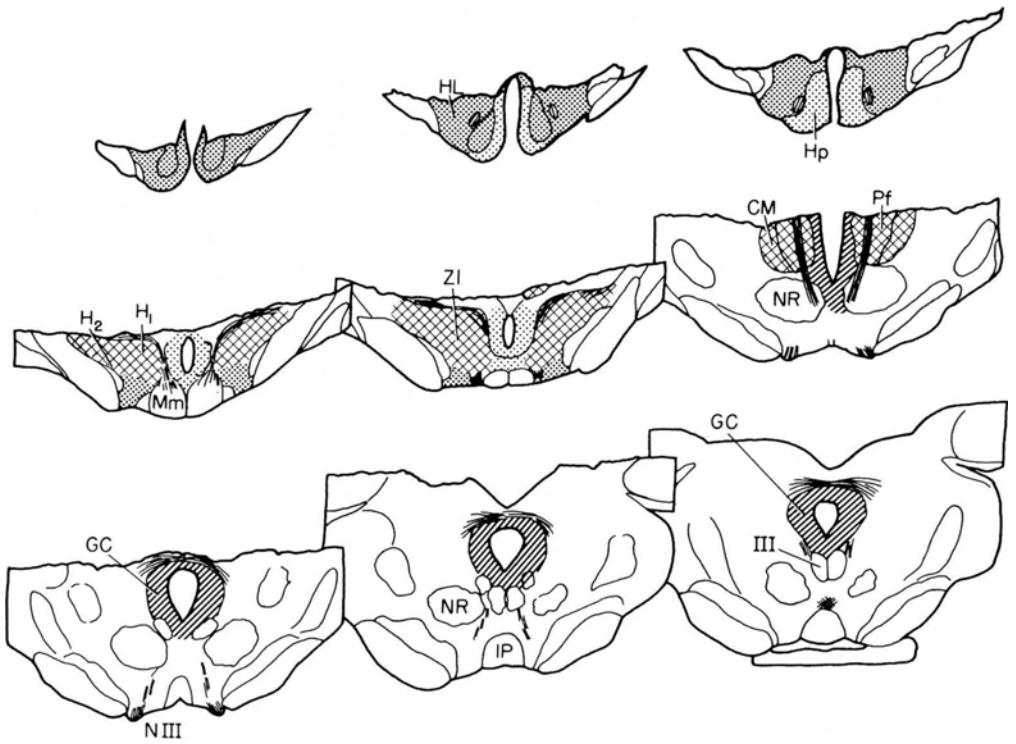

FIGURE 2 Transverse sections through the brain stem of a decorticate cat, showing the residual portions of diencephalon and rostral mesencephalon. The area marked by fine dots indicates the lateral hypothalamus and the tuber; the area marked by large dots indicates the posterior hypothalamus. The cross-hatched area indicates the subthalamic H_1 and H_2 fields of Forel and zona incerta, as well as the thalamic nuclei parafascicularis and centrum medianum. Central periaqueductal gray substance is the area covered by oblique lines. For other explanations, see text.

In this and the other figures the following anatomical abbreviations have been used:

A : anterior thalamic nuclei
Am : amygdala
AVT : ventral tegmental area of Tsai
CA : anterior commissure
CI : inferior colliculus
CL : thalamic nucleus centralis lateralis
CM : thalamic nucleus centrum medianum
CO : optic chiasma
CS : superior colliculus
GC : central gray substance
GM : medial geniculate body
Hb : habenula
Hip : hippocampus
HL : lateral hypothalamic area
Hp : posterior hypothalamic area
HPV : paraventricular hypothalamus
H_1 : H_1 area of Forel
H_2 : H_2 area of Forel
IL : intralaminar thalamus
IP : interpeduncular nucleus
LL : lateral lemniscus
LM : medial lemniscus
MD : thalamic nucleus medialis dorsalis
Mm : mammillary bodies
NCS : nucleus centralis superior
NGD : dorsal tegmental nucleus of Gudden
NGP : ventral tegmental nucleus of Gudden
NR : red nucleus
NRT : nucleus reticularis tegmenti pontis
N III : third nerve
PC : cerebral peduncle
Pf : thalamic nucleus parafascicularis
PM : mammillary peduncle
Pyr : pyramid
RPO : preoptic region
RS : rubrospinal tract
S : septum
SN : substantia nigra
VPL : thalamic nucleus ventralis posterolateralis
VPM : thalamic nucleus ventralis posteromedialis
ZI : zona incerta
III : third nucleus
IV : fourth nucleus

stance in the caudal hypothalamus down to the rostral portion of the fourth ventricle floor. This excision did not prevent the appearance of strong, well-integrated outbursts of rage after a prethalamic transection. This clearly shows that neither the periaqueductal gray substance nor, as it has been pointed out above, the lateroposterior hypothalamus can be considered as the only "seat" of the neural mechanisms for rage behavior. The mechanisms for rage expression appear to be diffuse in the caudal diencephalon and rostral midbrain, including lateral and posterior hypothalamus, subthalamus, the central periaqueductal gray substance, and the rostral reticular tegmentum. Although the hypothalamic areas are necessary for setting the system at its highest excitability, some of the basic circuitry for expression of rage seems to be within tegmental regions that are considered a part of the activating reticular system.

These conclusions do not mean to imply that structures located rostrally to the hypothalamus do not participate in the elaboration of emotional behavior, but simply that they are not strictly necessary for the appearance of this behavior or, to be more precise, for the appearance of those crude patterns of emotional behavior known as sham rage. It should be remembered that the emotional patterns occurring in the thalamic cat, although appearing to duplicate the rage behavior of the intact animal in all its motor and visceral manifestations, are either undirected or very poorly directed toward the environment. This is why the term "sham rage" was coined by Cannon.[35] As to attack behavior induced by hypothalamic stimulation, Masserman[36] maintained that it was not directed, that it could not be classically conditioned, and that it was only a motor automatism, unrelated to motivational or associational systems. On the other hand, Hess,[22] Hunsperger,[25,33] and Wasman and Flynn[37] agree that electrical stimulation of the hypothalamus in intact cats usually ends in directed attack, and more recently Roberts and Kiess[38] have shown that stimulation of the hypothalamic mechanisms for attack behavior is motivational.

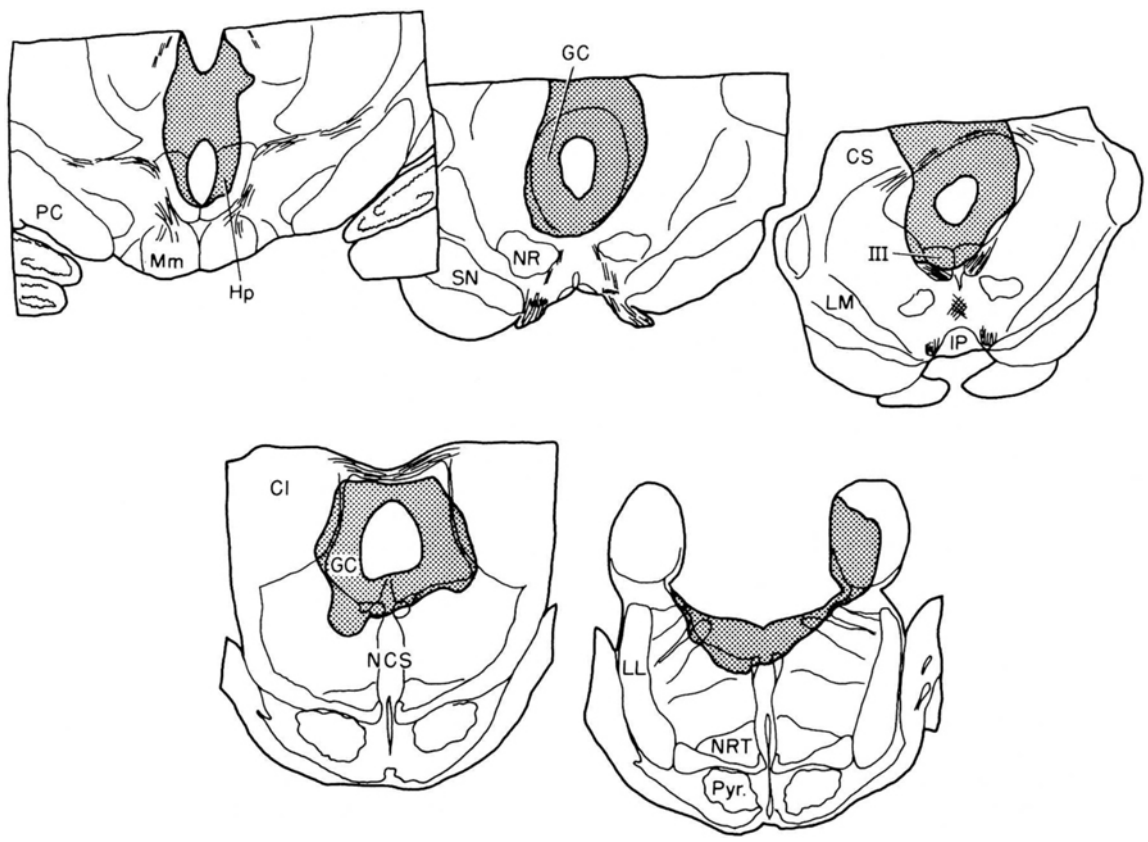

FIGURE 3 Wide electrolytic destruction of the central gray substance extends from the periventricular gray substance in the caudal hypothalamus down to the rostral portion of the fourth ventricle floor. This lesion did not prevent the appearance of strong, well-integrated outbursts of rage after a prethalamic transection. For anatomical abbreviations, see Fig. 2.

Coming back to the role of forebrain structures on emotional behavior, it is well known that they can exert both excitatory and inhibitory influences on the mesodiencephalic mechanisms of emotion. The excessiveness of the rage reaction of the thalamic animal first suggested to Cannon[21] and to Bard[8] that it might depend on release from a descending inhibition of cortical origin. Subsequently, attention was called to the importance of the so-called limbic structures. Figure 4 (A,B) summarizes the numerous descending connections from limbic structures to hypothalamus and midbrain, according to recent data by Nauta,[39] which provide a much more detailed pattern than an oversimplified one suggested in 1937 by Papez.[40] It can be seen from these drawings that both hippocampus and amygdala can articulate, either directly or through septal neurons, with neurons in the lateral preoptic region, and from this area through the medial forebrain bundle, with the lateral hypothalamus and the lateral and midline regions of the midbrain tegmentum (Figure 4A). Further hippocampal and amygdaloid connections articulate at the septal and lateral preoptic regions with neurons giving rise to a more dorsal pathway, which forms the stria medullaris, synapses in the habenula, and is continued by the fasciculus retroflexus onto midline and lateral regions of the midbrain tegmentum (Figure 4B).

A host of ablation and stimulation experiments have shown that emotional behavior can be altered, in either direction, by manipulation of various portions of the limbic system. Unfortunately, there is still no definite agreement on the part played by each structure. Although I do not intend to enter into details of this highly controversial subject, I shall cite the opposite effects of amygdalectomy described by Bard and Mountcastle,[41] who obtained increased susceptibility to rage behavior, and by Klüver and Bucy,[42] who observed placidity as a typical feature of their classical temporal lobe syndrome (see Note 43 for more references). Recent observations by Egger and Flynn,[44]

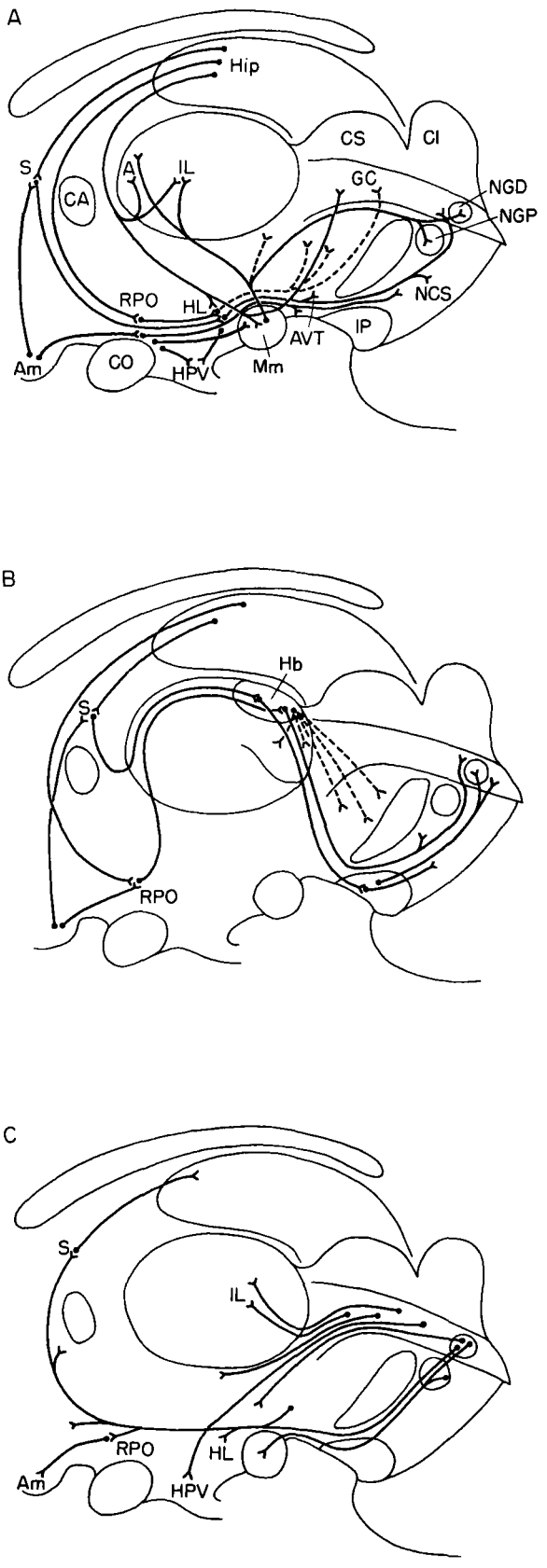

FIGURE 4 Diagramatic representation of the connections between some limbic forebrain structures and the midbrain. Broken lines indicate projections to central and lateral regions of the brain stem tegmentum. Pathways distributing to medial tegmental regions are indicated by solid lines. A: descending pathways from limbic system to midbrain—fornix, medial forebrain bundle, and mammillo-tegmental tract; B: pathways to stria medullaris and fasciculus retroflexus. C: ascending projections from midbrain to limbic system: dorsal longitudinal fasciculus, medial forebrain bundle and mammillary peduncle. For anatomical abbreviations, see Fig. 2. (From Nauta, Note 39, modified)

who have studied inhibition and excitation of rage behavior by stimulation of different regions within the amygdala, might provide, if confirmed by other authors, a basis for reconciling the opposite evidence obtained by lesions of this structure.

Ascending mechanisms for arousal of emotional behavior

Our next question is: "Through which pathways can the environment arouse emotional behavior?" Peripheral stimuli easily elicit outbursts of rage in the acute or chronic hypothalamic animal. This shows that afferent impulses can influence the mesodiencephalic mechanisms of emotional expression independently of neocortical or forebrain loops. Often trifling somatic stimuli are sufficient; a rage outburst can follow gentle touching of a forepaw. This effect is easily duplicated by electrical stimulation of low-threshold cutaneous fibers, which are known to be tactile in character.[45]

As peripheral somatic stimuli can induce rage behavior even in the hypothalamic preparation, that is, when the whole or almost the whole thalamus has been ablated, it follows that afferent impulses are likely to be conducted to the central mechanisms for emotional activity through other pathways than the classical lemniscal ones that project to the specific thalamic nuclei. Several ascending systems are to be taken into consideration: 1) the spinothalamic system,[46] as in most hypothalamic animals, at least part of the posterior group and the nuclei parafascicularis and centrum medianum are spared; 2) several pathways connecting the midbrain with the hypothalamus and the limbic system, which have been recently studied in detail by Nauta,[39] Guillery,[47,48] and Cowan, et al.,[49] and which make up what Nauta[39] has called the ascending portion of the limbic-midbrain circuit—these latter pathways include the mammillary peduncle and the ascending components of both the medial forebrain bundle and the dorsal longitudinal fasciculus (Figure 4C); 3) the anatomical substrate of the ascending reticular system.[15,50]

It is fortunate that these ascending pathways run partially segregated at the midbrain level, where the classical lemniscal and spino-thalamic systems are laterally placed; two of the pathways to the hypothalamus and limbic system (mammillary peduncles and medial forebrain bundle) run medioventrally and one (dorsal longitudinal fasciculus) runs mediodorsally. The reticular activating paths are spread through the tegmentum, with preferential distribution in its dorsolateral portions. Figure 5 shows that neither a lesion of the periaqueductal gray substance, including the ascending fibers of the dorsal longitudinal fasciculus, nor a wide interruption of the caudal component of the mammillary peduncle and medial forebrain bundle, nor a more complete interruption of the caudal and rostral components of the two systems, could prevent either the spontaneous outbursts of sham rage of the hypothalamic cat or those evoked by somatic stimuli. Neither was this type of behavior impaired by laterally placed lesions, which interrupted the medial lemnisci and the spinothalamic tracts.[51,52]

As to reticular lesions,[51,52] those that were not large enough to change the normally desynchronized electroencephalogram into permanently synchronized patterns were equally unable to impair the sham rage behavior that appeared after subsequent ablation of the forebrain (Figure 6). Only large tegmental destructions (including lateral and midline regions) sufficient to synchronize the electroencephalogram, constantly prevented the occurrence of spontaneous and somatically evoked sham rage behavior after decortication (Figure 7). Persistence of rage outbursts upon direct stimulation of the hypothalamus indicates that the disappearance of spontaneous and reflex outbursts of sham rage did not result from complete interruption of the descending pathways from the hypothalamic rage centers.

It seems reasonable, therefore, to envisage the activating reticular system as maintaining the central excitatory state of the diencephalon at the level essential for rage outbursts to appear. Sham rage would not be aroused in the hypothalamic animal, in spite of release from forebrain inhibition, without the tonic exictatory support and the phasic activation of the activating reticular inflow.

Motor pathways for emotional expression

Our third question concerns the motor pathways for emotional expression and, more particularly, whether emotional behavior is manifested through those descending chains of tegmental neurons that are so strictly intermingled and associated with the ascending arousal system and are grouped together under the definition of descending reticular system.[15]

There have been contrasting reports on this topic. As early as 1930, Beattie, Brow, and Long[53] maintained that efferent pathways from the posteromedial part of the hypothalamus could be traced, anatomically and physiologically, to the central periaqueductal gray and the adjacent portions of the dorsal tegmentum. More recently, Hunsperger[25] pointed out that damage to the mesencephalic periaqueductal gray led to a prolonged disappearance of the "affective defence reaction" induced by stimulating the perifornical areas of the hypothalamus in unanesthetized cats. On the other hand, Magoun, Ranson, and Hetherington[54] showed that pressor and respiratory re-

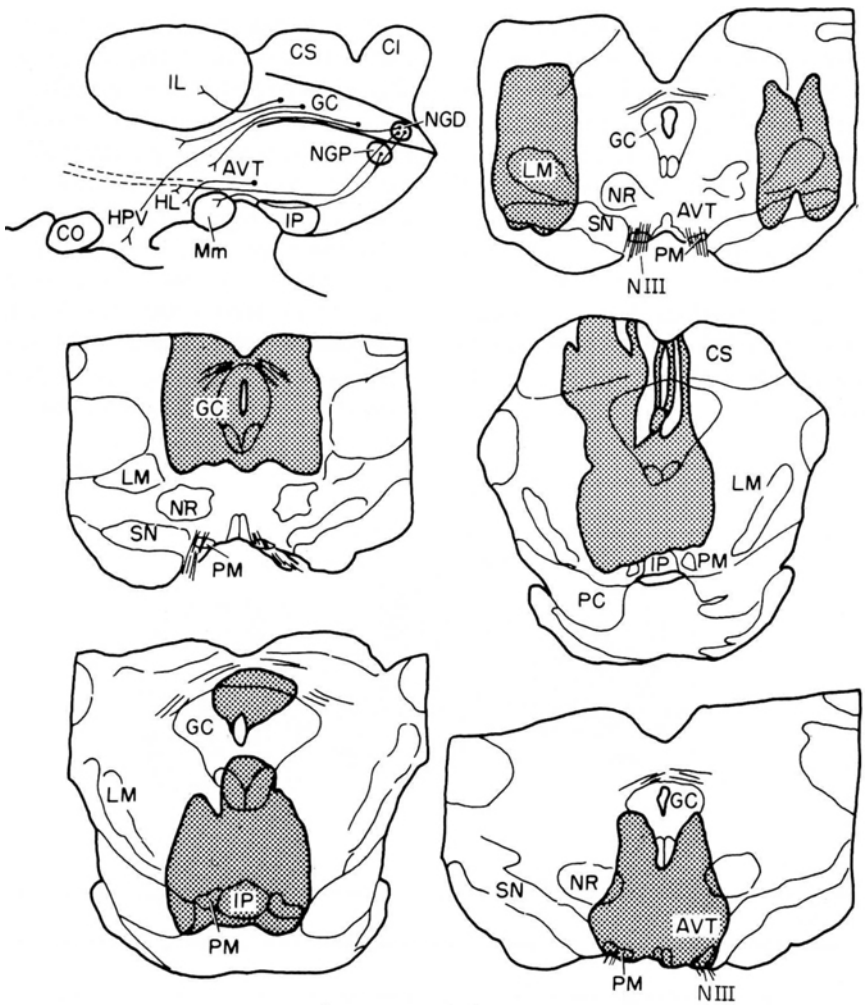

FIGURE 5 Midbrain lesions that do not prevent sham rage behavior in the thalamic cat. *Top left:* anatomical scheme showing the ascending connections to the hypothalamus; *Right:* interruption of the medial lemnisci and classical spino-thalamic tracts. *Middle left:* destruction of the rostral pole of the mesencephalic central gray substance with interruption of the dorsal longitudinal fasciculus; *right:* medial tegmental lesion. *Bottom left:* destruction of medial forebrain bundle and mammillary peduncles in the caudal mesencephalon; *right:* more complete interruption of the same ascending pathways in the rostral mesencephalon. For anatomical abbreviations, see Fig. 2. (From Zanchetti, Note 51)

sponses to lateral hypothalamic stimulation could survive a number of different lesions in the midbrain. They concluded that descending connections from the hypothalamus occupy a wide area in both the central gray and the mesencephalic tegmentum.

More definite conclusions, however, had to wait a more-detailed knowledge of descending hypothalamic projections, such as that recently provided by Nauta.[39] The drawings of Figure 4 (A,B) summarize the course of these pathways. 1) Fibers of the medial forebrain bundle originating from lateral preoptic regions (where their activity can be influenced by hippocampal, amygdaloid, and septal projections) and from the lateral hypothalamus distribute to the ventral tegmental area of Tsai; then they course caudally through the interpeduncular region, finally arching dorsally to be distributed to the nucleus centralis superior and to the caudal part of the periaqueductal gray substance, including both Gudden's nuclei. 2) The mammillo-tegmental tract, originating from the mammillary bodies (which receive massive hippocampal projections through the fornix), courses dorsally, ventral to the medial longitudinal fasciculi, and ending in the caudal-

most periaqueductal gray substance and Gudden's nuclei. 3) The fasciculus retroflexus originates from the habenula (where hippocampal and amygdaloid projections are conveyed through the stria medullaris) and terminates massively in the interpeduncular nucleus, where it articulates with a path directed to nucleus centralis superior and Gudden's nuclei. 4) The dorsal, longitudinal fasciculus connects the medial and periventricular hypothalamus to the periaqueductal gray and to the dorsal nucleus of Gudden. 5) Finally, the medial forebrain bundle also sends a massive lateral component, which spreads over the tegmental region occupied by both the ascending and descending reticular systems.

Briefly stated, experiments in which selective lesions of each of these pathways have been performed[55] have shown that lateral hypothalamic stimulation in the acute thalamic cat can as easily evoke rage behavior before and after complete interruption of all medially coursing descending pathways (Figure 8). On the other hand, large tegmental lesions, involving medial and lateral regions, had a much more dramatic effect. However, if some portion of the tegmentum was spared, such as the right dorsolateral region and the interpeduncular region shown in Figure 9, hypothalamic stimulation could still evoke some components of rage behavior, although the threshold had remarkably increased and the pattern of response had de-

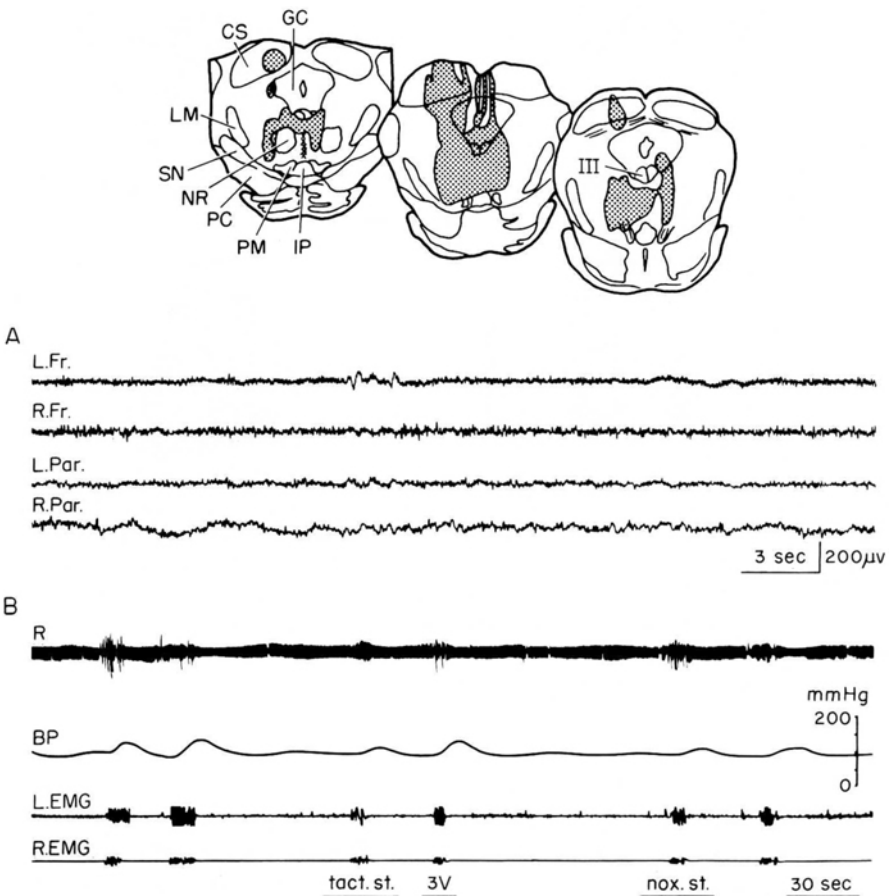

FIGURE 6 Persistence of spontaneous and evoked sham rage activity following a reticular lesion compatible with electroencephalographic patterns of arousal. A: electroencephalogram recorded two hours after the lesion shown in the anatomical drawings. Animal otherwise intact. L.Fr., left frontal; R.Fr., right frontal; L.Par., left parietal; R.Par., right parietal leads. B: rage behavior observed following subsequent decortication. In this, as well as in Figs. 7, 8, and 9, R is respiration; BP, blood pressure; L. EMG and R. EMG, electromyogram of the left or right triceps brachii; *tact. st.* and *nox. st.*, tactile or noxious stimuli of the body surface. When indicated, the voltage (V) is that of 100 cycles/sec, 1 msec pulses applied to the lateral hypothalamus. Rage outbursts not otherwise labeled are spontaneous. For anatomical abbreviations, see Fig. 2. (From Malliani, et al., Note 52)

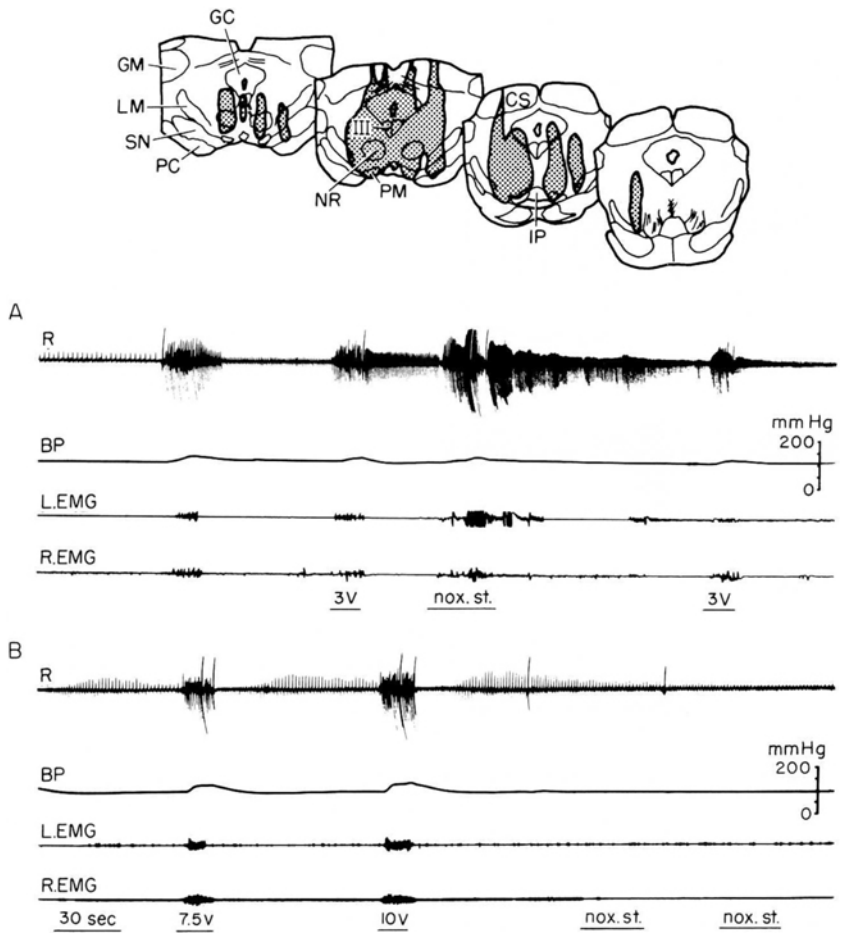

FIGURE 7 Disappearance of spontaneous and evoked sham rage activity following extensive damage of the midbrain reticular formation. A, before, and B, after the lesion shown in the anatomical drawings. For anatomical abbreviations, see Fig. 2. For other explanations, see Fig. 6. (From Malliani, et al., Note 52)

teriorated. On the whole, these data confirm that the pathways from the hypothalamus responsible for emotional expression are scattered through the midbrain, but are largely, although not exclusively, distributed upon descending reticular neurons.

Conclusions

The data I have discussed fit well into what Lindsley[5] defined as an "activation theory" of emotion. I have summarized them in the drawing of Figure 10, which is a modification of a somewhat similar figure of Lindsley.[5] Apart from specific influences conveyed to thalamus and cortex through the specific pathways, peripheral receptors of any kind activate the brain stem reticular formation, resulting in widespread electroencephalographic desynchronization and cortical arousal. Simultaneously, the activated reticular system excites subcortical structures involved in somato-visceral integration; these structures include large portions of the hypothalamus and the most rostral pole of the midbrain reticular formation, as well. These structures discharge toward the periphery by way of the descending chain of reticular neurons, so that the body is aroused simultaneously with cortical arousal. Normally, this does not result in emotional behavior, unless because of the strength or the nature of the stimulus, or because of the central excitatory state, hypothalamic and reticular neurons are not induced into an appropriate level of activity. In setting the level and patterning the discharge, excitatory and inhibitory influences from the limbic system and probably from the neocortex are likely to be of paramount importance, but their actual way of working is still poorly understood.

Does the "activation theory" of emotion imply a continuum of increasing reticular activity from coma through sleep, quiet wakefulness, arousal, up to emotion? One must admit that there are a few difficulties to overcome before accepting the continuum hypothesis. Indeed, it has been reported[26] that in the intact animal, electrical stimulation of the reticular formation at intensities higher than

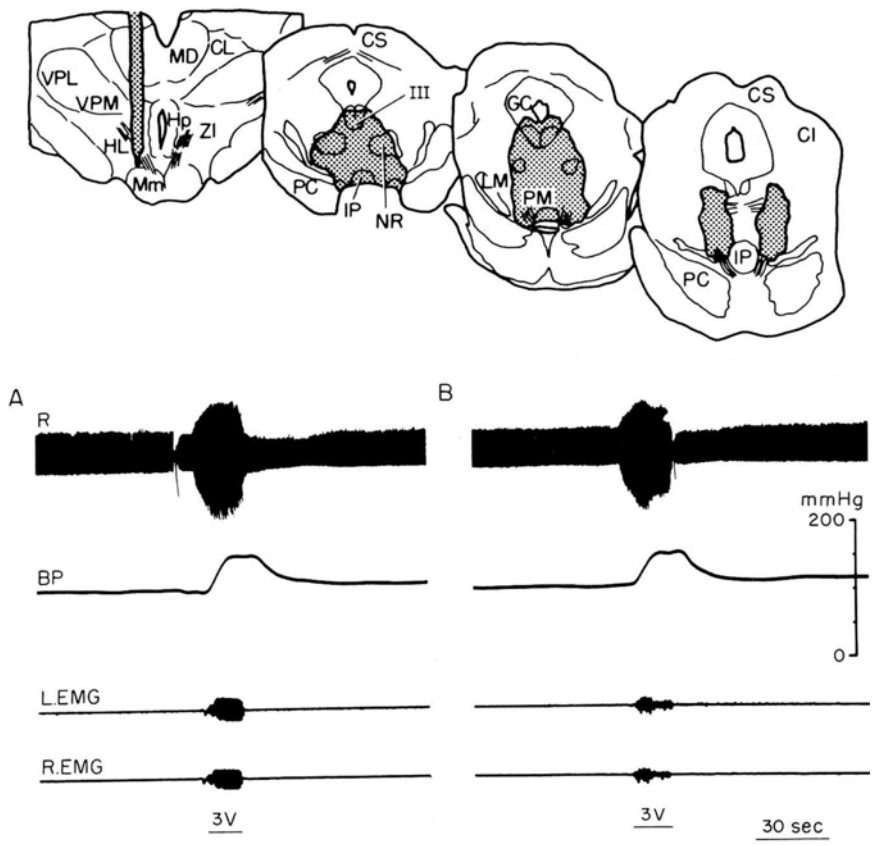

FIGURE 8 Effect of a medial tegmental lesion on rage outbursts induced by electrical stimulation of the lateral hypothalamus. A, before, and B, after the lesion shown in the anatomical drawings. For anatomical abbreviations, see Fig. 2. For other explanations, see Fig. 6. (From Carli et al., Note 55)

those inducing arousal does not evoke emotional behavior. This might be caused, however, by the activation of neocortical[56] or limbic feedback circuits, which prevent reticular excitation from rising too high. Indeed, in a hypothalamic animal in which all forebrain systems are ablated, electrical stimulation of the reticular formation easily elicits sham rage behavior.[57] Also, stimuli which in the intact animal are not even capable of inducing arousal, such as stimulation of group I muscle afferents,[58,59] can evoke sham rage when the excitability of hypothalamic and reticular neurons is increased by ablation of the forebrain.[60] Finally, those few visceral aspects of arousal and emotion that have been studied so far have been found to be remarkably similar,[61,62] although of greater magnitude during emotional behavior.

An alternative to the continuum hypothesis, or at least a way of phrasing it more cautiously, is to suggest, as did Dell,[63] that reticular activation simply "provides the active source of continued excitation" that is necessary for various types of behavior to occur. In order to define this reticular function in one word, Dell[63] has revived the term "vigilance" with the meaning originally used by Head[64] to indicate a raised background of central excitability.

So far, I have managed to avoid introducing the concept of motivation. At this point, however, it is obvious we must ask ourselves whether the "activation theory" of emotion implies that the reticular system itself is the motivational mechanism for emotion, and possibly for other behaviors. Once again, it is not necessarily so. The concept of vigilance, or of heightened activation, is perfectly compatible with the possibility that extrareticular mechanisms—hypothalamic, limbic, or even neocortical—may exert motivational effects on emotional behavior. The poorly directed behavior of the hypothalamic animal and the consideration that directed attack with motivational properties has been observed only by hypothalamic stimulation when the whole brain is intact, strongly suggest that arousal of emotional behavior normally depends on several interactions in which limbic and neocortical structures, as well as hypothalamic and reticular circuits, are importantly involved. In this viewpoint, it is not meaningless that limbic[39] and neocortical[15] areas project down onto the reticular system, which in its turn keeps these forebrain structures under its control.[15]

I am fully aware that I have provided an extremely oversimplified, and perhaps misleading, picture of the nervous mechanisms of emotional expression. Indeed, I

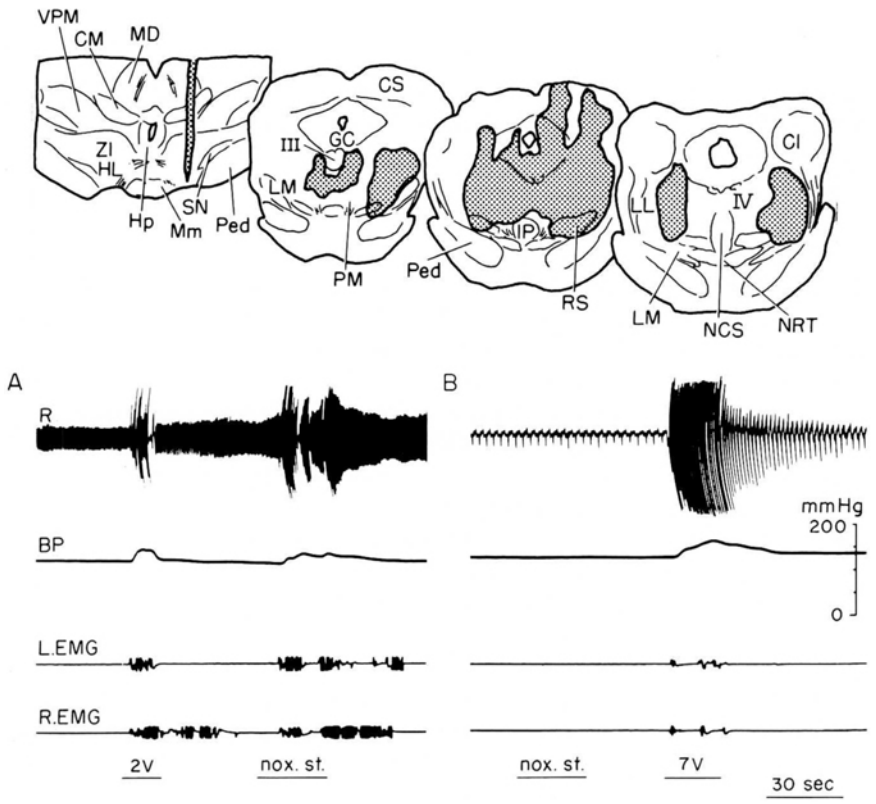

FIGURE 9 Effect of a wide midbrain lesion, involving both medial and lateral tegmentum, on rage outbursts induced by electrical stimulation of the lateral hypothalamus. A, before, and B, after the lesion shown in the anatomical drawings. For anatomical abbreviations, see Fig. 2. For other explanations, see Fig. 6. (From Carli et al., Note 55)

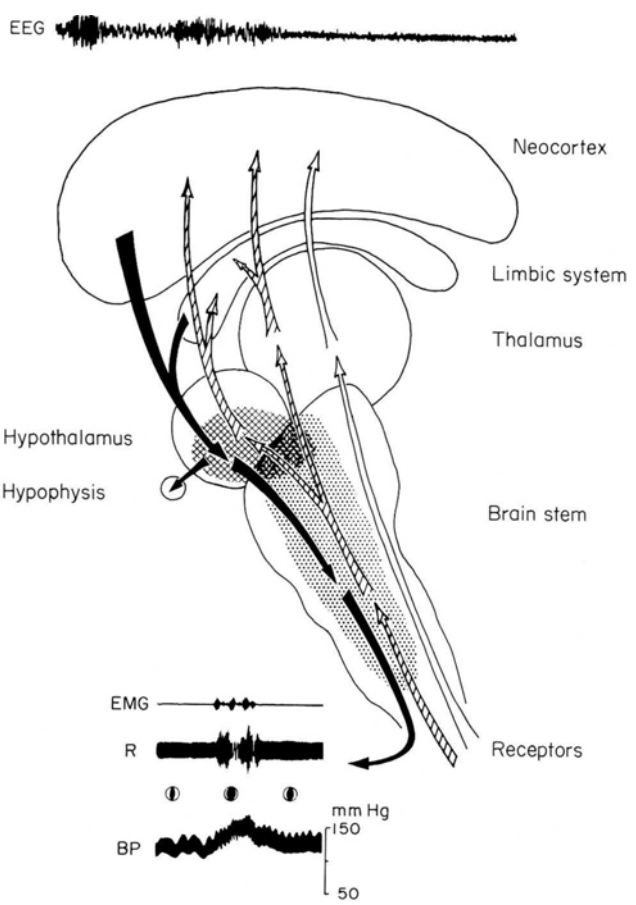

FIGURE 10 Importance of the brain stem reticular formation in mediating both emotional experience and emotional expression. Peripheral receptors, through specific sensory pathways (white arrows), influence specific thalamic nuclei and specific sensory areas of the cortex; these mechanisms are essential in sensory perception, but not in emotion. Peripheral receptors also influence the brain stem reticular formation (stippled area): ascending reticular influences (striped arrows) act upon limbic system and neocortex, eliciting an arousal reaction (see EEG at top of figure), which is the necessary excitability background for emotional experience. Reticular influences also act on the mesodiencephalic mechanisms for rage (cross-hatched area). Rage expression is induced through descending reticular pathways (black arrows), and consists in both somatic and visceral phenomena, as shown below figure. Ascending and descending interrelationships between hypothalamus, limbic system and neocortex are also indicated.

have limited myself mainly to discussing the rage of the hypothalamic animal—a crude type of emotional behavior —which is induced by trivial peripheral stimuli rather than by the very complex patterns, including learned associations, memories, etc., that act as stimuli in everyday life. However, the scheme of the "activation theory" of emotion (Figure 10) can easily be read upside down; the stimulus may well start from forebrain mechanisms and project downward to arouse the activating reticular system and the hypothalamus with consequent re-excitation of limbic and neocortical areas.

In spite of this flexibility, it cannot be denied that our schematic picture is likely to become less and less reliable as the emotional behavior to which it should apply becomes more and more complex. While the role of generalized activation is easily understood in the sham rage of the hypothalamic cat, in natural feline anger, and even in the easily displayed rage of the Homeric heroes, its role should become subtler, less unspecific, more directed, merged with inhibition, in order to fit into the emotional behavior of the self-restrained, frustrated *Homo sapiens* of the twentieth century. It is hoped, however, that knowledge of the simplest machinery for emotional expression in the carnivore brain may help us to anticipate the much more refined research that is still ahead.

Summary

This chapter deals with emotions only as patterns of response and with the crudest but more basic machinery of emotional expression. The rage behavior of the hypothalamic preparation is taken as an experimental model.

The summarized data show that the central mechanisms for rage expression interact with the reticular activating system responsible for arousal at three levels. First of all, the mechanisms for emotional expression belong in part to the activating reticular system. Second, emotional behavior can occur only when the activating function of the reticular system is not impaired. Third, the somatic and visceral manifestations of emotional behavior result largely from discharges running from the hypothalamus and rostral midbrain through the descending reticular system.

These data fit well into the "activation theory" of emotion and suggest that reticular activation must provide the continuous source of excitation necessary for emotional behavior.

Intrinsic Organization of Cerebral Tissue in Alerting, Orienting, and Discriminative Responses

W. R. ADEY

IF OUR STUDIES of brain functions are to lead ultimately to an understanding of the mechanisms of storage of information in cerebral tissue, and equally importantly, to ways in which its retrieval occurs, we must unrelentingly pursue processes which might have a causal relation to, and thus uniquely characterize, the laying down of a memory trace. It has not been clear to what extent, if any, electrical events in nervous tissue might bear a causal relationship to the initiation of those structural modifications in and around cerebral neurons that are presumably the basis of information storage.

In these circumstances, one might seek evidence in certain of the electrical signatures derived from brain tissue, that would reveal their preferential relation to phenomena of storage of information, as opposed to its mere transaction. That we should seek such differences should be made clear at the outset, for it will remain the central theme of this review. It may well be a herculean task, challenging the limits of current technologies and inviting our most earnest pursuit of new transducing methods in brain tissue. Yet without such a philosophy, there is no reason to suppose that even the most earnest study of ever-finer details in synaptology and axonology will afford us a frame for observation and comprehension of this most unique quality of brain tissue. The functional correlates of information storage demand consideration of an anatomical frame that might spell out with equivalent clarity structural interrelations in cerebral tissue consistent with findings in physiological studies of learning mechanisms.

Whether we have succeeded at this time in establishing even a minimal basis for the structural and functional basis of information storage in brain tissue must remain for future investigation. We shall review even finer evidence relating the wave processes in cerebral tissue both to intracellular events, and to specifiable behavioral responses in alerting, orienting and discrimination; and at the cellular level, the relations between intracellular waves and those recorded grossly from a domain of tissue in the EEG. In the latter area, we shall examine the premise that mere possession of a "wiring diagram" of characteristically simpler structures, such as the cerebellum or hippocampus, might provide us with that flash of insight by which the very fundaments of cerebral processes might stand revealed. Hopefully, we shall turn away from such notions, recognizing in both structural and functional organization of cerebral tissue aspects of uniqueness that would effectively delineate it from other quite complex central neural structures, not so endowed with a capacity for ready storage and retrieval of information.

In the very recognition of such a possibility has come an awareness that modification of state consequent upon information storage may not be the exclusive prerogative of the neuronal compartment in cerebral tissue. Impedance measurements in cerebral tissue have a regional specificity in changes that accompany both alterations in broad general states of sleep and wakefulness, as well as in brief impedance transients occurring in alerting, orienting, and discriminative responses. Further qualitative identification of the origins of these impedance changes has been gleaned following degenerative removal of the bulk of neuronal elements from cerebral nuclei, and has directed our attention to their possible genesis in mucoproteins and mucopolysaccharides distributed in intercellular fluid, where divalent cations, such as calcium, may modulate these macromolecular configurations, and thus indirectly influence ionic fluxes across the neuronal membrane.

Salient aspects of a tricompartmental model of cerebral tissue

THE NEURONAL COMPARTMENT Nerve cells in mammalian cerebral cortex are arranged in a pattern characteristically described as laminar. The degree of orderliness in this layering varies from one cortical region to another, and is often a highly subjective criterion.[1] A unique and characteristic feature of cerebral cortex, on the other hand, and also of cerebral ganglia of many invertebrates, is the great overlap of the dendritic branches of one neuron with those of adjacent neurons, with dendro-dendritic contacts as close as in synaptic junctions.[2] Possible functional interactions which might be initiated in this way are now

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being investigated, although numbers and locations of such contacts on any particular neuron remain unknown. Nevertheless, it is necessary that we consider such structural relations, since they may determine the degree of certain aspects of "coupling" between a neuron and others in its vicinity, in their mutual interaction in certain slow wave processes, to be discussed below.

THE NEUROGLIAL COMPARTMENT The interrelation between neuronal and neuroglial elements in cerebral tissue has been the subject of elegant reviews elsewhere in this symposium. Only certain features will be emphasized here. It appears indubitable that by their intervention between nerve cells and the vascular apparatus, neuroglial cells can exercise a regulatory function on neuronal metabolism. The neuroglial envelope has been described by some as essentially complete around individual neurons, while others have noted a more restricted "packeting" of glial elements around synaptic terminals, and with relatively bare intervening areas.[3] Enzymatic localization has been described in adjacent membranes where neuronal and neuroglial cells are in contact, and similarly, where neuroglial elements are in contact, but not where neuronal elements adjoin one another.[4]

THE EXTRACELLULAR SPACE There remains for consideration a third compartment, the extracellular, traditionally given scant attention in most histological descriptions, but which may rank equally with the other two in functional significance. This compartment might be considered of little importance if our notions of cerebral organization required it merely to contribute sodium ions. The size of the extracellular compartment has been variously estimated, but recent cytologic and chemical evidence has suggested a larger space than earlier electron microscope studies had indicated, and probably occupying as much as 15 to 20 per cent of the cerebral volume.[5] In sections of material prepared in ethylene and propylene glycol, and in the absence of calcium salts, Pease[6] has disclosed substantial amounts of material in intercellular clefts in cerebral cortex, not revealed in classical electron micrographs prepared from fixed material. This substance stains strongly with phosphotungstic acid at *p*H 3.5, thus differing from typical mucopolysaccharides, and its chemical identity remains undisclosed.

Movements of ions in the extracellular space will be substantially modified by the presence therein of macromolecules exhibiting ion-binding and fixed-charge characteristics,[7,8] so it is not possible to model ionic behavior in the perineuronal environment from consideration of a mere aqueous solution permitting unimpeded flow. Impedance phenomena to be presented below require consideration of such macromolecules in terms of dynamic interactions with cellular elements that they surround. Conductance characteristics in the narrow clefts between neuronal membranes and adjacent neuronal and neuroglial elements will depend on their macromolecular content.

We may consider the extracellular compartment and its macromolecular systems as susceptible to ionic fluxes from neuronal elements on the one hand and neuroglial on the other. Moreover, by the very nature of these macromolecular systems in this middle zone, which are exceedingly susceptible in their molecular conformation and ion-binding characteristics to the presence of divalent cations, such as calcium, they may exert an important influence on ionic exchanges across neuronal and neuroglial membranes.[9] Bennett[10] has termed these macromolecular envelopes "glycocalyces," and has suggested for them a direct role in determining differential entry of sodium and potassium to positions close to the plasma membrane. Others have proposed a role restricted to pinocytotic transfer of vesicular material, and specifically excluding a role in ionic mechanisms.[11]

It is proposed to consider this intercellular material from two aspects: firstly, as a source of fixed charges on the membrane surface, responsible for electrokinetic phenomena in the presence of an external EMF, and secondly, as the probable site of impedance changes in cerebral tissue accompanying transient alterations in its physiological state, including the recall of stored information.

THE PRESENCE OF FIXED CHARGE IN THE CELL MEMBRANE The ability of macromolecules at the cell surface to exhibit fixed charges has been extensively tested in our laboratory by Elul,[12,13] in movement of cells exposed to focal electric fields generated in the vicinity of micropipettes. Neurons, glia, and connective cells in tissue culture, as well as malignant cells and human erythrocytes in suspension, move toward the pipette when current is passed out of it, and repelled by passage of current in the opposite direction (Figure 1).

The focal electric field was generated by passing 5×10^{-7} to 1×10^{-5} A out of the micropipette, and induced movement of cells at a distance of 5 to 50 μ from the electrode tip. With cells embedded in culture, only local deformation of the membrane was observed, but once set free from the culture, cell movement replaced local deformation. Metabolic poisons had no effect on the movement, nor was it disturbed by rupture of the cellular membrane. Isolated membrane fragments showed movements similar to intact cells.

Such observations require careful exclusion of certain artifacts if the movements are to be attributed to a net membrane charge. Elul points out that the movements

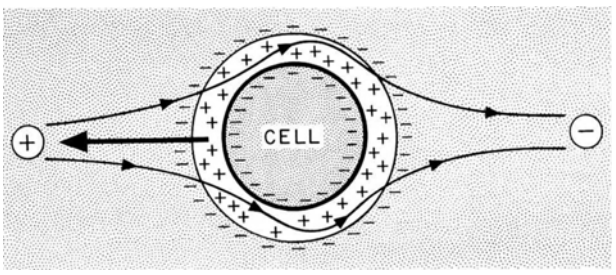


FIGURE 1 Induction of movement in a body having net fixed surface charge in the presence of a non-uniform electric field. (From Elul, Note 13)

are not dependent on concentration of solutions in the micropipettes nor on the composition of the culture medium. The possibility of artifacts due to the proximity of the focal electrode was considered and excluded, including electroosmosis, and electrophoretic effects due to chemical potential gradients, or electric potential gradients, or to electrochemical gradients. It is therefore postulated that the movement is caused by a force acting on cells placed in the electric field. Such a force might arise in two ways: either by a difference between the dielectric constants of a neutral solid body and that of the medium in which it is placed, or by the presence of a net electric change on the solid body. Analysis of the former possibility indicates that cells would exhibit a tendency to move to the locus of minimal electric field intensity, or in other words, to drift away from the focal electrode regardless of current polarity.

In summary, the only feasible mechanism appears to be that involving net electric charge, and subject to Coulomb's law. These charges must be firmly bound to the structural matrix of the membrane, since they persist in incubated membrane fragments. In such a scheme, selective permeability in the cell membrane may be envisaged as a two-step process. There would be an accumulation at the external surface of the membrane of potassium ions above their bulk concentration, and at a higher ratio to sodium ion concentration than in the solution. Transfer of potassium ions across the membrane would ensue by one of the mechanisms widely proposed for membrane transport, be it either by the Donnan mechanism, or a "pump," or an ionic exchange mechanism. It may be emphasized that in such a two-step mechanism, the requirements in ionic specificity would be a function of the macromolecular groupings, rather than of the intrinsic metabolic functions of the membrane.

The genesis of electric waves in brain tissue and their relation to cell firing

In extracellular records, paired microelectrodes separated by only 30 μ record a spontaneous electroencephalogram of apparently normal form and amplitude.[14] Moreover, no similarity could be found between monopolar and differential derivations of both spikes and slow waves recorded simultaneously with such small tip separations. This emphasizes that the dimensions of cortical dipoles generating the EEG are not necessarily larger than the dimensions of single cells.

THE GENERATION OF NEURAL WAVES In further investigations by intracellular recording, Elul[15] noted that, although no EEG could be recorded with a liquid-filled micropipette on approaching the membrane of a single neuron with recording sensitivities in the low millivolt range, large, rhythmic waves were seen immediately after penetration in unanesthetized cortical neurons, superimposed on the resting membrane potential (Figure 2). These waves have an amplitude of 5 to 15 millivolts, and are hundreds of times larger than the EEG recorded in adjacent extracellular tissue or on the cortical surface. Similar findings in neocortical neurons have also been reported by Creutzfeldt, Fuster, Lux, and Nacimiento[16] and Jasper and Stefanis,[17] and in hippocampal neurons by Fujita and Sato.[26]

Autospectral analyses of intracellular waves and the EEG recorded grossly from the same region have indicated a strong general relationship between the two

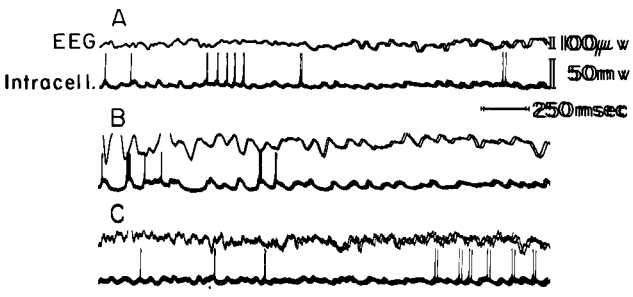


FIGURE 2 Typical examples of cortical surface (top trace) and intracellular (lower trace) records in the same domain of tissue. Initiation of action potentials in the intracellular records occurs on the depolarizing phase of the concurrent wave process, but not necessarily on the largest waves. Records A and B during sleep show slow waves in both extracellular and intracellular traces, whereas the alerted record (C) is faster in both. Calibrations for EEG channel, 100 microvolts; for intracellular records, 50 millivolts. (From Elul, Note 15)

phenomena, both in sleep states with large regular slow waves and in the rapid wave trains of the aroused state. The high resistance of the neuronal membrane in relation to the low resistivity of the enclosing extraneuronal medium substantially reduces the magnitude of the EMF appearing in the extraneuronal medium through transmembrane current flow from intracellular generators. Available data support the view that this attenuation would be at least 100:1, and are compatible with the observed EEG amplitudes of 50 to 200 μv at the cortical surface (Figure 3).

Despite the similarities in autospectral density contours between intracellular waves and the gross EEG, calculations of cross-spectral functions, and particularly the coherence, between the two trains clearly reveal that the gross EEG does not arise from the simple summation of phase-locked contributions from the intracellular wave process. Levels of coherence remain extremely low over

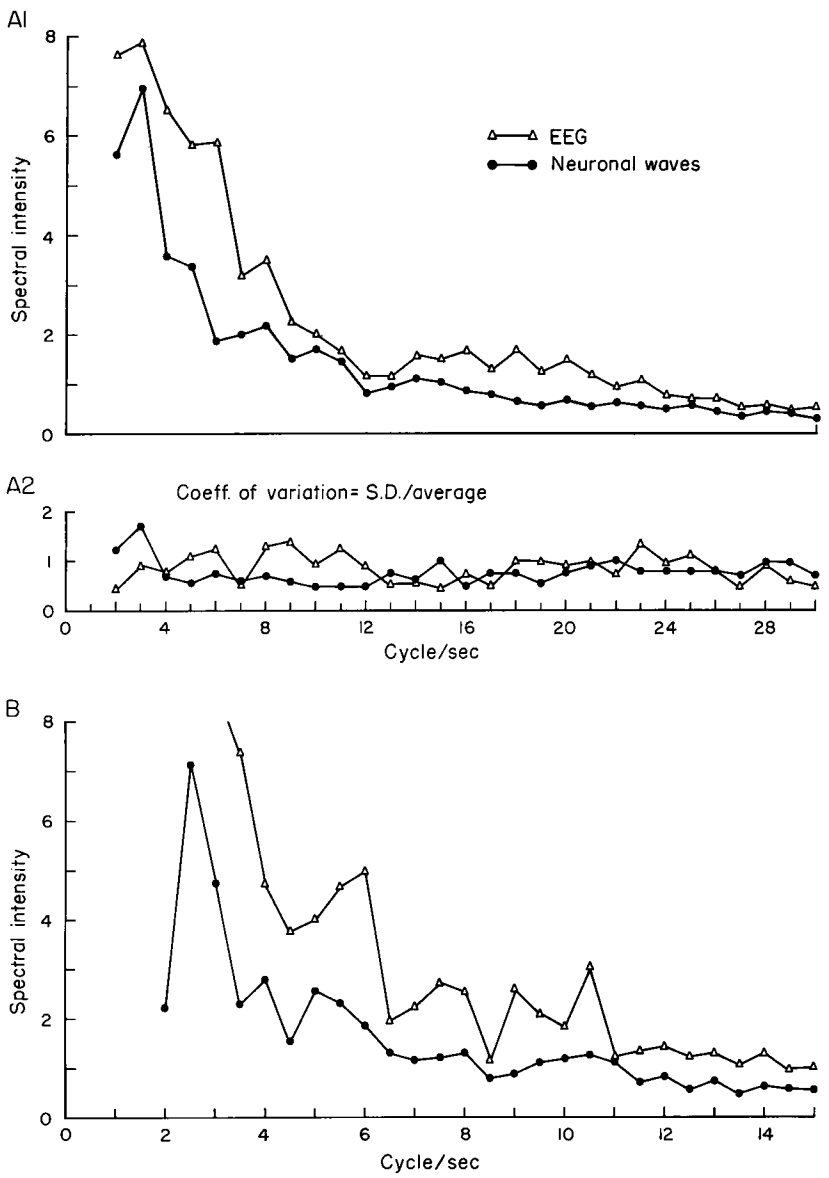


FIGURE 3 Auto-spectral density distributions for simultaneous intracellular and surface EEG records from the same region of suprasylvian cortex in the cat. Comparisons are for two different neurons, and show similarities in spectral contours between surface and cellular records. (From Elul, *ibid.*)

long periods (Figure 4). Elul's studies have indicated that the contributions from the intracellular generators occur on a statistical basis. The cortical generators appear to meet the requirements of the central limit theorem of Cramér,[19] exhibiting individual amplitude distributions, which are not linearly related, possess a mean, and a finite standard deviation.[20] Elul concludes that the EEG may be accounted for as the normal distribution ensuing from a combination of activity of non-linearly related neuronal generators, frequency characteristics of individual generators relating strongly to the gross EEG, but with loss of phase relations in the process of summation.

If such gross rhythmic waves arise in summed activity of neuronal generators, it is also necessary that we consider ways in which individual generators may be coupled in constantly varying degrees to other generators in the system.[21,22] We have therefore investigated amplitude histograms of the gross EEG as an indicator of the cooperative behavior of neuron populations. Whereas amplitude histograms of the gross EEG tended to reach a normal distribution, intracellular wave activity was not distributed in a Gaussian fashion. If, then, the gross EEG is derived through spatial summation of individual generators having non-Gaussian amplitude distributions, this is feasible, according to the central limit theorem, only if linear correlation is limited between the individual generators.

Recordings in human subjects with surface electrodes and electrodes chronically implanted in the hippocampus and amygdala have shown that, although convergence to Gaussian does occur in healthy brain tissue, it is not perfect, and markedly decreases during performance of demanding mental tasks. Since the sampled cortical neurons showed non-Gaussian activity even when the gross EEG was extremely close to Gaussian, it must be concluded that the changes observed in distribution of the gross EEG under varied behavioral stimuli cannot be wholly attributed to changes in amplitude distribution of the unitary generators. Rather, these changes in the amplitude distributions of the gross EEG could arise in terms of increased or decreased correlation between individual neurons in the sampled population. If this interpretation is correct, amplitude histograms of the EEG would provide an estimate of cooperative behavior of neuron populations.

THE RELATIONSHIP OF NEURONAL FIRING TO WAVES AND EVOKED POTENTIALS The firing of the neuron in relation to evoked potentials or induced wave trains in the same domain of tissue appears complex,[23] as is the simultaneously recorded activity of neurons in a single

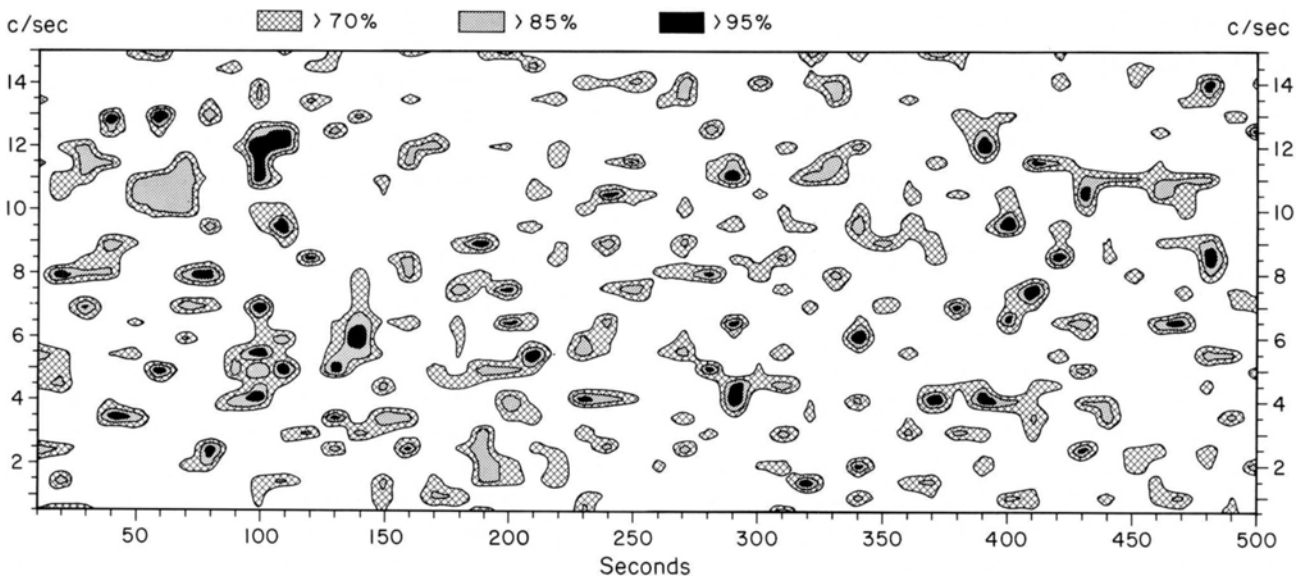


FIGURE 4 Plot of coherence over a 500-second epoch between intracellular wave records and EEG from cortical surface in same domain of tissue. Coherence levels are below statistically significant levels at all frequencies for the major part of the analysis epoch, and the incidence of significant levels of coherence (shown in black) remains around chance levels throughout the analysis. The findings are interpreted as indicating origins of the EEG in a population of independent neuronal generators (see text). (From Elul, *ibid.*)

region after a stimulus.[24,25] Fox and O'Brien[23] have found that variations in firing rate of a single cortical unit follow a contour similar to the evoked potential in the same region, but only after as many as several thousand repetitions of the stimulus. These findings may be interpreted in support of the view that the firing pattern of the individual neuron bears only a statistical relationship to the behavior of the integrated neuronal populations, as manifested by the essentially constant contour of the evoked potential.

In intracellular records, the firing of the neuron occurs near, but not necessarily at, the peaks of the depolarizing phase of the intracellular wave.[27] However, we have consistently observed that the level of depolarization reached on the depolarizing peaks of these waves is not the critical determinant of the initiation of firing. In many instances depolarizing peaks exceeded those on which firing occurred without initiation of a spike, suggesting that the relationship between intracellular waves and the spike output may not always be a linear one. The intracellular microelectrode may offer a restricted window on focal membrane potential changes that do not necessarily reflect changes occurring in the vicinity of the axon hillock, where impulse initiation presumably arises. The question of selective internal current paths to the spike triggering zone has been discussed elsewhere.[28]

The human electroencephalogram in basic behavioral states and task performance; establishment of population means

To this point, we have considered the EEG in a domain of tissue in terms of contributions from unitary generators. It seems clear that the EEG recorded grossly does indeed closely reflect aspects of cellular behavior within the neuronal population. If, however, we are to generalize across populations of subjects, and to relate the EEG, not only to basic states of consciousness, but also to ever finer grades of performance, we must confront the problem of substantial differences between EEG records from different individuals. Do they, nevertheless, conceal common characteristics not detectable by visual observation?

We have sought to establish by computer analysis the presence of common EEG factors in a population of 50 astronaut candidates, both in relation to task performances and assessment of basic states of sleep and wakefulness.[29] To ensure accurate timing in presentation of perceptual and learning tasks, a magnetic tape command system was used, and physiological data was recorded on magnetic tape, together with command signals. The EEG data was subjected to intensive spectral analysis, with calculation of auto- and cross-spectral functions for the 18 bipolar EEG channels from standard electrode placements in all subjects.

After the initial spectral analysis for each subject, the output data was synthesized by an averaging procedure, covering all 50 subjects in the various test situations, and in selected sleep epochs. These averages were made for each scalp region, and are presented as a series of bar graphs, covering the spectrum from 0 to 25 cycles per second. First, an average was prepared of spectral densities at each scalp recording site for all test epochs (Figure 5, top left) including sitting with eyes closed at rest, eyes closed during 1-per-second flash stimuli, during an auditory vigilance task, during visual discriminations at 3-second intervals, and a similar series of more difficult discriminations at 1-second intervals.

The contours of these "lumped" spectra were then used as the mean for comparison with the spectra for the individual situations. The subsequent graphs in Figure 5 thus show the variations about the mean established over 12 situations in the top left figure. Spectral densities above the mean at any frequency have bars above the baseline and vice versa. Lengths of these bars are in units of the standard deviation at that frequency, so that *relative* variation is emphasized.

Such a display clearly separates spectral density distribution for the 50 subjects in the 5 situations shown. Moreover, the distributions for the more difficult visual discriminations (Figure 5, lower right) exemplify trends that already characterize discriminations made in 3 seconds (Figure 5, lower middle). Pattern recognition techniques described below clarify differences between records in these two tasks. The method also allows comparison of an individual with the mean for the group, or with his own mean, using a two-color display technique.

Pattern recognition techniques applied to spectral parameters in defining attention states

If, then, a series of group means can be established for the EEG in a variety of states of attention, would it be possible to categorize with equivalent clarity the EEG of a single subject recorded during defined behavioral performances? A discriminant analysis was applied to spectral outputs in 4 subjects covering 5 situations: eyes open at rest, an auditory vigilance task, and the two visual discriminative tasks described above.[30] Data from 4 EEG channels was analyzed into 4 frequency bands, corresponding to the classical delta (1.5 to 3.5 c/sec), theta (3.5 to 7.5 c/sec), alpha (7.5 to 12.5 c/sec) and beta (12.5 to 25 c/sec) bands. In each band, measurements were made of the strength of activity in each channel, mean frequency within the

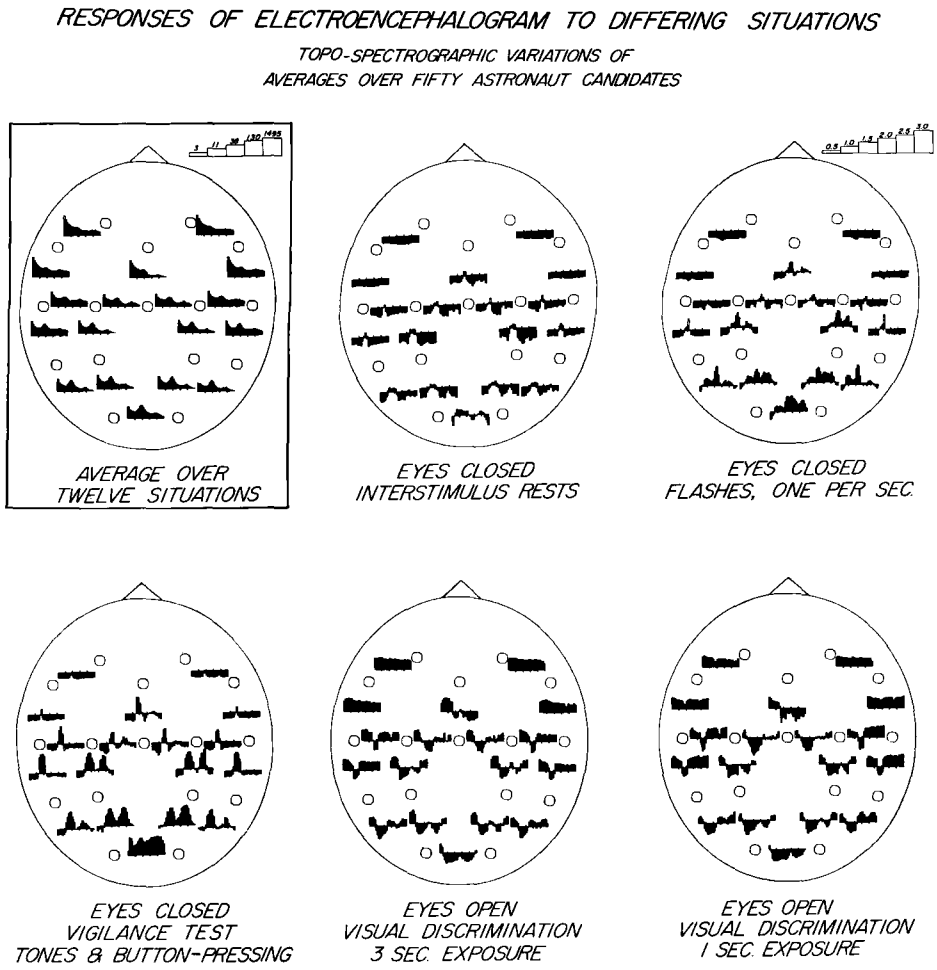


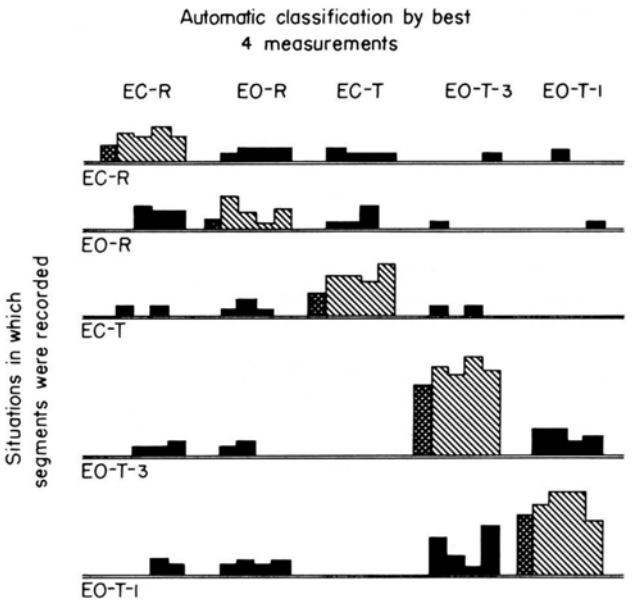
FIGURE 5 Averaged spectral densities over the range 0 to 25 cycles per second for a population of 50 subjects, with each spectrum presented as a series of bars at 1-cycle-per-second intervals, and placed at the appropriate location on the scalp. The top left figure is an average for all subjects across 12 situations (see text). The contour of this average was then used as the mean against which to measure deviations in the succeeding five situations, with powers above the mean at any frequency shown as a bar above the baseline and vice versa. Calibrations for average over 12 situations in microvolts squared per second per cycle; for the separate situations, in standard deviations. (From Walter, et al., Note 29)

band, bandwidth within the band, and the coherence between pairs of channels (Figure 6).

This discriminant analysis program first considers all the measurements for all the segments, and selects that parameter which best discriminates segments recorded in different situations. It then reexamines all measurements and chooses the parameter which will add most to the discriminating power of the first measurement, and then continues this iterative process until insufficient improvement is made by adding another parameter. The four variables which best distinguish among the five situations are: left parieto-occipital alpha intensity, the mean frequency of theta band activity in the vertex, the coherence in the theta band between left parieto-occipital and vertex, and coherence in the beta band between vertex and bioccipital leads.

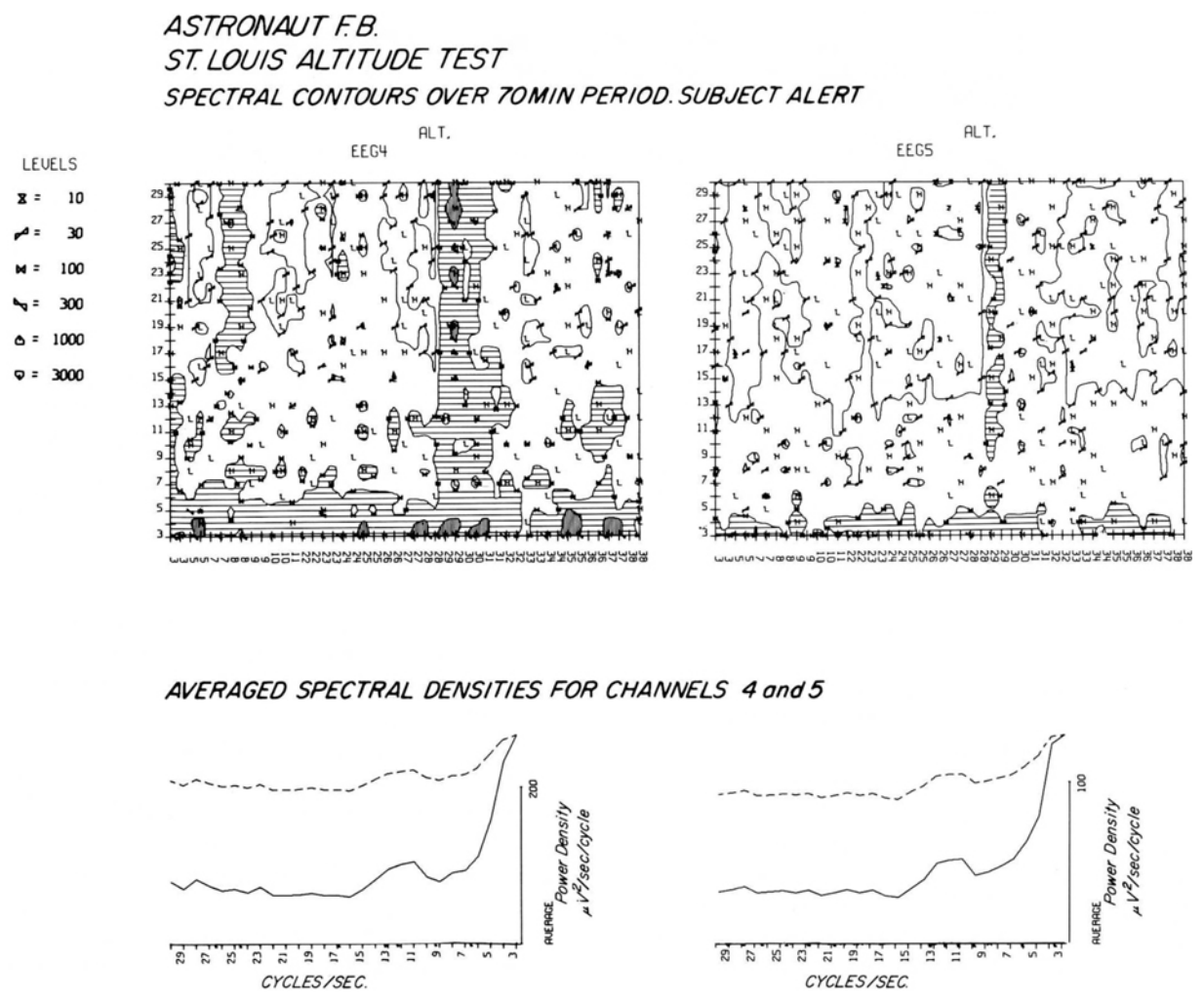
After 15 measurements were selected, the records from individual subjects were correctly classified on 95, 93, 96, and 90 per cent of the tests, compared with only 65 per cent for the group as a whole. It would thus appear that each subject may have a spatially and numerically characterized EEG "signature," as to which measurements are most effective in distinguishing different situations.

In these first, halting steps in the use of EEG pattern



recognition techniques, we nevertheless appear to have within our grasp a tool sufficiently fine to recognize the differences between the EEGs of individuals performing a moderately difficult visual discrimination in 3 seconds and a similar but substantially more difficult task in 1 second. It would seem reasonable to suggest that pursuit of such methods offers an opportunity not only to categorize the complexities of brain wave patterns, but to

FIGURE 6 Pattern recognition techniques applied to spectral outputs from 4 subjects, separately and jointly, with development of a matrix display of automated classifications for five situations: EC-R, eyes closed resting; EO-R, eyes open resting; EC-T, eyes closed while performing an auditory vigilance task; EO-T-3, performing moderately difficult visual discriminations in 3 seconds; EO-T-1, performing difficult visual discriminations in 1 second. (From Walter, et al., Note 30)



approach in a far more rigorous frame the underlying subtleties of cerebral system organization in processes of attention.[31]

Comparison of attention patterns in an astronaut during simulated flight and actual launch into orbit

The power of the EEG to manifest subtly shifting patterns relating to extreme requirements in focused attention is strikingly demonstrated in our analyses of records from Astronaut F. Borman in an altitude chamber simulation and during the period prior to and following launch in the Gemini GT-7 flight. This data was collected under the supervision of Dr. P. Kellaway and Dr. R. Maulsby.

In each study, identical electrode placements and tape recording equipment were used. Electrode placements for the two channels in the simulation and in space flight spanned a wide zone of scalp from vertex to occipital region, with one pair located in the midline, and the other spanning the left parieto-occipital area.[32]

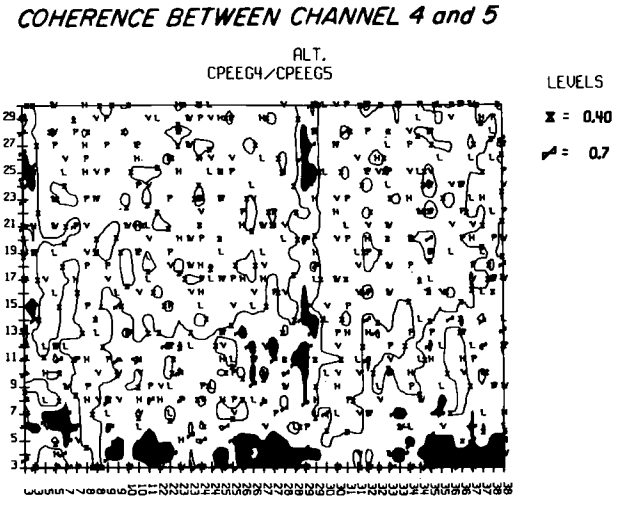


FIGURE 7 Analysis of EEG records from altitude chamber Gemini flight simulation. Electrode placements, amplifiers, and recording equipment were identical with actual flight systems. Left and middle contour plots show auto-spectral densities in the two EEG channels. Right-hand plot shows coherence between them. Averaged spectral densities for each channel were prepared from more than 40 epochs, each 20 seconds in duration. (Solid line in lower traces, linear plot; dashed line, logarithmic plot.) Coherence between EEG channels (right contour plot) reaches statistically significant levels only sporadically (black zones), mainly at frequencies between 3 and 5 cycles per second.

In the simulation (Figure 7), samples were analyzed over a 10-minute period characterized by typical alerted patterns, and occasional movement artifacts (epochs 28 to 30 in channel 4, and epoch 29 in channel 5). These contour plots show relatively low powers at all frequencies above 5 cycles per second, and lack any clear peak in the alpha range around 10 cycles per second. Simple averages of spectral density were prepared for each channel, covering the whole test epoch. The solid line shows a linear plot of spectral densities, and the dashed line a logarithmic plot, over the spectrum from 3 to 30 cycles per second. In both channels, a broad and ill-defined alpha peak at 9 to 13 cycles per second is overshadowed by higher powers in the theta range from 5 to 7 cycles per second. Coherence between the two channels reached significant levels (black areas in right contour plot) only intermittently, and almost solely in the range from 3 to 5 cycles per second. These findings contrast sharply with certain aspects of the flight records.

The data from the prelaunch period and a substantial part of the first orbit are shown in Figure 8, with the EEG spectrum plotted on the abscissa, and time on the ordinate. The prelaunch period was characterized by increased amounts of theta rhythms than was seen in baseline records. At one minute before lift-off, there was a further increment of theta activity and in the higher frequencies in the alpha and beta bands. This may be interpreted as relating to strongly focused attention and orienting responses in this novel situation. The power density of the EEG was augmented by a factor of 10 over many frequencies before and following launch, indicating a strong EEG "arousal reaction." Thereafter, there was a slow decline in these augmented densities, with recurrent epochs of higher powers in the higher frequency bands above 10 cycles per second in the first half hour of flight.

Coherence measurements between the two channels (right hand figure in each row) showed striking differences from the simulation baseline. Coherence grew to significant levels (black areas) at progressively higher frequencies in the range from 3 to 9 cycles per second up to the moment of launch. Thereafter it declined to non-significant levels at most frequencies for the next 30 minutes. This was followed by an enormous rebound (in the lower figure) to extremely high levels in the range from 3 to 11 cycles per second for the next 40 minutes, and a progressive decline thereafter. These high coherence levels were not seen in any of the ground-based recordings, nor in any subsequent part of the flight records.

It is rarely indeed that one has an opportunity to secure such data in circumstances requiring highly focused attention under conditions of severe environmental stress. The findings suggest that coordinated activity between

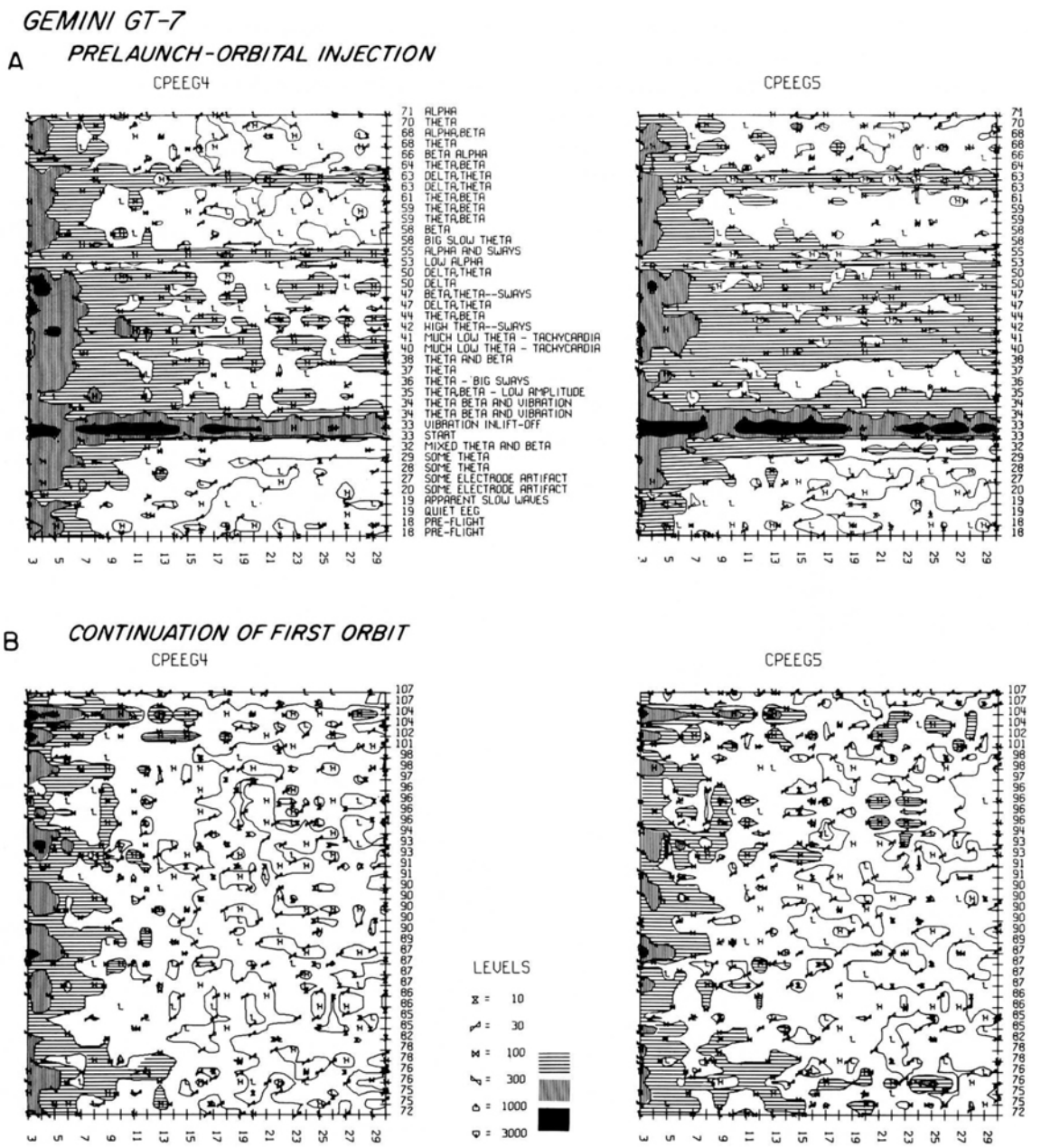
large masses of cortical tissue, as measured by the coherence between them, may depend critically on anticipatory or recapitulatory aspects of a novel and hazardous experience.

Electrophysiological studies of arousal, orientation, and discrimination

From the broad window on patterns of brain electrical activity available from scalp records, we may return to finer patterns to be discerned in cortical and subcortical structures as concomitants of alerting, orienting, and discriminate responses. The development of conditional firing patterns in cerebral neurons will be briefly reviewed prior to discussion of EEG processes also related to establishment and maintenance of conditional phenomena. These topics have been discussed in detail elsewhere.[28]

Unit Firing Patterns in Conditional Responses

Polymodal input to individual cells in brainstem and



diencephalic reticular structures[33,34] has suggested that "temporary connections" may be established via these cells. Despite great variability of responses in cortical structures, meaningful patterns have also been detected at this level.[35-37] The importance of reticular structures in conditioning has been established in both recording and lesion experiments.[38-40] Olds and Olds[36] have emphasized the relative ease of establishment of conditional behavior in paleocortical and subcortical units, by comparison with cortical units.

Medial thalamic units can be repeatedly extinguished and retrained during classical conditioning. Their behavior during repeated conditioning and extinction emphasizes the plasticity of responsiveness of single cells in such a paradigm, with gradual appearance of firing patterns which might be the converse of those initially elicited.[41] Extinction tests following each conditioning also exhibited progressive rebound phenomena, so that over a period of several hours it was possible to detect a series of gradual changes that increased in magnitude, as well as showing qualitative differences from those in the first extinction trial (Figure 9).

The "training" situation used by Kamikawa, et al.[41] involved pairing of a light flash as a conditional stimulus with an unconditional shock train to the sciatic nerve. The CS-US interval ranged from 300 to 800 msec. Tests with intervals shorter than 300 msec failed to elicit a conditional response. Training trials were given once every 10 seconds. A change in firing in the CS-US interval characteristic of a conditional response required a minimum of about 50 trials, presented over a period of about 20 minutes.

These findings offer some possibility of an understanding of processes that might underlie lasting structural changes associated with a memory trace. The required minimal CS-US interval of the order of 300 msec suggests a time scale comparable with electrophysiological events at the neuronal membrane, such as the prolonged inhibitory postsynaptic potentials seen in cortical neurons[42]

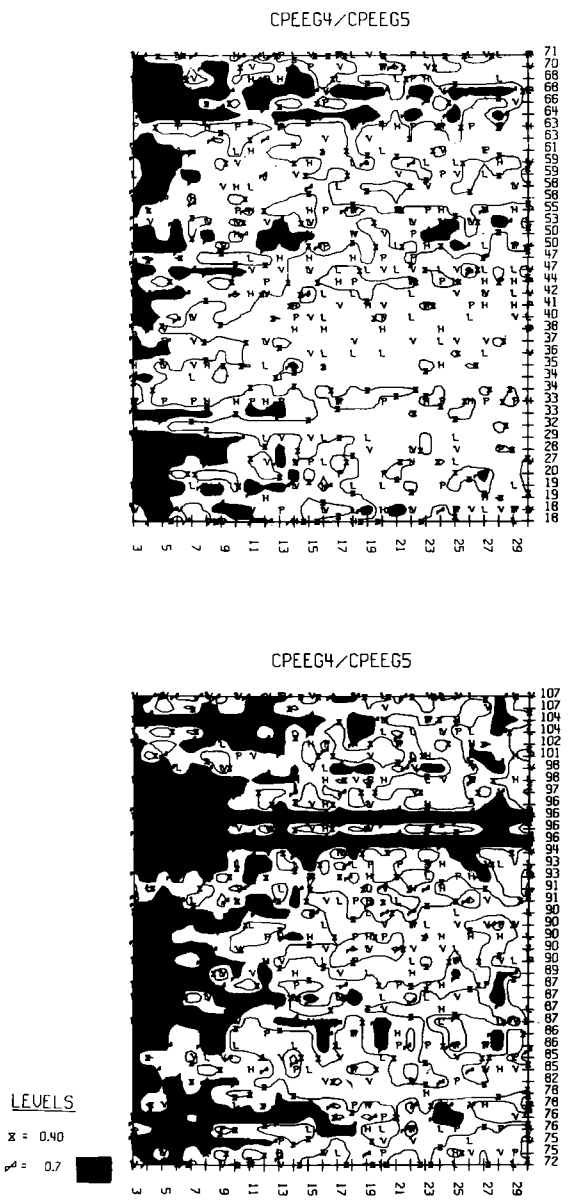


FIGURE 8 Contour maps of EEG data from F. Borman in Gemini Flight GT-7. Prelaunch and launch epochs are shown in the top row of maps. Auto-spectral densities in the theta range were augmented in the two EEG channels (CPEEG4 and CPEEG5) prelaunch, with great exaltation of many EEG frequencies immediately before and during lift-off. Coherence between the channels (CPEEG5/CPEEG5) became significant at progressively higher frequencies in the 15 minutes before launch, but declined sharply at lift-off for the first 5 minutes of flight. Thereafter the coherence in the range from 3 to 11 cycles showed a gradual return at progressively higher frequencies, and in continuation of the first orbit (B), attained very high values across the spectrum from 3 to 11 cycles, in a fashion not seen in any control records on the ground, nor in subsequent flight records. Calibrations in auto-spectral contours are in microvolts squared per second per cycle. Shaded contours are 100–300 μv^2/sec/cycle, horizontal shading; 300–1000, vertical shading; over 1000, solid black. In the coherence plots, values above 0.7 (statistically significant level) are in black.

lasting up to several hundred milliseconds, and without an equivalently persistent counterpart in spinal motoneurons or with wave processes in cortical neurons, which at this time are less definitely related to synaptic potentials.[27] On the other hand, the requirement that training trials have a time course of 20 minutes to generate a conditional response suggests a much slower process, perhaps the synthesis of a macromolecule, protein in nature, and located at the neuronal membrane, where it might directly influence the excitability of the cell by synaptic volleys.

EEG Correlates of Orientation and Discrimination The characteristics of spontaneous rhythm changes in classical conditioning have been reviewed in detail elsewhere.[28] These studies of the activated EEG in classical conditioning have contributed to the view that the desynchronizing response represents a stereotype that can scarcely be pursued farther in finer analysis of correlates with behavioral responsiveness and conditioning. Yet it has long been recognized[43] that certain manipulations of a classical conditioning procedure leads to trains of synchronous slow waves. This was observed where the CS-US interval was prolonged, or in cortical areas surrounding a focally activated response, and was attributed by Gastaut[44] to "internal inhibition" in the Pavlovian sense.

Recognition that a synchronous, rather than activated, pattern of cortical activity accompanies certain aspects of classical conditioning has led to a finer analysis of distribution of wave activity in cortical and subcortical structures regularly occurring in certain operant performances, and to extensive computer analysis of their patterns.[31,45-49]

Much attention has been directed to allocortical structures of the amygdaloid and especially the hippocampal systems in deposition of the memory trace.[50-53] The great antiquity of the hippocampal system in evolution of the brain and the essential stability of its basic structure in the face of immense evolutionary changes in the remainder of the cerebral mantle are in themselves a challenge to an understanding of its functional role. Despite strongly suggestive evidence for its participation in essential processes of memory even in simple brains, persisting difficulties in such an easy interpretation demand a cautious attitude. Thus, the memory trace may be laid down outside the hippocampal system,[54] but integrity of its interrelations with these seemingly unrelated cortical and subcortical regions may be vital to the appropriate recall of previously learned discriminative habits.[55] In consideration of sometimes incompatible and even contradictory findings from a variety of lesion studies, Drachman and Ommaya[56] have concluded that the essential defects after hippocampal lesions are impairment of acquisition and loss of retention, rather than impaired short-term memory.

Our studies in the hippocampal system in the course of learning a discriminative task have sought evidence of altered electrical patterns closely correlated with acquisition of a learned task, and more fundamentally, whether such modifications might suggest anything about the essential processes of storage in cerebral tissue.[45-47,49,55,57] The neuron may be considered in terms of its ability to sense complex spatiotemporal patterns of waves induced at its surface. Such a frame of functional organization would assist in defining the possible uniqueness of integrative processes in cortical systems, characterized by dendritic overlap in a palisade arrangement of cells as described above, and with wave phenomena as a concomitant of electrotonic processes, which appear largely localized in dendritic structures.

We have extensively studied hippocampal EEG activity in the cat in the course of acquisition of a visual discriminative performance, in a modified T-maze, with approach to a concealed food reward on the basis of a visual cue. Whereas alerted behavior was accompanied by a wide spectrum of activity at 4 to 7 cycles per second, with a 4 cycles per second dominant, the period of discriminative performance was characterized by a very regular burst of "theta" waves at an essentially single frequency around 5.5 cycles per second in the dorsal hippocampus, and in the entorhinal area of the pyriform cortex. Concurrently, less regular and less constant rhythmic processes were frequently noted in subcortical structures, including midbrain reticular formation and subthalamus. The latter are under continuing investigation.[58]

Assessment by Computed Averages of Hippocampal and Midbrain Reticular EEGs During Task Acquisition and After Cue Reversal in the Cat When discriminative ability was still at chance levels, but a relatively stable response pattern and approach latency were already established, computed averages of hippocampal EEGs during 30 or 40 daily trials showed some rhythmicity at 5 cycles per second. In the course of subsequent training, there was frequently a decline in rhythmicity of the average at performance levels between 80 and 90 per cent. At performance levels around 100 per cent, a greater degree of regularity was noted than at any previous stage of training (Figure 10). The transient decline in rhythmicity at mid-training did not relate to a decline in regularity of the 5 cycles per second burst in individual records. It thus appears to have resulted from either a loss of phase-locking of these bursts to the onset of the situational presentation at mid-training, or, possibly, to the appearance of significant degrees of frequency

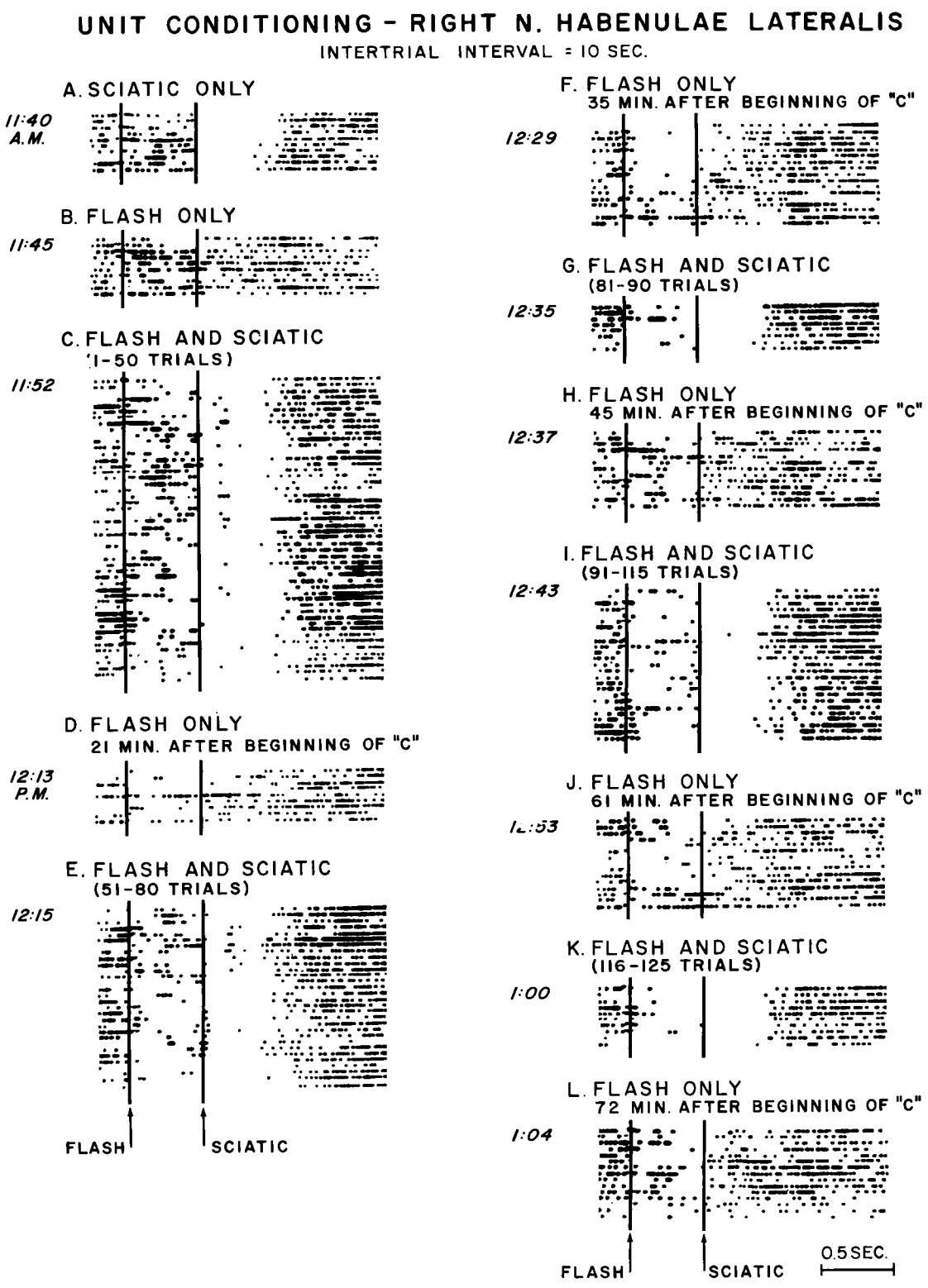


FIGURE 9 Development of an inhibitory conditional response in an habenular unit. Each dot represents a unit discharge, and each horizontal row of dots, a single trial. Trials are grouped according to stimulus conditions. A: sciatic nerve stimulation only (US) as control; B: flash only (CS) control; C: flash and sciatic praised in first sequence or training trials. Left vertical line indicates time of CS presentation. Vertical line marked "sciatic" indicates time of onset of US. (From Kamikawa, et al., Note 41)

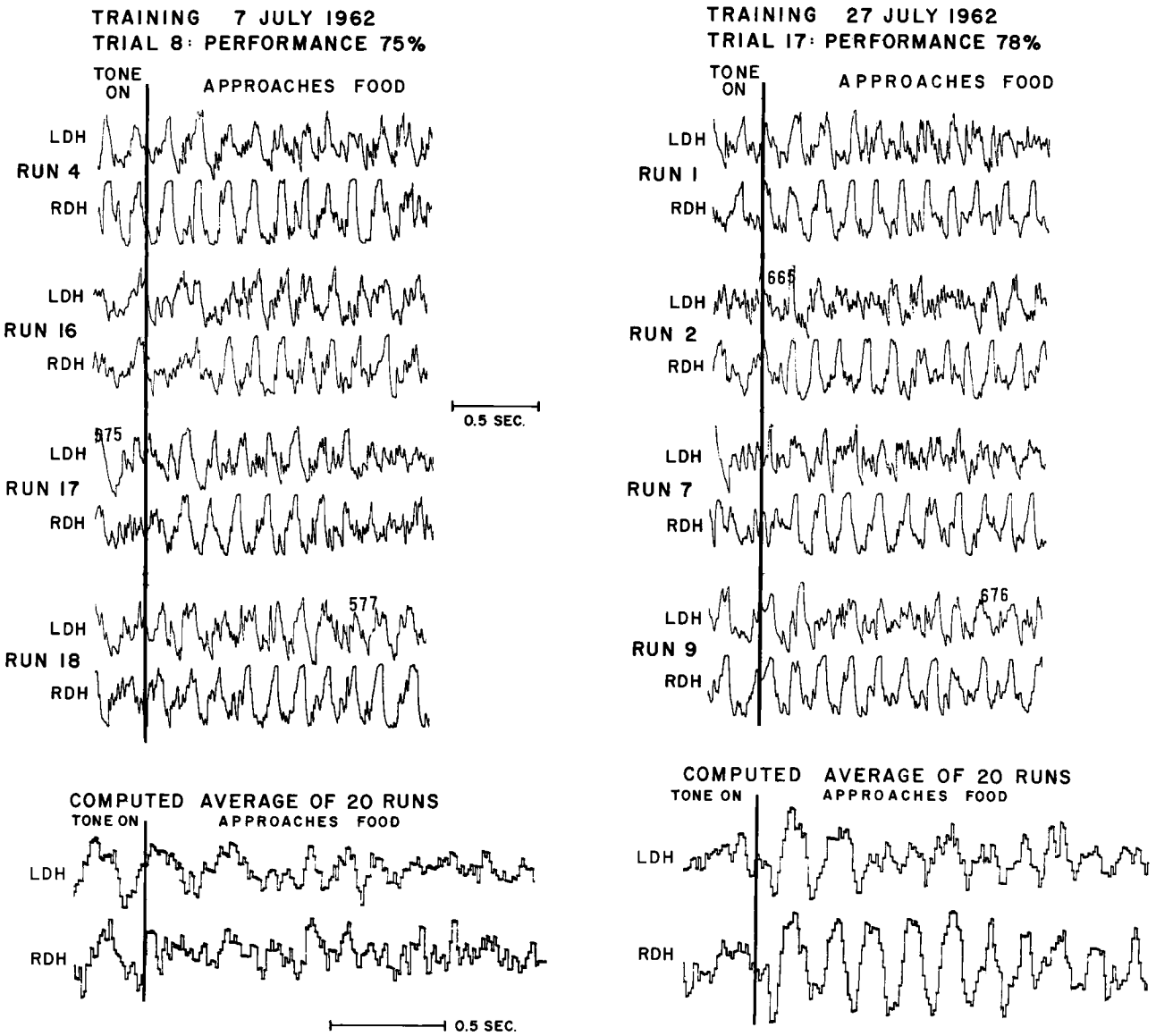


FIGURE 10 Representative EEG records with computed averages from 20 pairs of hippocampal traces at mid-training after first cue reversal (left). Note irregular character of averages. Later in training, rhythmic average appeared (right) and was sustained into overtraining. (From Adey and Walter, Note 46)

modulation on the 5 cycles per second bursts, as detected by sensitive digital filtering techniques.[46] By contrast with subcortical structures, such as the midbrain reticular formation, the regular components of the hippocampal EEG persisted into substantial overtraining, although the duration of the regular burst was often abbreviated.

On the first day after cue reversal, averages of hippocampal activity were extremely regular at 5.5 cycles per second, higher in amplitude than before cue reversal, and sustaining throughout the approach. The increase in averaged output apparently resulted from diminished scatter in phase patterns in consecutive performances, rather than from increased amplitude in the individual bursts.

In ensuing training days, with performances from 50 to 75 per cent, there was a progressive decline in amplitude and regularity of the computed hippocampal average. On attainment of a performance level around 90 per cent in

the new paradigm, a highly rhythmic average again appeared. Repeated cue reversals with retraining to high performance levels, or substantial overtraining in a particular paradigm, led only to shortening of the length of the regular average.

By contrast, subcortical structures, such as the midbrain reticular formation, showed after each cue reversal a decline and then a gradual reestablishment of a regular average at high performance levels. Beyond the fifth or sixth cue reversal over a six-month period, a sophistication in the situation appeared, with a rapid rise in performance in the first few training days after cue reversal. Reticular records did not regain a rhythmicity comparable with that in earlier tests, even at performance levels over 95 per cent. It may be surmised that information essential for discrimination may have reached minimal proportions, and that appropriate behavioral performance may occur with little more than fleeting attention to behavioral cues. A high scatter might once again appear in phase patterns of successive records, but subtly different from the irregular patterns in early training.

COMPARISON OF EEG PATTERNS IN ORIENTING AND DISCRIMINATIVE BEHAVIOR The mammalian response to a sudden stimulus runs a gamut from the "startle response," with arrest of ongoing behavior, through various investigative reactions, to an almost infinite variety of complex coordinated motor patterns, constituting "fight or flight" responses.[59] It is in the second category that we may group the behavioral components of the orienting reflex, as first characterized by Pavlov.[60] Its uniqueness rests on certain "principles" in the intimate behavior of its component reflexes, including their non-specificity with respect to both quality and intensity of the stimulus, and the selectivity of various properties of the stimulus with repeated presentation.[25,61]

Although a specific relationship has been postulated between hippocampal theta wave trains and orienting behavior,[62] the exquisite plasticity of hippocampal theta rhythms in changing behavioral states, including the appearance of bursts of waves in a narrow spectral range during performance of a visual discriminative task, have suggested more subtle and specific relations to discriminative functions and judgment capability.[45,49,55,63]

Radulovački and Adey[49] found it possible to distinguish hippocampal EEG activity in three basic states in the cat; in alert but non-performing animals, in the course of discriminative performances, and during orienting behavior. Alert but non-performing animals exhibited a wide spectrum of "theta" waves in the range 3 to 7 cycles per second on first introduction into the test situation, without overt aspects of orienting behavior. During T-box discriminative performance, theta waves regularized at 5 to 6 cycles per second, as described above. Computed averages in orienting trials, given in the same numbers on each test day and randomly interspersed with discriminative trials, showed slower and less regular averages at 4 to 5 cycles per second (Figure 11).

These studies indicate that in the cat, hippocampal wave trains relate in clear and specifiable ways to the performance of a discriminative task, and in different, but equally recognizable patterns, to aspects of orienting behavior. Collectively, these studies in hippocampal, sensory cortical, midbrain and subthalamic areas have suggested that the deposition of a "memory trace" in extrahippocampal systems may depend on such wave trains, and subsequent recall on the stochastic reestablishment of similar patterns.[46]

Although no causal relationships can be established at this time between the decision-making process and a particular EEG pattern, the detection of differences in wave patterns in correct and incorrect responses,[47,55] using cross-correlation and cross-spectral techniques, has emphasized the strong possibility of a stochastic mode of operation in the handling of information on the basis of a wave process. Such a scheme would envisage the excitability of the individual neuron as depending not only on its previous experience of complex spatio-temporal patterns of waves, but would also suggest that the effectiveness of any subsequent wave pattern in eliciting neuronal firing might depend on its multivariate relationship to an "optimal" wave pattern, capable of inducing firing of that neuron at its lowest threshold.

Impedance changes in alerting, orienting, and discrimination in the cat

In a series of studies, we have measured the impedance of focal volumes of cerebral tissue with chronically implanted coaxial electrodes at 1000 cycles per second.[9,64-67] The technique has been described in detail elsewhere[68] and uses a current density of 10^{-13}A per μ^2 of electrode surface, with a differential sensitivity one hundredth of that current level. The probable pathways for these low-level measuring currents lie through the extracellular fluid and neuroglial cells, since both may be presumed to offer paths preferred over the substantially higher impedance route through neuronal membranes.

Electrical impedance was measured in the hippocampus, amygdala, and midbrain reticular formation during alerting, orienting and discriminative performances.[9] The impedance of small volumes of cerebral tissue changed differentially at different sites in the course of this repertoire of alerting and learned responses. The magnitude of

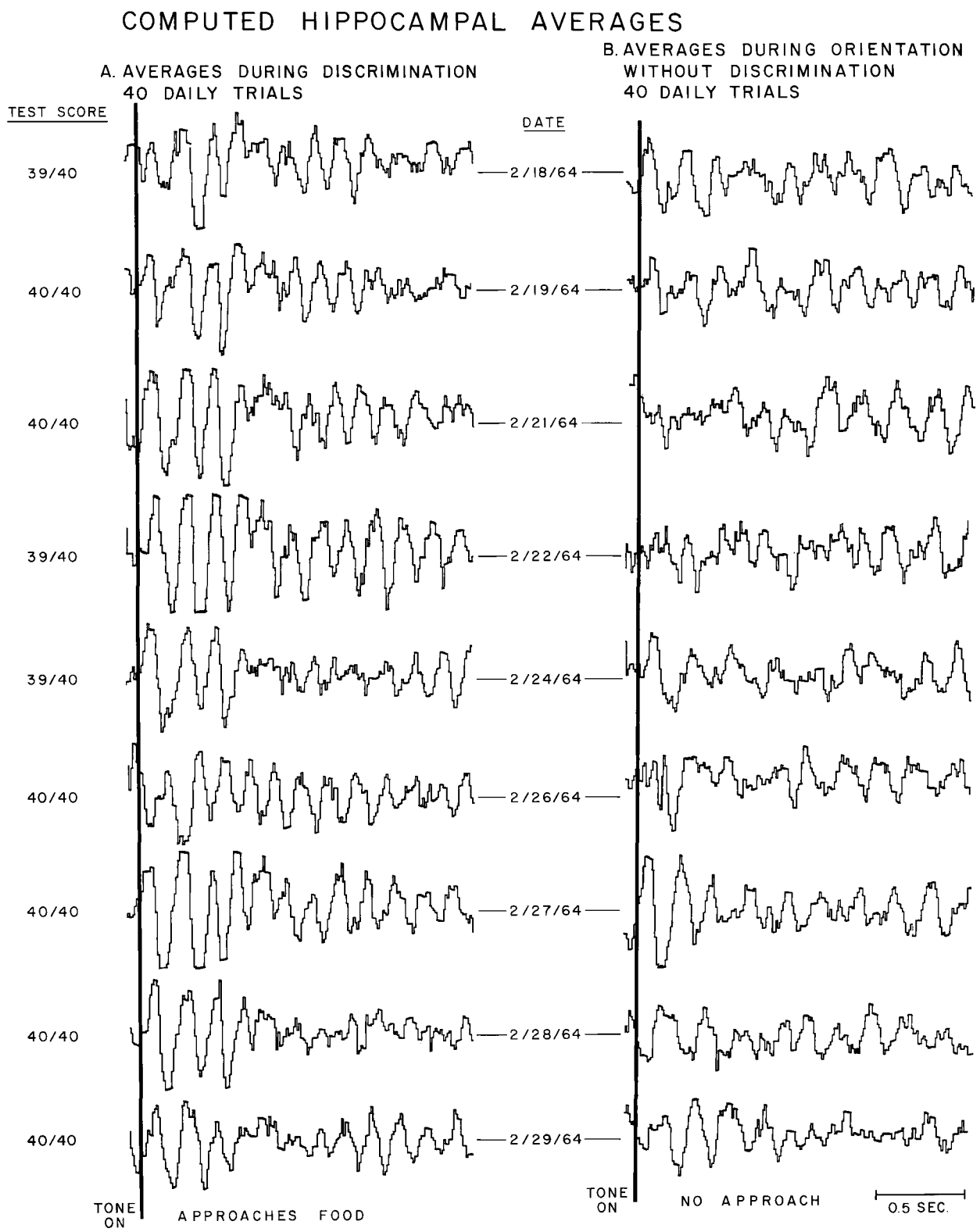


FIGURE 11 Effects of introduction of orienting trials (daily w=40) into training schedules of a cat already at a high level in discriminative task performance. Computed averages during discrimination (A) showed high amplitude waves at 6 cycles per second. Randomly interspersed orienting trials (B) exhibited a lower amplitude to 4 to 5 cycles per second rhythm in later parts of analysis epoch. (From Radulovački and Adey, Note 49)

these impedance responses increased as levels of performance rose progressively above chance. They were susceptible to cue reversal and subsequent retraining. Variability, as indicated by standard error of the mean, was large in records from initial training trials, decreased progressively with training, reverted to wider levels on cue reversal, and decreased once more with retraining (Figure 12).

In the fully trained animal, computed averages of hippocampal impedance decreased by as much as 8 per cent of baseline during visual discrimination, whereas alerting and orienting responses immediately preceding were not accompanied by comparable impedance changes. Similar measurements in the rostral midbrain reticular formation showed small responses during orientation and discrimination, and less constantly during alerting responses. The amygdala exhibited consistent responses only in the alerting epoch.

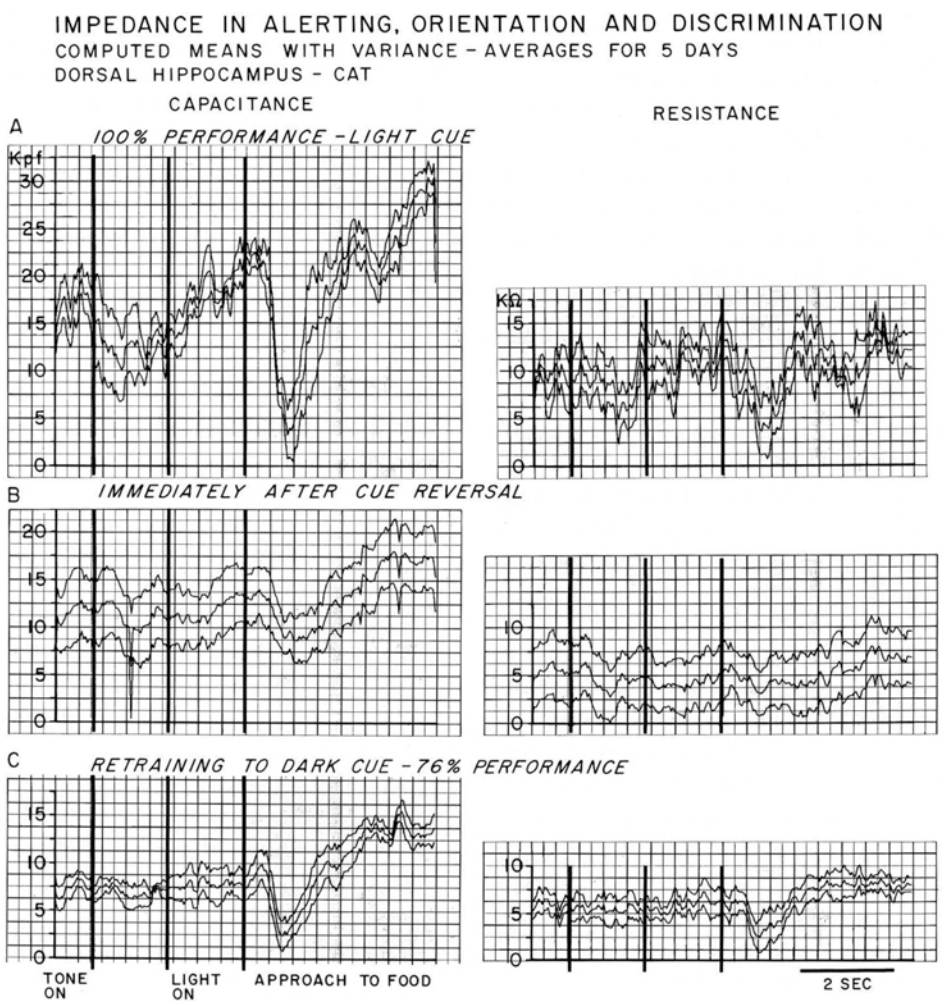


FIGURE 12 Calculations of means and variability in hippocampal impedance over 5-day periods at various levels of training, with successive presentations of alerting, orienting and discriminative stimuli. In each graph, middle trace indicates mean, with upper and lower traces showing one standard deviation from the mean. Calibrations indicate 50 picofarads, with mean baseline at 11.1 kilopicofarads throughout the training maneuvers; and 100 ohms, against a mean baseline of 16.0 kilohms for the same period. Variability was low at 100 per cent performance (A), increased substantially immediately after cue reversal (B), but decreased again after retraining (C). (From Adey, et al., Note 9)

The nature of the impedance response: the role of neuronal elements and the influence of divalent cations on extracellular macromolecules

From the foregoing, these impedance responses appear to relate to changes in intrinsic characteristics of cerebral tissue. Our studies have indicated that regional differences in impedance responses occur with shifts in carbon dioxide levels induced by hypercapnea or hyperventilation, and that they do not arise in simple relationship to alterations in blood pressure, cerebral blood flow, or brain temperature.[66,67]

It is generally agreed that extracellular space offers a high conductance pathway, but its precise relationship to the observed impedance requires consideration of its extent in cerebral tissue, and its content of macromolecular and ionic material, as discussed above. If it is the site of the impedance changes described here, then we must seek evidence of a temporary and presumably reversible movement of ions into it, a movement presumably initiated in neuronal elements, but capable of modulation by neuroglial cells, or of influencing neuroglia.

To test the role of neuronal elements, retrograde degeneration was induced in the lateral geniculate body by unilateral resection of the visual cortex. This led to loss of about 80 per cent of lateral geniculate neurons, and was followed by perturbations in geniculate impedance baselines from 10 to 30 days postoperatively. Subsequently, impedance responses to alcohol and to a cyclohexamine drug were reduced to about 20 per cent of those in the intact nucleus, thus indicating a requirement for an intact neuronal population in the normal manifestations of impedance responses.

The findings suggest that the impedance responses are mediated through perineuronal compartments, with modulation by either neuronal or neuroglial elements of conductance in an intercellular fluid containing a matrix of macromolecules. The extent to which changes in neuroglial membrane resistance might contribute directly remains uncertain.[69]

The susceptibility of intercellular macromolecules to divalent cations, such as calcium, has led us to investigate the effects of injection of small quantities of calcium salts into the lateral ventricle in the cat, while recording with chronically implanted coaxial electrodes the impedance in periventricular structures, including hippocampus, caudate nucleus, and amygdala.[10]

Injections of calcium chloride solution (40 to 60 microequivalents in 0.1 ml) were made, preceded by control injections of normal saline. No changes followed the control injections. A sharp decline occurred in both resistive and reactive components, beginning 15 to 30 minutes after injection of 40 to 180 microequivalents of calcium solution, in the structures named above, bounding the lateral ventricle (Figure 13).

Impedance readings were shifted from baseline values by as much as 25 per cent for periods that exceeded 36 hours in some cases. Changes were largest and earliest in structures closest to the tip of the cannula, and were delayed by 30 to 50 minutes in reaching peak values in symmetrically placed leads in the opposite half of the brain. Maximum shifts occurred within two hours of injection. The intraventricular injection was regularly followed by a topographically determined sequence of impedance changes, consistent with diffusion from the injection site via the cerebrospinal fluid. On the other hand, direct injection of up to 120 microequivalents into periventricular structures was without comparable effects, except at electrodes immediately adjacent to the injection site. The onset of these impedance shifts in hippocampus and amygdala was accompanied by seizure-like discharges, but the impedance shifts were prolonged many hours beyond cessation of gross EEG abnormalities.

Thus the evidence is consistent with the view that cerebral impedance changes accompanying physiological responses may arise in perineuronal fluid with a substantial macromolecular content, and that calcium ions may modulate perineuronal conductivity, as well as fluxes of sodium and potassium across the neuronal membrane. In such a frame, the disclosure of impedance changes in cerebral tissue in the course of alerting, orienting, and discriminative responses, their selective regional distribution, and dependence on levels of learning, all invite consideration of the role of perineuronal elements in aspects of transaction and storage of information in brain tissue.

Summary

This review has considered the gamut of neural organization, ranging from subcellular events in the genesis of intracellular waves, to the patterns in scalp EEG records characterizing a population of human subjects in states of focused attention and visual discrimination. A tricompartmental model of cerebral tissue is described, with neuronal, neuroglial, and extracellular divisions. The role of macromolecular systems at the neuronal surface and in the intercellular fluid is considered. Evidence is presented that mucoproteins and mucopolysaccharides may be responsible for net fixed charges at the cell surface, and may thus play a role in ionic fluxes across the membrane. Divalent cations, such as calcium, may modify these macromolecular configurations. Impedance changes in

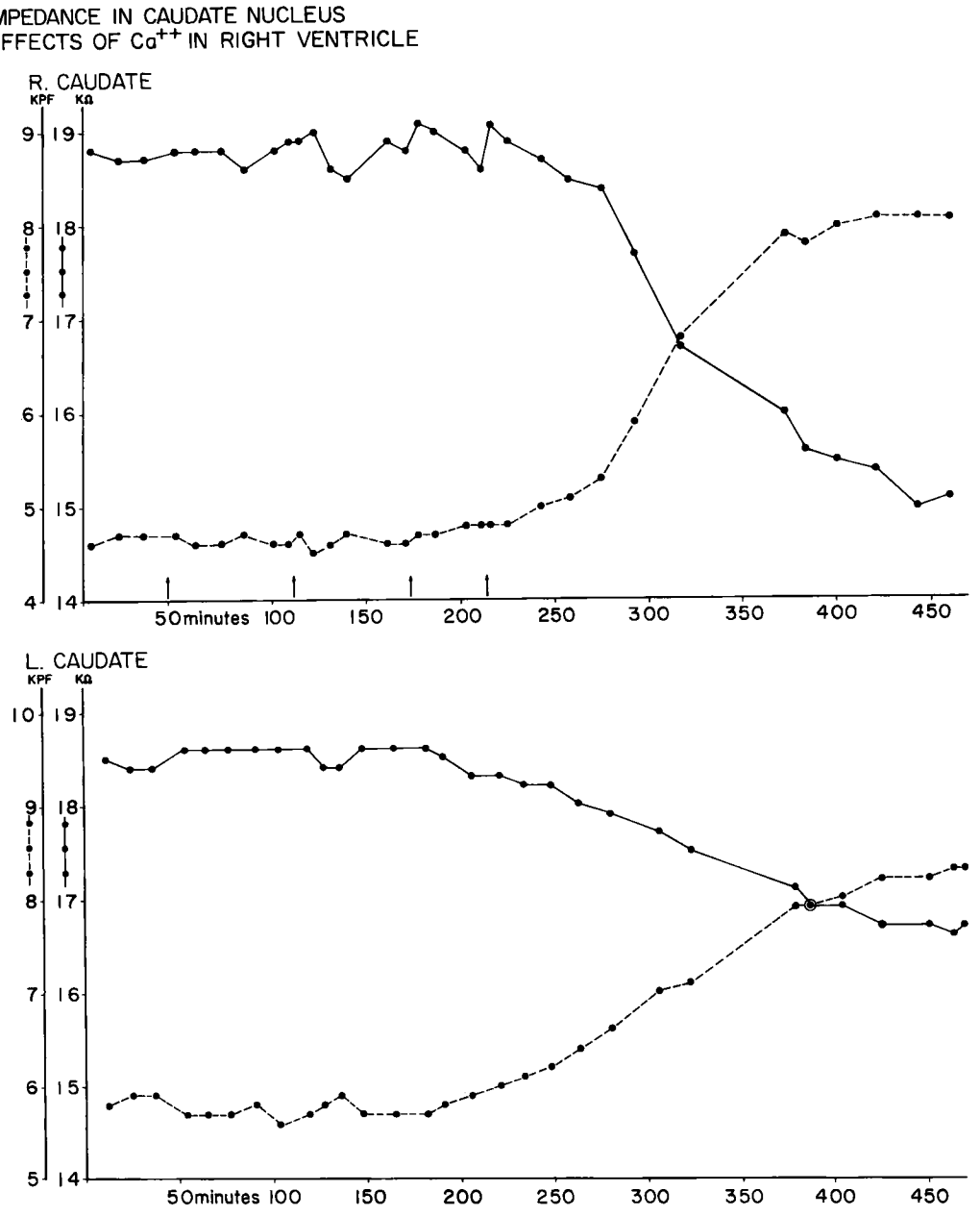
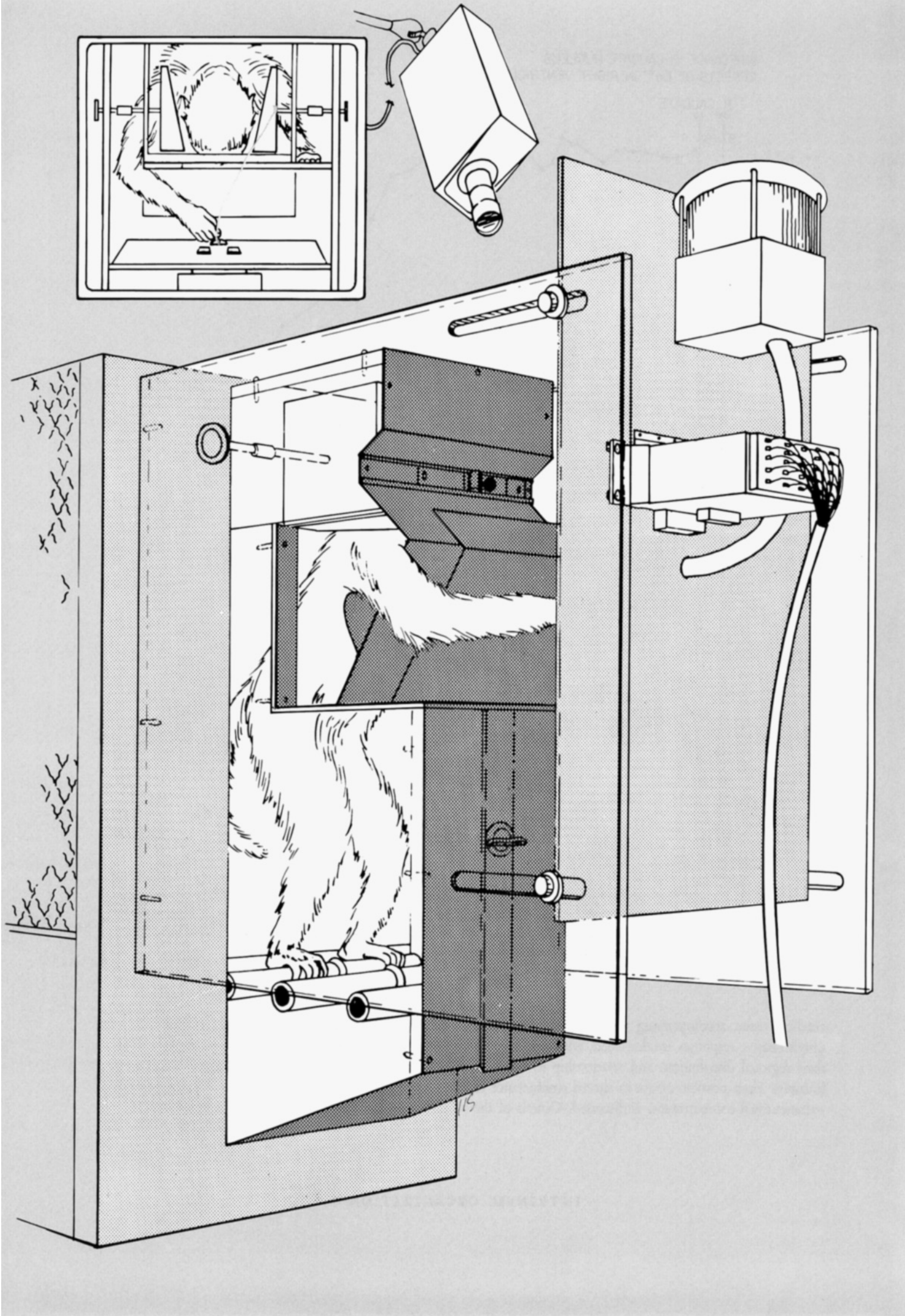


FIGURE 13 Impedance measurements in left and right caudate nuclei, with chronically implanted coaxial electrodes. Control injection of 0.1 ml normal saline at 50 minutes was followed by 3 injections of calcium chloride (each 60 microequivalents in 0.1 ml) at 110, 175 and 210 minutes. Resistive (solid line) and reactive (dashed line) readings are shown for both structures. Ordinate scales: resistance in kilohms (Kr) and capacitance in kilopicofarads (kpf). (From Nicholson, Note 70)

cerebral tissue accompanying alerting, orienting, and discriminative responses are described, with emphasis on their regional distribution, and relationship to levels of learning. Their possible origin in altered conductance in extraneuronal compartments is discussed. Genesis of the electroencephalogram in a population of neuronal wave generators is reviewed, and evidence presented that these generators are non-linearly related, with the EEG arising as a normal distribution from the combined activity of such non-linearly related neuronal generators.



BRAIN CORRELATES OF LEARNING

"When a response regularly follows a stimulus in behavior, the existence of some specific and durable mechanism for connecting them together must be inferred. This mechanism, or memory, resides in morphological, physiological, and biochemical states in part laid down during embryology and in part altered through experiences during life." GALAMBOS, PAGE 643.
This drawing illustrates an experimental study of learning in monkeys with disconnected cerebral hemispheres, PAGE 719.

INTRODUCTION

ROBERT GALAMBOS

Brain Correlates of Learning

WHAT FOLLOWS is an attempt to discuss brain correlates of behavioral responses in a way that will prove useful to scientists who are not biologists. Hopefully, the professional psychologist and physiologist will not find the account wholly useless, even though most of the same ideas and experiments are covered in any good textbook of physiological psychology (see, for example, note 1). What I have done is to select facts and concepts, however elementary, that epitomize the problems behaving organisms present for analysis; devise for them a conceptual framework that may be maximally useful; and compress the result into a few thousand words. If the reader finds some outrageously simple-minded points in the account, I trust he will in spite of this still be inclined to reflect upon the fundamental problems posed. The fact is that animals do fascinating things whenever we look at them; how can these behavioral phenomena be classified, what methods are useful in studying the underlying mechanisms at work, and how far along has the analysis been pushed?

The S → R equation

Much study of animal and human behavior has yielded the following equation to which virtually everyone now adheres:

Stimulus → Response

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This S–R equation has become nearly as strict a dogma in psychology as has the DNA → RNA → protein equation in molecular biology.

For 75 years a substantial fraction of experimental psychologists have been concerned either with discovering and quantifying the Stimulus or with defining and measuring the Response in this equation. Physiological psychologists (or psychological physiologists), on the other hand, deal particularly with the arrow, which, of course, sums up diagrammatically the laws of the brain by which a stimulus becomes connected with a response. Any organism that possesses an organized nervous system can readily be shown to link R's with S's—that is, to display behavior.

Diagram of an organism

Figure 1 expands the simple S–R equation into the shape of a model organism. Its body surface is clothed with receptors specifically tuned or sensitive to physical dimen-

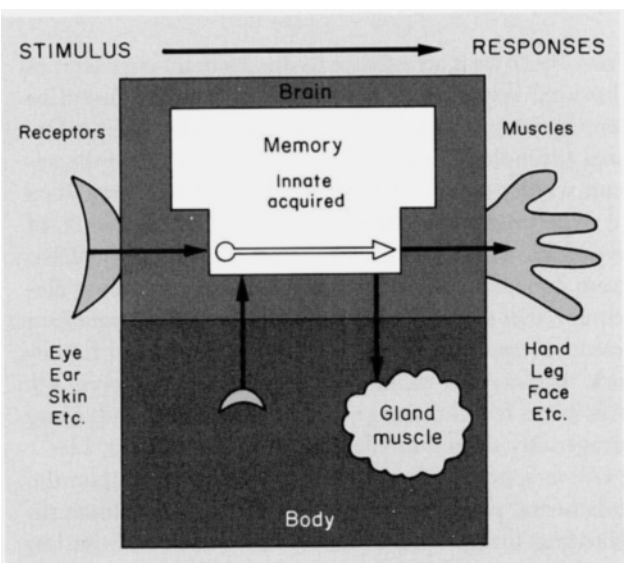


FIGURE 1

sions of the environment. Within the eye, ear, skin, nose, and tongue energy interchanges between these receptors and the environment culminate in the nerve impulses that enter the brain; in this way the S in the equation arises and the input into the organism is defined. Note that receptors also exist within the body itself where they transduce mechanical, thermal, and chemical energy associated mainly with body housekeeping: blood vessels, hollow organs like the bladder, and even the brain substance itself contains classes of such receptors (e.g. in hypothalamus and medulla).

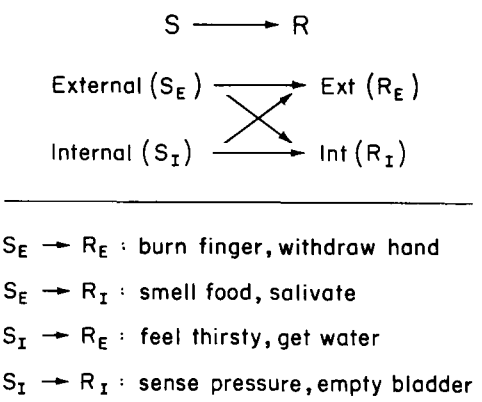
The R in the equation almost invariably results from muscle action, for behavior is synonymous with activity, and animal activity is practically synonymous with muscle contractions. Most of the verbs we use, such as walk, talk, smile, drink, etc., simultaneously describe observed behavior and summarize pattern sequences of muscle contractions. It is, in fact, difficult to think of an example of objectively measured behavior free of muscle responses. Note, again, that hidden from direct view within the body are structures such as glands, heart, stomach, and bladder that can and do make responses to appropriate stimuli, as anyone with a peptic ulcer that acts up in the presence of stressful stimuli will testify.

It is currently fashionable to view an organism as an information-processing system with its inputs, outputs, central processing, and memory store. Our model emphasizes the memory, that property of organisms we infer from observation of their behavior. Inserted between S and R, every input enters it, and all output exits from it. What goes on in it, what we know about the processing, storage, and retrieval of information by brains, is the general problem addressed by this entire book and the specific issue of the chapters that follow.

Types of behavioral responses

Figure 2 shows all possible S–R connections, given the two classes of stimuli (external, internal), and the two of responses contained in our model. The behavioral examples in the figure, showing a given stimulus to activate only one response category, grossly oversimplify most real-life situations, of course; organisms rarely if ever do only one thing in a stimulus situation. Our man in trouble with his ulcer may at the same time be walking slowly away from the source of his irritations and stress.

The various response types of Figure 2 for which we



would like to have brain correlates can actually be discovered in any small story, like the following one, which describes the everyday activity of an organism.

> Each of you woke up this morning, went to the bathroom, dressed, ate breakfast, and walked to his lecture room; now you sit here, digesting the breakfast, taking notes, and learning something new.

Every S–R category shown in Figure 2 appears, I believe, many times in this account of your activities—and if I am wrong, you can think up an appropriate verb phrase that corrects me.

Before accepting the S–R equation as a wholly complete and satisfactory formulation of behavioral responses, one or two difficulties with it should be made explicit. First, it is not always easy to define either S or R, and sometimes we are in trouble with both.

Consider, for example, going to sleep. The internal S for sleep is still unknown, although many possibilities have been suggested. The idea that it is a fatigue chemical that builds up in the blood once had great appeal, dashed, unfortunately, by movie film records showing one of a pair of Siamese twins deeply asleep while its partner, who shares the common blood supply, is obviously wide awake and playful. Very well, if not in the blood, is the chemical stimulus to be found in the cerebrospinal fluid? Or is it not chemical at all, but rather the spread of an inhibitory process across the cortex, as Pavlov thought? Or is it some circadian excitability change of the sort Strumwasser described (this volume) in certain cells of the reticular system that Jouvet and Zanchetti (this volume) implicate in sleep?

As for R called sleep, it displays numerous subtle features that make it hard to measure in toto. Verbs of inaction, not of action, describe sleep best: closed eyes, relaxed face, deep regular breathing, etc. Yet the specific brain organization that underlies and establishes this complex, coordinated behavioral response engages many serious scientists, as earlier chapters in this book demonstrate (Zanchetti, Evarts).

At another level let us take the puzzle of what stimuli and responses operate in recall and reminiscence. Suppose you give me, unasked, the name of the pet dog that brightened your childhood days; how does one conceive the S–R equation to operate in this case? The original S, the real dog, and the final R, your naming him now, are clear enough; what needs explication are the internal "responses" by which the dog and his name became stored in memory a long time ago and the internal "stimulus" that immediately preceded retrieval of his name just now.

It is a common experience for information to become stored simply because one sees, hears, or otherwise receives stimuli from events in the environment; such "responses" must equate with whatever morphological, physiological, and biochemical events take place within the brain during learning. It is an equally common experience to retrieve remembered material in reminiscence and recall; the "stimulus" here presumably emerges at the end of unknown brain processes that (figuratively) search the memory store, select appropriate items, and match them for identity. The S–R formula, by this analysis, would denote as responses certain events within the brain itself ("memory storage") and also include as stimuli the output retrieved from that memory store by some process not as yet known to us. Some psychologists prefer to lump these unknown processes (storage, retrieval) into the arrow in the equation, while others postulate a sequence S–R–S–R–S . . . R of indefinite length proceeding within the brain; the formula fits the facts either way. The problem is to specify the unique brain processes, however formulated, that go on when one recalls the name of a dog long dead.

Innate vs. acquired responses

No one teaches a newborn baby how to go to sleep or the way to empty its bladder. He comes fully equipped to do such things. The baby reaches for his bottle, however, only after he learns through experience what pleasure its contents give. Such facts illustrate the two types of memories, inherited and learned, that have been the subject of polemics for at least a century.

By far the largest amount of attention has gone to the analysis of learned responses; most actual examples of behavior, however, seem neither wholly learned nor wholly inborn, but rather a bit of both. A proposition that appeals to me holds that it is rarely possible, and frequently undesirable, to draw a firm line between innate and acquired behavior; experiments that discover what they have in common may prove far more useful than those that try to find out what separates them.[2] More of this will come later, but first let me present the distinctions historically recognized between them.

An acquired or learned response is one that clearly results from an interaction between the organism and its environment. (Technically, it is the increase in the likelihood of an R to an S as consequence of experience.) Two groups of them can be distinguished. First, organisms learn many kinds of things depending upon whether they receive reward or punishment for their acts. Thus rats learn to run mazes correctly for food reward, or they learn to avoid one alley of a maze because shocks punish them there; Pavlov's dog, which salivated when the bell rang, is another example. The dog learned to salivate because positive reinforcement, food, was appropriately juxta-

posed to the stimulus. Countless thousands of conditioned reflex, instrumental, and discrimination learning studies of this sort reported during the past 50 years fill the psychological literature.

By contrast, healthy, active organisms seem to learn many things merely through contacts, manipulation, and experience—simply by exposure to the richness of the normal environment around them, so to speak. No apparent reinforcement is required for this type of learning. Thus rats[3] and dogs[4] reared in environments filled with objects perform better on animal "intelligence tests" (such as solving a complex maze problem) than do their littermates reared in barren surroundings. What subtle but crucial brain events go on when young animals merely look, listen, satisfy curiosity, and manipulate objects in play remains to be worked out.

Imprinting is a particularly interesting case of learning without apparent reinforcement.[5] Many birds and mammals pass through a phase near birth when they orient strongly toward, and tend to follow, moving objects. Usually this is the mother animal, but in numerous experiments neutral objects such as wooden boxes pulled by a string have been substituted for the mother. Experimental birds turn out in later life to prefer the substitute mother over the real one and will follow the moving box despite the frantic attempts of the mother bird to lure the young one to her side. In imprinting, then, learning of a durable kind—it lasts a lifetime—becomes possible during a critical period in development near birth.

Innate responses, unlike these learned ones, emerge fully formed, or nearly so, the first time a particular stimulus is applied. Training is not required before they appear, and for them questions relating to reinforcement seem irrelevant. Into this category go all behaviors as characteristic of the organism as is the number of its legs. The repertoire of the newborn baby contains numerous complex examples (breathing, crying, etc.) as well as many simpler ones (knee jerk, pupil constriction to light, etc.). In displaying them the organism reveals, one may say, some fraction of its ancestral behavioral heritage.

Instincts, as defined by the ethologists,[5] represent the most complex examples of all: the fish that viciously attacks an object that resembles a rival male, even though that object be a bit of wood with a characteristic red dot painted upon it; the nesting bird that retrieves egglike objects placed nearby, and so on. In such cases a single trigger stimulus, or an ordered sequence of them, where experienced for the first time, evokes a fully formed response, stereotyped in pattern, and species-specific.

To these innate and acquired response classes, one must add a final class of actions, or propensities to act, that organisms display. Poorly defined, perhaps largely innate, they still defy proper classification, let alone analysis. Here belong, on the one hand, the curiosity that drives children, apes, and scientists to explore; the search for novel stimuli that can readily be measured in animals and men; the differences in native intelligence revealed by how well two children perform in the classroom. Here also belong motivation, alertness, and attention, those accessory conditions for learning that reflect aspects, or states, of brain activity that seem to prime and prepare the brain for deposition and retrieval of memories.[6] Brain correlates for some of these behavioral states, capacities, and propensities required for storage and retrieval have been discussed (see Teitlebaum, Livingston, Jouvet, Scheibel in this volume) and will receive no further attention here. As might be suspected, traps for the unwary lie everywhere in the path of those who would analyze such matters as native intelligence, attention, and curiosity.

Some generalizations and a definition

I should now like to state a view about innate and learned responses intended to erode away the historical distinctions between them and to provide a possibly more useful conceptual framework for those doing experiments upon memory. To begin, let me tell another story.

The ethologist Eibl-Eibesfeldt once took a young squirrel away from its mother and raised it in isolation. In the fall of its first year he put a nutlike object on the concrete floor of the laboratory, released the squirrel from its cage and watched to see what would happen. The squirrel approached the pseudonut, lifted it into its mouth, dug an imaginary hole in the floor, stuffed the imaginary nut into the imaginary hole, and then covered it over with imaginary dirt. His account (with which I have taken some minor liberties) demonstrates the existence of a remarkably complex innate motor pattern released initially by a sign stimulus (the nut), to use the ethological terminology.

My story continues. One day a boy approached his father complaining that no matter where he hid his marbles his brother always found them. His father thought a moment, then took the boy outdoors, dug a small hole under a rosebush, dropped his son's newest and best marble into it, and covered it over with dirt. His aim, of course, was to teach the boy, who had watched the proceedings with great interest, something he did not already know.

Some days later the father looked out the window and saw a squirrel dig a real hole, stuff a real nut into it, and cover it over with earth. He thought to himself; "Here is a clear example of an innate, species-specific response somehow built into the organism through the readout of the genetic code." Looking in a different direction he saw his son, who had just acquired a new marble, in the process of digging another hole under the bush to bury it. "There, on the other hand," he thought, "is a good example of memory and learning, the result of an interaction

with the environment, stored and retrieved by mechanisms about which we have no knowledge."

This story is intended to illustrate three generalizations that I must assert without proof, since there is none. First, it is not possible to distinguish clearly between innate and acquired responses on the basis of their complexity, durability, utility for the organism, ease of evocation, or any other of the dimensions that have been discussed at one time or another. This means we are not likely to find in such concepts as "higher (as opposed to lower) nervous activity," or in innate vs. acquired, any useful guides to experiments on memory. Every sample of behavior reveals an aspect of the past history of the organism that has been stored within the brain. These memories arise both via the genes and through experience; I suggest, in the absence of conclusive data to the contrary, and out of respect for the principle of parsimony, that all of them come into existence, are stored, and receive their expression through fundamentally the same mechanisms. Figure 1, which lumps all memory together, emphasizes this concept of their basic similarity.

The second generalization follows from the first and leads to a number of the experiments appearing later in this book. We suppose, with good reason, that the impressive behavioral repertoire of a squirrel, or a newborn baby, results from the correct assemblage of brain parts by the DNA architect and the RNA workmen. A baby sleeps, cries, suckles, salivates, swallows, makes a fist, kicks its legs, and performs every other response it is born with because human genes can be counted on to generate an object that not only looks human, but acts human as well. Experimental data supplement this common sense view. For instance, if behavioral deficiencies appear at birth or later, gross abnormalities may appear in the chromosomes, as in the mongoloid idiot, or the individual may be an enzymatic cripple, as in the case of some 20 known "inborn errors of metabolism." Thus both inference and evidence implicate genetic mechanisms in the monotonously regular appearance of behavior characteristic of the species. We may still be largely ignorant about the way the genetic readout assembles a brain so that it works so well, but embryologists studying organogenesis are beginning to dispel it. (Edds, Ebert in this volume).

If despite lack of exact knowledge of the details we can agree that genetic mechanisms read into the brain memories characteristic of the species, what about the probability that they also contribute to memories arising out of experience? To me it seems very high. Once cells discover how to organize themselves in a baby's brain so that hunger leads to crying, it is unlikely they would devise some other mechanism, based on entirely different principles, to enable the baby to learn that crying commands attention. The original solution is so elegant, simple, and exact; why should nature (or physiologists) abandon it in favor of some other?

The third generalization complements the other two. To dichotomize responses into innate and acquired tends to obscure the persistent interactions between them. A squirrel probably learns many relevant things before its nut-burying instinct can emerge in fullest form, while boys learn as they do because they are innately equipped with intelligence and curiosity, among their many other native endowments. This interactionist position would place a few purely innate responses opposite a few purely learned ones as terminal points on a continuum, with most samples of behavior lying somewhere in between, the true product of heredity and environment. The available evidence, at any rate, has led all except the most obdurate to this conclusion.

Let me summarize the viewpoint just presented with this definition: memory is the inferred collection of brain processes by which responses are linked to stimuli in behavior. Such a definition, in making no distinction between a knee-jerk reflex and the recall of one's own name, frankly recognizes our inability to state criteria by which the central mechanisms operating in acquired and innate responses can be differentiated. Until such criteria do develop there seems no reason (other than a subjective "feeling") to argue that the mechanisms responsible for reminiscence differ from those for reflex.

Mechanisms of memory

The objective of the remaining chapters in this book is to provide data, however incomplete, on the specific question of where and in what form a brain stores memory. The authors are trying to find the locus of the engram and/or to specify mechanisms by which responses become acquired, stored, and retrieved. The various methods they apply fall into the well-known three major groups: anatomical, physiological, and biochemical. Any given experiment, of course, may fit into more than one of these categories.

Historically, the anatomical approach came first because, one may suppose, good microscopes appeared on the scene before vacuum tubes and sophisticated biochemical analyses, and experimenters invariably put to use whatever good tools they have available. By about 1900 the anatomists had clearly formulated three morphological questions still debated, and still unresolved by experiments. First, are memories stored in particular brain regions, or centers, that increase in size with use? Second, does synaptic efficiency rise in learning because nerve terminals became larger, or more numerous, or more ef-

fective? Third, do glial cells join with the neurons in making memories by providing essential support, by their mechanical activities (motility), or in some other manner? Sperry and Chow deal with efforts to localize brain areas essential for learning—to specify "centers," whereas Altman takes up particular brain cellular changes that are, or may be, connected with memory storage and readout. Kandel's essay on altered synaptic efficiency measured by physiological techniques also belongs here, even though no morphological correlates for his phenomenon are available. The idea that one might actually see cells change shape during their physiological activity is at least 75 years old, but the necessary light or electron-microscope techniques for making the required observations in living brain have still not been developed.

Since for the past half century the electrophysiological approach has yielded the richest harvest of new information about the brain, its advocates have naturally assumed it will also contribute significantly to the eventual solution of the memory problem. Electrical events mirror ongoing metabolic and membrane changes in the brain cells, and so those who take and read electrical records hope to deduce from them the sequence of cellular events by which memories are acquired, stored, and retrieved. E. Roy John, using large electrodes, measures global events under way in mammalian brain, whereas Kandel, using probes inside single invertebrate cells, carries the method to the ultimate in the opposite direction. The high degree of methodological sophistication and data analysis attainable by modern exponents of the technique is beautifully represented in these papers.

Electrophysiologists have frequently speculated upon the fundamental processes underlying memory.[6] The most popular idea centers around altered "synaptic efficiency" in learning and forgetting. Increase in the size of presynaptic contacts, or in the amount of transmitter they release, or in the sensitivity of the postsynaptic membrane have all been suggested as possible mechanisms. Because relatively large numbers of brain cells seem to be altered with acquisition of a memory, most hypotheses include the creation of new chains (reverberating and otherwise) of neuronal activity secondary to the altered synaptic transmission. In this way cell assemblies of varying size, each unique for a given memory, are supposed to come into existence during an experience; retrieval of the memory at a later time is equated with re-creation of the original unique activity within the ensemble. Many variants on these fundamental ideas are to be found in the literature, including the thought that short-term memory represents purely electrophysiological conduction of impulses through the assembly of neurons involved, with gradual entry of DNA-dependent biochemical events to fix the activity, and therefore the memory, into permanent form.

Biochemists, too, have offered their ideas about how memories might come about, as the final three chapters in this book demonstrate. These applications of modern biochemistry to our problem provide whatever common grounds chemists, psychologists, and physiologists share. They give us the newest, and in some ways the most interesting, developments in research on the problem. Conceptually arising out of the remarkable advances in molecular biology, they represent, in one way or another, attempts to apply to brain cells the techniques and methods that have yielded such brilliant results with other cell types. Quarton, Agranoff, and Hydén fairly and judiciously sample the accomplishments realized to date.

Considered as a whole, these chapters illustrate all the modern methods of brain research available today. One can take the brain apart (Sperry, Chow) and see how it is put together (Altman); he may put electrodes into it and give it electrical shocks (Eisenstein), or make recordings of electrical responses within it (John, Kandel); he can analyze it chemically (Hydén); heat, cool, or poison it (Agranoff); and even transfer part of it to another animal by syringe (Quarton). No little boy curious about what makes a clock tick ever showed greater ingenuity in his efforts to find out.

As you read these carefully prepared summaries on the morphological, electrophysiological, and biochemical events so far related to the memory problem you may wish to bear in mind certain other dimensions of thinking that lie behind the actual experiments. Some investigators, you will discover, prefer to use simple preparations containing few synapses, while others study entire brains in complicated animals like the monkey; behind these choices lie convictions, sometimes strong ones, regarding the shortest path to that understanding of memory processes we would all like to possess. Another difference in viewpoint shows up in the relative emphasis placed upon glial cell participation in learning; Hydén, for instance, conceives of the neuron and its attending glial cells as somehow participating jointly in the enterprise of laying down and storing memories, while other investigators give something less than enthusiastic support to this idea, advocating instead the view that the neurons alone take care of the job through specific connections due to altered synaptic events. And, finally, you should be warned that while all the authors seem convinced, more or less, that experiments will eventually show learning to be closely tied to biochemical change in brain cells, this feeling is not universally shared by all students of the problem. For better or worse, no real sceptic—or perhaps I should say extreme sceptic, or true disbeliever—has been asked to present his views to you.

Just 10 years ago, Clifford T. Morgan and I wrote a chapter on the Neural Basis of Learning.[6] When recently I reread it, I was impressed by the contrast between what one can say about memory mechanisms now as compared to what one could say then. There has been an immense flowering of new concepts, new information, and new data during the past five years. Despite this, the chapters that follow are not likely to convince you that the problem of memory is solved; you may, however, agree that good progress in that direction is being made.

Summary

When a response regularly follows a stimulus in behavior, the existence of some specific and durable mechanism for connecting them together must be inferred. This mechanism, or memory, resides in morphological, physiological, and biochemical states in part laid down during embryology and in part altered through experiences during life. Where experiential factors are minimal or absent, as in reflexes and instincts, the genes through their readout assemble and organize brain cells to establish the memory and enable its expression. For learned responses, also, the genes probably (but not yet certainly) play a crucial role. Thus all memories, whether phylogenetic or ontogenetic, display similar if not identical basic properties, probably require participation of the genes, and may well be laid down and expressed in fundamentally the same manner. However, the detailed understanding of the way any memory comes into existence, even the clearly species-specific ones, awaits the results of future research.

Certain Facts of Learning Relevant to the Search for its Physical Basis

NEAL E. MILLER

AN ENORMOUS AMOUNT of detailed experimental work has been done on learning and memory. A recent book on conditioning alone has 1,700 references, although it does not attempt to cover the entire field of learning.[1] This chapter is a highly selective summary covering only certain facts of learning that I believe are likely to be most relevant to the search for its physical basis. One may hope that a consideration of the kinds of evidence needed to put useful constraints on the search for that physical basis may lead future investigators to reevaluate old experiments on learning critically and to design quite different and illuminating new ones.

Some defining characteristics of learning

GRADE-A CERTIFIED LEARNING Because the greatest part of the work on learning has been done on mammals, and because that is the type with which I am most familiar, I deal here only with mammalian learning. Furthermore, I deal only with phenomena that all investigators will agree are clearly learning. We might call this "Grade-A Certified Learning." To the extent that lower animals show the same phenomena, we shall be reasonably confident that we are dealing with something like the kind of learning that occurs in mammals. It is entirely possible that certain insects and other animals display radically different types of learning, the physical basis for which could be different from that for the kind we see in mammals. It is also possible that there are other phenomena—the habituation of an innate response when the stimulus for it is repeated; the more rapid production of enzymes with repeated demand; or possibly even immune reactions to foreign proteins—that may have some elements in common with learning and that may provide the key to an understanding of its physical basis. After we achieve this understanding, we shall be in a much better position to define learning and its relationships to other phenomena. In our present state of ignorance we cannot afford to discard any approaches.

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Although I believe we can be reasonably sure that certain phenomena are learning, we cannot always be as sure that certain other borderline phenomena are *not* learning. With this clearly understood, I shall attempt to proceed with one approach—that of concentrating on phenomena which show most clearly the distinctive characteristics of mammalian learning.

Learning is a relatively permanent increase in response strength that is based on previous reinforcement and that can be made specific to one out of two or more arbitrarily selected stimulus situations.

Each of the criteria contained in the foregoing definition will now be discussed.

1) "Relatively permanent" means that the change lasts for days or months rather than seconds or minutes. Perhaps some of the physical processes involved in learning are transient, but under normal circumstances the final product of the entire sequence should be relatively permanent. This characteristic will be discussed further in a later section.

2) "Increase in response strength" means an increase in the prepotency of the response; in other words, in its ability to occur in competition with other responses. If the strength of the competing responses remains equal, there is an increase in the probability that the response will occur. But learning can also result in a decrease in the probability that a specific response will occur. It is obvious that such decrements are frequently the product of learning new responses that are incompatible with the one that is becoming less probable; we are *tentatively* assuming this is the case with all learned decreases in the probability of response occurrence.

3) "Reinforcement" involves association by temporal contiguity and can be produced in two ways:

(a) In *classical conditioning,* a conditioned stimulus (CS) is reinforced when it is followed promptly by an unconditioned stimulus (UCS) that elicits the response to be learned. However, as we shall see later, some unconditioned stimuli are better than others. Because association by contiguity is required, classical conditioning should be produced during trials when the CS is paired with the UCS, but not during trials when they are each presented separately.

(b) In *instrumental learning* (also called trial-and-error learning, type II conditioning, or operant conditioning), a response is reinforced when it is promptly followed by a reward. Escape from a noxious stimulus can serve as that reward. In instrumental learning, following a given response (R_1) by reward should produce more of an increase in R_1 than of R_2; on the other hand, following R_2 by reward should produce more of an increase of R_2. In this last test, one must, of course, use suitable controls to make certain that the effect is indeed the product of *previous association* between the response and the reward rather than of some transient after-effect of the reward or some product of the reward that affects the animal's behavior by permeating the present physical environment—for example, the smell of food in the goal box on the correct side of a T-maze or, in a different example, the CO_2 excreted into the water by the bacteria used to feed a paramecium.[2]

4) The final criterion—that a learned response can be made specific to an arbitrarily selected stimulus—is believed to be the most fundamental one. When the response is not, initially, reasonably specific to the arbitrarily selected stimulus, it can be made so by trials on which it is reinforced to that one stimulus and not to others. To illustrate a test of this criterion in some detail, let us suppose that we have divided a sample of rats into two randomly selected groups and trained one group to turn right in a white T-maze and left in a black T-maze; at the same time, we have trained the other group in the opposite responses of turning left in the white and right in the black T-mazes. Controls have shown that these responses are indeed dependent on the blackness and whiteness, rather than on other factors, such as the odor left by preceding rats. Stated abstractly, this extremely stringent test means that our instrumental learning or classical conditioning procedures should be able to either cause S_1 to elicit R_1 and S_2 to elicit R_2, or to cause S_1 to elicit R_2 and S_2 to elicit R_1. Only new responses (as contrasted with innate right or left turning tendencies) capable of being made specific to arbitrarily selected stimuli could allow us to produce such a pattern of results. I believe everyone will agree that when the test has been passed learning has been demonstrated.

As far as I know, phenomena that will meet the foregoing test of criterion 4 also will be found to satisfy all of the other criteria. This increases our confidence that we are dealing with a fundamental aspect of nature rather than with a purely arbitrary and relatively useless definition. Criteria 1 and 2 are necessary but not sufficient; criterion 3 may also be used as a test of learning. Actually, all of the laws of learning are a part of its definition, and the more of these a particular example is known to satisfy the more confident we can be that it actually is Grade-A Certified Learning.

In the foregoing definition, stimulus and response are defined broadly as independent and dependent variables, which may include direct electrical stimulation of certain neural structures and direct recording from them. I have discussed this liberalized definition in more detail elsewhere.[3] According to this definition, the sound of a friend's voice (as a CS) followed by the sight of his face

(as a UCS eliciting a perceptual response) could be the basis of a conditioned response causing an image of the face to be elicited when subsequently the voice was heard.

EXAMPLES NOT QUALIFYING AS LEARNING We have already given an example of learning in which practice involving the association between the color of a maze and the reward for a specific turn caused the rats to learn to make that turn in a maze of that specific color. Let us now contrast this example with certain phenomena which, at a superficial glance, may be thought to be learning, but which will turn out to be at least somewhat different.

If a subject is given trials of lifting a weight with one of his fingers, the response will undergo progressive change until the subject stops responding. This change is not, however, specific to a given stimulus situation (although under some circumstances it may have more specificity than one might expect), and hence fails to meet criterion 4 of our definition. Furthermore, it is relatively transient in contrast with the permanence of the true learning in the T-maze, and fails to meet criterion 1. In this way we distinguish physical *fatigue* from learning.

With sufficient exercise of a given response system, such as a set of muscles, its capacity to respond may be increased. This increase may be relatively permanent, but does not appear capable of being made specific to a given stimulus situation. Thus we distinguish the effects of exercise on *physical fitness* from learning.

If a stimulus that innately elicits a response is repeated in a series of closely spaced trials, the amplitude of the response is greatly reduced. This reduction may be relatively specific to the stimulus that was presented; on the other hand, since the response is innate, the various arbitrary stimulus-response combinations specified in criterion 4 and illustrated in our example of the T-maze, cannot be achieved. Furthermore, the effect is rather transient. It is called *habituation* rather than learning, although some people believe that the two phenomena may share some elements in common. (There are some cases in which the decrement in an apparently innate response is relatively specific to the stimulus selected for habituation and is relatively permanent. Such cases may be found to be true learning.)

An experimenter may give the subject several presentations of a strong unconditioned stimulus such as an electric shock, which elicits a strong unconditioned response (UCR), such as running or leg withdrawal. After this he may find that certain other reasonably strong and sudden stimuli that were neutral, in that they did not previously elicit this response, will now elicit it even though they were not present immediately before the unconditioned stimulus and response. Because this new tendency was not dependent upon the reinforcement of the UCS promptly following the CS (criterion 3), it cannot be made specific to either one or another arbitrarily selected neutral stimulus in the way demanded by criterion 4 and illustrated in the example of the T-maze. Furthermore, the phenomenon is relatively transient, and hence fails to pass criterion 1. It is called *sensitization* or, sometimes, *pseudoconditioning*.

The typical test for sensitization is to contrast the results for an experimental group in which the CS is paired with the UCS and UCR, with those of a control group, in which both CS and UCS are presented an equal number of times but never paired. If the CS has an equal tendency to elicit the response in both groups, the results clearly are not learning, but sensitization. If the paired group elicits the response reliably more often than the unpaired one, learning is involved.

Actually, the learning may not only increase the tendency to respond in the paired group, but may also decrease it in the unpaired group. In some cases, what seems to be sensitization may actually be conditioning that is relatively specific to the room, the apparatus, or other features of the experimental situation, and is also permanent enough to be called learning. However, it is not learning to respond specifically to the particular cue that the experimenter first throught was serving as the conditioned stimulus. But these are refinements that need not concern us at this moment.

Sensitization is a fairly common phenomenon, so it is necessary to control for it. For example, in Kandel and Tauc's experiments on *Aplysia*,[4] the large cells showed only sensitization, while some of the small ones passed the foregoing test for learning.

SIMILARITIES BETWEEN CLASSICAL CONDITIONING AND INSTRUMENTAL (OPERANT) LEARNING You will remember that both instrumental learning and classical conditioning meet exactly the same criteria of learning except that, in the reinforcement specified in criterion 4, the obvious temporal association is between CS and UCS for classical conditioning and between response and reward for instrumental learning. As we shall see later, these two apparently different associations may actually function in the same way.

In both classical conditioning and instrumental learning the nonreinforced repetition of a response weakens the tendency for that response to occur, a process called *experimental extinction*. As we have already pointed out, in both cases the response can be made more specific to a given stimulus by discrimination training that involves reinforced trials to one stimulus and nonreinforced trials to another one.

Because Skinner[5] originally thought the stimulus situation was irrelevant in the absence of specific discrimination training, he spoke of the instrumental response as being "emitted" rather than "elicited," and maintained that this was a primary difference between instrumental and classically conditioned responses, which he calls operant and respondent, respectively. Recent work makes it seem probable that such a fundamental distinction does not occur; both instrumental and classical conditioning procedures necessarily involve a certain amount of inherent discrimination training, and in both cases the response can be made more specific by additional training.[6]

The traditional view has been that only skeletal responses mediated by the cerebrospinal nervous system are subject to instrumental learning and that visceral and glandular responses, mediated by the autonomic nervous system, are subject only to classical conditioning.[1,5,7,8] Thus these two types of learning were thought to be fundamentally different, involving different parts of the nervous system. Recent work in my laboratory, however, clearly shows that visceral responses, under the control of the autonomic nervous system, are subject to instrumental learning.[9-12]

There are many other similarities between the laws of classical conditioning and instrumental learning, and such differences as seem to remain may not be fundamental qualitative differences, but, rather, quantitative differences analogous to the effects that can be produced within each type of learning situation by varying certain parameters.[9] It may develop that classical conditioning and instrumental learning are two different types of training situations rather than two fundamentally different types of learning.

In view of the similarities between the learning in these two situations, I have advanced the hypothesis that rewards for instrumental learning act via a "Go Mechanism" that facilitates any neural activity that immediately precedes the reward.[13] This Go Mechanism is presumed to have exactly the same effect (an increased rate of firing) on the motor neurons, which have just produced a given rewarded response in instrumental learning, as is produced in classical conditioning when the UCS elicits that response as a UCR. It should be possible to test this hypothesis by recording the activity of the proper motor area during the moments immediately after the first time its activity is strongly rewarded.

DRIVE AND REWARD It should be noted that the unconditioned stimulus serving as a reinforcement in classical conditioning must be able to elicit the response to be learned. Thus, Pavlov[14] conditioned the salivation of a hungry dog to a previously neutral bell by immediately following the bell by meat powder that elicited salivation. But when the same meat powder is used to train a hungry dog in an instrumental response, such as pressing a bar, we are not using an innate reflex tendency to elicit the salivation response—in fact, the meat powder produces no such tendency. Instead, we are using its ability to strengthen any immediately preceding response. However, the unconditioned stimuli, which are most effective in classical conditioning, are also effective in instrumental learning. We have just pointed out that, with a hungry dog, meat powder can be used as a UCS to elicit salivation or as a reward for an instrumental response, such as pressing a bar. Similarly, the same electric shock that can be used as a UCS to condition a response of footlifting can be used to train an animal to press a bar, but in this case the escape from shock serves as the reward. The stimuli that are good unconditioned stimuli for reinforcing classical conditioning also seem to function either as rewards, to get which an animal will learn an instrumental response; or as aversive stimuli, to escape which it will learn an instrumental response. In the latter case it is the escape from the aversive stimuli that functions as a reward.[15]

Until recently, one of the striking exceptions to the foregoing generalization appeared to be direct stimulation of the sensory-motor area responsible for pawlifting, which Doty and Giurgea[16] showed could be used as a UCS for such responses, even though it apparently had little rewarding or aversive value. However, conditioning reinforced in this way is weak, in that it can be established only with widely spaced trials and only with certain dogs. Furthermore, Thomas[17] has shown that, if dogs that had learned such a CR were given a UCS to the motor cortex without a preceding CS, they tended to fall down, although they had not done so while performing the CR. On the basis of this observation, he suspected that the avoidance of sudden postural disturbance could function as the motivational basis for such conditioning. To test this hypothesis he required dogs to stand with each foot on a little individual platform. To emphasize the aspect of postural adjustment, he moved the platforms further apart, and he found that this change favored this kind of conditioning. Wagner et al.[18] secured additional confirmation of the hypothesis by showing that the opportunity to make a suitable postural adjustment before receiving this kind of UCS could be used as a basis for instrumental learning; dogs learned to press the one of two panels that always caused the UCS to be preceded by a CS, rather than the one that caused the CS and UCS to be unpaired, so there was no opportunity to make an anticipatory adjustment.

While there is still some controversy about whether any learning can occur in the complete absence of all motivation and reward, it is quite clear that learning is better if some motivation and reward are present.[19] Indeed, it seems reasonably certain that some selective process must be involved in learning and/or retrieval, or we all would be doomed to be mired hopelessly in total recall, and continually to add new central connections to our repertoire until our brain finally would be overwhelmed by one grand convulsion.

One of the early notions was that learning was the product of mere frequency, like the wearing of a path, but we now know that this is inadequate as a sole explanation. The nonreinforced repetition of a response does not strengthen it, as would be predicted if use were the sole factor; instead the response becomes less likely—a phenomenon called *experimental extinction*. Similarly, when hungry animals are run to food in a D-shaped maze, some of them start out by taking the long alternative considerably more frequently than the short one; with further practice the probability of their choosing the long path to the reward decreases instead of increasing, as it would if mere use were the primary factor producing learning.[20]

We are not yet certain of the exact roles of motivation and reward as selective factors in learning and/or retrieval. It is conceivable that they act directly to facilitate the strengthening of a connection or to protect it from being weakened during a crucial early stage of consolidation. On the other hand, it is also conceivable that they achieve their effects in indirect ways, such as by providing a necessary level of general arousal, by causing the animal to pay attention to the relevant cues, or by facilitating the retrieval and performance of the learned response in competition with other responses. These matters need further clarification by more penetrating research.

We have discussed the theoretical role of motivation and reward in producing efficient learning; they also have great practical significance. Anyone trying to secure specific types of learning in order to work on its physical basis will be well advised to see that the animal is motivated and rewarded so that efficient learning will be more likely to occur.

For example, attempts to classically condition single neural units have produced a discouragingly low proportion of such conditioned units. Perhaps such conditioning would be considerably more efficient if a rewarding brain area were electrically stimulated immediately after the unit was fired by the recording microelectrode. With a curarized animal, a CS not originally capable of firing the neuron in question should be followed by stimulation via the microelectrode firing it, in turn followed by stimulation of a rewarding area of the brain. Such a procedure should increase the probability of producing learning. If conditioning is produced, it seems probable that the changes involved in such learning will be in the stimulated neuron or in the synapses of its dendritic tree. This type of rewarded conditioning procedure should be contrasted with the purely instrumental learning procedure used by Olds and Olds,[21] in which a rewarding area is stimulated every time the single unit fires. The learned changes responsible for such spontaneous firing can readily occur in groups of neurons elsewhere, which in turn affect the recorded one indirectly via a number of intermediate links.

Other relevant characteristics of learning

We have been discussing characteristics that are especially useful in producing and identifying learning; let us now consider other characteristics especially relevant to the search for its physical basis. Some of these will also refine its identification.

CAN OCCUR QUICKLY Under many conditions learning can appear to be a painfully slow process, but under favorable conditions it can occur very quickly. Certainly many cases of slow learning involve situations in which many different stimulus-response connections, or habits, have to be learned in order to cope with complex and varying circumstances. They also may involve situations in which the conditions favorable for a specific element of learning occur only occasionally during the training trials. In fact, many workers believe that all instances of learning occur suddenly in an all-or-none fashion on a single trial, and that the gradual learning curve is a statistical artifact of averaging together a large number of such instances.[22,23] Although this may turn out to be a somewhat extreme position, experimental evidence makes it perfectly clear that under favorable conditions learning can occur in one trial. The physical mechanism must be one capable of accounting for such instances.

For example, in one experiment a tame albino rat was placed in a short, narrow compartment leading out into a considerably larger one, and within a few seconds he moved into the larger compartment where he was immediately given a strong electric shock. When given a test trial without shock 24 hours later, he did not step out of the small compartment within the time limit of 180 seconds; he had learned in a single trial. Almost all of the rats showed this same behavior. Since their exploratory behavior was not greatly disturbed in other stimulus situations, this one-trial learning must have been reasonably specific. Even if rats were taken completely out of the situation within 2 seconds after the termination of the

2-second, 1-milliampere shock, they still remembered on the next day. Therefore, the learning did not depend on extensive rehearsal, during which after-effects of the shock were repeatedly associated with the physical presence of the stimulus situation.[24]

The speed of the initiation of at least the first steps of the physical changes involved in this learning can be inferred from experiments on the amnesic effects of a strong electroconvulsive shock (ECS) administered to the brain after one-trial learning of the type that has just been described. Although ECS administered to the brain within one second after the painful shock to the feet usually will wipe out the effects of the learning, however, this amnesic effect falls off rapidly, so that approximately 50 per cent of the rats remember if the ECS is given as long as 15 seconds after the shock to the feet, and practically all of them remember if it is given 60 seconds post-shock.[24,25] For our purposes it is interesting to note that there is considerable variability in the effects of the ECS; some of the rats, which have been given a strong ECS (100 milliamperes for 0.3 seconds) as soon as 0.1 second after the shock to their feet, fail to step out into the large compartment on the memory test 24 hours later. Better controls are needed to be certain that this is not an effect of the ECS on the exploratory tendencies of a certain proportion of the rats receiving this treatment. Such a control is inherent, of course, in the comparison between the groups receiving the ECS 0.1 second and 15 seconds after the shock to the feet. We can be quite certain that because an increased number of rats in the latter group refuse to go to the place where they received the foot shock, better memory for that painful experience is indeed indicated.

Electroconvulsive shock produces immediate unconsciousness in human subjects, and appears to have the same effect on the rats. The massive way in which it disrupts the normal patterns of conduction in the brain seems almost certain to disrupt any memory that was being stored by the perseveration of activity in a reverberating neural circuit. Thus some other persisting effect of the learning experience must have been initiated in a relatively brief interval.

In the experiments just described, we have seen that the biological mechanisms for learning must be capable of so consolidating the memory within a short interval that in some cases it can survive severe electroconvulsive shock given only 15 seconds after the learning experience. There is a suggestion that there may be cases in which it survives with an even shorter opportunity for consolidation. In this short interval the mechanisms must be able to translate the information from neural impulses to some other form of storage.

On the other hand, under different circumstances the gradient of retrograde amnesia may extend over a much longer period of time. With exactly the same strain of rats and the same learning situation used in a previous experiment on ECS, Paolino, Quartermain, and Miller[26] found that the gradient of retrograde amnesia produced by CO_2 anesthesia extended over a period between five and ten times as long as that produced by ECS. Control tests showed that these differences could not be attributed to differences in the intensity of the treatments. Other experiments employing different types of learning situations and/or different types of amnesia-inducing treatments have secured considerably longer gradients of retrograde amnesia.[27-29] Because most of these experiments differ in more than one respect—details of the training situation, types of amnesia-inducing treatment, and strains or species of animals used—it is difficult to draw any detailed generalizations from the results, except for two limiting ones: the mechanisms responsible for the consolidation of the memory trace must be able to account for (a) some cases in which it can survive strong ECS, given as briefly as 15 seconds after the learning experience, and (b) other cases in which it can be affected by treatments given hours or days later.

Now that we are beginning to see some of the outlines of the problem, additional studies, which are more systematic and analytical, should yield more definitive information.

PERMANENCE OF LEARNING There is a great deal of evidence that the effects of learning can last a long time. Using the simple one-trial learning situation that has just been described, Miller and Kushel[30] found that every single one of 60 rats given the single, brief electric shock in the large compartment were slower in stepping into it when tested 23 days later. Furthermore, there was no appreciable difference between the rats tested one day after training and those tested 23 days later. Thus, the learning produced by that single, brief exposure to the electric shock survived.

Other experiments in which animals have been trained for a number of trials show similar results. During World War II, Skinner[31] trained pigeons to peck at a very specific point on a rather complex aerial photograph. After they had learned, he did not reward every peck but used a schedule designed to keep the pigeons pecking for long periods without rewards. After this particular project was discontinued, he set these pigeons aside as breeders. Four years later he restored the birds to a feeding schedule and put them back in the same apparatus, to be tested again. All of the pigeons showed excellent retention, in that they did not start to peck until the picture was

projected and then promptly pecked at the precisely correct place on the photograph. Although they were not rewarded during the test, they pecked many times at the correct place. One pigeon gave 700 responses before it quit. There was some forgetting, in that the birds had given from 2 to 4 times as many responses before their four-year holiday, but there was also excellent memory, as indicated by the persistence and accuracy of their pecking. Other experiments on dogs, sheep, and raccoons in a variety of test situations have yielded similar evidence for the persistence of learning.[32-34]

Most of the carefully controlled laboratory experiments on memory do not involve long periods of retention, for obvious reasons. Many commonly used types of laboratory animals are not long-lived, and with long-lived species, any long-term experiment on retention involves tying up considerable cage space, the continued expense of maintaining the animals, and no early prospect of completing a publication! It is difficult to have rigorous control over the activities of human subjects during any long retention period, and to find them again when it is over. For these reasons, most evidence of good retention of learning over really long intervals is anecdotal. Therefore, I may be pardoned for mentioning one bit of evidence of which I have especially accurate knowledge. When inducted into the Air Force in 1942, I stopped playing tennis and did not have a racket in my hand until 1963, when my son had grown up and urged me to play with him. Although speed and endurance were considerably reduced by increased age and weight, when I stepped onto the court for the first time in 21 years, I found to my delight that there had been very little deterioration in the basic strokes. While it is difficult to say exactly how much loss had occurred, it was perfectly clear that most of the elements of the complex skills were retained, although they could not have been practiced without court, ball, and racket during the 21 intervening years. Such retention seems to be characteristic of many motor skills. It is also characteristic of other situations in which it is more difficult to rule out rehearsal completely—for example, the tendency of the aged to return to memories of their early childhood, which they do not seem to have rehearsed for many years.

It is quite clear, then, that the physical mechanism for learning and memory must be capable of retention and retrieval after long periods of time, during which most of the components of the body have turned over many times. Thus the physical basis for learning must reside in one of the relatively rare bodily components that do not turn over with time or in some self-maintaining structure, self-replicating molecule, or self-maintaining system. Considering this requirement, it may be no accident that neurons are among the rare types of mammalian cells that show very little, if any, cell reproduction after early stages of development. This unique characteristic may well be related to the mechanism of memory storage. If memories are indeed stored in specific connections between neurons, one can imagine that the reproduction and growth of new neurons would confuse the effects of earlier learning.

Relatively Little Effect from Mere Disuse

We have seen that the effects of learning can be long-lasting. But we all know about rapid forgetting; in fact, most of us personally know far more than we really care to about this phenomenon. The common sense idea is that memory, like a muscle, atrophies with disuse. Like many common sense ideas, this one appears to be largely wrong. The effects of learning do not seem to atrophy appreciably with disuse, but are interfered with by new and opposing types of learning.

It has been found that if a subject learns the association A-B and then subsequently is taught the interfering association A-C, the contradictory training will greatly reduce his retention of the original learning. This phenomenon is called *retroactive inhibition,* and has been the subject of many careful experimental studies.[35] While the greatest interference is produced if the new stimulus element is identical to the original one and the new response is completely different, appreciable amounts of interference are produced if the stimulus elements in the interfering learning are only somewhat similar and the response elements are only somewhat different. Hence, one of the reasons I remember tennis so well is probably that I was engaging in no other similar activities in the meantime. If I had been playing other racket games, such as squash or paddle-tennis or, worse still, playing tennis with an opponent who was so poor that I had merely to pat the ball to him, the skill would have deteriorated more than it did with complete disuse. Similarly, the animals in the previously reported experiments would have shown more forgetting if they had had an opportunity to learn different habits in the same apparatus. One of the requirements for good memory seems to be the absence of interfering learning.

Not all interference need come from subsequent learning; some can be produced by preceding learning, and this is called *proactive inhibition.* As with retroactive inhibition, the deleterious effects are greater the more similar the stimuli that were attached to different responses before the learning in question occurred.[35]

To give a simple illustration, the first time one teaches a class he has no trouble remembering whether he has already used a particular illustrative example with that

class; but after one has taught the same subject for several years it becomes much more difficult toward the end of the course to remember whether one has used the illustration with that class or with some preceding one. And if one has several sections of the same course on the same day, the difficulty is compounded. The memory for the given instance depends on preceding ones with which it can be confused. It is as if the discrimination of whether the story has been told to this particular class is based partly on time, so that it becomes more difficult as the relative difference in time is reduced. This particular hypothesis about the time factor may or may not be correct, but there have been many experimental studies of proactive inhibition that indicate it plays a considerable role in forgetting. A review of the literature[36] suggests that the first time subjects are confronted with the somewhat novel task of learning a list of nonsense syllables, they remember approximately seven times as much of the material after a given rest interval as they do if they have previously learned 15 or 20 somewhat similar lists.

As would be expected from the interference theory, little forgetting occurs once a subject is soundly asleep, and forgetting is retarded by a quickly acting anesthesia administered after an interval long enough to allow the learning to be consolidated.[37,38]

While it is difficult to be certain that absolutely no forgetting occurs with mere disuse, the general conclusion from a large amount of experimental work is that most forgetting that occurs under normal circumstances is probably the product of interference, and that the interfering habits outcompete rather than remove the forgotten one. Thus, even after a habit seems to be completely forgotten, there typically is some trace, as evidenced by an appreciable saving in relearning. Therefore, it seems unwise to look for considerable disappearance of the physical basis for memory with the mere passage of time, to devise hypothetical mechanisms to account for most forgetting in terms of automatic disappearance, or to design experiments in which one hopes to produce central phenomena analogous to true forgetting by inducing disuse through such a treatment as cutting the tendon to a muscle.

ASYMPTOTE OF LEARNING One of the characteristics of learning is that, after a possible period of initial acceleration, it seems to proceed with diminishing returns, eventually reaching an upper limit—an asymptote.[39] If rats are taught to run down a short alley for food, their speed will increase rapidly during the initial trials but eventually will level off, so that additional trials do not produce any additional increase. That they are not really running as fast as they can may be demonstrated by making them hungrier or by increasing the amount of food used as a reward, in which case they will run still faster. There are other types of evidence for some kind of an asymptote. After a certain amount of rewarded training in approaching a goal, further training will not continue to increase the resistance of the approach to disruption by conflict with the avoidance induced by electric shocks at the goal. Paradoxically, the resistance may even be decreased.[40] Similarly, a point is reached beyond which an increased amount of rewarded training does not produce an increase in the persistence of the habit during nonrewarded trials and, in fact, may produce some decrease in its resistance to experimental extinction.[41] Similarly, there is some evidence for an asymptote in the effects of overtraining on resistance to forgetting and to retroactive inhibition.[37]

That an asymptote of learning shows up with such a variety of measures suggests that it is a real phenomenon. If so, it should be useful as a control for the type of nonlearning changes that may be produced in the brain by mere exercise of a specific system. If animals show a different pattern of protein or RNA synthesis during learning, and the effects are truly unique to the learning process, they should not occur while a thoroughly overlearned animal is receiving additional practice in the same situation.

Of course, it is possible that a thoroughly skilled habit involves less physical work and neural firing than do the clumsy attempts during early stages of learning. Therefore, it will be necessary to investigate the effects of different rates of performance of the skilled habit, some of which should be high enough to involve more work and central activity than did the performance during learning.

There is yet one more way to be misled. It is conceivable that the parts of the brain being used during the early stages of learning are subjected to a new level of activity, hence that the changes observed are merely those induced by the adaptation of the enzymatic systems to greater demands for performance, and actually have nothing to do with the mechanism of the learning that is occurring at the same time.[42] However, such adaptation of enzyme systems should eventually regress with disuse, while we have just seen that learning is not greatly affected in this way. If the changes observed during the early period of learning are truly specific to that phenomenon, they should not be observed (or at least should be greatly reduced) if the measurements for change (e.g., uptake of labeled precursor) are made after the animals have been brought to an asymptote of performance, given several weeks of rest, and then required to start performing again. To be absolutely on the safe side, one might give the animals several periods of rest followed by performance,

so they will be at their asymptote of learning to respond to any new features of being required to perform again after a rest. However, changes produced by the sudden demand of an increased level of activity should reappear when performance is required after a sufficiently long rest period.

FORWARD ASSOCIATION A number of studies indicate that conditioning is most effective when the UCS follows the CS by an interval of approximately a half-second. Progressively longer delays are progressively less effective. Absolute simultaneity is definitely poorer, usually producing little or no learning, and there is considerable question about whether backward association (with the UCS preceding the CS) produces any learning at all. The early experiments, which seemed to indicate a transient phase of backward conditioning, did not control adequately for sensitization, and very probably showed only this phenomenon, which was mistaken for true conditioning specific to the CS used. Beritoff[43] believes that certain later experiments reported in the Russian literature show that backward conditioning is possible if the strength of the CS is increased and that of the UCS is decreased, but Konorski (personal communication), who is also familiar with this literature, is convinced that no true backward conditioning occurs. From the way the experiments are reported in translation, it is extremely difficult to judge whether adequate controls were used. The unidirectionality of associations (or the conditions under which true bidirectional associations are learned) is such an important point that it should be clarified unequivocally.

Two experiments carried out in my laboratory by Nagaty[44,45] failed to show any evidence that reinforcement given before the performance of an instrumental response had any effect on the maintenance of that response. In the first of these, rats were trained to rotate a wheel to avoid electric shocks. Then they were given extinction trials, during which the shocks, given if the rats did not turn the wheel in time, were omitted. Half of the rats received a brief shock immediately before the CS and the other half received a shock between trials. They all reacted to this shock by rotating the wheel, but the close association between the shock and the immediately subsequent warning signal had no effect on the extinction of the wheel-turning response.

In the second experiment, hungry rats were trained to press a rod as soon as it was inserted into the experimental apparatus. Each time they pressed they received a pellet of food. During the latter part of training this rod was withdrawn if the rat did not press it immediately. Rats sometimes received a pellet of food immediately before the rod was inserted. When the delivery of the pellet after pressing the rod was delayed 2 seconds, the habit was maintained indefinitely. On the other hand, when the pellet was always given 2 seconds before the rod was pressed and none afterward, the habit extinguished. Both these experiments agree with the apparent results on classical conditioning, in showing that the effects of reinforcement act only in one direction.

Similarly, results on human verbal learning indicate that forward associations are much stronger than backward ones, and that the latter may possibly be accounted for by associations (analogous to sensitization) with general, persisting features of the experimental situation or by rehearsal during a brief memory span, so that they actually are acquired, or retrieved, in a forward direction.[37] If you have never specifically practiced saying the alphabet backwards, you can test this for yourself by closely observing your own behavior while trying to proceed from the letter "L" first in a normal forward direction and then in the opposite backward one.

If forward associations are indeed the only kind possible with a single pair of neurons, it suggests that a connection from neuron 1 to neuron 2 is made if 2 is fired by some other source (UCS or Go Mechanism[13]) after 1 has been fired and has partially depolarized the postsynaptic membrane of the synapse between 1 and 2. Although we cannot be certain that only forward associations are possible, it seems worthwhile to ask what unique physical-chemical conditions are present at the synapse between neuron 1 and neuron 2 when the CS has been followed by the UCS, or a response has been followed by a reward. For example, it is conceivable that when neuron 1 is fired, the initial depolarization of the membrane on the presynaptic side of the synaptic cleft releases certain molecules which have opposite effects, depending on whether they are incorporated on the outside or the inside of the postsynaptic membrane of neuron 2 on the other side of the cleft. Unless the depolarization of neuron 2 is prolonged (by a UCS of Go Mechanism), these molecules might be incorporated on the outside of a postsynaptic membrane where they block receptor sites and produce extinction. If it is prolonged, they might have time to diffuse through to the inside of that postsynaptic membrane, where they act to induce new receptor sites and produce learning.

SPECIES DIFFERENCES—QUALITY VS. NUMBER OF UNITS There are vast differences in learned behavior between man and monkeys and between monkeys and rats. These differences do not appear to be in the ability to form simple associations under optimal conditions; in fact, it would be hard to improve on the one-trial learning shown by the rats in the first experiments described in

this paper. Even in the more complicated test of learning a multi-unit spatial maze, rats do as well as college students. The superior learning ability of humans seems to be primarily a function of other capacities, such as the ability to process sensory information in such a way that we respond to more complex and abstract aspects of the environment, process information at higher rates, have a wider span of attention and a longer span of immediate memory, and are aided more by the cumulative effects of previous learning. Thus, rats can learn a simple alternation problem (turn first right then left, then right then left) rather easily, but it is impossible for them to learn a double alternation problem (right right, left left, right right, left left). But the latter problem is very easy for people who, as we have just seen, are not especially superior to rats at learning by rote a completely illogical sequence of turns in a maze.

It seems reasonable to suppose that these peculiarly human abilities are not the properties of a simple association or a simple connection between neurons, if indeed that is the basis for learning. Instead they would seem to be the properties of complex networks, many of which may be innate and many of which may be the product of previous learning by a brain with the capacity for forming a larger number of associations because it is composed of a larger number of elements—much as a big computer may be able to accommodate more complex and powerful programs than a small one, not because its elements are different, but because it has more of them. It has been estimated that the human brain has at least 10^{10} neurons, which have thousands of complex branches, resulting in a total of at least 10^{13} potential synaptic connections. This number of connections could be combined in a large number of combinations and still have capacity to spare for considerable redundancy.

In looking for the properties of learning that provide clues to its physical basis, we must try to distinguish between two kinds of learned behavior. One is most likely to be determined by the primary properties of a single connection (or whatever the fundamental unit of memory storage may be). The other is more likely to be determined by those secondary properties of complex neural networks that process the information after it has been received but before it is stored, or after it has been retrieved from the store and is involved in the initiation of behavior. In selecting the properties of learning to be emphasized in this chapter, I have made certain assumptions about which are primary and which are secondary. Specifically, I have assumed that the following properties are secondary, and refer more to capacities for performance than to the primary process of learning: stimulus generalization, positive and negative induction and behavioral contrast, secondary reinforcement, effects of schedules and correlated reinforcement, concept formation, insight, reasoning and problem solving, and (probably) delayed reactions and short-term memory. But it is necessary to examine carefully other assumptions about the primary properties of the fundamental mechanism of memory storage and the secondary properties of neural networks for processing information.

Finally, it should be remembered that this chapter is only a brief summary. A true sophistication can be acquired only by becoming familiar with the great amount of work that has been done on various aspects of the phenomena of learning.

The Use of Invertebrate Systems for Studies on the Bases of Learning and Memory

E. M. EISENSTEIN

INVESTIGATIONS INTO THE NEURAL BASES of behavior often seem to be hampered by a peculiar paradox. As we study progressively simpler systems and thus increase the likelihood of uncovering fundamental mechanisms, we move progressively away from a system that is acknowledged as demonstrating the phenomenon—the behavior whose bases we are trying to determine. In this paper I will consider in some detail the procedural requirements for demonstrating one kind of behavior—learning in intact organisms—and to propose a more abstract formulation than is usually given. This may be useful as a guide to molecular investigations involving simple systems far removed from intact organisms. I will also discuss some of the invertebrates that have been studied and suggest the advantages each offers for certain kinds of investigations.

The concept of learning

Most commonly, learning is said to occur in a system when it can be shown that an association between a stimulus and a response is developed *de novo* or that a previous association has been strengthened. The procedure giving rise to this association is termed a learning procedure. The development of an association is commonly measured in terms of some behavioral change, such as response frequency, latency, or magnitude. Two broad classes of procedures give rise to the development of an association between a stimulus and a response. These are the classical conditioning and instrumental learning procedures.

CLASSICAL CONDITIONING This procedure is also known as respondent, Pavlovian, type I, or type S conditioning. A stimulus that invariably elicits a particular reflex (such as food in the mouth reflexly producing salivation) is presented repeatedly, together with a stimulus that initially does not elicit the reflex or does so only weakly (such as a bell or a light). After several presentations of the two stimuli, the originally ineffective or conditioned stimulus (CS, bell or light) now elicits a response similar to that originally produced by the unconditioned stimulus (UCS, food). This strengthening of the relationship between the conditioned stimulus and the response is defined as learning.

An adequate control for changes resulting from the sheer number of stimulus presentations often is achieved by comparing behavior resulting from a forward conditioning procedure with that resulting from a backward conditioning procedure. In the former, the onset of the CS *precedes* the onset of the UCS. In the latter, the onset of the same CS always *follows* the onset of the UCS. Generally, the response level is much higher in the former.

INSTRUMENTAL LEARNING This learning procedure is often referred to as operant, type II, type R, trial-and-error, and simple problem solving. The animal is placed in a situation in which the experimenter has designated that a given response will be followed by a stimulus selected to alter the probability that the response will reoccur. The change in probability is termed learning. The stimulus event that follows the response and is responsible for the change is termed the reinforcer. (The reinforcer with a Pavlovian procedure is the UCS, because it is the stimulus event that alters the occurrence probability of a particular response to the CS.) The reinforcer may be either positive or negative, depending on whether the behavioral change is in the direction of attaining the reinforcer more rapidly (positive) or of avoiding it (negative).

Suppose a rat is placed on the white side of a two-chamber compartment and is shocked until it jumps over a hurdle to the black side. Following several such trials, the rat, when placed on the white side, will jump over to the black side more rapidly than it did initially. To be able to call this change—or any part of it—learning, it is generally considered necessary to demonstrate that the response of hurdle-jumping is caused by the presence of the white-shock-jump pattern in the apparatus and not just to shock, independent of this sequence. Thus, if one group were shocked only on the white side of the hurdle box and another group received the same number of shocks either on the black side or outside the training situation, we

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would expect that the group receiving shocks on the white side, if later placed on that side without shock, would make more crossings to the black side than the other groups if, indeed, there were any association between shock onset on the white side and the hurdle-crossing response. The difference between two such groups indexes the occurrence of learning.

In both of these examples the organism must demonstrate the ability to generate a difference in output or response to a given stimulus input (CS in the Pavlovian experiment and the white side of the hurdle box in the instrumental experiment) as a function of the previous sequence of stimuli and responses to which it has been exposed, before the phenomenon of learning is generally acknowledged to have occurred. With a Pavlovian procedure, the response to the CS when a forward conditioning sequence has been used must be different from that to the same CS when a backward conditioning sequence has been employed. The probability of hurdle jumping from the white side also must be different in animals when shock has followed the placement of the animal on the white side, compared to animals in which shock has occurred independently of whether the animal was on the white or black side. These relationships are schematized in Figure 1, and form the basis of the reformulation of learning discussed below.

TOWARDS A REFORMULATION OF THE CONCEPT OF LEARNING I am basing this definition of learning on an abstraction of factors generally considered important in experiments accepted as demonstrating learning and including both of the learning procedures just discussed. FORMAL STATEMENT A system is said to demonstrate learning when its output (Response) to a given test input (Stimulus) is a function of the total previous input-output pattern of which the test input was a part. That is, a system can be said to have learned if its output to a given test input is a function of the specific input-output pattern to which it has been exposed. (Whether any given example of learning involves the coding of a pattern of inputs and outputs [stimuli and responses], or just the stimulus input pattern alone, is not known. The present formulation is stated in operational terms denoting both the stimulus and response elements whose temporal sequence is actually varied with learning procedures, and is not a theoretical commitment to the actual elements being associated.[1])

The coding of some temporal sequence of events seems to me to be the most fundamental aspect of learning. An input-output pattern difference is interpreted as any distinguishable temporal feature between two sets of stimulus and response elements. Commonly, this will be a difference in the temporal order of the stimulus and the response events. It may also include other temporal variations, such as spacing of the events. The pattern difference may also show up over several trials rather than on any single given trial, as when a partial versus a 100 per cent reinforcement schedule is employed. It is critical, in demonstrating that differences in output to a test stimulus are

GROUP		TRAINING	TEST
Classical (Pavlovian) Conditioning	1	S[CS, bell or light] ⟶ S_{rf}[UCS, food] ⟶ R[salivation]	S[CS] —?→ R
	2	S_{rf}[UCS] ⟶ R ⟶ S[CS]	S[CS] —?→ R
Instrumental Learning	1	S[white side] ⟶ S_{rf}[shock on white side] ⟶ R[hurdle crossing]	S[white side] —?→ R
	2	S_{rf}[shock before placement on white side] ⟶ S ⟶ R	S[white side] —?→ R

FIGURE 1 Scheme showing that with both classical and instrumental training procedures, the essential variable manipulated in experimental and control groups to demonstrate learning is the same, i.e., input-output sequence. (If responses are the same during the test period, no learning; if different, learning is said to occur.) S is the stimulus to which the system learns to respond, i.e., appropriate cues in a maze or hurdle box, or bell or light with a Pavlovian procedure. R is the response designated as correct, i.e., turning right or left in a maze, jumping a hurdle, or salivating to a bell or light. Srf is the "reinforcer" responsible for strengthening the association —commonly food, water, or electric shock. Often with an instrumental procedure as in maze learning, variation in temporal pattern of stimuli and responses between experimental and control groups is achieved by following a right turn with reinforcement in one group and a left turn with reinforcement in the other group. (Right turn → reinforcement, left turn → no reinforcement, leads to higher probability of right turns than in group in which sequence has been left turn → reinforcement, right turn → no reinforcement.) Ability of animals to discriminate such sequences allows conclusion that learning has occurred.

determined by the previous pattern, to show that the output varies although the *same total amount of stimulus input in the same time period* occurred for both patterns.

"System" is being used in its broadest sense to include any arbitrary integrating unit from a single cell to a whole organism, or any subdivision thereof. Such a formulation considers changes as learning when the inputs may be direct electrical stimulation of peripheral sensory nerves and the output is measured by discharges or some other appropriate response—for example, from a motor nerve. (Elsewhere in this volume, Dr. Kandel discusses his work on *Aplysia* ganglia.)

This concept of learning has two key points: (1) traditional procedures are seen as varying input-output sequence, and the coding of sequence is the basis of learning; (2) there is no control group in the sense of a group showing nonlearned changes, because to show a system's ability to code sequence, one must vary the temporal pattern in at least two groups to demonstrate that it is the responsible factor. The "control group" receives the second pattern, and its difference from the first group in response output shows that the system under consideration can undergo learning. Such a formulation of learning considers experimental and control groups of both instrumental and Pavlovian procedures in terms of the one variable that distinguishes them most consistently. That variable is the temporal pattern of some input-output function.

Such a formulation of learning, it is hoped, will allow investigations of systems previously used only transiently in learning studies. That is because the terminology frequently employed in discussing learning includes concepts such as motivation, incentive, reward, approach, avoidance—in a word, adaptive behavior. Such terminology has meaning in relation to intact, evolutionally advanced systems, but little or none for isolated subsystems, such as a ganglion in higher or lower organisms. Often it is even difficult to employ such words when discussing the behavior of intact lower invertebrates. Thus, systems most likely to make important contributions to the understanding of fundamental mechanisms of pattern storage are often not acknowledged as showing the phenomena of learning, primarily because the above terminology does not always easily apply.

Before turning to a consideration of a number of invertebrate systems, I would like to review several terms commonly used in discussions of learning.

1) *Habituation* is used to describe the progressive decrease in response resulting from repeated elicitation by an intermittent stimulus. Thus, if a loud bell is sounded for the first time, an animal may show a startle response, indicated by a host of autonomic and skeletal reactions. Following several presentations of the bell, the animal shows progressively less response, and in some cases the response may diminish to zero. That this decrease is a central phenomenon and not caused by fatigue of the effector apparatus can often be demonstrated through reinstatement of the original response by changing the stimulus.

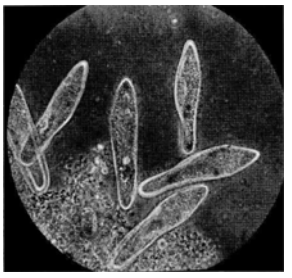
2) *Sensitization* In Pavlovian conditioning, the CS often is not completely neutral at the start of an experiment. It may reflexly produce a small response of the kind to be conditioned, such as a galvanic skin response to sound. After several pairings of sound and a noxious stimulus such as electric shock, the galvanic skin response to the sound becomes much larger, and it is argued by some that this is not "true" conditioning because the response to be conditioned initially occurred to some extent to the conditioned stimulus.

3) *Pseudoconditioning* is used to denote the change in response to a stimulus that produces no initial response until after several presentations of another unpaired stimulus. Thus, if a mild sound produces no eye-blink response until after several unpaired puffs of air to the eyeball, the response is termed pseudoconditioned, i.e. it is not based on a specific pairing of the sound and a puff of air.

4) *Extinction* designates the decrease in magnitude of a learned response to a stimulus when the stimulus is presented repeatedly and the response is no longer reinforced. Thus, if an animal has been taught to press a lever to obtain food and at some point food stops coming, the frequency of pressing the lever will decrease.

5) *Spontaneous recovery* refers to the increase in response magnitude following a rest period after extinction.

A number of the systems to be considered may offer opportunities for establishing the bases of these learning-related phenomena.[2,3]



PARAMECIA

PROTOZOA The main body of work on behavior modification in protozoa has been done on the paramecium. Early work by Day and Bentley (1911)[4] showed that if a

paramecium was drawn into a glass capillary tube whose inside diameter was less than the animal's length, the paramecium, after swimming to one end, would make repeated attempts to turn around. Over a period of one to two hours, it would take progressively less time to complete a turn and, in addition, make fewer abortive turning attempts. Furthermore, if the paramecium was removed from the tube after such training, placed in an open dish of culture water, and replaced in the capillary tube after a ten- to twenty-minute interval, it showed a "savings"—that is, it took less time to complete a reversal and made fewer abortive attempts per successful reversal than it did the first time it was placed in the tube.

Is this change in behavior evidence of learning? It certainly demonstrates at least two characteristics seen in most kinds of learning: (1) there is a progressive change in response to a given stimulus situation over time; and (2) there is evidence of retention of the response change (at least over a twenty-minute interval). A criticism of this work as evidence of learning was made by Buytendijk,[5] who argued that paramecia, when subjected to mechanical stimulation, lost "tonus" and became more flexible, suggesting that only physical effects were involved. However, such an explanation may be misleadingly simple. It would be of interest to know, for example, what molecular rearrangements in the paramecium allow this behavioral change to occur, and whether such molecular changes associated with "use" bear any relationship to long-lasting changes that may occur in neural networks with use.

The most systematic efforts to explore learning-related behavior in paramecia have been those of Gelber.[6-11] Her general procedure is to isolate a small culture of paramecia in a depression slide, lower a sterile platinum wire into the center of the culture, and observe over a several-minute period how many paramecia attach to or are within the immediate vicinity of the wire. This yields a pretraining measure of the attachment tendencies of the culture. She then introduces a training procedure during which the wire is baited with the bacterium *Aerobacter aerogenus* (food material for *Paramecium aurelia*), lowered into the culture for fifteen seconds, and then removed. Twenty-five seconds later, the wire is lowered again. Generally, forty training trials are given; the wire is baited with bacteria only on every third trial.

This procedure gave the following results: (1) spaced training trials led to more wire-clinging than did massed trials; (2) a decrease in the clinging behavior occurred if, following training, several trials of wire without food were given (analogous to an extinction procedure); (3) if the culture was tested two hours after such an extinction procedure, there was greater wire-clinging than immediately after the extinction procedure (analogous to spontaneous recovery); and (4) the behavioral change lasted up to at least 10 hours after training and apparently survived fission within the culture.

Jensen[12-14] has been the most systematic critic of interpreting the above behaviors as evidence of learning. His major thesis, based on his own work and on earlier studies by others, is that the procedures used to demonstrate learning in paramecia have produced changes in the environment, rather than in the paramecia. Jensen, and Jennings[15] earlier, demonstrated that paramecia show a clinging response to an object in their environment, and that this clinging, as well as aggregate behavior, is increased in an acidic environment. Jensen contends that the feeding procedure develops a zone rich in bacteria that produce CO_2 during metabolism. The paramecia are drawn into this area, and in the presence of CO_2 increase their clinging behavior, themselves producing CO_2 and thus further trapping themselves in an acidic zone. During a later test period, he contends, they would be expected to cling more to a sterile wire—not as a result of learning, but because of changes in the acidity of the environment.

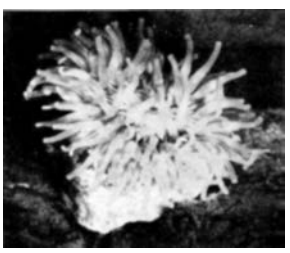
As is often the case in such controversies the evidence for both positions is suggestive rather than conclusive. That CO_2-rich zones may have been a critical factor in Gelber's early work is supported because control cultures were not fed during the training period, and their environment was therefore less acidic. On the other hand, that such metabolic differences are not the full explanation of Gelber's findings is suggested by the results of her work just noted. The safest conclusion one can draw at this time regarding learning in protozoa is that it remains a most provocative possibility.

This controversy raises the question of just what kind of experiments could demonstrate learning in this group. The essence of the problem, I feel, is to show that a system can generate a different output or behavior to a given stimulus as a function of the preceding total input-output sequence, of which both stimulus and response have been a part on previous occasions. None of the protozoan work has conclusively separated the effects on output that may be a result of total amount of stimulation in a given time period, from that resulting from specificity of the input-output pattern occurring in the same time period. Gelber's results[11] showing that "spaced" training trials lead to more responses than "massed" trials come close to demonstrating temporal specificity. However, spacing requires a longer training period for the same number of trials than does massing, so there is the possibility that, during a later test period, effects caused by the *specificity* of the distribution of trials over time could be confused with those that may be caused by the total *amount* of

stimulation occurring in a given time period.

The ability to differentiate temporal pattern is the unmistakable earmark of learning in higher organisms; if it can be shown in simpler systems, it seems reasonable to assume that one is on the right track in uncovering mechanisms associated with the class of integrative phenomena of which learning is a part.

The protozoan may offer unusual opportunities for correlating histological and behavioral changes in cells that are discrete, genetically identical, and relatively simple by comparison to even the simplest nerve net. It allows us to consider whether the ability to learn is a general property of all protoplasm or whether, in fact, it is unique to nervous tissue.



SEA ANEMONE

COELENTERATES Bullock[16] has pointed out that the coelenterates represent the most simply organized phylum containing members that possess true nervous systems. The neural elements do not appear fundamentally different from those occurring in more highly evolved animals. Furthermore, according to Bullock, "integration between conducting systems" in the marginal ganglia of medusae represent "the first integrating concentration of nervous tissue in the animal kingdom."

Evidence for Pavlovian conditioning at this phyletic level is suggestive. Ross[17] has attempted conditioning in the sessile anemones, *Metridum senile* and *Stomphia coccinae*. In experiments on the former, he used a drop of food extract to produce a mouth-opening reflex. This was chosen as the unconditioned response, as it occurred almost without fail, and could be elicited as often as twice an hour for many hours without any appreciable weakening of the response. For a stimulus that was initially ineffective in producing this response, he used repeated electric pulses delivered at the rate of one per five seconds through electrodes attached to the base of the animal. On a given trial a drop of food extract was introduced into the oral disk, and at the same time electric pulses were applied to the base of the animal as long as the mouth remained open. In successful experiments, mouth opening to the electrical stimuli alone was seen after ten to fifteen trials. It should be pointed out that of 45 experiments, only seven showed responses to the electric stimuli alone.

Another group of experiments by Ross on *Stomphia coccinae* are perhaps more suggestive of "true" Pavlovian conditioning. He observed that *Stomphia,* found in Puget Sound, would swim when touched by substances removed from the aboral surface of only two of the twelve species of starfish that are found in the same waters. He then observed that strong mechanical pressure on the base of the anemone caused it to retract and close. If he applied strong mechanical pressure to the base of the anemone within two seconds after applying the starfish substance, he could inhibit swimming. If he waited longer than two seconds it became difficult to stop the swimming response when the mechanical stimulus was applied. He attempted to pair these two stimuli to see if he could induce retraction and closure to the starfish substance (Figure 2).

After five days of training eight animals, the starfish material alone was applied at half-hour intervals. The results showed that although all eight animals had given a swimming response to the starfish substance before training, none of the eight swam on the first test trial after training. Rather, they showed a retraction response, which Ross described as similar to that shown to a sharp mechan-

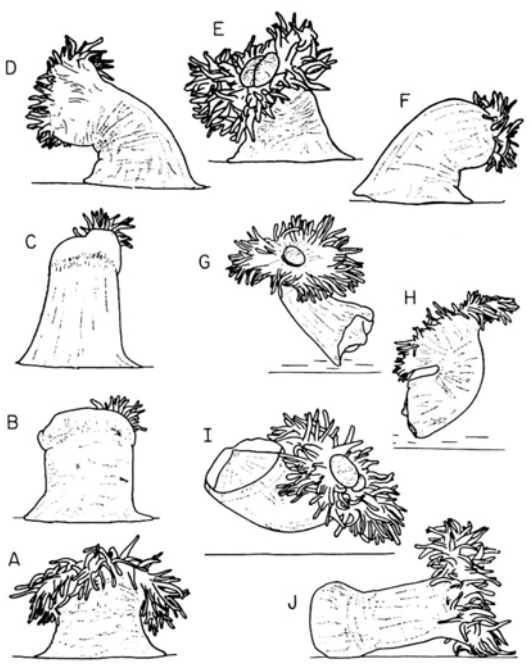


FIGURE 2 The swimming sequence in *Stomphia coccinea*. A: Normal, attached position. B: Response to stimulation by contact with starfish. C: Extension of column by circular muscles after contraction of longitudinals. D,E,F: Lateral bending by parietobasilar muscles. G,H,I: Lateral bending. J: Inactivity after swimming. (From Bullock and Horridge, Note 16)

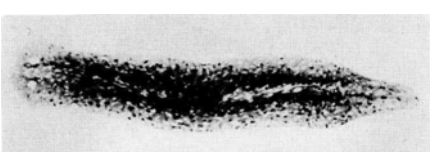
ical stimulus. In addition, those animals receiving fewer training trials began to swim sooner after the stimulus pairing ceased than did those given more training trials.

Here we are dealing with two stimuli that produce mutually exclusive behavior, in which the innate response to one stimulus is apparently inhibited, and a response appropriate to the other stimulus is substituted. This is surely rather complex learning.

There are other interesting experimental procedures that may be applied at this phyletic level and may be useful in pinning down the morphological bases of modifiable behavior. Rushforth,[18] working with hydra, demonstrated that these animals contract when shaken. When the amount of mechanical agitation was standardized and delivered intermittently, the percentage of contracting animals gradually diminished over a six- to eight-hour period. This response decrement was found to last up to four hours after training; it was not the result of fatigue, because contractions could be evoked by a light stimulus after the animals were habituated by mechanical agitation.

Rushforth also discovered that one species, *Hydra pseudoligactis,* was relatively insensitive to both light and mechanical stimulation, while another species, *H. pirardi,* was sensitive to both of them. As these species were capable of being grafted to each other, Rushforth transected two animals through the gastric region and grafted them together, using apical-aboral grafts. He noted that, initially, the two portions contracted independently, but after a few days the halves appeared highly coordinated in their responses. His results showed a responsiveness midway between that of the two parent species.

Such preparations might be most useful for combined histological and behavioral studies in determining the types of cellular changes that are responsible for this behavior.



PLANARIAN

PLATYHELMINTHES Our discussion of this phylum will center around work on the turbellarian flatworm, *Planaria.* Platyhelminthes represents the lowest group of organisms demonstrating bilateral symmetry. As Bullock has noted, at this level we see the first centralized nervous system with a distinct brain; also present are the nerve cord with an outer rind of cell bodies and an inner fiber core, a feature typical of all higher invertebrates. We also see neuroglia and neuropil and "at least six differentiated types of sensory neurons."

Perhaps the best-known effort to study learning in this class of organisms is that of McConnell and his collaborators working on the planarian *Dugesia.* In their first study they attempted to demonstrate Pavlovian conditioning in *Dugesia dorotocephala.*[19] They presented a light as the conditioned stimulus and an electric shock as the unconditioned stimulus. The shock produced a number of responses, including longitudinal shortening, turning, and a combination of the two. They demonstrated that the group receiving both stimuli gave significantly more responses to the light than did two control groups receiving light alone and shock alone, respectively. One may ask whether the increased responsiveness in the stimulus-paired group represented an association based on a specific temporal sequence or was caused by something more akin to spatial and temporal summation based on total amount of stimulation.

A later paper by McConnell et al.,[20] concerned itself with the provocative question of whether trained planarians, cut in half transversely and allowed to regenerate, would show retention of previous training. Of particular interest was how retention in the tail regenerate would compare with that in the head regenerate. They used three groups of planarians. One, given paired light and shock training as in the previous experiment, was then cut transversely in half and allowed four weeks to regenerate. The control group, given no previous training, was cut in half and allowed to regenerate, and a third group was trained and then left alone for the four weeks during which the two other groups were regenerating. Following this period, all animals were given light-shock training to a criterion of 23 out of 25 responses to the conditioned stimulus of light. The results were startling. Both the head and tail regenerates of the originally trained group took significantly fewer trials to relearn than did the original group. Furthermore, there was no response difference between the head and tail regenerates. These results were not caused by cutting and regeneration, because both the head and tail regenerates of the untrained group, which had been cut in half and allowed to regenerate before training, took significantly longer to learn the criterion than did the head and tail regenerates of the experimental group.

Do these findings indicate that Pavlovian conditioning

can survive regeneration? Perhaps a more meaningful question, and one raised by the last experiment, is whether the savings represent the storage of a total amount of light-shock stimulation or a stimulus-response pattern. It would be most interesting to compare the savings of a forward-conditioned group with another group to which a random or backwards sequence of light–shock was given during training. Both groups would be cut in half, allowed to regenerate, and then trained with a forward-conditioning procedure. If both regenerated groups showed a savings over the original forward-trained group, but no longer differed from each other following regeneration, it would suggest that quantity of stimulation is stored but specificity of temporal pattern is lost.

In 1962, McConnell published a paper entitled "Memory Transfer Through Cannibalism in Planarians."[21] He reported that if untrained, cannibalistic planarians devoured trained ones, they became responsive to light sooner than had the devoured planarians.

Accepting the phenomenon of transfer as demonstrated, two questions are raised: what is the molecular basis of the transfer, and, perhaps more important, what is the interpretation of the transfer effect? There are at least two possible answers to the latter question: (1) memory of light-shock association can be transferred via ingestion; and (2) metabolic, humoral, and other nonspecific changes are transferred to the cannibals, making them more reactive to these stimuli.

A recent study of Hartry, et al.,[22] asked whether such nonspecific factors as those mentioned could produce a transfer effect. They reported no difference in the more rapid learning among cannibalistic groups that devoured planarians exposed only to light, only to handling, or given light-shock training. They conclude that their results do not disprove that memory transfer may occur, but suggest that hypotheses other than that of memory transfer may explain the results.

Westerman[23] has investigated transfer of habituation by cannibalism. He showed that cannibals, fed planarians habituated to light, themselves habituate more rapidly. These and other studies indicate two points about the transfer phenomenon: (1) it may be either excitatory or inhibitory—so to that extent there is specificity; and (2) the transfer effect can be the same as the original training effect—that is, if the response is increased or decreased during training, the cannibals show a faster increase or decrease, respectively.

Perhaps new insights into the specificity of the transfer effect might be achieved by feeding a group of forward-conditioned and a group of backward-conditioned planarians to two different groups of cannibals. Any observed differences in cannibal learning would indicate transfer of a specific pattern of training, which perhaps is more akin to what is meant by memory transfer. (Since writing this paper, an article by Jacobson et al.[24] has come to my attention. In a study related to that suggested here, they demonstrate specificity of the transfer effect in planaria.)

A recent study of Kimmel and Yaremko[25] leaves, I think, little doubt that planarians can demonstrate Pavlovian conditioning and that, at least with respect to the pattern of reinforcement, their behavior follows the lawful relations seen in higher vertebrates (Figure 3). There is also evidence now that planarians can learn by instrumental[26] as well as Pavlovian training procedures.

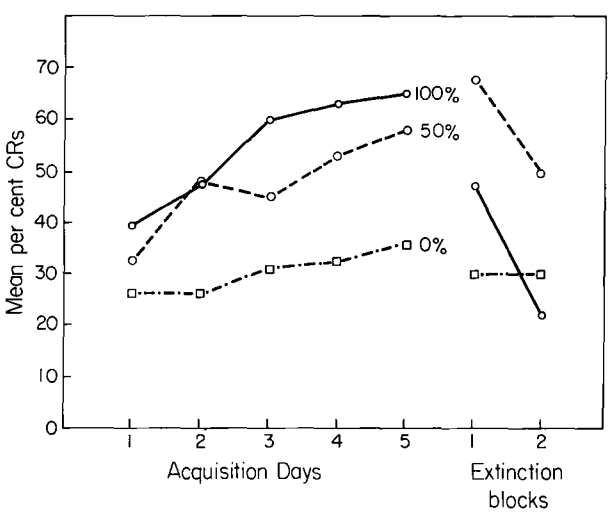


FIGURE 3 Mean number of CRs in the 100%, 50%, and 0% groups of six planarians each on each day of conditioning and in two blocks of 25 extinction trials. Light was used as CS and electric shock as UCS. Forty-eight light and shock exposures were given daily for five days. The 100% group received only paired light and shock. Second group received same total number of both exposures; half were paired, half unpaired, with at least 15 sec between light and shock. Third group received all unpaired stimuli; all presentations had at least 15 sec between them. Curves show mean responses of groups to the first two seconds of light presentation on each day. On day six, extinction trials were run, consisting of light presentation alone. The two paired groups were significantly more responsive than the unpaired group. Extinction took longer in the 50% group than in the other two. The superior resistance to extinction of a group receiving partial as compared with 100% reinforcement during training is a common finding in higher vertebrates. (From Kimmel and Yaremko, Note 25)

EARTHWORM

ANNELIDS Experiments with annelids (discussed here) involve two classes of worms—the polycheates and the oligochaetes. The nereid polycheates have been shown capable of habituation to the withdrawal reflex through repetition of mechanical shock, a moving shadow, and sudden increases or decreases in light intensity. They are also capable of shock avoidance training; thus, Clark[27] has reported that if the worm is placed at the entrance to a glass tube and is shocked when it crawls through to the other side, it crawls more slowly on successive trials, often reversing in the tube to return to the entrance, and eventually refusing to enter. There is considerable retention for six hours, but almost no retention after twenty-four hours. If the animals are rewarded by darkness when they make the correct turn in a T-maze, there is improvement with repeated trials. The learning will persist overnight without training, and there appears to be species differences in the rate of learning the T-maze. Removal of the brain after training abolishes the correct choice behavior, and the animals revert to a random selection of the arms of the T-maze. Furthermore, naive worms that have had their brains removed fail to learn the maze. These results indicate the importance of the brain for such learning and retention.

A number of studies using both Pavlovian and instrumental training procedures have shown learning in the oligochaetes. The earthworm, *Lumbricus terrestris,* is most commonly used. The results of Wyers et al.,[28] using Pavlovian training procedures to study learning in these animals are shown in Figure 4.

Yerkes[29] studied maze learning in a single earthworm, *Allolobophora foetida.* One arm of the T-maze led to a dark, earthy area and the other arm led to electric shock. If the anterior five segments of the worm were cut off following learning, thus removing the brain and subesophageal ganglion, the worm could choose the correct arm in the maze immediately after the operation; however, once the brain grew back, the learning was lost. If the intact animal rested after training for the same length of time it took for regeneration, the same response decrement was not produced. This raises the question whether the new brain erases the previous learning stored in the ventral cord or only suppresses it. Jacobson[30] has suggested that an answer

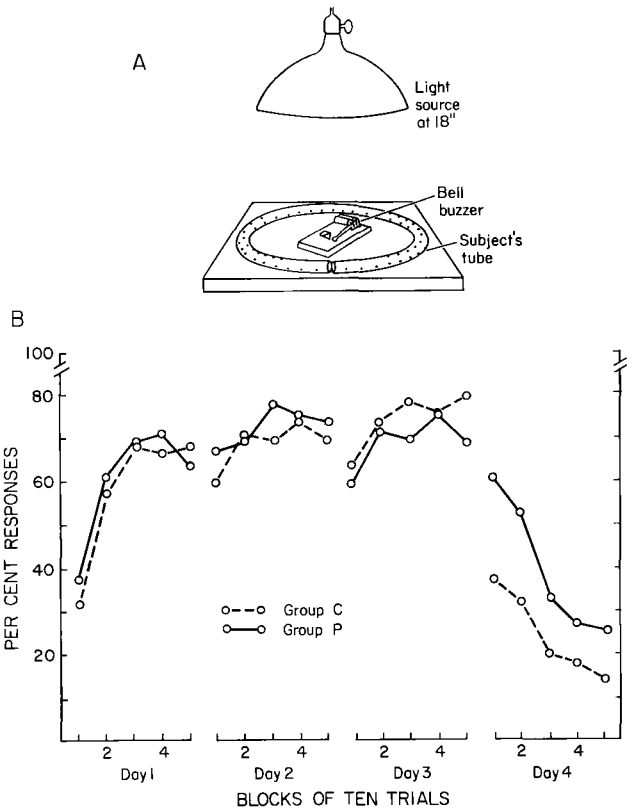


FIGURE 4 Percentages of withdrawal responses for blocks of five trials for two groups of worms (B). (Group C—100% reinforcement; Group P—50% reinforcement.) Group P was more resistant to extinction than was Group C. Removal of the brain abolishes differences during an extinction period. UCS was a bright light turned on for 2 seconds; it produced a longitudinal contraction and a rearing of the anterior end. CS was a buzzer serving as a vibratory stimulus. Training procedure (A) consisted of turning on buzzer for 6 sec with light coinciding with last 2 sec. Responses were measured during the 4-sec period between buzzer onset and light onset. (From S. C. Ratner and K. R. Miller, 1959. Classical conditioning in earthworms, *Lumbricus terrestris, J. Comp. Physiol. Psychol.,* Vol. 52, pp. 102–105; and from Wyers, et al., Note 28)

might be supplied if the regenerated brain were again removed immediately to see if the response returns. There was no apparent cerebral dominance in planarians with respect to learning, as head and tail regenerates responded equally well. Therefore, it would be of interest to know if this apparent dominance arises at the level of the annelids.

There are groups among the polycheates with true ganglia and cell-free connectives between them, while the oligochaetes have a medullary cord—that is, cell bodies distributed throughout the cord in addition to those in

ganglia. We do not yet know in what ways such a difference is reflected in the integrative abilities we have been considering.

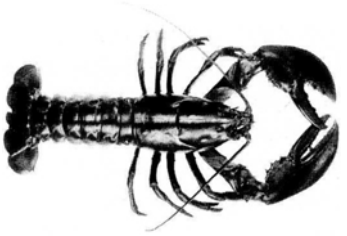
MOLLUSKS A great deal of work has been done on learning, memory, and perception in the octopus, and it is summarized with relevant references in Volume II of *Structure and Function in the Nervous System of Invertebrates,* by Bullock and Horridge.[16] The octopus studies deal primarily with changes in behavior associated with ablation of various parts of the nervous system. Many of the learning experiments employ some positive reward, such as a crab, or a negative one, such as electric shock. The animals are capable of learning rapidly under conditions that lead to learning in vertebrates, demonstrating also the distinction between short-term and long-term memories; that is, it is possible, by making selective lesions in the brain, to abolish recently acquired memories, leaving older ones intact. Because of the highly developed visual system of these animals, much work has been done on visual perception, particularly on shape recognition.

OCTOPUS

Bullock has noted that "the conditions for behavioral and histological tests are so favorable that it seems likely we may soon understand for the first time the system of neural elements to accomplish an abstraction, even if it is a simple one."

Kandel (this volume) has worked on pairing electrical inputs directly to nerves in the ganglion of the gastropod *Aplysia,* and Strumwasser (this volume) has studied that animal's cellular circadian rhythms. Their experiments demonstrate the amenability of such preparations to cellular studies of complex integrative processes in relatively isolated regions of nervous tissue, and will undoubtedly aid greatly in specifying how nervous systems acquire and store information.

ARTHROPODS The basic plan of the arthropod nervous system is similar to that of annelids. In the two classes to be considered—the crustaceans and the insects—there is a bilateral longitudinal arrangement of ganglia.



LOBSTER

Crustaceans A number of studies[27-30] have shown that crustaceans are capable of learning, and there is evidence that they may retain learning for many days.[33] Evidence also indicates that they use chemical, tactile, visual, and proprioceptive inputs in learning.[31-34] There is particular advantage in using this group for neurophysiological investigations of learning, because they possess peripheral inhibitory fibers known to modulate both sensory input and motor output.[16] These animals may be especially useful for studying the role of inhibition in the learning process.

Skeletal muscles of crustaceans show multiple innervation of single muscle fibers by the same neurons, and polyneuronal, i.e., fast and slow, axon innervation. In addition, the graded electrical and mechanical responses provide a potentially rich region for integration in the periphery, in this sense not unlike CNS neuropil. "Pattern-sensitive" neuromuscular synapses are also known; in these, the magnitude of muscle contraction may be greater in response to shocks in doublets initiated in the motor nerve than to equally spaced shocks of the same frequency.[35] This is evidence of pattern recognition at a neuromuscular level. The possibility that higher integrative processes akin to learning occur in the neuromuscular region of crustaceans should not be overlooked.

Insects Four preparations seem to offer particularly interesting possibilities for establishing the neural basis of learning in these systems. Working with the cockroach,

COCKROACH

Blatta orientalis, Luco and Aranda[36-38] noted that the animal normally holds the antenna it is cleaning with an opposite foreleg. If both forelegs are amputated, the animal at-

tempts to hold the antenna with one of the middle legs; it takes from four to seven days before the animal learns to use three legs to support itself while holding its antenna with one of the middle legs.

The investigators removed the central nervous system after such learning. Applying a single, presynaptic shock just anterior to the first thoracic ganglion, and recording from the major leg nerve of the third pair of legs, they noted a delay from stimulation to nerve impulse of about 2 milliseconds. In normal cockroaches the delay was considerably longer, averaging about 4.4 milliseconds. Such a shortened delay in trained animals apparently is not caused by leg amputation, because animals that had only one foreleg or both middle legs removed (in which cases no learning was required) showed the same delay as did the normal animals. The difference in delay between normal and leg-amputated roaches occurs in the third thoracic ganglion, as it still is present if the stimulus is applied just anterior to this ganglion. If this reduction is causally related to the postural learning of the insect, it becomes important to know the basis for the changed efficacy of the pathways involved.

Another study is provided by Hoyle,[39] using primarily the locust, *Schistocera gregaria.* Recording with a microelectrode from a single muscle fiber in either the anterior or posterior metathoracic adductor, he was able to demonstrate that if he shocked the leg when the spontaneous nerve discharge to the muscle *decreased,* he could drive the discharge frequency up; although the results were somewhat more variable, a shock delivered following spontaneous *increases* in discharge frequency could drive the frequency down (Kandel, this volume).

Horridge[40,41] has described a procedure for studying the learning of leg position in headless locusts and cockroaches (Figure 5). In initial training procedures, corresponding legs of two animals are connected in a series curcuit using 25-micron silver wire. One of the animals, called the positional, or P-animal, could complete the circuit by lowering its leg lead into a saline bath. When this occurred, both animals received shocks until the P-animal lifted its leg. The random, or R-animal, could therefore receive shocks when its leg was in a variety of different positions, while the P-animal was only shocked when its leg was extended. Following an approximately one-hour training period, the circuit was altered so that each animal received shock independently when its leg lead was extended into its own saline bath. If any association between leg extensions and shock occurred during the initial training, during the test period the P-member of the pair would be expected to take less shock than the former R-member. This is because leg extension was always associated with shock in the P-animals and only occasionally in the R-animals. Horridge found that the P-animals take significantly less shock than the R-animals during the testing period. He interprets this as a demonstration of learning by the insect's ventral nerve cord. Apparently, the brain is not necessary for this kind of learning.

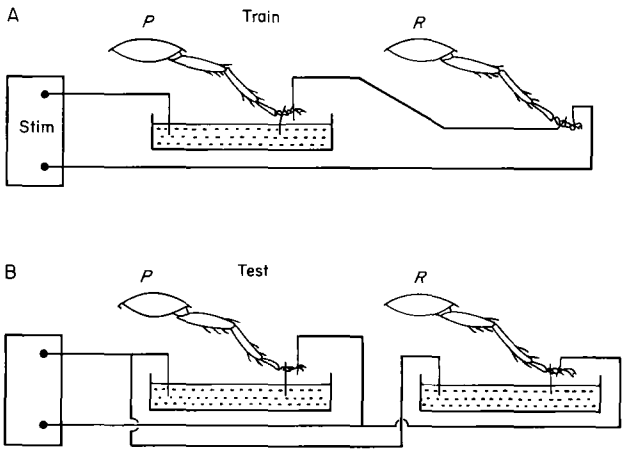


FIGURE 5 The arrangement of the connections to P and R animals from the stimulator. A: In the initial training the two animals are arranged in series and both animals receive shocks when P lowers its leg below the critical level. B: For retest, animals are connected so that either receives a shock when it lowers its leg below the critical level. Any of the six legs may be employed and the animal may be trained on one leg but tested afterward on a different leg. (From Horridge, Note 41)

Dr. M. J. Cohen and I[42] were interested in whether this type of learning could occur in a single, thoracic ganglion of a cockroach when the ganglion had been isolated from the rest of the central nervous system while the peripheral sensory and motor innervation to the legs were left intact (Figure 6). Although shock was delivered across the femoral-tibial joint, the predominant response was flexion at the coxal-trochanter joint (Figure 7). The R-animals received the same shock pattern as the P-animals because of the series connection between them. During testing (Figure 8) the P-animals held their legs out of the saline for longer periods than the former R-group, and therefore took fewer shocks. The difference between the two groups in number of shocks taken over the first twenty-eight minutes of testing is significant. If the improvement seen during training represented only a general shock effect, then no difference between the two groups would be expected during testing, as both groups received an identical number of shocks. That the P-group took significantly fewer shocks than the former R-group, indicates that during training the P-group established a

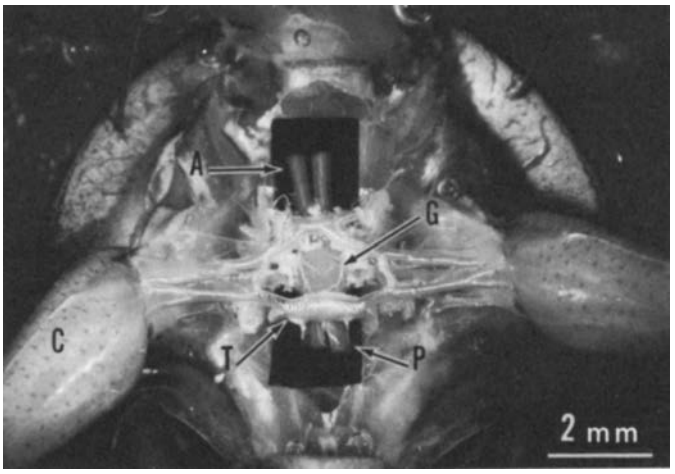


FIGURE 6 Ventral view of the isolated prothoracic ganglion as used in both P and R animals. The head has been removed and the posterior connectives between the pro- and mesothoracic ganglia have been cut. A, anterior connectives; C, coxa; G, ganglion; P, posterior connectives; T, tracheole.

specific association, i.e., it learned to lift the leg to avoid shock; also, improvement for the P-group was more rapid during testing than during training.

The predominant trend of the R-group during the first eighteen minutes of testing was toward an increase in the number of shocks received per minute. This cannot be a deterioration or fatigue effect, as the P-group received the same number of shocks during training and yet showed rapid and maintained improvement during testing. We think that the upward trend of the R-group curve and the difference between the two groups are the result of one experimental variable—the relationship of leg position to shock during training. The P-group relationship was always constant; the R-group had no control over the shock during training. When the R-group was given the same task as the P-group during testing—that is, to avoid shock by lifting the leg—it first may have had to extinguish any previous chance associations between leg lift and shock onset that had occurred during training. Such a competing response tendency would have to be extinguished before avoidance learning by leg-lifting could occur. That the R-group performance gets worse early in the testing period and then improves supports the idea of an initial period during which competing responses

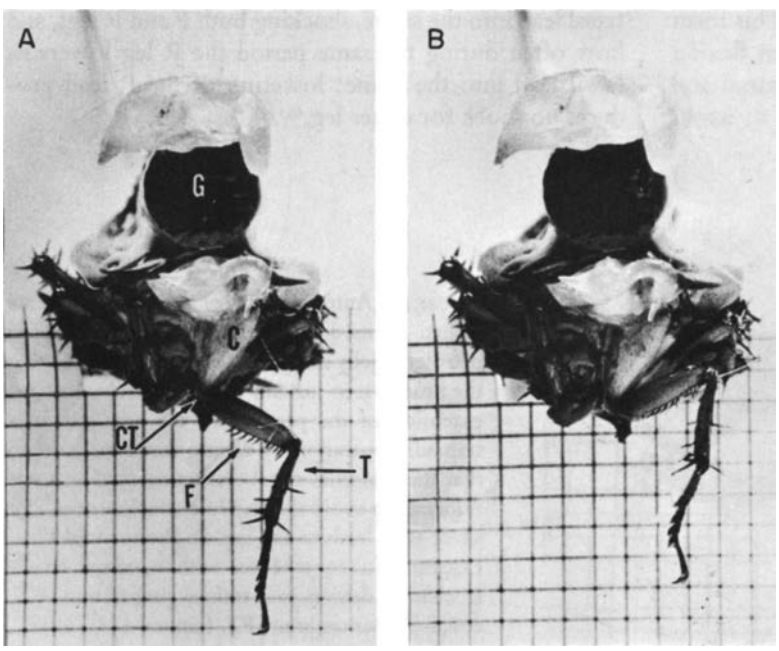


FIGURE 7 Anterior view of the preparation attached by its dorsal surface to a glass rod. The petroleum jelly sealing the cut made by removing the head is seen just above the coxa. A: Position of the left prothoracic leg before training. B: Position of the leg in the same preparation at the end of training. Leg is lifted by flexion at the coxal-trochanter joint. C, coxa; CT, coxal-trochanter joint; F, femur; T, tibia; G, glass rod. Grid squares, 1.5 x 1.5 mm.

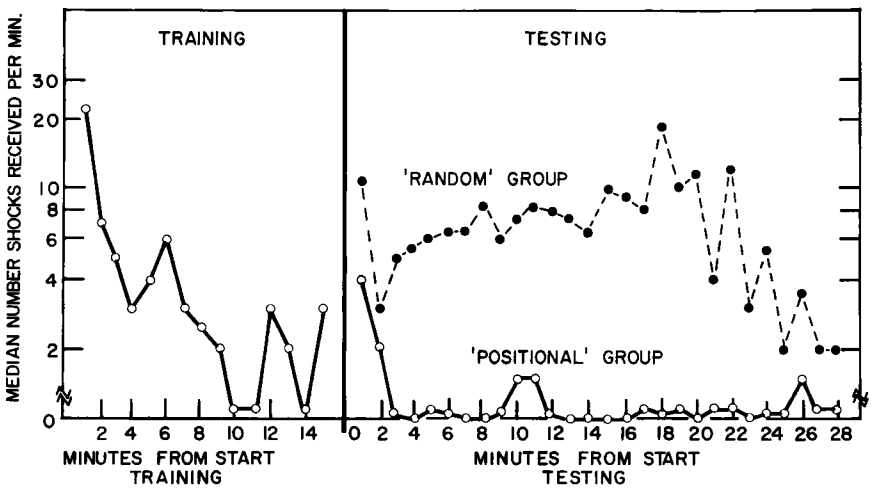


FIGURE 8 The left curve illustrates the decrease in the median number of shocks taken by the P animals during training. The right curves show the difference in the median number of shocks taken by P and R animals during testing.

are extinguished, followed by relearning. After twenty-eight minutes of testing, the R-group closely approaches the performance level of the P-group. One interpretation of these phenomena is that both P- and R-groups learn. What is learned, as shown by their different behavior, is dependent on the input-output sequence to which this system was exposed during training.

The avoidance behavior in P- and R-animals showed considerable variability, as seen in Figure 9. The predominant avoidance response, as has been mentioned, was a flexion of the coxal-trochanter joint, frequently accompanied by extension of the femoral-tibial joint. This resulted in elevation of the tibia and tarsus. Flexion at this latter joint occurred only rarely. Occasionally, the response involved complex coxal movements, in which the leg was extended laterally or anteriorally. This form of avoidance response generally appeared when flexion of the coxal-trochanter joint, together with tarsal and tibial elevation, did not raise the leg enough to avoid shock. Hence, the behavior was by no means stereotyped. The isolated ganglion was capable of generating a variety of leg movements when necessary.

For quantitative studies of such learning it is desirable to use P and R preparations with minimal genetic variability, and to obtain behavioral information from both during training, rather than during a test period when the learned difference in behavior has been abolished —especially if one wishes to examine the histological correlates of behavior.

We are currently investigating the use of a single preparation in which one leg is wired as the P leg, controlling the onset of the shock as before, and the contralateral leg is wired as the R leg. It is possible to record how often during the training period the P leg lowers its tarsal lead into the saline, shocking both P and R legs, and how often during the same period the R leg lowers its tarsal lead into the saline; lowering of the R lead produces no shock for either leg.[43]

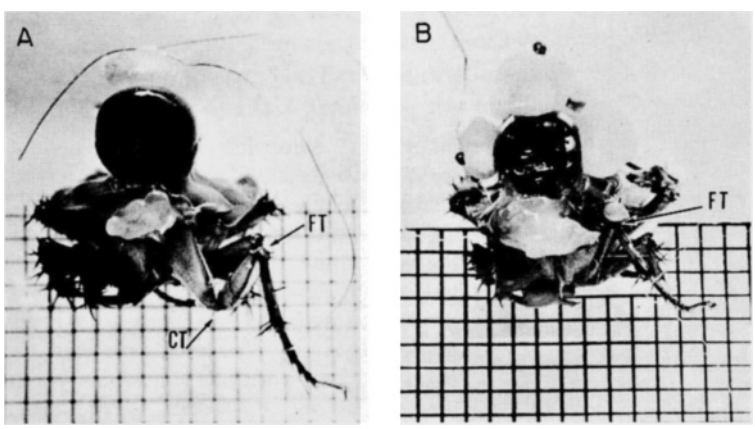


FIGURE 9 Anterior view of the preparation attached by its dorsal surface to a glass rod. The petroleum jelly sealing the cut made by removing the head is seen just above the coxa. A: Anterior extension of the prothoracic leg produced in a trained preparation by raising the saline level so that the maximal coxal-trochanter flexion is not sufficient to avoid shock. Note attachment of 25μ silver wire leads to the leg. B: Pronounced tibial extension in conjunction with maximal coxal-trochanter flexion in a trained preparation. CT, coxal-trochanter joint; FT, femoral-tibial joint.

In view of the recent mapping of the motor cells in the metathoracic ganglion of this cockroach,[44] *Periplaneta americana* (demonstrating marked bilateral symmetry on a cellular level), it may be possible to determine cellular changes underlying avoidance learning using bilaterally matched legs by examining bilaterally matched cells and their associated neuropil.

Finally, a class of experiments that might be of great significance in specifying the neuroanatomical substrates in learning and memory storage are those which, in the context of this discussion, might be incorporated under the question: Will the butterfly remember what the caterpillar learned? That is, perhaps advantage should be taken of the remarkable phenomenon of metamorphosis, demonstrated so widely among insects, and which may involve such changes as coalescing of ganglia and disappearance of certain cell types. Such studies may help shed important light on the anatomy of memory storage.

Coding of temporal sequence appears to be a characteristic of most, if not all learning. A system learns if it can code temporal pattern as manifested by its ability to generate a differential output to the same test input as a function of the total previous input-output pattern of which the test input was a part. This is not to say that long-lasting changes from stimulation per se are not learning; nor does it mean that all systems that code temporal pattern involve identical mechanisms and therefore represent the same kind of learning. Such questions await analysis at a more molecular level. The concept of learning has been phrased here in terms of the essential variable manipulated in experiments in which there is unmistakable learning, so that one may still deal with this variable in even the simplest system chosen for investigation.

Learning phenomena may embrace one or a few subclasses of a larger class of integrative phenomena in which systems acquire and store temporal sequences of events in some three-dimensional form. If such is the case, one would expect certain as yet unknown molecular properties to be common to this class of integrative events with different properties uniquely defining the various subclasses.

Cellular Studies of Learning

ERIC R. KANDEL

THE STUDY OF THE CELLULAR MECHANISM of learning is barely beginning, and one can well argue it has yet to begin. This disappointing situation is caused by two factors. First, the cellular neurophysiological, morphological, and biochemical techniques for undertaking such a study have been developed only in the last decade. Second, the neurophysiological study of learning is highly dependent upon anatomical and physiological knowledge of connections in the nervous system; yet knowledge of neuronal interconnection is so meager that no single function of the central nervous system, even the simplest, has been completely analyzed in terms of its "wiring diagram."

The second deficiency becomes immediately apparent when one seeks a preparation suitable for studying learning. Ideally, one would like a preparation in which a reflex can be conditioned, either by the development of new reflex (classical conditioning) or by the change in efficacy of a previous existing reflex (alpha conditioning), and in which all the anatomical pathways mediating the conditioned and unconditioned responses are known and can be examined at the cellular level. At present, these simple requirements cannot be met in any system. In vertebrates, the only reflexes that have been completely worked out are the monosynaptic reflexes of the mammalian spinal cord. Yet, even here, current knowledge is inadequate to account completely for such a simple behavioral act as the patellar reflex these monosynaptic connections subserve.[1] More complex (polysynaptic) reflexes, such as the flexion or the scratch reflex, are far from worked out. In the spinal cord, one at least has the advantage of being able to record from the final common pathway, the motoneurons, involved in these reflexes. In many systems, even this limited advantage is lacking.

The situation ought to be better in invertebrates, but actually it is not. These animals do possess numerically simpler nervous systems. Instead of 10^{12} cells of the vertebrate nervous system, the nervous system of mollusks, arthropods, and worms generally contain about 10^4 neurons. Also, some invertebrate nervous systems, particularly those of mollusks and crustaceans, have large central neurons that are directly accessible for microelectrode and biochemical investigation. Therefore, much progress has been made in understanding the biophysics of cellular function and of synaptic transmission in these large invertebrate cells. In addition, there have been remarkable advances in the study of transmitter chemistry and pharmacology in the nervous system of the crustaceans and of mollusks (see Notes 2–4, and Kravitz, this volume).

Although it is my belief that, in the long run, invertebrate preparations offer great promise for the cellular study of learning, for the moment their advantages are counterbalanced by a striking paucity in knowledge of their anatomy and behavior. Little is known of the learning capacity of mollusks, insects, and crustaceans, and only slightly more about their reflex capabilities. The invertebrate nervous system has, until recently, not proved overly attractive to experimental psychologists, hodologists, and integrative neurophysiologists. For this reason, the recent anatomical studies by Coggeshall[5] in *Aplysia* and by Cohen and Jacklet[6] in insects, the studies of reflex coordination by Wiersma[7] and by Kennedy[8] and their associates in the crayfish, and the behavioral studies by Kupfermann[9] and by Lickey[10] in *Aplysia* are particularly welcome.

In giving this rather harsh description of the currently tenuous foundation of the cellular study of learning, I do not wish to imply that a complete electro-anatomical understanding of the connections of the brain need be worked out before the cellular physiology of learning can be tackled in a particular animal, but I do think it necessary that some reflexes be worked out in detail. Those preparations that lend themselves most readily to an analysis of reflex function are quite likely to prove most suitable for a cellular study of learning.

If no ideal preparation is available for the cellular study of learning, why study the cellular mechanism of learning at all? At least two reasons can be given. One: while it is not yet possible to study the cellular details of actual learning, one can study cellular concomitants of learning, and one can develop and analyze cellular analogs of learning. Such studies also direct themselves to the larger problem of how long-term (plastic) changes in neuronal function occur. Because an understanding of neuronal plasticity is essential to an understanding of the integrative function of the nervous system, approaches which contribute to the analysis of plasticity are worthy of experimental interest independent of their relevance to learning. Two: although

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imperfect, the application of cellular neurophysiological techniques to larger problems such as learning is bound to bring a fresh perspective to these traditional psychological problems. Even in their faltering beginnings, these approaches raise interesting conceptual and methodological questions about the relationship to each other of cellular function, the distinctive properties of neural aggregates, and actual behavior.

Figure 1 lists several approaches which have been developed in the cellular study of learning, and which I will discuss below. It is useful to draw a distinction between studies of *actual learning* and studies of *analogs of learning*. Actual learning involves the use of natural stimuli and behavioral (effector) responses. Analogs of learning utilize artificial (electrical) stimuli and/or responses. A third category consists of studies of use and disuse and of the trophic functions of neurons. Although these studies are often not specifically directed toward elucidating learning mechanisms, they have pointed up a number of highly interesting plastic properties of neurons that may be shared by psychologically relevant processes.

One can also draw subdistinctions among some of these approaches. For example, two types of studies of actual learning can be distinguished. One concerns the *cellular determinants* of learning. This involves an analysis of the cellular mechanisms underlying a learned response, an ideal case yet to be approached experimentally. The other type of study concerns the *cellular concomitants* of learning, of which there are now several excellent examples.

Cellular studies of actual learning

CONCOMITANTS OF ACTUAL LEARNINGS The first study of the cellular concomitants of learning was by Jasper, Ricci, and Doane, who examined a conditioned avoidance response in monkeys.[11] The conditioned stimulus (CS) was a series of light flashes repeated at five per second; the unconditioned stimulus (UCS) was an electrical shock to the hand, five to six seconds after the light flashes. Learning consisted of pressing a switch to turn off the shock when the CS appeared. Unit activity was recorded extracellularly in the frontal, motor, sensory, and parietal cortex. Simultaneous recording of surface EEG activity permitted comparison with the change of firing patterns of individual cortical neurons during conditioning.

The cellular concomitants of this conditioning situation proved complex. In all cortical regions, units showed increments or decrements in their firing patterns when the CS was initially presented. In most cases, these responses tended to decrease (habituate) with repeated presentation of the CS alone prior to pairing. During and after pairing, the response to the CS varied considerably among different cells, even when these were located near each other in the cortex. Two such examples are illustrated in Figure 2A. The cell illustrated in part 1a shows a marked acceleration of its firing pattern following four flashes of light. The acceleration begins several seconds prior to the onset of the delayed conditioned response (CR) and continues considerably beyond the period of the CR. In the

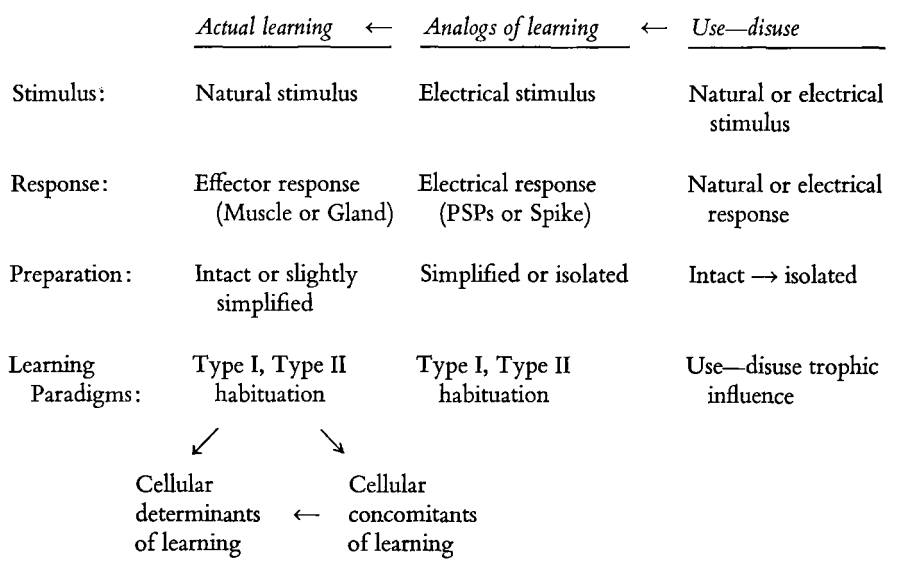


FIGURE 1 Cellular neurophysiological approaches to the study of learning. See text for details.

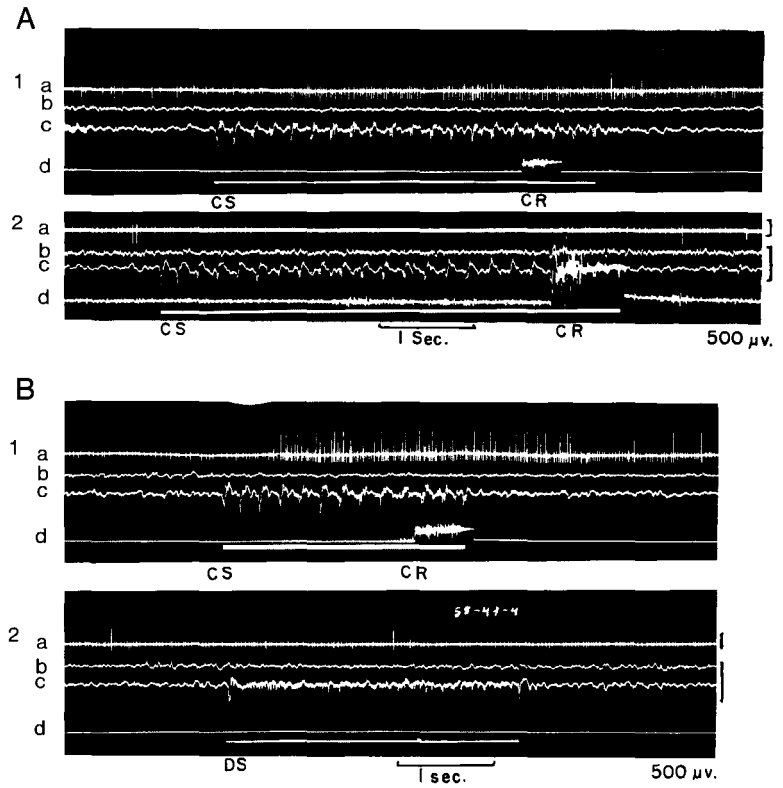


FIGURE 2 Cellular concomitant of conditioned avoidance learning in the monkey. A: Unitary discharge from motor cortex of the monkey during two conditioned responses (CR) to a visual stimulus (CS) at 5 per sec. Microelectrode record is shown in a; surface ECG from motor cortex in b; and from visual cortex in c. EMG and motor response to the CS is shown in d. The same conditioning procedure produces the acceleration of discharge of the cell illustrated in A1 and inhibition of the cell illustrated in A2.

B: Discharge of a unit in the motor cortex during a conditioned response (B1) and during a differential stimulus (DS) without motor response (B2). The CS was an intermittent photic stimulus at 5 per sec. The DS was the same stimulus at 30 per sec. (From Jasper, et al., Note 11)

cell illustrated in part 2a of Figure 2A the opposite effect is observed: the CS produces an inhibition of cell's discharge and firing resumes when the CR is initiated. The response pattern of single units during conditioning also bore little relationship to the surface EEG (trace b in Figure 2A1 and 2A2). For example, while the cell illustrated in part A1 is accelerated and that in part A2 is decelerated, in both instances the EEG is desynchronized and shows low-voltage fast activity.

Despite the over-all complexity of the results, certain cells showed responses that were strikingly parallel to the behavioral conditioned response. The cell illustrated in Figure 2B1 showed a response to the CS similar to that shown by cell illustrated in Figure 2A1, an anticipatory acceleration of discharge, prior to the conditioned response, which continued for several seconds after the termination of the conditioned response. When a new, previously unpaired, light stimulus was presented (a 30 per second instead of 5 per second light flash), it produced no conditioned motor response and no change in cell firing (Figure 2B2). Therefore, this cell showed a differential response to the input stimulus, responding to the paired stimulus (5 per second) but not to the unpaired one (30 per second), which parallels the differentiation of the motor response to the CS.

Comparison of units in different brain regions also yielded interesting findings. The anticipatory acceleration, illustrated in Figures 2A1 and 2B1, was characteristic of units in the arm area of the *motor* cortex. This is consistent with the cells' participation in an effector mechanism of the CS. Units in the arm area of the *sensory* cortex tended to show a later excitation consistent with activation by proprioceptive feedback from the responding limb. Only 30 per cent of cells in the parietal lobe responded to the CS, but those that responded were closely linked to CS. They responded with brief burst of spikes to each of the 5-per-second light flashes and with inhibition to the differential (30 per second) stimulus, suggesting to Jasper, et al., that the "switching mechanism" for this learning task occurred in the responding cells of the parietal cortex.

Studies of cellular concomitants of learning are therefore highly useful in comparing cellular responses in different brain areas during conditioning. Also, studying unit activity overcomes some of the interpretive difficulties encountered in studying EEG and DC potential. However, this approach has also encountered certain problems. First, it is difficult to know what sample one obtains with microelectrodes, and whether one records some cells to the exclusion of others (see Note 12 for a new approach to this problem). Second, it is difficult to specify the anatomical

and physiological relations of any given units to the behavioral conditioned response. Third, it has not been possible to analyze the cellular mechanisms that underlie the alterations in unit activity, because these studies have been carried out on chronic animals, using extracellular recordings and complex natural stimuli.

To overcome some of these difficulties, several investigators have attempted an alternate cellular approach to the study of learning. They have tried to develop cellular analogs of learning in a number of simplified preparations that can be approached with intracellular microelectrodes and with the analytic techniques of cellular neurophysiology.

Cellular analogs of learning

The aim of this approach is to determine whether the experimental simulation of stimulus sequences, which are capable of generating learning in the whole animal, can produce long-term changes in the behavior of single nerve cells. The stimulus sequences used have been modeled upon habituation and upon Type I and Type II conditioning. These three learning paradigms have some obvious features in common: (1) they generate what psychologists consider elementary forms of learning; (2) they are quite general and apply to a wide variety of animals from mollusks to man; (3) they are operationally well defined. This last aspect makes the paradigms translatable into direct neurophysiological terms. *In the most general terms, the learning paradigms specify a series of rules for patterning a stimulus over time or combining it with other stimuli.* Consequently, they may be viewed as a series of stimulus sequences that can be applied even to simplified and isolated preparations, using electrical instead of natural stimuli.

A behavioral rationale for this approach is, in part, provided by work on Type I and Type II conditioning, indicating that in some simple situations conditioning can occur independently of the nature of the reinforcing stimulus. *In these cases, conditioning is dependent only on the pattern or schedule with which the stimuli are presented.*[13-15]

Figure 3 illustrates the stimulus sequence associated with the three paradigms. The top part of the figure is, in each case, a conventional statement of the paradigm. The middle part illustrates examples of how these statements can be applied to isolated preparations, using electrical stimuli instead of natural ones. The bottom portion illustrates some of the intracellularly recorded electrical indexes that have been used as responses in these cellular analogs.

The habituation paradigm is concerned with the consequences of the repeated or monotonous presentation of a stimulus of constant strength. With such repeated stimulus presentations, a decrement in response occurs. Interjection of extraneous stimulus via another modality produces re-

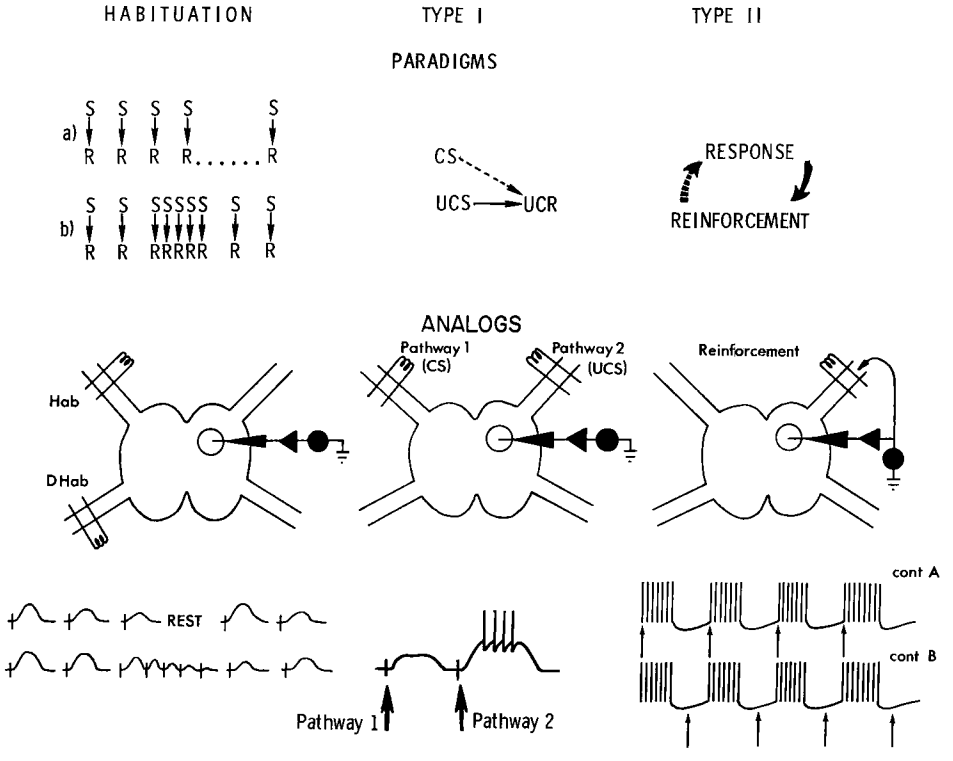


FIGURE 3 Cellular analogs of learning. See text for details

sponse restoration (dishabituation). In the analog, a pathway to a preparation can be stimulated in a constant manner and alterations in the responsiveness of some behavior of a cell, such as an excitatory postsynaptic potential (EPSP) or an inhibitory postsynaptic potential (IPSP), can be measured. Another pathway can be stimulated to produce dishabituation.

Type I conditioning is based on the temporal association of two stimuli: an initially ineffective conditioning stimulus (CS) becomes effective after a period of pairing with an initially effective unconditioned stimulus (UCS). In the analog, two different afferent pathways are chosen. The stimulus parameters to one pathway are adjusted to produce a small excitatory synaptic potential (the less effective, or "conditioning," stimulus), while the parameters to the other are set to produce a train of spikes (the more effective, "priming" or "unconditional," stimulus). The intracellularly recorded response of an otherwise quiescent cell can be considered an analog of the conditioned and unconditioned responses. The two inputs are paired repeatedly, with the test stimulus (analogous to the CS) preceding the priming (analogous to the UCS) stimulus by about 300 milliseconds, and the effects of stimulus pairing on the test response are examined.

In Type II (operant or instrumental) conditioning, the reinforcement is contingent on a particular behavioral response. In a highly simplified way, this procedure may be viewed as a stimulus ("reinforcement") contingent upon response. In an analog, one of the several spontaneous responses of an active cell—i.e., the generation of spontaneous IPSPs or EPSPs, the generation of bursts of spikes, or even changes in the background firing rate—can be selected as an appropriate electrophysiological response suitable for reinforcement. A nerve stimulus can then be made contingent upon the occurrence of the particular response selected.

Analogs are useful for specifying the necessary stimulus patterns required to produce these three types of learning, as well as for examining possible cellular interrelationships among them. For example, in its simplest form the Type I paradigm is concerned with changes in the amplitude domain of evoked responses, while the Type II paradigm is concerned with changes in the frequency domain of ongoing activity. It is worthwhile in this context to recall that in the simplest case—that of a single neuron—frequency at the output is a simple and invariant transform of amplitude at the input. Thus a Type I analog can be turned into a Type II analog by making the CS (operant) pathway spontaneously active. Similarly, the dishabituation and the Type I analogs are related to each other, because they both involve an increment in the efficacy of one pathway following a highly effective stimulus to a second pathway.

Analogs of habituation

Even the simplest organisms show habituation to the repeated presentation of a stimulus that is parametrically similar to the habituation found in higher organisms. The possibilities of developing a cellular analog of habituation are made particularly attractive (Figure 3) because response decrement seems to be a common feature of different types of neural aggregates. Recently, a number of efforts have been made to develop this approach, using two different types of preparations: the isolated spinal cord of the cat and the isolated nervous system of *Aplysia*. Because the parametric details of this analog and its relevance to actual behavior are better understood in the isolated cat spinal cord than in *Aplysia,* I will first consider the spinal cord studies.

Sherrington described decrement of the flexion reflex to closely spaced stimuli in the spinal animal as early as 1898.[16] His findings were subsequently confirmed by Prosser and Hunter.[17] In addition, Prosser and Hunter pointed out that a strong extra stimulus restored the response amplitude following decrement. The demonstration of "dis-habituation" and the finding that weak, but not strong, stimuli produce the decrement, established the parametric similarity of this type of response decrement to other types of actual (effector) habituation.[18]

Recently this problem has been investigated with modern cellular neurophysiological techniques. In an elegant series of studies Spencer and his collaborators have simplified the spinal preparation progressively to permit a more detailed examination of the mechanisms involved.[19-21] Spencer, et al., turned from the chronic to the acute spinal cat and showed, in this radically simplified preparation, all the features of response decrement seen in the chronic animal.[18] An experiment from the acute spinal cat is illustrated in Figure 4, which is a continuous record of an isometric myogram of the tibialus anterior under low initial tension. The test stimuli were brief trains applied to the skin of the rump and delivered at 2-minute intervals during the control period in order to establish a base line of responsiveness. During the habituation period (indicated by arrows), the frequency of the train presentation was increased twelvefold, from one per 2 minutes to one per 10 seconds. The increased frequency of stimulus presentation produced decrement to the phasic response of the tibialus anterior. After the decrement had occurred, spontaneous recovery could be plotted by delivering the stimulus trains at the slower intervals used to establish the initial baseline responses (Figure 4A). In most cases, decrement reached a plateau only after dozens or even hundreds of stimuli. The period of recovery was also variable, ranging from 30 seconds to 30 minutes and averaging several

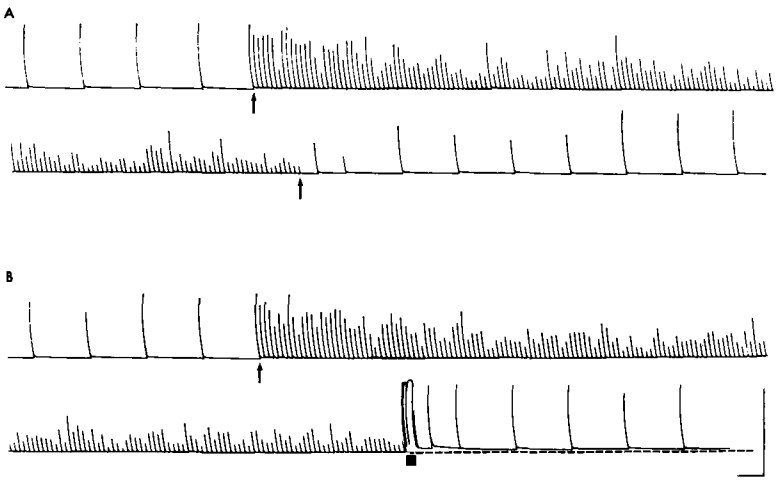


FIGURE 4 Essential features of habituation (response decrement) and dishabituation (response restoration) in the acute decerebrate-spinal preparation. Continuous records of isometric myograms of tibialis anterior under low (<50 g.) initial tension. Test stimuli were brief trains (0.5-sec. duration) of high frequency (50/sec.) pulses applied to skin of rump at 2-minute intervals. A: Decrement and spontaneous recovery. Period between arrows indicates response decrement following increase in stimulus frequency from 2 per minute to 10 per second. B: Restoration of the previously decremented flexion reflex (dishabituation) by introduction of a strong extra stimulus. Test stimuli similar to those in A. Response restoration (during period indicated by shaded rectangle) produced by brief pinch to digits of same leg. Time index = 1 min.; tension index = 50 g. (From Spencer, et al., Note 19)

minutes. In addition to spontaneous recovery, restoration of reflex responsiveness could be produced by the presentation of extra stimulus as indicated in part B. The most effective stimulus for restoration of the decremented response was a pinch to the digits or a brief, intense, high-frequency (50 to 100 per second) electrical stimulus to a cutaneous nerve of the responding hind leg.

This simplified preparation thus shows the two most interesting features of the habituation paradigm: (1) response decrement, and (2) response restoration by a strong extra stimulus. Spencer and his colleagues first directed themselves to the response restoration. Restoration of the decremented response by a strong extra-stimulus (dishabituation) generally has been attributed to a disruption of the process responsible for the decrement. But Spencer and his colleagues found that the extra stimulus often increased the magnitude of the decremented response well above the initial control level (upper graph, Figure 5). In fact, the response amplitude increased above control level even when the extra stimulus was given during initial control tests and prior to decrement by high-frequency stimulation (lower graph, Figure 5). These findings indicate that the effect of the extra stimulus represents not a disruption of a pre-existing process, but rather an independent facilitation prôcess superimposed upon the decrement.

For the analysis of response decrement, Spencer and his colleagues abandoned electromyographic recordings and recorded postsynaptic potentials (PSPs) from the motoneuron with intracellular microelectrodes. Instead of natural stimuli, they used electrical stimuli to the cutaneous nerve from the hind limb.[21] They therefore moved from studying a simplified preparation showing actual habituation to studying an even more simple neural analog of habituation. The data they obtained with intracellular recordings fell into two categories, those instances in which spiked discharges were produced by the test volley, and those in which they were absent. Both results are qualitatively similar, but I will focus only on the second case because it permits a clear examination of the changes in configuration and amplitude of the polysynaptic excitatory postsynaptic potential. In those motor neurons in which the EPSP was subthreshold for firing, change in repetition rate from 1 per 30 seconds to 1 per second produced a clear decrement of the EPSP (Figure 6–1A and 1B) and an extra stimulus (1C) or spontaneous recovery (1E) produced EPSP restoration.

Spencer, et al., next showed that the decrement in the polysynaptic EPSP represented a change in the responsiveness of the polysynaptic pathway, not an alteration in the properties of the motoneuron membrane, because the monosynaptic EPSPs and membrane threshold measurement remained constant during the period in which the polysynaptic EPSPs decremented (Figure 6–2). Spencer and his collaborators therefore postulated that the response decrement of the polysynaptic pathway probably represents a homosynaptic depression of excitatory synaptic transmission in an interneuronal pathway. Its restoration by extraneous stimulus was caused by a superimposed facilitation resulting from either a post-tetanic afterdischarge in the interneuronal pool, or presynaptic facilitation. To test these ideas further requires the development of a monosynaptic system among the interneurons in the spinal cord. This has not yet proved possible.

An opportunity to test these ideas has recently been provided in the pleural ganglion of the marine mollusk

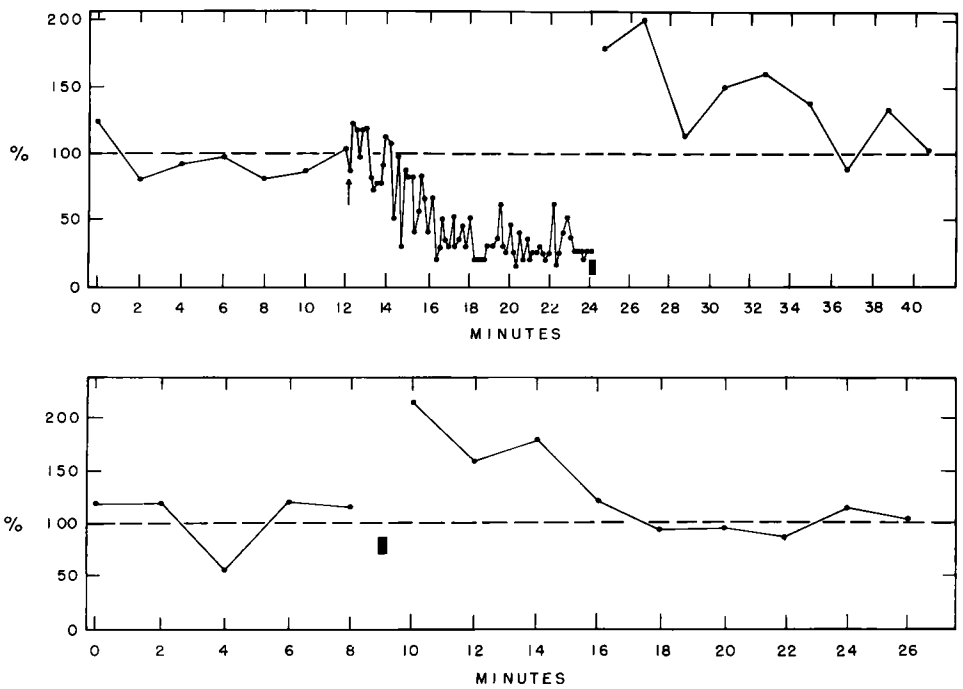


FIGURE 5 Dishabituation effects of strong extra stimuli in acute spinal preparation. Upper graph: Myographic responses of tibialis anterior muscle to stimulation of foot pad at a rate of one per two minutes. At arrow, stimulus frequency was increased to 1/10 sec. Shaded rectangle indicates time of application of strong pinch to same leg, followed by test stimuli delivered at initial frequency. Note that responses immediately following the extra stimulus were well above the control level. Lower graph: Stimulus site and recording conditions same as above. Test stimuli delivered at 2-min. intervals throughout. Arrow represents application of extra stimulus, as above. Note the increase in response amplitude above the control level. (From Spencer, et al., Note 19)

Aplysia. In this ganglion, Bruner and Tauc[22,23] have developed an interesting analog of habituation which is quite similar to that developed by Spencer, et al., in the isolated spinal cord. Stimulation of the tentacles of *Aplysia* with drops of sea water produced a compound, polysynaptic EPSP in the pleural ganglion giant cell; this EPSP decreased rapidly in amplitude. If stimulation is stopped for a few minutes, spontaneous recovery results. As in the isolated spinal cord, the EPSP response decrement is also produced by repeated stimulation of one of the cerebral nerves, and response restoration can be produced either by rest (spontaneous recovery) and or by interjecting a strong extra stimulus via another nerve or connective. Moreover, Bruner and Tauc demonstrated decrement and restoration in a unitary, presumably monosynaptic EPSP (Figure 7), providing further support for the notion that decrement involves a depression of synaptic transmission localized to the presynaptic terminals (for further details see review by Bruner and Tauc, Note 23). The restoration was attributed to presynaptic facilitation similar to that previously shown by Kandel and Tauc in the right upper quadrant giant cell (R2) of the abdominal ganglion with the analogs of the Type I paradigm[28] (see below).

A second possible mechanism for response decrement, differing from EPSP depression, has been suggested for mollusks by Holmgren and Frenk,[24] who showed that in certain giant cells of the parietal ganglion of the snail, repeated ipsilateral mantle nerve stimulation, at a rate of 1 to 5 per second, produced a characteristic pattern of response. The first few stimuli produced excitatory responses, but subsequent stimuli produced an oscillatory type of hyperpolarization that either builds up gradually during stimulation or appears abruptly after one of the series of stimuli. After stimulation was discontinued, the hyperpolarizing oscillation and a consequent inhibitory effect subsided in the following 100 to 200 seconds. Holmgren and Frenk suggested that behavioral habituation in the snail could result from a build-up of this kind of inhibition, but their data did not permit them to specify the nature of the hyperpolarizing oscillations.

Waziri, Frazier, and Kandel[25,26] have recently found a phenomenon in the abdominal ganglion of *Aplysia* that is

similar to that encountered by Holmgren and Frenk in the snail. In the left hemi-ganglion, a number of identified cells show prominent spontaneous IPSPs caused by the activity of several inhibitory interneurons.[26] (For a discussion of the identified cells in this ganglion, see Notes 26a-b and Strumwasser, this volume.) In some of these cells (L7, L8, and L9) a stimulus to the connectives or to the branchial nerve produces first an excitatory response followed by a recruitment of IPSPs. The IPSP recruitment is often progressive and prolonged from several minutes to over one hour following 1 to 20 stimuli at a rate of 1 per 2 minutes. The consequence of this IPSP recruitment is twofold: it produces first a response decrement in the early excitatory response to the stimulus and, second, a prolonged decrement in the spontaneous firing frequency of these cells (Figure 8). These results are similar to those of Holmgren and Frenk in showing a build-up of a hyperpolarizing oscillation. In *Aplysia*, this build-up is more prolonged, and it is possible to demonstrate that it results from a recruitment of inhibitory postsynaptic potentials.

The results from these two types of experiments—the EPSP decrement in the cat spinal cord and in the pleural ganglion of *Aplysia* and the IPSP recruitment in the snail and the abdominal ganglion of *Aplysia*—indicate the existence of two different mechanisms for response decrement, one a diminution in size of the excitatory synaptic potential, and the other an increased frequency (recruitment) of ongoing inhibitory synaptic potentials. The existence of two different analogs for such an apparently simple form of neural plasticity emphasizes the likelihood that a variety of cellular mechanisms may be able to produce the same type of effector change.

The mechanism underlying IPSP recruitment is still not worked out, but there is some reason to believe that it may represent a prolonged increase in the firing rate of spontaneously active interneurons capable of endogenous pacemaker activity[26] (see, for example, Figures 18 and 19). I introduce this speculative interpretation here because of evidence I will consider later for the possibility of storing information by changes in pacemaker activity.

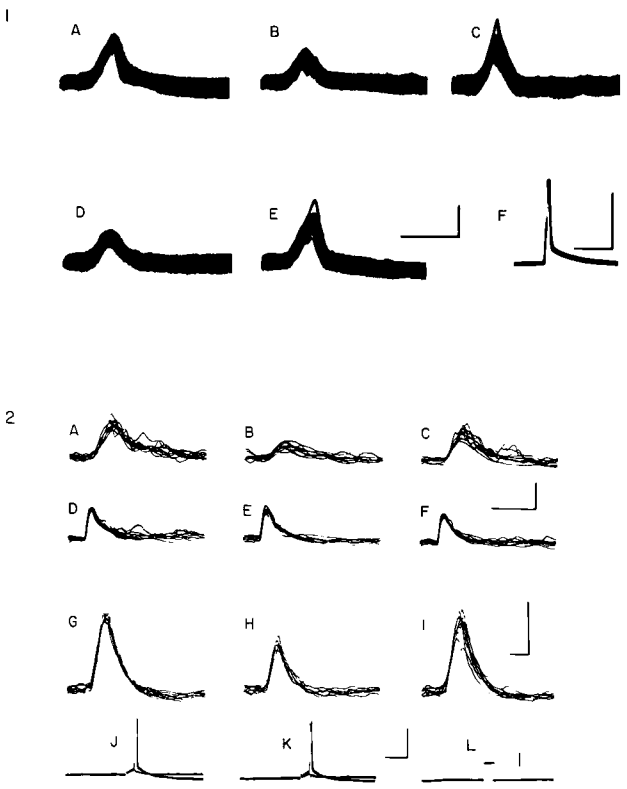


FIGURE 6 Analog of habituation in acute spinal animal. 1: Intracellular recordings of polysynaptic PSPs decrement from deep peroneal motoneuron (identified by antidromic activation as shown in F) in response to single shock stimuli delivered to the superficial peroneal nerve. A: polysynaptic EPSPs during control period established by stimulating at 30-sec. intervals; B: PSP decrement during stimulation at 1-sec. intervals; C: restoration of PSP amplitude following an extra stimulus delivered to the tibial nerve (tetanus at 100/sec. for 4 sec. duration); D: subsequent PSP of decrement produced by continued stimulation at 1-sec. intervals; E: spontaneous recovery of PSP amplitude, stimuli at 30-sec. intervals. Time and voltage calibrations for A to E = 10 msec. and 2 mv.; for F = 5 msec. and 50 mv.

2: Demonstration of constant amplitude of interpolated monosynaptic PSPs and stability of threshold to applied depolarizing currents during period of polysynaptic PSP decrement in deep peroneal motoneurons. A-C: polysynaptic PSPs to single shock stimuli delivered to posterior femoral cutaneous nerve. A: polysynaptic EPSP during control period established by stimulating at 30-sec. intervals; B: decremented PSP during stimulation at 1-sec. intervals; C: recovery of PSP amplitude, stimuli at 30-sec. intervals; D—F: monosynaptic PSPs to stimuli delivered to deep peroneal nerve at 30-sec. intervals corresponding to those in A, B, and C, respectively. Note constancy of monosynaptic responses during polysynaptic PSP decrement. G, H, and I are polysynaptic EPSPs obtained during periods identical to those of A, B, C, but from a different motoneuron in the deep peroneal group. J and K show responses to depolarizing current pulses of shape shown in L during control and decrement periods corresponding to G and H. Note that pulse continues to be of threshold-straddling intensity, indicating constancy of threshold in the presence of PSP decrement. Calibrations A-F: time = 10 msec., voltage = 2 mv.; G-I: time = 10 msec., voltage = mv.; J, K: time = 5 msec., voltage = 50 mv., L: vertical bar represents 1 x 10^{-8} A. (From Spencer, et al., Note 21)

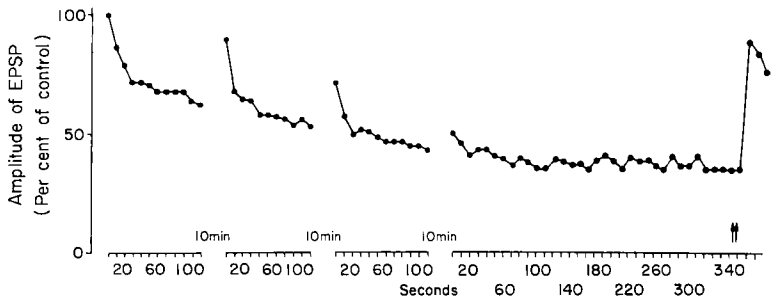


FIGURE 7 Analog of habituation in the giant cell of the left pleural ganglion of *Aplysia*. Response decrement and restoration of a unitary, presumably monosynaptic EPSP. The stimulus is presented 1/10 sec. Note decrement with repeated presentations and partial recovery following 10-minute rest periods. There appears to be a cumulative effect with repeated trials. A three-second tetanus of left posterior pedal nerve (arrows) produces restoration of the response. (From Bruner and Tauc, Note 22)

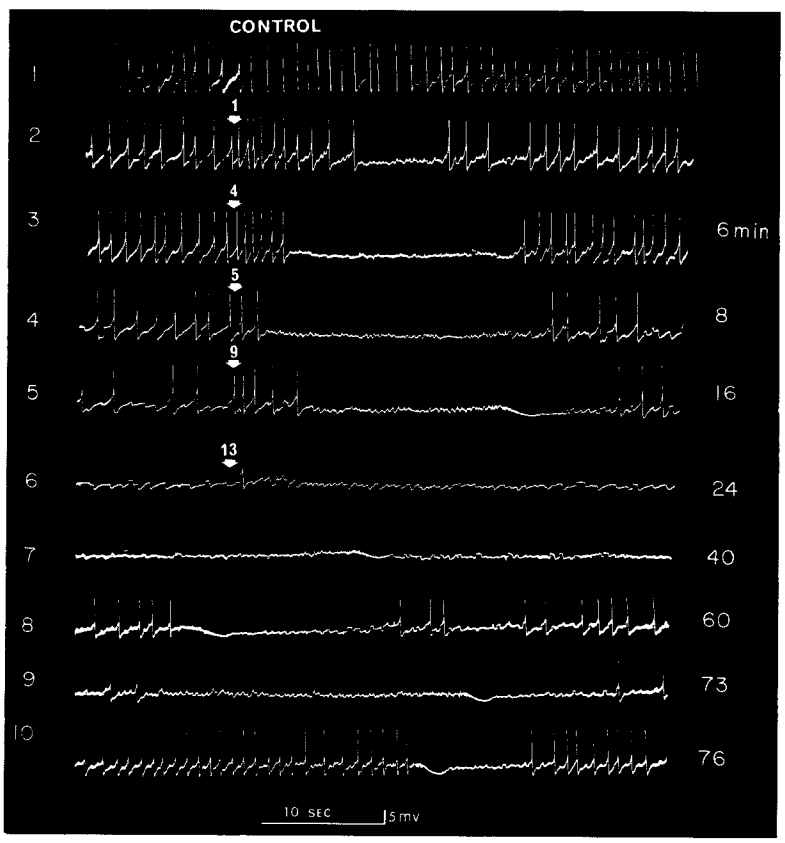


FIGURE 8 Analog of habituation in an identified cell (L8) of the left hemi-abdominal ganglion of *Aplysia*. Response decrement of spontaneous and evoked response following recruitment of inhibitory PSPs. Line 1 is from the pre-stimulus control period and shows regularly recurring spikes with interposed IPSPs. Lines 2 to 6 are data showing the consequences of the 1st, 4th, 5th, 9th and 13th stimulus to the left connective. Time elapsed following the first stimulus is indicated in the column at the right. Note the decrement in the early excitatory response to the stimulus and in spontaneous firing with the progressive increase in the number of IPSPs. At maximum IPSP recruitment cell firing was completely suppressed. Line 7 is taken from data 40 minutes after first stimulus and 16 minutes after last stimulus, and shows the continued suppression of spike activity. Lines 8, 9, and 10 show the gradual decrease of IPSP rate and the intermittent return of spikes. (FromWaziri, et al., Note 26)

One of the themes I should like to develop is that two general classes of neuronal plasticity—changes in pacemaker activity and changes in synaptic efficacy—seem to account for a number of different processes involving short-term information storage. Whereas neural analogs of behavioral pheonomena such as habituation may, in different settings, partake of one or a number of different specific cellular mechanisms, many of these may prove to be variants of two general classes of plastic change.

Before considering changes in pacemaker activity in the context of analogs of operant conditioning, let us return to another example of changes in synaptic efficacy by considering an analog of Type I conditioning.

Analogs of Type I conditioning

The Type I paradigm consists of the temporal association of two stimuli that differ in their efficacy (Figure 3). This type of approach can be illustrated by considering a series of experiments by Kandel and Tauc on the isolated abdominal ganglion of *Aplysia*.[27,28] Intracellular recordings from single cells were obtained and the stimulus parameters to two different afferent nerves were controlled so that one produced a relatively small EPSP (the "test" stimulus, an analog of the CS) and the other, usually a brief train of stimuli, produced a burst of spikes (the "priming" stimulus, an analog of the UCS). The two stimuli were paired once every ten seconds for one to several minutes, with the test preceding the priming by about 300 milliseconds.

In most of the ganglion cells examined, input pairing produced no facilitation of the test EPSP. However, in the right upper quadrant giant cell (R2) and in some unidentified cells located near the medial border of the giant cell, the test EPSP was augmented during pairing, and the facilitation declined only slowly during the ten to forty minutes following the pairing procedure. During peak facilitation the initially ineffective test PSP became effective in triggering an action potential. Figure 9 illustrates the facilitation in the giant cell. In this experiment, the EPSP to the less effective test input increased in amplitude as a result of pairing and reached the critical level for spike generation. Because it is the *difference* in PSP amplitude and in the generation of action potentials that forms the basis for distinguishing the relative efficacy of the test and of the priming responses, we observe that intermittent presentation of a highly effective reinforcing (priming) stimulus to one nerve has increased the efficacy of the response to the test stimulus to another nerve so that after pairing the test response resembles somewhat more the response to the reinforcing stimulus. The PSP facilitation encountered by pairing stimuli of different efficacy to two separate nerves has been called *heterosynaptic facilitation*.[27]

By introducing controls for quasi-conditioning analogous to those used in behavioral experiments, it appeared possible to divide heterosynaptic facilitation into two types. One type occurred in the giant cell (R2) and was not specific to pairing and to paired input; it was therefore analogous to quasi-conditioning. Because this nonspecific facilitation occurred in one of the identified cells, it was studied in detail. The second type occurred among a few unidentified cells to the left of the giant cell. Some of these cells showed specificity to pairing and to the paired input in several ways analogous to true behavioral conditioning. However, the data on these cells is very limited. We will therefore first consider the studies of nonspecific facilitation in the giant cell.

A number of parametric studies were undertaken.[27]

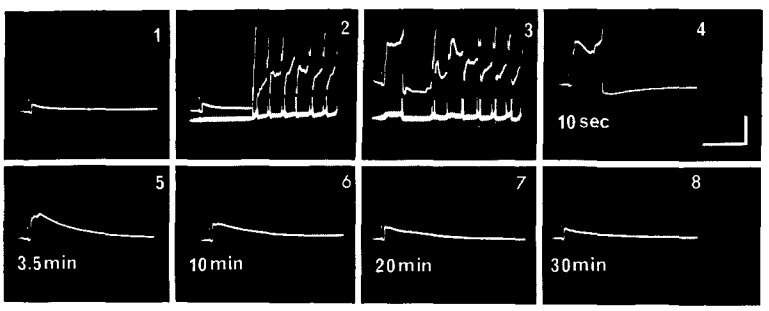


FIGURE 9 Analog of nonspecific Type I conditioning in *Aplysia*. Heterosynaptic facilitation in the giant cell (R2) of the abdominal ganglion. The lower trace in 2 and 3 are simultaneously recorded in low gain records. 1: Test EPSP produced by stimulation of genital nerve before pairing. 2: First of nine pairing trials of test EPSP and response to priming stimulus (6/sec train of 1-sec duration to the siphon nerve). 3: Seventh pairing trial. 4 to 8: Test PSP 10 sec, 3, 5, 10, 20, and 30 min. after pairing. The voltage calibration is10 mv for the upper and 100 mv for the lower trace. The time calibration is 500 msec; intertrial interval 10 sec. (From Kandel and Tauc, Note 27)

These showed that the magnitude of the EPSP facilitation in the giant cell was less a function of the test than of the reinforcing stimulus. Both small and large test PSPs were facilitated, and there seemed to be relatively direct relationship between the strength of the priming stimulus and the degree of facilitation. With optimal priming stimuli, the test PSP was facilitated up to 700 per cent and during peak facilitation an initially ineffective PSP became effective in triggering an action potential. Thus, not only did the PSP facilitate (as in quasi-alpha conditioning) but it also produced a new response following priming, analogous to quasi-classical conditioning. The facilitation was not dependent upon paired presentation of the test and priming stimulus. Facilitation of the test PSP was as great following unpaired presentations of the priming stimulus alone as after paired presentation of the test and the priming stimulus. These parametric data are quite similar to those obtained in behavioral studies of quasi (nonspecific) conditioning.[29]

Experiments were also performed on the whole animal, pinned rigidly in a dissecting dish, while recordings were obtained from the axon of the giant cell.[27] In these experiments, a physiological priming stimulus (stroking the peri-siphon area) rather than an electrical one was used. Nonetheless, heterosynaptic facilitation was obtained, indicating that this phenomenon can occur under somewhat more natural conditions.[27]

The availability of a large identifiable cell in which a number of experimental manipulations could be performed permitted an analysis of the mechanism of the nonspecific form of heterosynaptic facilitation.[28] First, it was possible to exclude the contribution of components of the postsynaptic cell. By measuring the membrane resistance of the cell before and after facilitation, it was possible to show that facilitation occurred without a change in the passive properties of the postsynaptic cell (Figure 10A). Because facilitation could not occur if a directly initiated train was used as a priming stimulus, it was also possible to exclude a contribution of the postsynaptic spike-generating mechanism to the facilitation.

Facilitation could be demonstrated despite a blockade of postsynaptic inhibition by curare, thereby eliminating the possibility of disinhibition. The response configurations to different test stimuli were next compared before and during facilitation. In most cases, the test PSPs retained their configuration during peak facilitation, indicating an increase in the efficacy of the units initially making up the test pathway. This suggested that the facilitation might involve a presynaptic mechanism whereby the priming pathway interacted with the presynaptic terminals of the test pathway in addition to synapsing on the postsynaptic cell. This hypothesis was tested by use of an elementary, presumably monosynaptic, PSP (Figure 10B). Such an elementary PSP was facilitated by 100 per cent and for periods of 15 minutes after pairing with a priming stimulus (10C). The data are therefore consistent with a presynaptic facilitation hypothesis, although the alternative possibility of post-tetanic facilitation could not be excluded completely.[28]

Further support for the presynaptic facilitation hypothesis has recently been provided by Tauc and Epstein,[30] who showed that heterosynaptic facilitation can be demonstrated under conditions in which post-tetanic facilitation cannot be developed, such as low temperature or lithium substitution for Na^+.

Kandel and Tauc's data on heterosynaptic facilitation among the unidentified cells is more fragmentary.[27] Of 80 unidentified cells examined, only 15 cells, located in a restricted region in the right upper quadrant, showed facilitation. Controls for specificity to pairing were carried out on only three cells and for specificity to paired input on two other cells. Although all five cells showed specificity, the number of cells examined was small, and the possibility that some of the unidentified cells showed nonspecific facilitation or that transitional forms exist was not excluded. Indeed, recent studies by Von Baumgarten and Djahnparwar,[31-33] by Cohen and Pfaff (unpublished observations) and by Kupfermann and Kandel (unpublished observations) have revealed a number of cells showing nonspecific facilitation and no cells with complete specificity.

The most detailed of the recent studies of the unidentified cells is by Von Baumgarten and Djahnparwar.[31-33] About 15 per cent of the unidentified cells examined showed heterosynaptic facilitation. In no cell was the specificity complete. However, ten or twelve cells showed a partial specificity with paired presentation producing 20 to 100 per cent greater facilitation than unpaired presentation. Von Baumgarten and Djahnparwar suggest that specificity is related to the parameters of the priming stimulus and not to cell type, as Kandel and Tauc[27] had suggested. Stronger stimuli gave less specificity than weaker ones. With appropriately selected stimulus strengths, even the giant cell showed a partial specificity to pairing. Von Baumgarten and Djahnparwar also provided independent evidence for specificity to pairing.[31,32] A strong priming stimulus, when continuously presented alone for a long period of time, eventually became incapable of facilitating an unpaired test PSP. However, if the test PSP was then paired with the priming stimulus, an unusually large and transient facilitation followed the second pairing trial.

The mechanisms for the specificity in heterosynaptic facilitation in the unidentified cells is obscure. It could involve a special type of presynaptic mechanism in which the

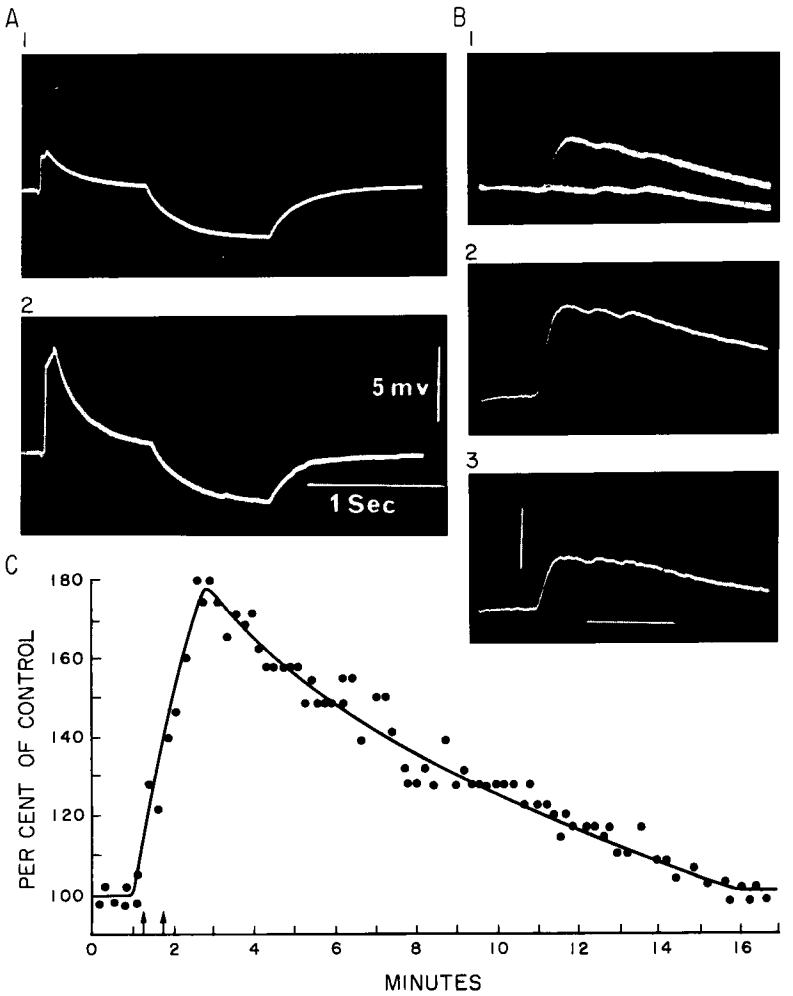


FIGURE 10 Analysis of nonspecific heterosynaptic facilitation in the giant cell. A: Conductance measurements during facilitation. The electrotonic potential used to measure changes in steady state membrane conductance is produced by a constant current pulse applied through a second microelectrode A-1 before pairing and A-2 during peak facilitation after pairing. Note increase in the PSP and absence of change in the amplitude of the electronic potential. B: Test of presynaptic facilitation hypothesis. Heterosynaptic facilitation of unitary, presumably monosynaptic, EPSP from right connective. B-1, control, superimposed sweeps illustrating all-or-none property of PSP and the presence of two late synaptic components; B-2, peak facilitation after pairing—the late components have also increased; B-3, return to control 14 min. after pairing. Voltage calibration is 2.5 mv. Time calibration, 2.5 msec. C: Graph of percentage increase in a unitary, presumably monosynaptic, test EPSP (right connective) during and after four pairing trials with a suboptimal priming stimulus to the siphon nerve (arrows). (From Kandel and Tauc, Note 28)

heterosynaptic facilitation of the test pathway is effective only if the test unit has been active just previously.[28] The data of Von Baumgarten and Djahnparwar[32] are consistent with this notion. Alternatively, one could obtain specific facilitation from a nonspecific mechanism by appropriate arrangement of connections. One of several such circuits has recently been described by Burke.[34]

Two different approaches, one based upon response decrement in a habituation analog and the other based upon nonspecific heterosynaptic facilitation in a Type I conditioning analog, have attributed the resultant prolonged changes in synaptic efficacy to alterations in the presynaptic terminals. While the evidence is not decisive in either case, there is increasingly good reason to believe that alteration in the presynaptic terminals may be important for some kinds of short-term information storage.

In addition to response decrement (homosynaptic decrement) and heterosynaptic facilitation, other changes attributable to presynaptic terminals of excitatory neurons have been studied. These are homosynaptic (post-tetanic)

facilitation[35,36] and heterosynaptic decrements (presynaptic inhibition).[35,37-39] These four possibilities are listed in the upper part of Figure 11.

Some interesting consequences derive from these considerations. First, synapses may differ by responding either with decrement or with facilitation to a given homosynaptic volley. In fact, the *same* synapse may show facilitation at one frequency of stimulation and decrement at another. Second, synaptic efficacy is determined by at least two processes. One of these is endogenous and determined by use (frequency) or by a pattern of impulses in the presynaptic fibers; this process underlies homosynaptic change in efficacy.[35] The other process is exogenous, and appears to be determined by inhibitory and facilitatory axonal synapses in the terminals. The evidence for the exogenous presynaptic influence is much less direct, but there is good indirect evidence that such synapses on synapses exist and serve as governors controlling the efficacy of synaptic transmission. Figure 11B is a schematic diagram illustrating an idealized neuron and two possible types of presynaptic governors. In reality, no single neuron has as yet been shown to have both types, but there is clear evidence for presynaptic inhibition in a number of neurons[35,37-39] and suggestive evidence for presynaptic facilitation in others.[28,30,40]

The mechanism underlying the capacity of the presynaptic terminals for information storage has not been determined. The most likely hypothesis is that it involves the availability of the chemical transmitter substance. Following tetanic stimulation, many synapses show post-tetanic facilitation, which has traditionally been attributed to a presynaptic mechanism. The exclusiveness of this facilitation to chemically operating synapses was, until recently, only inferred. An elegant demonstration of the importance of chemical transmission in synaptic plasticity has now been provided by Martin and Pilar[41] in a study of post-tetanic facilitation in the chick ciliary ganglion. The cells in this ganglion receive innervation from fibers mediating conjoint electrical and chemical transmission. In this conjoint synapse Martin and Pilar provided direct evidence that following a tetanizing volley only the chemical synapse was facilitated, the electrical synapses showed no facilitation at all (Figure 12).

Long-lasting changes in excitatory synaptic efficacy attributed to a presynaptic mechanism

1. Homosynaptic facilitation
2. Homosynaptic depression
3. Heterosynaptic facilitation
4. Heterosynaptic depression

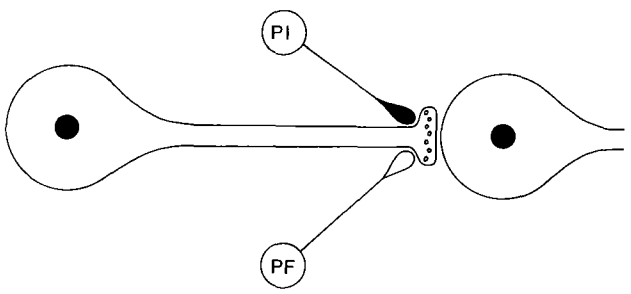


FIGURE 11 Schematic diagram illustrating possible presynaptic controls of synaptic efficacy. PI and PF denote neurons mediating presynaptic inhibition and facilitation.

This type of experiment has encouraged cellular neurophysiologists in their growing belief that the abundance of chemical over electrical transmission in the CNS may be related to the ability of chemical synapses to undergo long-term alterations in efficacy following activity, and thereby to serve as an elementary mechanism for information storage. Other advantages of chemical transmission are amplification[35,42] and a ready means for providing synaptic inhibition.[35]

Experimental approaches based upon the Type I paradigm have now also been applied to neurons of the mammalian brain by Bureš and Burešová,[43] by Adám, Adey, and Porter,[44] and by Morrell.[45] The studies by Morrell (this volume) are of particular interest because they show specificity of pairing using complex natural stimuli.

In addition to providing some biological models of Type I conditioning, studies of these analogs are useful in describing possible relationships between the paradigms. For example, the analogs of dishabituation in the spinal cord and in *Aplysia* and those of Type I nonspecific condition in *Aplysia* seem to involve similar facilitory mechanisms, a finding consonant with data from actual behavior indicating that dishabituation and nonspecific conditioning are parametrically interrelated.[18]

Type II conditioning analogs

Whereas the Type I cellular analog involves changes in the amplitude of the evoked PSP, the Type II analog involves changes in the cells' ongoing activities (Figure 3). Stated another way, the Type II paradigm is concerned with how ongoing activity can be altered by afferent stimuli, and whether this alteration is contingent upon the temporal relationship between the stimulus and the ongoing response.

A particularly important analog of Type II conditioning has recently been developed by Hoyle[46] in an extension of Horridge's work[47] on the headless insect. For a detailed discussion of Horridge's and other work on learning in insects, see Eisenstein.[48] Hoyle approached this

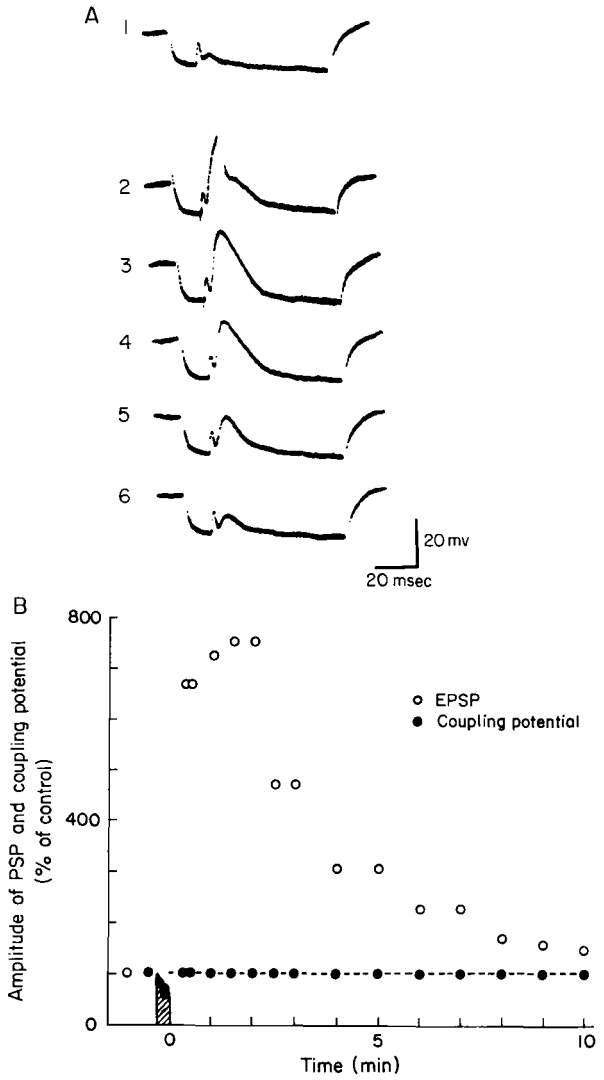


FIGURE 12 Comparison of the effect of a conditioning tetanus on amplitude of a chemically mediated EPSP and an electrical coupling potential. A 1-6 illustrate selected records from one experiment. The first rapid response is the coupling potential; the second, slower response is the chemically mediated EPSP. 1: Response recorded before tetanus. EPSP was reduced in amplitude by adding D-tubocurarine chloride to the bathing solution in a concentration of 5 g/ml. 2: Response recorded 15 sec. after a tetanus of 50/sec. was applied to the presynaptic nerve for 20 sec. 3, 4, 5, 6: Responses recorded 1, 3, 5, and 10 min. after end of tetanus. EPSP is potentiated, coupling potentials unchanged. B: Time course of post-tetanic potentiation of EPSP in ganglion cell. Measurements of EPSP (open circles) and coupling potential (filled circles) expressed as per cent of control amplitude (initial points). Hatched vertical bar shows tetanus duration. EPSP is potentiated by almost 800 per cent, coupling potential is unchanged. Tetanus frequency 50/sec. (From Martin and Pilar, Note 41)

preparation with the following hypothesis: "If following a sharp change in frequency in a motoneuron whose continuous output is associated with significant posture, a burst of sensory impulses occurs in the nerves of the limb, then the frequency of that motor discharge will undergo a prolonged alteration in the direction to counter the change."

To test this hypothesis, Hoyle obtained intracellular recordings from a leg muscle that showed spontaneous activity in the absence of external stimulation and that was involved in a leg flexion response of the sort studied by Horridge.[47] Hoyle assumed that an increase in frequency of motor fiber discharge (as indicated by increased frequency of the end-plate potentials) signaled leg withdrawal and a decrease in frequency signaled failure to withdraw. Whenever the frequency of discharge decreased below a certain minimal or "demand" level, Hoyle shocked the leg. Although Hoyle does not focus on this aspect, his approach is essentially that of developing cellular analogs of operant avoidance conditioning.

In selecting a muscle for experimental use, Hoyle found that several of those involved in leg flexion had no spontaneous background activity and therefore were not suitable for his purposes. The muscle he chose was one of the coxal adductor muscles: the metathoracic anterior adductor of the locust *S. gregoria*. The cockroach was rejected because it consistently failed to give good results with this experiment. The question of whether the metathoracic anterior adductor in the locust was involved in leg flexion could not be fully determined, but from a model system point of view this was not an essential requirement.

Hoyle found that when a demand level of spontaneous activity was set and the leg was shocked every time the average frequency (over a 10-second period) fell below that level, the shock invariably produced an increased frequency (Figure 13A). After 10 to 12 shocks, the level of firing was maintained at a higher level—some 25 per cent above the previous mean—for up to 15 minutes and longer. In some cases, the change persisted for the duration of the experiment (several hours). When the firing frequency had reached a new level and was maintained there, the demand level could be increased and would generate a further and greater (100 per cent) increase in the maintained firing frequency. Further increase in the demand level caused further increases in firing rate, until a ceiling was reached at which point further increments in the demand level gave rise to decrements in the response.

Hoyle controlled for contingency by using randomly applied shocks in some experiments (Figure 13B). These experiments did not lead to an increase in firing rate. However, when the animals were later placed on a

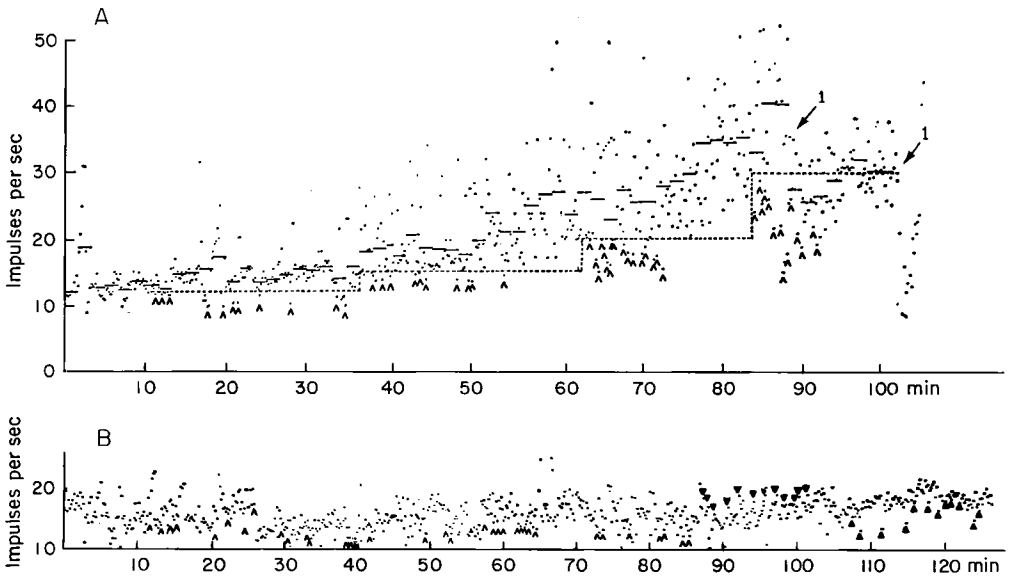


FIGURE 13 Analog of Type II conditioning in an insect preparation. A: Contingency-specific alterations in the fixing rate of an insect motoneuron. Results of an experiment in which a shock was given to the leg of *S. gregaria* each time its mean frequency over a 10-sec. period fell below a prescribed arbitrary level ("demand" level), indicated by the broken line. Moments of stimulation are indicated by ∧. The dots show the mean frequency over 10-sec. periods; the bars show the mean frequency over 100-sec. periods to illustrate the general trend. After a time the demand level was raised. Note that after a few stimuli have been received, the mean frequency rises. In this experiment major inhibitions (indicated by arrows and numeral 1) did not occur until the demand level was raised to 30/sec. B: Noncontingent stimuli not correlated with output. Stimulus times (indicated by ∧) were taken from a successful experiment of the kind given in A. They were thus not correlated with output for the test animal, and no maintained increase in frequency resulted. Subsequent stimuli selectively timed to follow rises in mean frequency (indicated by ▼) failed to depress the background and stimuli timed to follow a decline in mean frequency (▲) caused only a small rise in the background frequency. (From Hoyle, Note 46)

contingent schedule, they still failed to show a consistent rise, as would have been expected had the initial random shocks not been given. Despite the uncertain relation of the response under study to leg withdrawal, this study provides an elegant model of Type II avoidance conditioning. The value of the approach is all the more enhanced by the direct relationship of the analog to the peripheral mechanism. In this case, the analog becomes almost isomorphic with actual effector behavior.

It is of great interest to know what these stimuli are doing to the insect motor and interneurons involved in the reflex pathway. Unfortunately, the insect nervous system consists of small cells, and it has not yet been possible to study the cellular alterations that underlie changes in the firing patterns of the motoneurons.

Since the Type II paradigm involves changes in ongoing activity, one way to develop a cellular analog is to examine the effects of noncontingent and contingent nerve stimuli on the spontaneous activity of pacemaker cells. This approach has recently been explored in *Aplysia* by Frazier, Waziri, Pinsker, and Kandel.[49-51] The experiments were carried out on one of the identified cells in the abdominal ganglion (cell L3), which for long control periods produces a fairly regular rhythm consisting of alternating bursts of spikes and quiet periods. Thus two output behaviors of the cell—bursts and quiet periods—could be examined and nerve stimulation could be made contingent on one or the other.

An analysis of the cell's rhythm[51] showed that the burst generation was an endogenous process resulting from an unstable membrane potential similar to that which exists in pacemaker cells of the heart,[52] which are believed to have a high resting Na^+ conductance. The quiet period between bursts was caused by a hyperpolarizing afterpotential following the burst of spikes, and its duration was a function of the number of spikes per burst. The decay of the afterpotential, or pacemaker potential, represents a time- and voltage-dependent decrease of a high conductance (presumably to K^+) turned on by the burst.

This endogenous rhythm was readily modified by nerve stimulation.[49] A single strong train of nerve stimuli produced changes in the bursting rhythm lasting up to 20 minutes, while six to eight repeated stimuli (one per two minutes) produced greater effects (Figures 14 and 15). These effects were independent of the position of the stimulus in the burst cycle and were therefore noncontingent. These noncontingent effects parallel those seen with some behavioral forms of the Type II paradigm. Indeed, prolonged alterations in the firing rhythm of a spontaneous active cell are readily produced, and their occurrence in inhibitory neurons might account for IPSP recruitment discussed under the analogs of habituation.

In exploring the effects of different stimulus parameters on the bursting rhythm of this cell, Frazier, Waziri, and Kandel[49,50] found it possible to reinforce selectively either bursts or quiet periods on a contingency basis and to produce opposite effects with the same stimulus. When the stimulus was placed during the onset of the burst, it produced a reduction in the burst-onset interval and more burst per unit time. The same stimulus placed late in the burst or during the silent period produced a prolongation of the burst-onset interval and fewer bursts per unit time (Figure 16A and B). The increase in number of bursts per unit time with contingent burst onset stimulation was due to a reduction, by the stimulus, in the number of spikes per burst resulting in a briefer post burst hyperpolarization and a shorter burst-onset interval (Fig. 16A). The decrease in the number of bursts per unit time, with the stimulus placed at the end of the burst or during the quiet period, was due to the lengthening of the quiet period by a direct, as yet unspecified effect of the stimulus on the developing pacemaker potential.

In early experiments stimuli were applied to the connectives, so the reinforcing stimulus for contingency specific affects were complex. Although the best stimulus consisted of a train of EPSPs, these were often followed by a small hyperpolarizing synaptic component.

To simplify the reinforcing stimulus, Pinsker and Kandel[53] have recently used an elementary, monosynaptic IPSP produced by intracellular stimulation of an identified interneuron. With this elementary input it was also possible to produce contingent-specific effects. These effects were larger and more consistent than with nerve stimulation.

In experiments such as the ones illustrated in Figure 16B (using short periods of stimulation), the burst onset interval returned to control values almost immediately after the withdrawal of the stimulus. Longer periods of contingent stimulation with both the complex and the elementary PSPs are often followed by residual effects lasting from one to fifteen minutes (Figure 16A).

These results indicate that in the isolated ganglion of *Aplysia,* as in the insect ganglia, an operant conditioning stimulus sequence is capable of producing both noncontingent and contingent-specific changes in the frequency of the spontaneous activity of single neurons, and that some of these changes may persist for some minutes after the termination of the stimulus. How this occurs is still not clear. The first factor that needs to be investigated is whether this represents a change in the endogenous rhythm of the cell, or a change in a tonic synaptic modulating drive on these cells. In preliminary experiments, Pinsker and Kandel[53] have not seen changes in a tonic modulating drive. That some persistence can be obtained with a single interneuron also argues in favor of a change in the cell's exogenous rhythm. Although the evidence on this point is incomplete, these experiments raise the possibility that cells having endogenous rhythm may possess intraneuronal mechanisms for sustaining a plastic change in their firing pattern. A suggestion similar to this has previously been made by Strumwasser on the basis of a different set of experiments.[54,55] Therefore, the interesting possibility exists of a second mechanism of short-term information storage—that of an alteration in the endogenous rhythm of a pacemaker cell. The intriguing aspect of this possibility is that although the alterations are mediated by synaptic transmission, the ultimate site for information storage is not the synapse but the postsynaptic cell.

Cellular studies of use and disuse

Other approaches to the problem of neural plasticity, not based upon actual learning or analogs of learning, have also been taken. In particular, a number of studies have examined the trophic influences, mediated from nerve to muscle during development and regeneration, which determine the extent of the chemosensitive area in the muscle and the contractile property of the innervated muscles (for reviews, see Kravitz, this volume, and Notes 33 and 64). Although these studies may not appear immediately relevant to learning, they are of great importance for understanding long-term synaptic function. The results from these studies indicate that neurons can influence each other through their synapses in a number of relatively permanent ways besides the immediate effects of synaptic transmission. The study of trophic interactions of neurons therefore offers important clues as to how relatively permanent types of information are stored. As the most interesting forms of learning involve long periods of retention, studies of trophic interactions are likely to prove highly useful models for the study of long-term information storage.

An instructive transitional case between long-term

trophic influence manifest during development and regeneration and the short-term phenomena considered under the neural concomitants and analogs of learning is the effect of prolonged physiological use and disuse of synapses in the CNS. A particularly interesting variant of a use and disuse experiment has recently been applied by Wiesel and Hubel[56-58] in an imaginative series of studies on the effects of unilateral and bilateral lid closure and artifical squint in new-born kittens on unit responses in the striate cortex. Wiesel and Hubel showed that if a kitten is raised from birth with one eye sutured shut, recordings made in units of the striate cortex at three months show loss of binocular interaction. Only a very few cells respond to stimuli applied to the deprived eye.[56] From these results one would expect that, in new-born animals with bilateral lid closure, most cells in the

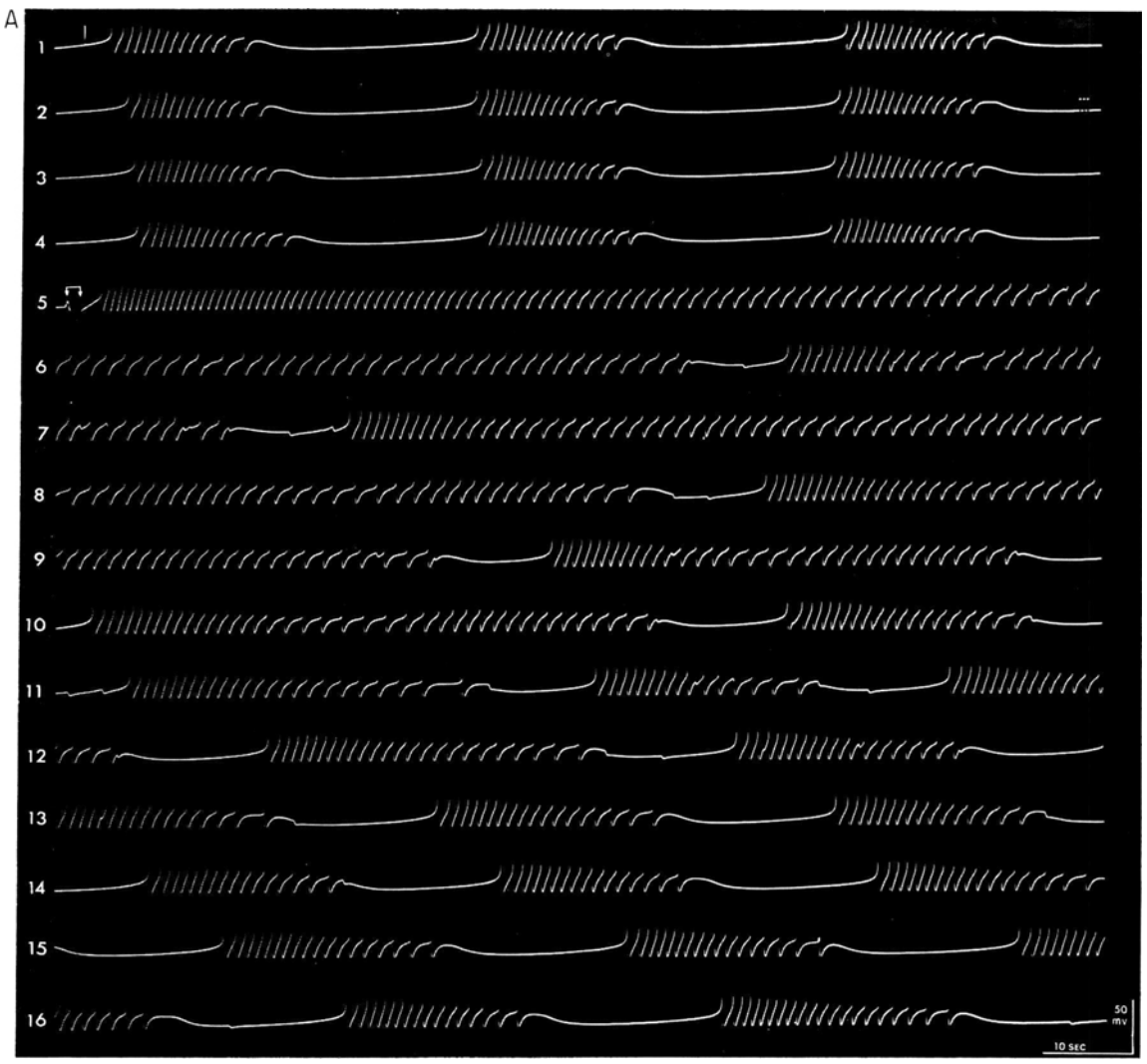
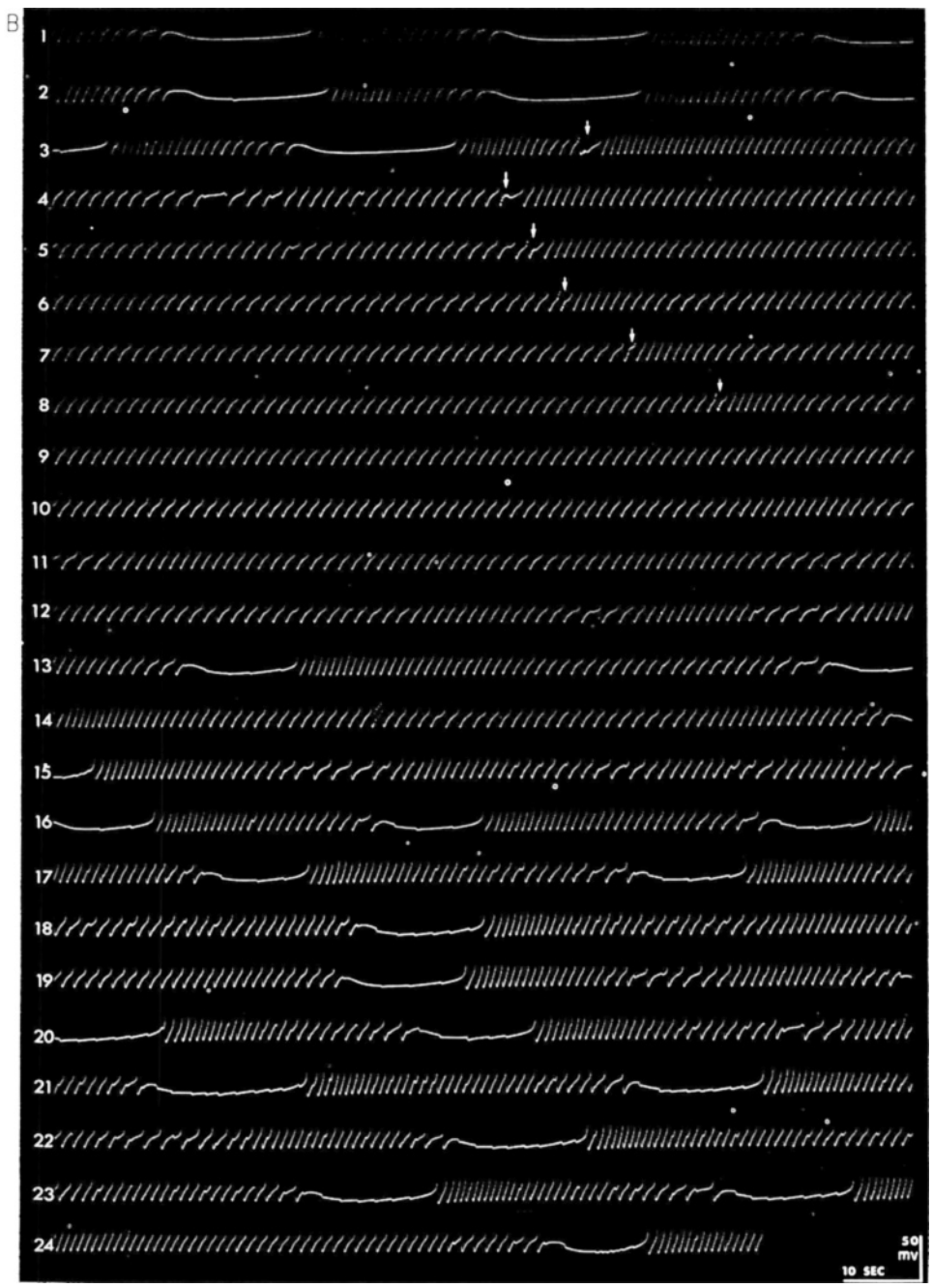


FIGURE 14 Noncontingent (Type II) effects of *single* and *repeated* stimulus trains on burst rhythm of an identified bursting cell (L3) in *Aplysia*. A: Effect of a single strong noncontingent stimulus (6/sec. train of 1 sec. duration) to the left connective. Lines 1–4 are the last part of the control period. The stimulus is indicated by the arrows at the beginning of line 5. Note the prolonged time for return to normal burst cycle (lines 5–16). B (*facing page*): Effect of a *repeated* strong noncontingent stimulus identical to that used in A. The control period (lines 1–2) followed soon after that indicated in A, line 16. Six stimuli (6/sec. train of 1 sec. duration) to the left connective were applied, 1 per 2 min. (lines 3–8). Burst onset interval did not return to control during 30 minutes following the last stimulus (lines 9–24). Each line in A and B is a two-minute period. (From Frazier, et al., Note 49)

striate cortex would be unresponsive to stimulation from either eye. Surprisingly, this proved not to be the case.[56] Cells responsive to binocular stimulation were found in most penetrations. Over half the cells from which records were made appeared normal, but many responsed either abnormally or not at all. Both unilateral and bilateral lid closure of animals showed similar atrophic histological changes in the appropriate layers of the lateral geniculate. The cells in layers receiving input from the closed eye decreased in cross-section area by about 30 to 40 per cent. When the lid of the deprived eye was opened and vision examined, the deprived eye proved blind, although the pupillary response remained normal. Even when assessed 3 to 15 months after lid opening, animals that had lid closure from birth to three months of age had only a very slight functional recovery.[57]



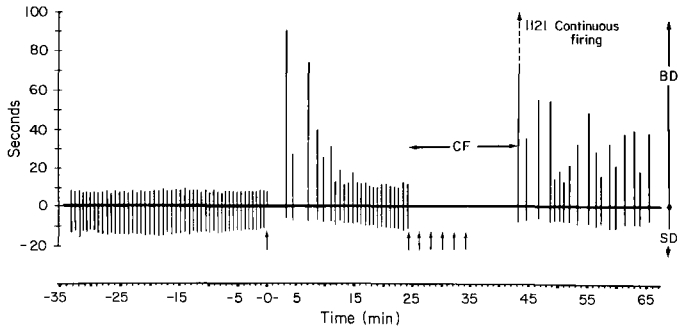


FIGURE 15 Graph of data illustrated in Fig. 14. The duration of each burst cycle is plotted along the ordinate as a single line. The burst duration (BD) is the part of the line above the horizontal baseline, and the silent period duration (SD), the part of the line below the baseline. CF indicates a period of continuous firing of 18 min. 41 sec., 10 minutes of which occurred during the period of stimulation and 8 min. 31 sec. of which followed the last stimulus. This is indicated by the line above the baseline that goes off the ordinate (1121). (From Frazier, et al., Note 49)

Kittens with artificial squint (produced by cutting the right medial rectus at the time of normal eye opening) showed no behavioral defects.[58] Cells in the striate cortex responded normally, but there was a marked decrease in the proportion of binocularly driven cells. Instead of a normal value of 90 per cent, only 20 per cent could be influenced from the two eyes. There was no obvious histological change in the lateral geniculate. A similar result was obtained when an opaque contact occluder was placed on each eye on alternate days (Figure 17). These experiments on bilateral lid closure and on an artificial squint are particularly instructive because they suggest that the integrity of certain striate cortical pathways may be dependent on the normal, presumably synchronous, interrelationship between activity in at least two and perhaps several converging pathways.[57] The studies also illustrate the possibility of producing radical and permanent changes in synaptic efficacy by alteration in the convergence of inputs along several pathways. Although it is only possible to produce these alterations in newborn animals, the interesting possibility exists that qualitatively similar although quantitatively more subtle changes in synaptic efficacy might also occur in adult animals following prolonged periods of altered usage.

At present there is little understanding of the manipulations that produce permanent changes in neuronal function in adult animals and of the mechanisms that underlie them. Do these permanent processes simply involve extensions of the types of mechanisms for short-term change (e.g., synaptic efficacy and pacemaker alteration) discussed above, or do permanent changes utilize entirely new processes? As yet there are no answers to these questions, but some clues are emerging from a number of different experiments.

An important beginning was made by Spencer and Wigdor in acute experiments investigating the effects of prolonged use on the monosynaptic reflex.[36] They wanted to know whether tetanization at frequencies high enough to produce significant post-tetanic facilitation (100–500/second) might produce permanent increments in the monosynaptic reflex if the tetanus was maintained for sufficiently long periods of time. By tetanizing for 15 to 30 minutes they prolonged post-tetanic facilitation so that it persisted for 1 to 2 hours. But even with longer periods of stimulation (up to one hour) the response always returned to control values (Figure 18A).

In a parallel study on the effects of tetanization on the monosynaptic reflex in the cat spinal cord, Beswick and Conroy[59] found, in agreement with Spencer and Wigdor, that long periods of tetanization produced post-tetanic facilitation lasting over an hour. In addition they found that *intermittent* trains of stimuli were more effective than *continuous* tetanization, suggesting that pattern may be more important than usage per se. Most interesting was their finding that even when the monosynaptic reflex had returned to control value, following a prolonged period of tetanization, a concealed residual effect was still present and became manifest when tetanization was attempted a second time. With the second tetanus the facilitation appeared earlier, rose more quickly, and reached a maximum in a shorter period of time than when it was first given. This residual effect could be demonstrated as long as 1½ hours after the reflex response had, by all other criteria, returned to control (Figure 18B).

These results raised the interesting possibility that some phenomena that might be involved in short-term information storage are capable of leaving residual effects not manifest in the transient response. Even after transient response has returned to control level, a pathway may remain altered, in some subtle way, for a considerably longer period of time.

Summary and discussion

I have considered several approaches to the cellular studies of learning. Since none of these involve a direct analysis of the cellular concomitants of learning, they all are rather incomplete and somewhat unsatisfactory ap-

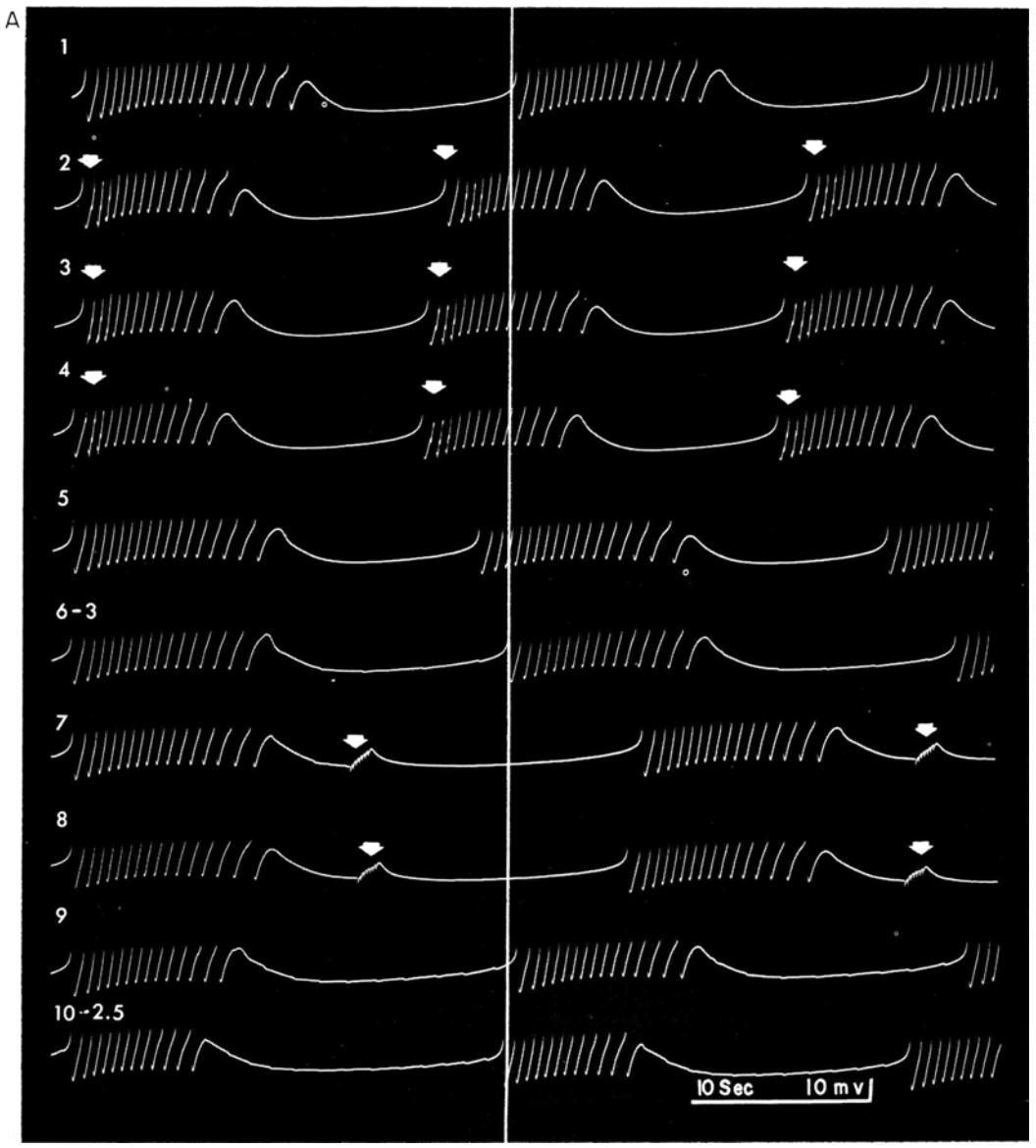


FIGURE 16 Analogs of Type II conditioning in *Aplysia*. Contingency-specific effects in identifiable cell (L3). The reinforcing stimulus is indicated by arrows. With two exceptions, a 3 and 2.5 minute interruption before lines 6 and 10 respectively, these are continuous data with sections at the ends of some lines omitted in order to line up the first burst in each line. This permits comparison of burst onset intervals before, during, and after contingent stimulation. The time from the first spike of the first burst in line 1 to the vertical line indicates the control burst onset interval. A: Line 1 is the last part of the control period. The same reinforcing stimulus (a weak 6/sec. train of 1 sec. duration) is first applied at the beginning of the burst (lines 2, 3, and 4), and later during the silent period (lines 7 and 8). Note opposite effects produced by the two types of contingencies.

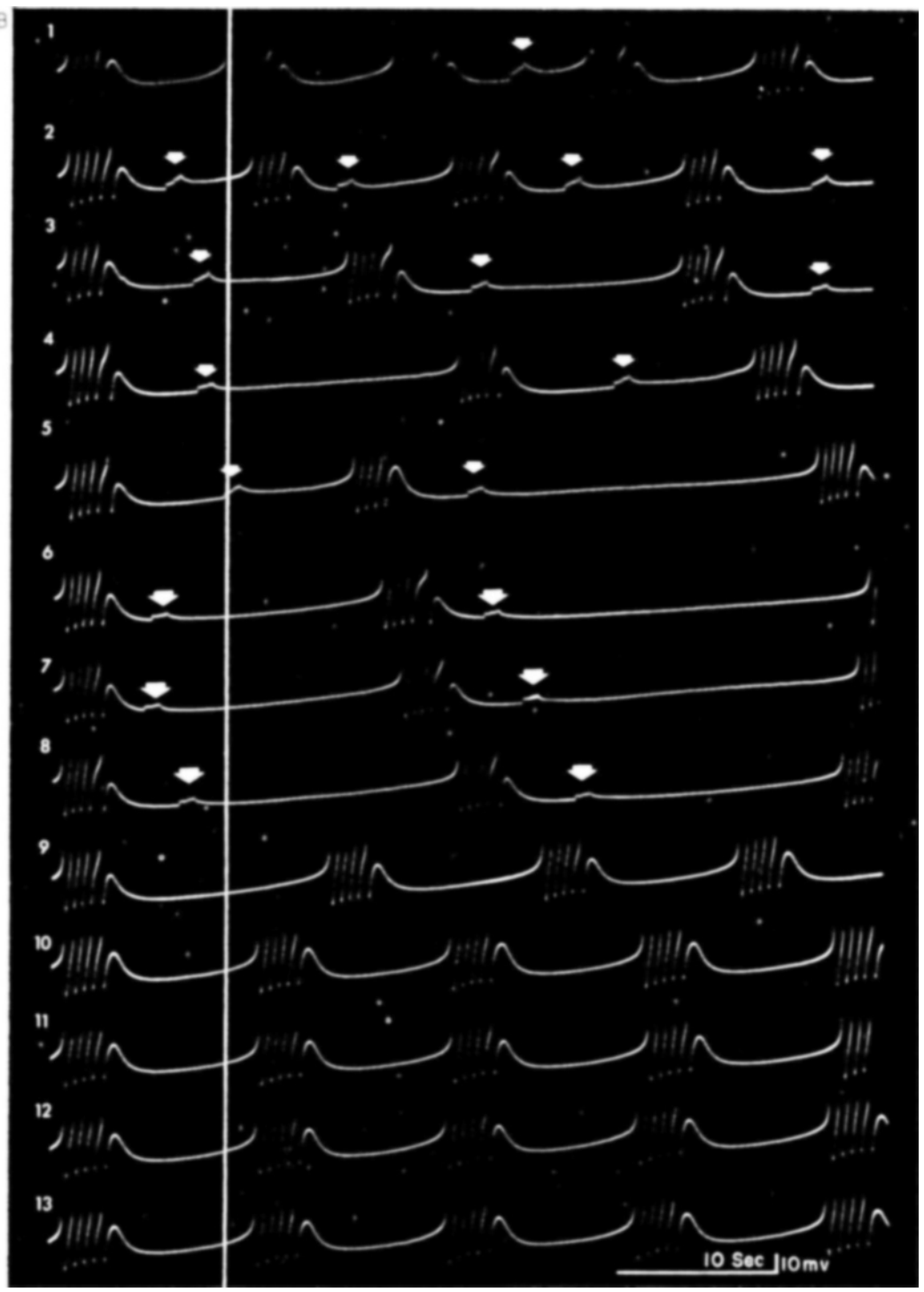


FIGURE 16B Residual effect following contingent stimulation of the silent period. There is a gradual build-up of response. Following termination of stimulus the cell assumed a burst onset interval of 11.8 sec., compared to the control value of 10 sec. (From Frazier, et al., Note 49)

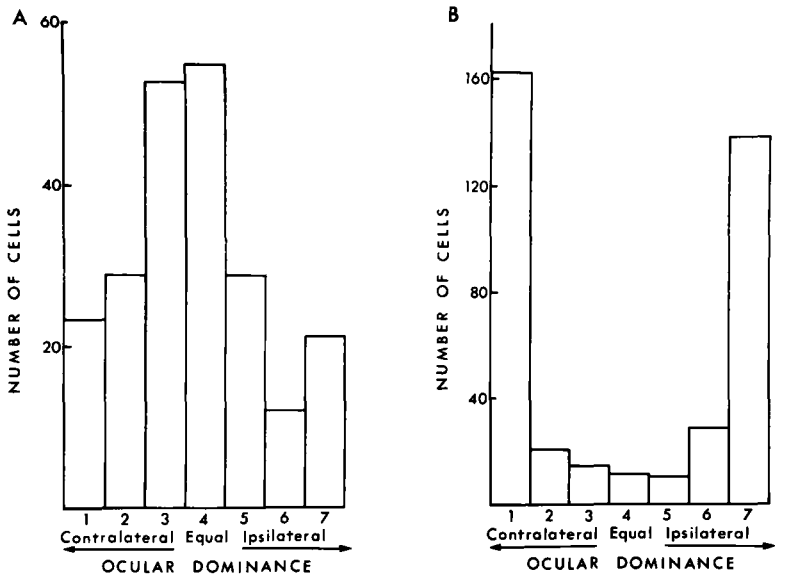


FIGURE 17 Altered pattern of synaptic usage in the cat visual cortex. Comparison on ocular dominance in normal animal and animal with artifical squint. A: Ocular dominance histogram of 223 cells recorded from a series of normal cats. Cells of group 1 were driven by the contralateral eye, for cells of group 2 there was a marked dominance of the contralateral eye, for group 3, a slight dominance. For cells in group 4 there was no obvious difference between the two eyes. In group 5 the ipsilateral eye dominated slightly, in group 6 markedly. In group 7 the cells were driven only by the ipsilateral eye. B: Ocular dominance of 384 cells recorded from four strabismus experiments. (From Wiesel and Hubel, Note 57)

proaches. However, each approach has its own advantages, and to some degree the several approaches complement one another and permit one to gain a wider perspective on this complex problem.

The main disadvantage of studying the cellular concomitants of learning is that the relationship of the cellular response to the conditioned behavioral act is undefined. In addition, these studies have used extracellular recordings and complex inputs, and have therefore not lent themselves to an analysis of the cellular mechanism. Such studies do, however, have the great advantage of being carried out under natural conditions and promise to be useful electroanatomical adjuncts in studying the pathways involved in a particular conditioning procedure.

The deficiency in the analog approach is primarily the artificial nature of the electrical stimuli and responses employed. As a result, the relationship of these cellular response patterns in isolated preparation to conditioning in the intact animal is as yet undetermined. Consequently, the proposed isomorphism between these cellular analogs and behavioral conditioning in the intact animal should not be taken too literally. Effector conditioning involves a change in the output of a population of cells, and with current techniques it is still difficult to predict the aggregate output from the behavior of some members of the population. It seems safer to look at these models primarily from a neurophysiological point of view. Here they represent attempts to produce long-lasting change in the behavior of single nerve cells by applying higher order stimulus sequences of the sort used in conditioning experiments.

Moreover, it is important, even in the development of analogs, to separate conditioning, as parametrically defined, from the conditioning that is both parametrically defined and intuitively interesting. While most parametric definitions demand specificity (to pairing or to contingency), none specify time course. Yet the factor of time separates, in large part, the important aspects of learning from the trivial ones. Insofar as the phenomena so far encountered in these isolated preparations generally endure at most one to two hours, they must be considered as fairly trivial analogs.

Despite these reservations, the cellular analog approach represents a type of "biological model building" that seems to have considerable promise. First, this approach has shown that the stimulus sequences used in learning

paradigms can be highly effective in producing relatively long-term alterations in neuronal activity. These paradigms are therefore likely to continue exerting a liberating influence on the selection of stimulus patterns available to the neurophysiologists. Second, analogs can be developed in relatively simple preparations that can be approached with the analytic technique of cellular neurophysiology. Consequently, cellular analogs and other cellular studies of neuronal plasticity have been useful in specifying a number of cellular mechanisms capable of undergoing prolonged alteration in function as a result of synaptic input.

Whereas the cellular analogs have primarily provided possible mechanisms for short-term information storage, cellular studies on nerve cells in response to regenerative change indicate that trophic substances carried from one neuron to another via the synapse can produce long and even relatively permanent alterations in neuronal function. The data of Hubel and Wiesel[58] on the requirement of synchrony for the maintenance of normal function of two or more independent pathways also suggest some trophic interaction. Needless to say, a great deal is yet to be learned about changes lasting days or weeks, but it may develop that in normal function, as in ontogenetic and regenerative processes, neurons can influence one another through their connections in a number of ways beside the

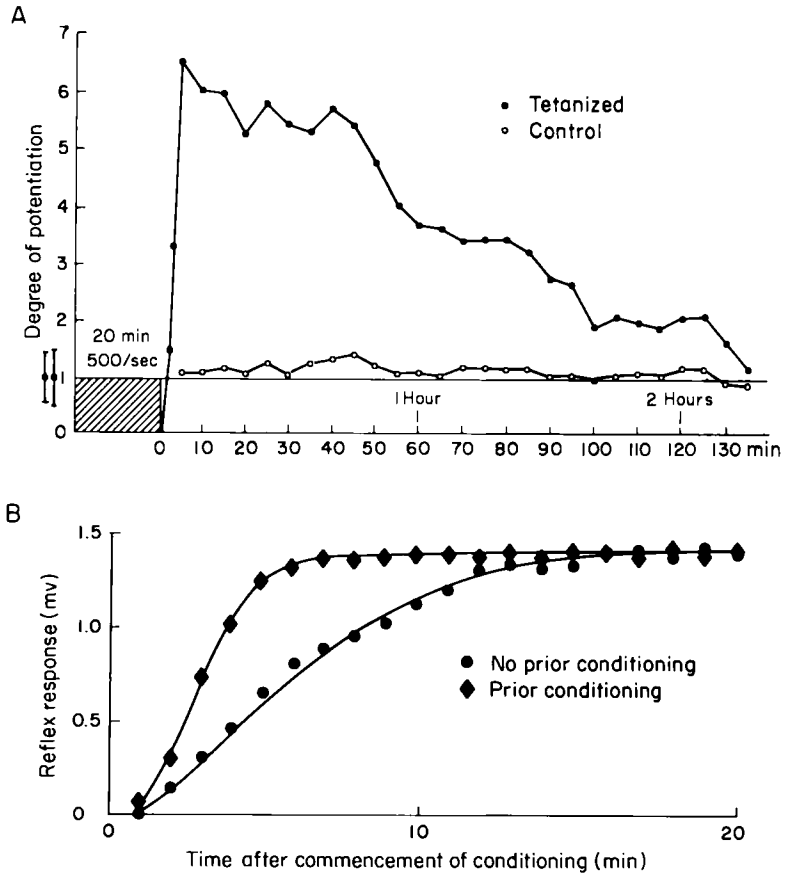


FIGURE 18 A: Prolonged post-tetanic potentiation following 20 min. tetanus. The stimulus was applied to the tibial nerve; recordings were obtained from the L_7 ventral root. Filled circles, left (test) side. Open circles, right (control) side, which was treated exactly the same as test side except that the tetanus was omitted. Each plotted point represents an average of 10–20 individual responses. Each test stimulus was delivered at 10-sec. interval. The degree of potentiation is indicated on the ordinate; time in minutes on the abscissa. (From Spencer, in preparation)

B: Concealed residual potential. Increase in size of monosynaptic reflex response in absence of prior conditioning (●). Conditioning was then discontinued at 20 min.; 30 min. after testing response reached zero, conditioning was recommenced—and reflex response plotted (♦). Responses from each minute averaged: stimulus applied to medial gastrocnemius; recording from lateral gastrocnemius-soleus. (From Beswick and Conroy, Note 59)

usual type of synaptic transmission. A possible beginning is the residual efficacy in a pathway following subsidence of facilitation.[59]

Based on cellular studies, it is possible to consider four categories of neural phenomena that are likely to be involved in information storage (Figure 19). Three of these have been discussed: changes in synaptic efficacy lasting about an hour; changes in pacemaker activity; and more permanent trophic influences. Also listed, although they have not been considered here, are possible changes in the spike-generating capacity of the neuron. For example, dendritic trigger zones of the sort that normally exist in hippocampal neurons become evident in motoneurons when they undergo chromatolysis.[60,61] Conversely, cortical neurons in neonatal kittens have dendritic trigger zones and these become suppressed with maturation (Purpura, this volume). Thus the number of trigger zones that a neuron has may not be fixed. Whereas chromatolytic and maturational changes are radical, there is evidence in motoneurons that the immediate extent of the excitable dendritic membrane may be dependent upon ongoing synaptic input.[62] As a result, Nelson and Frank[62] have suggested that the dendritic extent of the spike-generating membrane in motoneuron might change in a relatively permanent way following appropriate patterning of input, and serve to enhance the effectiveness of remote synapses.

I. *Changes in synaptic efficiency directly related to synaptic transmission*
(presynaptic)
   1. homosynaptic ⟨ EPSPs / IPSPs
   2. heterosynaptic ⟨ EPSPs / IPSPs (?)

II. *Synaptic modulation of pacemaker cells endogenous rhythm*
(postsynaptic)
   1. increments and decrements in pacemaker activity
   2. turning on and turning off of pacemaker activity

III. *Changes in the spike generating capacity of a neuron*
(postsynaptic)
   1. change in the predominance of a remote trigger zone
   2. changes in the extent of the excitable dendritic membrane

IV. *Changes in the properties of the postsynaptic cell mediated by trophic substances*
(postsynaptic)
   1. spatial extent of the chemosensitive area
   2. functional properties of the post-synaptic cell

FIGURE 19 Possible mechanisms for information storage in the CNS.

When the data now emerging from cellular studies of learning are examined in the context of recent work in genetic neurology (especially that of Sperry[63] and of Wiesel and Hubel[56-58]) they lead most cellular neurophysiologists to the following tentative hypothesis. Most connections in the brain develop in a highly specific manner under genetic control. Experiential factors, and specifically learning, produce their effects not by specifying new connections but by increasing and decreasing the functional efficacy of previously existing connections. Changes in functional efficacy can be produced in a number of ways (Figure 19) and may involve synaptic and nonsynaptic processes. The evidence is clearly limited, but what evidence does exist argues for a selective rather than an instructive mechanism in learning.

Electrophysiological Studies of Conditioning

E. R. JOHN

UNDERSTANDING THE MODIFICATION OF BEHAVIOR by experience requires knowledge of how information is stored in the brain and retrieved to interact with subsequent input to modify response. Experimental studies of the brain mechanisms of learning and memory have long utilized the conditioned response as a simple example for analysis. In the *classical* conditioned response paradigm, a sensory input, which is called the *conditioned stimulus* (CS), elicits a new efferent output, which is called the *conditioned response* (CR), as a result of systematic pairing of that CS with some other stimulus; this is referred to as the *unconditioned stimulus* (UCS), which causes that efferent output as an innate or *unconditioned response* (UR). In *instrumental* conditioning, an animal's performance of some activity selected by the experimenter is systematically *reinforced* by presentation of a positive reward, such as food or water, or by termination of a noxious stimulus, such as shock. This systematic alteration of the animal's environment as a result of performance of the stipulated act leads to a marked increase in its frequency of occurrence. Performance of this conditioned behavior can be brought under *stimulus control* by utilizing the presence or absence of a conditioned stimulus to signal a period in which this behavior will result in reinforcement not otherwise available.

Thus, classical CRs and stimulus-controlled, instrumental CRs have in common the feature that, after a learning experience, some specific input to the nervous system elicits an output that did not occur previously. Therefore, it seemed plausible to many workers that learning must consist of the establishment or facilitation of a pathway from the region of the nervous system excited by the sensory input to the region responsible for control over the efferent outflow. The residual effect of an experience—*memory*—consisted of the preservation of the newly built pathway, or increased synaptic efficiency. From this viewpoint, the subsequent modification of response—*remembering*—was accomplished by the firing of the nerve cells which comprised the connection that had been formed between the input and output regions.

Although the details of the cellular neurochemical and neurophysiological mechanisms currently postulated to account for the establishment and preservation of these hypothetical pathways have changed somewhat during the last thirty years, these fundamental assumptions have long characterized the prevailing theories of learning. Such theories might be defined as *connectionistic,* in that they assume that the essential feature involved in learning is establishment of a new functional pathway between brain regions, and *deterministic,* in that the discharge of a particular cell or set of cells is viewed as necessary and sufficient for a learned performance or remembering to occur.

It is not within the scope of this paper to summarize the arguments against connectionistic, deterministic theories of learning. Some of the shortcomings of such theories were pointed out long ago by workers such as Lashley[1] and have recently been reformulated.[2] Suffice it to say here that both theoretical and experimental reasons have led a number of workers to propose alternative statistical formulations, in which neural networks are considered to have the capacity to generate processes that are functionally equivalent although they differ with respect to the actual cells used. The brain can be viewed as a statistically organized system producing certain classes of lawful and invariant behaviors. Current examples of such theories have been summarized by Rosenblatt[3] and by Moore, et al.,[4] and are additionally to be found in a number of recent symposiums.[5,6] In these theories, the informational specificity of neuronal activity is assumed to reside in the statistical properties of the spatio-temporal configuration of firing in the neuronal population, rather than in the discharge of any specific cell or set of cells within that population.

The attempt to resolve the contradictions between deterministic and statistical theories of memory would seem to be of the highest priority for the formulation of future strategies of neurochemical and neurophysiological research in this field. It seems most likely that both sorts of mechanisms are involved in learning. Electrophysiological studies of learning are particularly relevant, because these techniques provide intimate insight into the detailed processes of nerve cells and populations that underlie and presumably mediate the behavioral performance. This chapter will not attempt a comprehensive review of the vast number of publications in the field, but will focus on some salient features of the processes that have been observed.

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Detailed surveys of this research area have been provided by John[7] and by Morrell.[8]

Changes in level of synchrony

Alterations in levels of synchrony or the characteristics of intrinsic brain rhythms have been observed by numerous workers during conditioning. When initial reinforcement of a CS takes place, widespread changes from slow rhythmic, high voltage, alpha activity to low voltage, high frequency potentials appear in the surface electrocorticogram. As training proceeds and the CR becomes fully established, this activation, or desynchronization, pattern becomes limited to only a few circumscribed regions of cortex. The widespread irradiation of desynchronization early in learning was adduced as evidence of involvement of the mesencephalic reticular formation, while the consolidation, or localized desynchronization, later in learning was interpreted by some to indicate a shift to dominance by the thalamic reticular formation. Studies of the habituation of the so-called "arousal" response led to concepts of phasic thalamic accommodation to repeated inconsequential events, followed by tonic mesencephalic adaptation resulting in the gradual diminution and disappearance of desynchronization.

Changes in theta rhythm

A slow, synchronous, hippocampal rhythm appears in response to ambiguous stimuli early in conditioning, and is often associated with the orienting response. This "theta" activity disappears in later stages of training, when the stimuli acquire an unambiguous meaning. Studies of changes in hippocampal response have led to the suggestion that replacement of the dominance of the mesencephalic reticular formation by the thalamic reticular system may be caused by hippocampal inhibition of the mesencephalic reticular formation. Analysis of theta rhythms displayed by animals at choice points has revealed a change in phase relationship between the entorhinal cortex, hippocampus, and the dentate gyrus—a change apparently related to the appropriateness of the behavior.[9] These findings were interpreted as evidence for a phase comparator mechanism, in which phase relationship in these structures is dependent upon the similarity between present input and previous experience.

Steady potentials

A body of evidence suggests relationships between steady potentials and the neural activity involved in learning, and has been reviewed elsewhere.[2] Many observations indicate that local direct current polarization of an anatomical region may strikingly alter its responsiveness to previously ineffective stimuli, apparently facilitating the establishment of new functional relationships. Positive shifts in local potential tend to facilitate, while negative shifts seem to impair acquisition of conditioned behavioral or electrical responses. Imposition of local DC shifts can disrupt performance of previously established conditioned responses. Shunting effects have been demonstrated in which a conditioned stimulus, previously capable of eliciting a particular CR, elicits a new response, rather than the conditioned response, during polarization. Local DC shifts have been conditioned by pairing with sensory stimuli. DC shifts in various brain regions have been observed after sensory receptors or particular neural structures have been stimulated. Furthermore, changes in the DC effects caused by the CS have been observed during conditioning. Considerable interest has recently been aroused by the finding that in man the expectation of a meaningful event is attended by a marked DC shift recordable from certain regions of the head.[10]

Some of these various effects are probably not caused by unspecific sensitization, as they do not appear when "pseudoconditioning" controls are provided, and differential responses have been obtained.[11] Thus, the application of appropriate DC shifts to certain brain regions seems to facilitate the establishment of new responses, while the acquisition of new responses seems to be accompanied by the appearance of DC shifts in certain brain regions. Perhaps the facilitating effects of polarization are the result of the establishment of an organized set of neurons capable of coherent discharge, while the new DC shifts, observed in response to the CS after conditioning, reflect the coherent discharge of an organized set of neurons.

Although experiments of the sort discussed have provided a large and interesting volume of data suggesting a number of general conclusions, it has been difficult to interpret such findings in terms of detailed underlying neural mechanisms. Additional electrophysiological insights into these processes have been forthcoming primarily from two other types of experimentation.

Single unit studies

The first method is the study of changes in the responses of single nerve cells to conditioned stimuli during learning. An excellent review of single unit studies can be found in Dr. Kandel's chapter in this volume, and striking additional examples have been presented by Morrell, Strumwasser, and Adey. We will here summarize some features of findings from single unit studies that seem relevant to the consideration of deterministic and statistical processes.

Single nerve cells in the central nervous system are spontaneously active. Such cells respond to presentation of a variety of stimuli with a change in firing pattern. Burns and Smith have concluded that most cortical cells display a detectable change in firing pattern in response to almost every stimulus, whether it is delivered peripherally or centrally.[12] Different stimuli elicit different patterns of discharge, but the details of cellular response to the same stimulus also often vary among repeated presentations.

Yet, although the responses of a single neuron to a specific stimulus display significant variability, averaged responses to repeated stimulation display fairly good reproducibility. As the monitored cells can be assumed to be representative of some proportion of the population, Burns and Smith have proposed that certain kinds of information may be represented in the nervous system by the average response of an ensemble of cells, rather than by the output of any individual neuron.

The unit studies reported here earlier show clearly that the response patterns of single neurons change during conditioning. These results provide an exciting and important insight into the nature of neuronal changes related to learning. Such changes must take place, whether the contribution of the unitary neural event is of informational significance in a deterministic or in a statistical fashion, and we can expect fundamental information about relevant cellular mechanisms from further single unit analysis.

Many cells respond to stimuli in several modalities, although some respond only to one sensory class. The response patterns of the "polyvalent" cells to the CS can be changed readily by conditioning procedures, while the responses of "monovalent" neurons are resistant to change.[13] The changed response patterns can be seen readily by use of such statistical techniques as averaged poststimulus frequency histograms, although they may be difficult to discern in any single example.

Perhaps particular interest attaches to the observation that after conditioning the response pattern of polyvalent neurons often resembles the algebraic summation of the response to the CS and UCS alone before conditioning. Such findings suggest that the neural network can reproduce the temporal pattern of activity that occurred during a particular sensory experience when, subsequently, stimulation by only a part of the stimulus complex occurs.

It may be worthwhile to raise some questions about the implications of these unit studies. Yoshii and Ogura[13] have reported that about 30 per cent of so-called polyvalent neurons, or about 10 per cent of all units studied, displayed changes in response during conditioning. This figure agrees with the 12 per cent modifiability reported by Morrell. The ease with which changes in unit response can be detected in learning suggests that a high proportion of neurons is affected during any learning experience. This indicates that any particular cell may be altered by a wide variety of events, and may therefore be involved in representation of multiple past experiences. Because the firing of a neuron may be caused by a variety of stimuli involved in a multiplicity of past experiences, the ambiguity of the information in mere neuronal discharge is very large.

Further, the cells that fire after conditioning also fired before conditioning. How is novelty distinguished from familiarity in the discharge of an active pathway? As shown clearly by the data presented by Morrell, it is the *pattern* of firing during a time interval which changes (See also Eisenstein, this volume). Such observations seem difficult to reconcile with concepts of learning based upon the establishment of a new pathway. Those same data show that the responses of a neuron to successive presentations of a conditioned stimulus display appreciable variability. This variability raises the question whether the firing pattern of a single neuron is unique and stable enough to provide unambiguous information to the nervous system. These various considerations, together with the results of ablation studies (Chow, this volume), indicate some of the reasons for suggesting that the informational content of a neuronal ensemble may be specified by the statistical properties of the aggregate activity, rather than by the discharge of any selected subset of cells.

Tracer technique

The major data considered in the remainder of this chapter come from studies in which a so-called tracer technique was used to enhance the signal-to-noise ratio in the electrophysiological activity recorded from performing animals. In this method, some intermittent stimulus with a characteristic rate of repetition is defined as the *tracer conditioned stimulus* (TCS). Electrical activity recorded from chronically implanted electrodes is analyzed for rhythmic responses at the frequency of the TCS. These frequency-specific events are called *labeled responses*. The appearance of labeled response in a structure is interpreted as sufficient but not necessary evidence that the activity of the structure is influenced by the presentation of the TCS.

When tracer technique is applied in electrophysiological studies of conditioning, complex and wide-spread changes can be observed in the amplitude and distribution of labeled responses. Labeled responses disappear from some structures where they are initially present, change in form or amplitude in some other structures, and appear in certain regions where they are seldom observed in the naive animal. The details of this configuration change somewhat in different training situations and from task to

task. Labeled responses increase in amplitude and distribution during simple conditioning, and diminish markedly as the new behavior becomes fully acquired, especially with overtraining. However, in differential conditioning, the distribution of labeled responses stabilizes, and further training brings little additional change.

As has been shown by the pseudoconditioning studies of McAdam,[14] some of the changes that take place are unspecific, and are properly attributed to the general effects of increased arousal and motivation, rather than to specific reflections of changes in information processing. However, some changes seem to relate to the development of new neural processes activated by the stimulus. Realization that a portion of the constellation of response to the stimulus might reflect sensitization has led to the development of a number of methods to provide continuing assessment of those changes that relate specifically to the altered informational significance of the stimulus. Emphasis has become focused upon differential changes specific to the reinforced conditioned stimulus but not to neutral indifferent stimuli. Labeled response characteristics have been analyzed in animals fully trained to one class or modality of conditioned stimulus, before and after *transfer* to a second stimulus. Marked changes in the distribution and waveshapes of labeled responses to new stimulus have been observed upon transfer of conditioned response from steady tone or light to flicker,[15] from flicker to click,[16] from click to flicker,[17] and from peripheral sensory to central electrical stimulation.[18]

Additional evidence, indicating the specificity of many of the observed changes in labeled response, has been forthcoming from comparisons of activity during correct and incorrect behavioral performance.[19] Similar results have been obtained in so-called conflict studies, in which differential responses are elaborated in each of two sensory modalities and the effects of simultaneous presentation of contradictory signals are analyzed,[16] and also in studies of "split-brain" cats.[20] The results have revealed that the distribution and form of labeled responses alters drastically, depending upon the behavioral performance of the animal. Such findings have been summarized elsewhere.[2]

No attempt will be made here to catalog the diversity of changes in anatomical distribution and form of labeled responses that have been observed during conditioning. Suffice it to say that such changes are extremely widespread and have been observed in most anatomical regions of the brain, particularly since the introduction of average response computation. In the remainder of this chapter, I discuss some particularly striking findings from such studies, which seem to provide an insight into the mechanisms involved in the processing of information as new responses are learned and performed.

Assimilation of the rhythm

In 1945, a phenomenon called "assimilation of the rhythm" was described.[21] This term referred to the observation that, when an animal was being trained to perform some conditioned response to a tracer CS, marked electrical rhythms at the frequency of the CS dominated the intertrial activity *between presentations of the stimulus*. Assimilated rhythms have been observed in rat, rabbit, cat, dog, monkey, and man, in a wide variety of behavioral situations. Such activity appears earliest, lasts longest, and is most pronounced in nonsensory-specific brain structures. It appears early in conditioning; it is most noticeable during the middle period, when performance improves most rapidly; and it tends to disappear after the conditioned response becomes fully established, returning only if an error occurs and the animal receives an unexpected consequence to his behavior.

The specific relevance of assimilated rhythms to the acquisition of informational significance (cue value) by the conditioned stimulus during training is indicated by recent observations of Majkowski.[20] He implanted electrodes symmetrically into corresponding structures of "split-brain" cats in which the two halves of the brain had been substantially separated by surgery. Subsequently, one eye and the corresponding half brain were trained to perform a conditioned avoidance response to a flicker CS, while the other eye was covered by a contact lens occluder. Assimilated rhythms appeared during the intertrial intervals, but were restricted to structures on the trained side. In these animals, the section included the optic chiasma, corpus collosum, the posterior part of the commissura fornicis, and the anterior part of the commissure of the superior colliculus. The midline thalamic nuclei were not damaged.

When we observed these spontaneous rhythmic electrical waves between conditioning trials, our first thought was that they might be hippocampal theta rhythms related to orientation. A number of findings made us abandon this interpretation. First, they often appeared when no behavioral sign of orientation could be observed. Second, they corresponded approximately to the frequency of the conditioning stimulus being used, although it was not presented. These frequencies were often beyond the range of theta rhythms. Third, they were often observed in such regions as the mesencephalic reticular formation at times when hippocampal electrodes showed no rhythmic activity. Fourth, many times we observed that animals would perform the conditioned response spontaneously shortly after the appearance of strong assimilated rhythms. Other workers have observed a similar relationship.[22]

We next considered the possibility that such rhythms

were related to a reverberatory process representing the sustained aftereffects of the preceding presentation of the rhythmic conditioned stimulus. This idea also had to be abandoned. Trained animals, which had just been placed in the conditioning apparatus, displayed striking rhythmic activity of this sort while awaiting the first presentation of the conditioned stimulus, although assimilated rhythms were absent in the home cage. Similarly, when intertrial intervals were made quite long relative to the usual spacing between stimulus presentations, such rhythmic waves would appear spontaneously from a desynchronized resting activity. Therefore, it was not plausible to suggest that they were merely persisting reverberatory activity.

We have gradually come to the conclusion that assimilated rhythms arise from the release of characteristic patterns of neural activity representing the response of certain neural structures to the repeated presentations of the conditioned stimulus during the training procedure. Analogous observations of the release of temporal patterns of activity corresponding to previous stimulation have been obtained, including data from the chronically isolated cortical slab,[23] single units of the visual cortex,[11] and the so-called cortical conditioning procedure.[24]

Figure 1 shows the assimilation of rhythms in a single unit of the visual cortex, as studied by Morrell et al.[25] The post-stimulus frequency histogram reveals a gradual growth of a discharge pattern with maximum discharge rate at intervals of 100 milliseconds. When a 1-cps flicker is presented, the cell continues to display a 10-cps fluctuation in firing probability; this gradually diminishes as stimulation is repeated. The cell fires throughout the post-stimulus interval, and displays the fluctuation in firing rate as a feature that is readily discernible when a fairly large number of responses are averaged. This shows clearly that a previous pattern of stimulation can be released by a neural element. A central problem is whether this phenomenon depends upon the establishment of a resonance-like tuning of a network, or pacemaker properties in the discharging neuron.

One of the early attempts to assess the functional significance of this phenomenon involved both cortical and behavioral conditioning.[26] First, cats were conditioned to perform an avoidance response to a 10-cps flicker CS. Next, steady tone was paired with 10-cps flicker until the onset of the tone caused 10-cps electrical rhythms to appear in the visual cortex (Phase II of cortical conditioning). Finally, the animals were replaced in the avoidance apparatus and the behavioral and electrophysiological effects of presentation of steady tone were investigated. Although steady tone elicited frequency-specific discharge in the visual cortex, closely resembling the effects of the flicker CS, performance of the avoidance response did not occur.

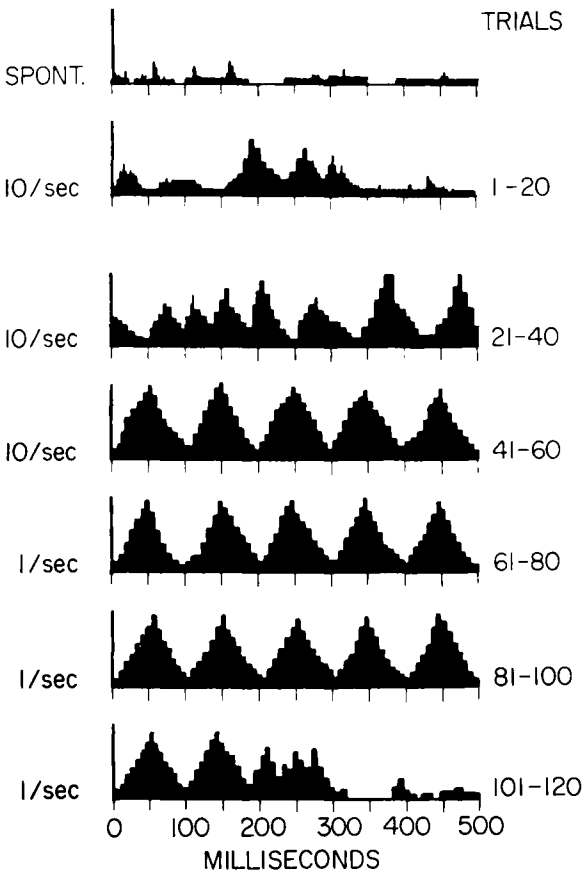

FIGURE 1 Poststimulus frequency histograms from single neuron in visual cortex subjacent to 10-microampere surface-positive polarization. Current flow was maintained throughout the interval. Top record shows spontaneous firing pattern. Notice how "driven" response of unit improves from first to third block of 20 trials in response to 10-cps flicker. Stimulus frequency was then changed to 1 cps. Poststimulus frequency histogram shows that unit continued to display a 10/sec response pattern, which dominated activity during next 40 trials, gradually lessening by 120th trial, as seen in last histogram. (From Morrell, et al., Note 25)

However, in subsequent work,[27] in which the order of the first and second experimental steps was reversed, presentation of a steady tone elicited *both* frequency-specific visual cortex potentials and conditioned response performance on a substantial proportion of test trials. It has also been observed that spontaneous performance of conditioned responses is often preceded by strong assimilated rhythms at the frequency of the absent conditioned stimulus.

Other evidence suggesting that assimilated rhythms might play a functional role came from comparison of the electrical activity observed during correct and incorrect

behavioral responses to two different frequencies of flickering light, which were the discriminative stimuli for performance of an approach response and an avoidance response in differentially trained animals.

An example of such data is provided in Figure 2. At the top are recordings taken from various brain regions of a cat that had learned to get milk by pressing a lever. The presentation of a 6-cps flicker was merely an environmental event of no relevance to the acquisition of food by the animal. Little labeled response was evident.

The animal was then trained to press the lever for milk during a 10-cps flicker and to withhold the conditioned response during a 6-cps flicker. The middle records show the labeled responses elicited by the negative stimulus during correct inhibition of lever pressing. There was a widespread enhancement of labeled responses after differential training.

The lower records illustrate recordings obtained during an *error of commission,* in which the cat treated the signal as though it were the 10-cps positive CS. In a number of structures—particularly visual cortex, reticular formation, and hippocampus—the 6-cps flicker elicited activity that did not correspond to the signal frequency, but was approximately 10 cps. When the lever was pressed erroneously, the labeled response disappeared from the lateral geniculate, although rhythmic activity persisted in some other structures. A second later, as milk was not forthcoming, the lever was released. The labeled responses in reticular formation, hippocampus, and visual cortex then came into correspondence with the actual stimulus frequency. The converse phenomenon has been observed during *errors of omission* to the positive signal.[19]

This kind of evidence led us to propose that some of the electrical activity observed during the presentation of a conditioned stimulus was *exogenous,* evoked by the stimulus, while a portion of the activity was *endogenous,* arising in certain regions from the release of stored temporal patterns of response established during the conditioning procedure. That is, the electrical rhythms inappropriate to the actual stimulus—those which were observed during errors in behavioral performance—were attributed to the release of information stored in the memory of the animal. Endogenous patterns were seen to vary as the motivational levels of such animals were manipulated by drugs or by satiety, suggesting that the ease of activation of these presumed memory patterns was influenced by the state of the organism.

It was suggested that a coincidence-detector mechanism achieved recognition of sensory input by comparison of the endogenous and exogenous patterns of activity.[19] Subsequently, it was demonstrated that electrical interference with late (but not early) components of the cortical potential evoked by the conditioned stimulus blocked the conditioned response performance; this was interpreted as compatible with the hypothesis.[28] Implicit in these formulations was the assumption that the temporal sequence of electrical events in a brain region constituted usable information for the nervous system. This assumption received support from our finding that differential conditioned responses could be established between two different temporal patterns of otherwise identical electrical pulses delivered to the same pair of electrodes, even though the pulse trains were equated for energy.[28] These findings have since been confirmed by other workers.[29]

A particularly interesting study on central conditioned stimuli was carried out by Leiman.[18] He conditioned cats to respond to a 10-cps flicker, and subsequently transferred the conditioned response to direct 10-cps electrical stimulation of the lateral geniculate and the mesencephalic reticular formation. Although transfer to lateral geniculate stimulation occurred slowly, transfer to reticular formation stimulation was exceedingly fast, sometimes requiring only one trial. At each stage of learning, electrodes in a wide variety of brain regions were stimulated at 10 cps, and generalization was studied. After flicker training, no central stimulus sufficed to elicit conditioned response. After lateral geniculate training, ipsilateral visual cortex stimulation would infrequently elicit performance. However, after reticular training, performance could be elicited regularly by stimulation of a variety of regions, including ventralis anterior, centralis lateralis, centre median, globus pallidus, nucleus ruber, substantia nigra, nucleus entopeduncularis, and even the pyramidal tract. Yet, stimulation of sensory-specific regions, including the untrained lateral geniculate body on the other side, failed to elicit generalization. These findings are difficult to reconcile with the idea of memory storage in a discrete neuronal pathway.

Figure 3 illustrates electrical activity recorded from an animal trained to perform a conditioned avoidance response to a 4-cps flickering light. These records were obtained during generalization to a 10-cps flicker presented to this animal for the first time. At the onset of the novel stimulus, evoked responses at the 10-cps flicker frequency clearly showed in the lateral geniculate body and the visual cortex. After a few seconds, a much slower rhythm appeared in the visual cortex. Shortly thereafter, the animal showed a behavioral startle response, stood up, walked across the apparatus, and performed the conditioned response. During this period, slow waves at about the frequency of the 4-cps conditioned stimulus appeared in the visual cortex and the reticular formation, although the lateral geniculate continued to respond to the 10-cps stimulus frequency. These data show that some regions of the

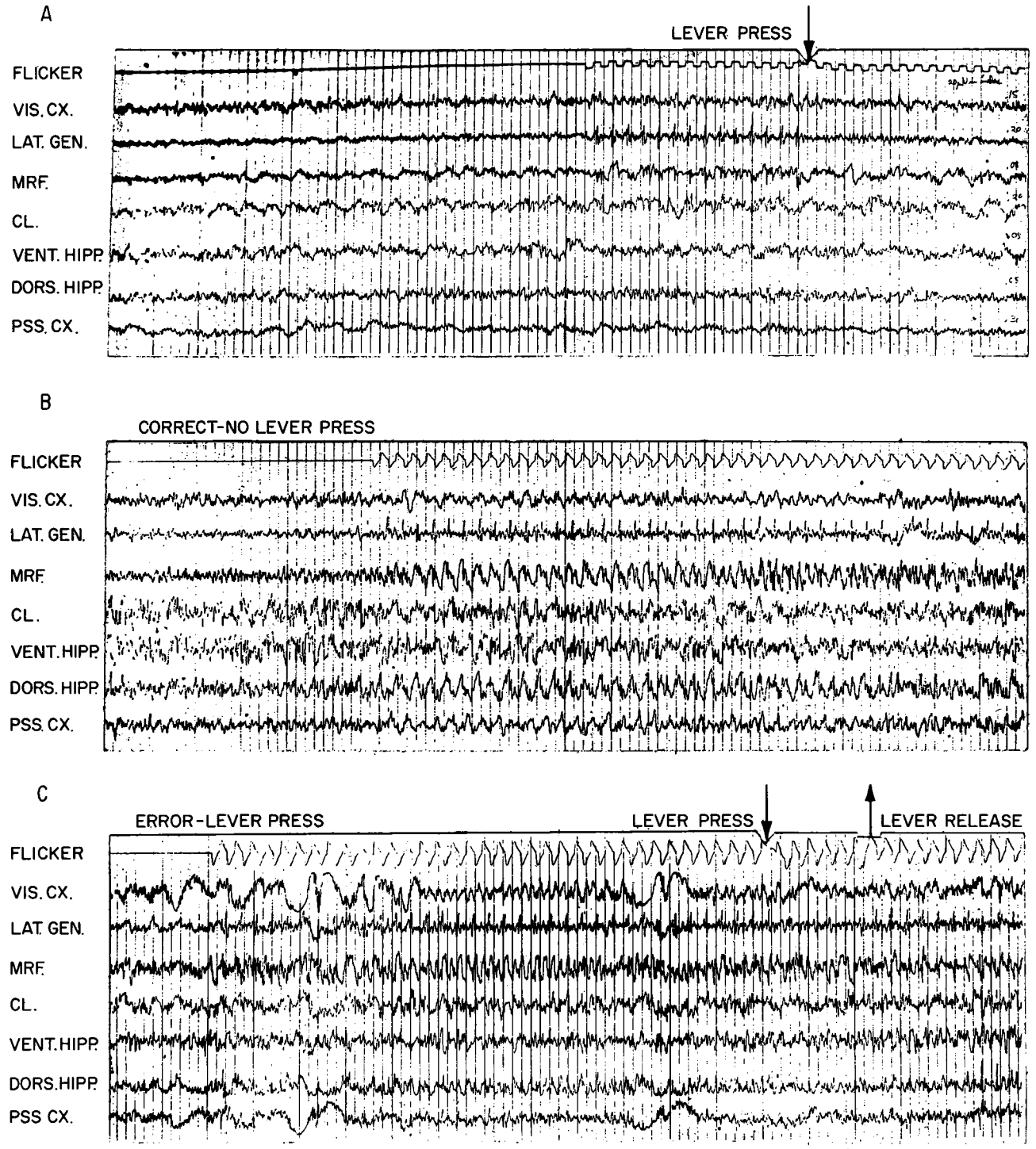

FIGURE 2 A: Effect of 6-cps flicker presentation to cat after it had learned that milk could be obtained whenever a lever was pressed. Flicker had no signal value at this stage. Notice how little labeled activity was elicited by the flicker except in the lateral geniculate. Note disappearance of response in lateral geniculate due to internal inhibition, as cat presses lever and waits for milk.

B: Effect of 6-cps flicker after elaboration of frequency discrimination, using 10-cps flicker as positive signal and 6-cps flicker as negative signal. These records were obtained

brain display electrical activity at the frequency of the conditioned stimulus actually used in training when the conditioned response is performed as a result of presentation of a new signal. The new signal does not cause these electrical rhythms directly; rather, they seem to be released from a neural system established during the conditioning experience. Similar observations have been reported by Majkowski,[20] who has noted that this phenomenon is restricted to the regions on the trained side of a split-brain cat.

Another example of the phenomenon, observed in a different animal, is seen in Figure 4. The electrical activity was recorded during generalization to a novel 10-cps flicker after an avoidance response was established using a 4-cps flicker as the conditioned stimulus. Although the lateral geniculate showed a regular response to the 10-cps-stimulus frequency, a slower rhythm was seen in several other structures, particularly the visual cortex. In the visual cortex tracings, a faster negative component was superimposed on the slow positive wave. The animal was then

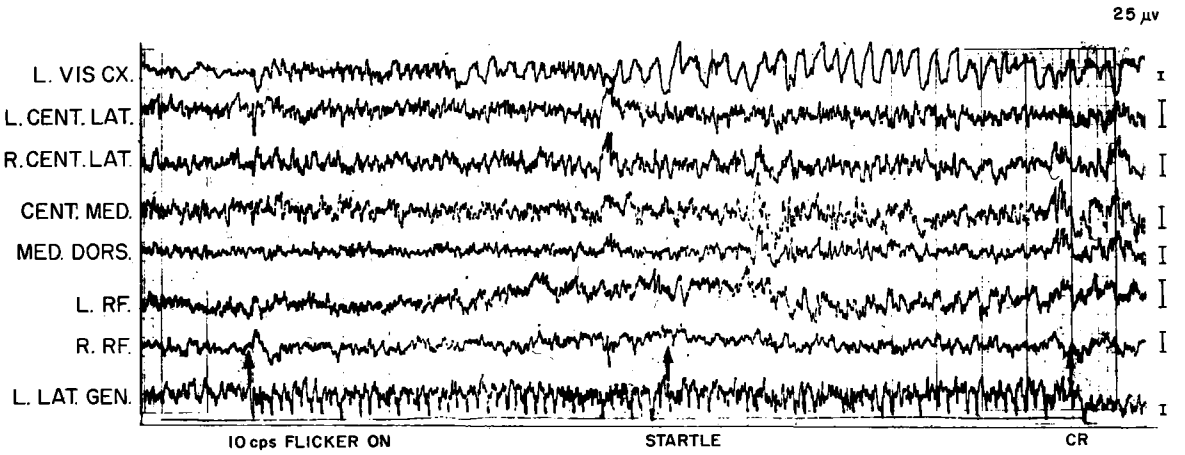

FIGURE 3 Electrical responses to 10-cps flicker during generalization of the conditioned avoidance response after avoidance training with a 4-cps flicker tracer conditioned stimulus. All leads bipolar. L. VIS. CX., left visual cortex; L. CENT. LAT., left centralis lateralis; R. CENT. LAT., right centralis lateralis; CENT. MED., centre median; MED. DORS., medialis dorsalis; L. RF., left mesencephalic reticular formation; R. RF., right mesencephalic reticular formation; L. LAT. GEN., left lateral geniculate. (From John, Leiman, and Sachs, Note 40)

during correct inhibition of lever press during 6-cps flicker. Note marked enhancement of labeled potentials at the stimulus frequency after differential training, and frequency specificity of the brain activity.

C: Effect of 6-cps flicker during an *error of commission,* in which the cat pressed the lever during the inhibitory 6-cps signal. At stimulus onset the cat moves restlessly, causing movement artifacts, which can be seen in first part of the tracing. As the animal settles down, clear potentials at the 6-cps flicker frequency can be seen in the lateral geniculate. However, in other structures, little activity corresponding to the signal frequency can be seen. In the visual cortex, mesencephalic reticular formation, and dorsal hippocampus, marked 10-cps electrical rhythms appear and are followed by performance of the lever-pressing behavior, which would be appropriate to a 10-cps signal. Note the disappearance of 6-cps activity in the lateral geniculate while the animal holds down the lever and looks in the dish for milk to appear. The 10-cps reticular activity continues during this interval. Finally, the animal releases the lever, 6-cps activity reappears in the lateral geniculate, and the mesencephalic reticular formation begins to show activity corresponding to the actual stimulus frequency. This rhythm then appears in dorsal hippocampus and in the visual cortex. All leads bipolar. FLICKER, stimulus artifact; VIS. CX., visual cortex; LAT. GEN., lateral geniculate; MRF., mesencephalic reticular formation; CL., nucleus centralis lateralis; VENT. HIPP., ventral hippocampus; DORS. HIPP., dorsal hippocampus; PSS. CX., posterior suprasylvian cortex. (From John and Killam, Note 41)

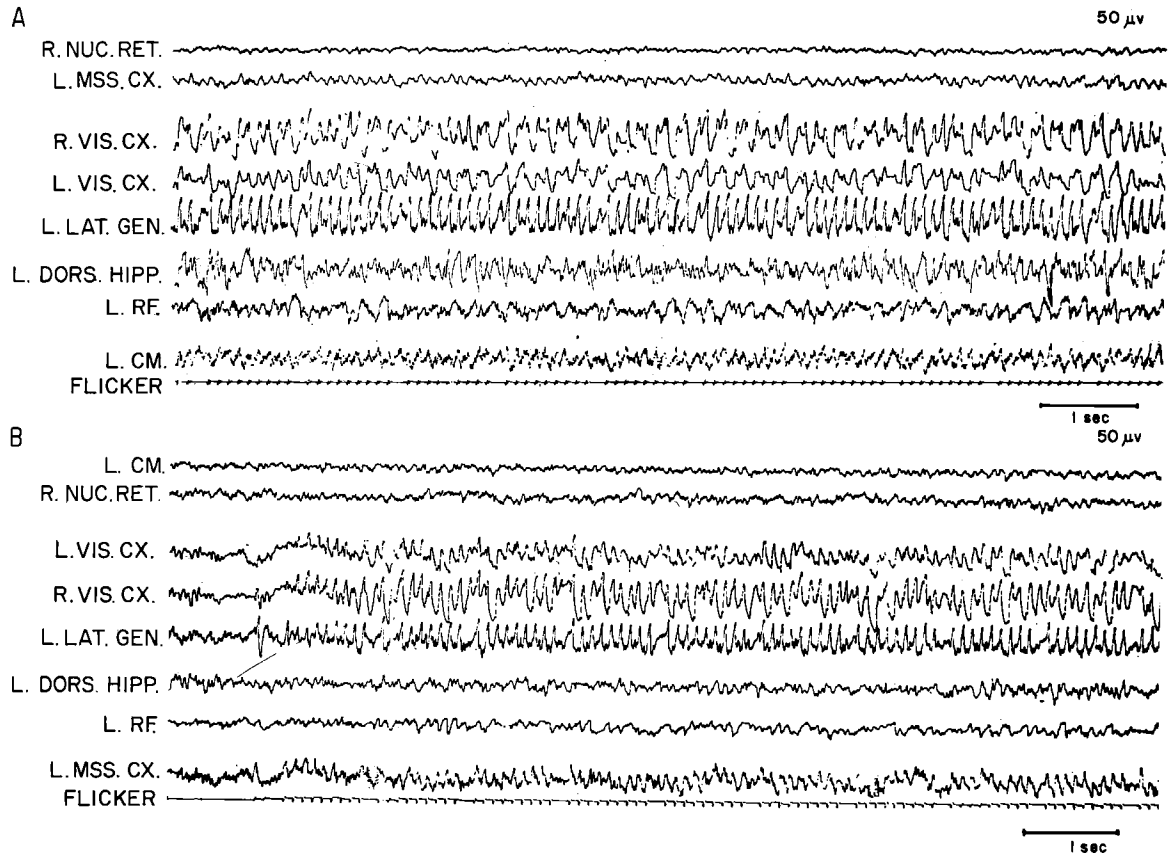

FIGURE 4 A: Electrical responses to 10-cps flicker during generalization, after avoidance training using a 4-cps flicker tracer conditioned stimulus. B: Electrical responses to 10-cps flicker following differentiation of avoidance response. R. NUC. RET., right nucleus reticularis; L. MSS. CX., left medial suprasylvian cortex; R. VIS. CX., right visual cortex; L. LAT. GEN., left lateral geniculate; L. DORS. HIPP., left dorsal hippocampus; L. RF., left mesencephalic reticular formation; L. CM., left centre median. (From M. Weiss, Note 42)

taught to differentiate between a 4-cps and a 10-cps frequency, receiving punishment if it performed the conditioned response to the 10-cps flicker. The records at the bottom of Figure 4 were obtained following completion of differentiation, as the avoidance response was inhibited during presentation of the 10-cps signal. The dominant rhythm of the electrical activity in most structures now corresponded exceptionally well to that in the lateral geniculate, which reflected the actual stimulus frequency.

Figure 5 shows the average response evoked in the lateral geniculate body during generalization to the 10-cps flicker. The evoked potential accurately corresponds to the stimulus frequency.

Figure 6 shows average response computations obtained from the visual cortex under various conditions. The first waveshape (A) is the average response of the visual cortex to the 4-cps conditioned stimulus after training. The second waveshape (B) is the average response of the visual cortex during generalization to the new 10-cps flicker. This waveshape does not possess a periodicity at 100 milliseconds, although a component at the stimulus frequency can be seen clearly. The third waveshape (C) is the average response evoked by the 10-cps signal after completion of differentiation. Now the waveshape is periodic at 100 milliseconds, corresponding accurately to the stimulus frequency. The slow component, which was so marked in waveshape B, has now disappeared, as the informational difference between 10- and 4-cps flicker became established. These data show that when a flicker at one frequency is processed behaviorally as if it possessed the same informational significance as a flicker at a second frequency, the electrical activity of the visual cortex resembles an interference pattern containing two components, as calculated in line D. One component seems to represent the

actual repetition rate of the stimulus. The other seems to correspond to the frequency of the familiar conditioned stimulus, presumably released from memory storage. After differentiation, this inappropriate release no longer occurs. Similar phenomena have been demonstrated in the mesencephalic reticular formation.[28]

The evidence presented thus far shows that rhythmic electrical activity, with a temporal pattern corresponding to the frequency of repetitive conditioned stimuli, appears in the brain under certain conditions. Analysis of the characteristics of such activity during erroneous performance and generalization suggests that it may arise from the release of stored information that influences the behavior. In other words, these electrical rhythms seem to reflect, with corresponding frequencies, the activation of memories about the stimuli.

FIGURE 5 Average response computed from lateral geniculate during generalization to 10-cps flicker, after avoidance training using a 4-cps flicker tracer conditioned stimulus. (From M. Weiss, Unpublished data cited in John, Note 28)

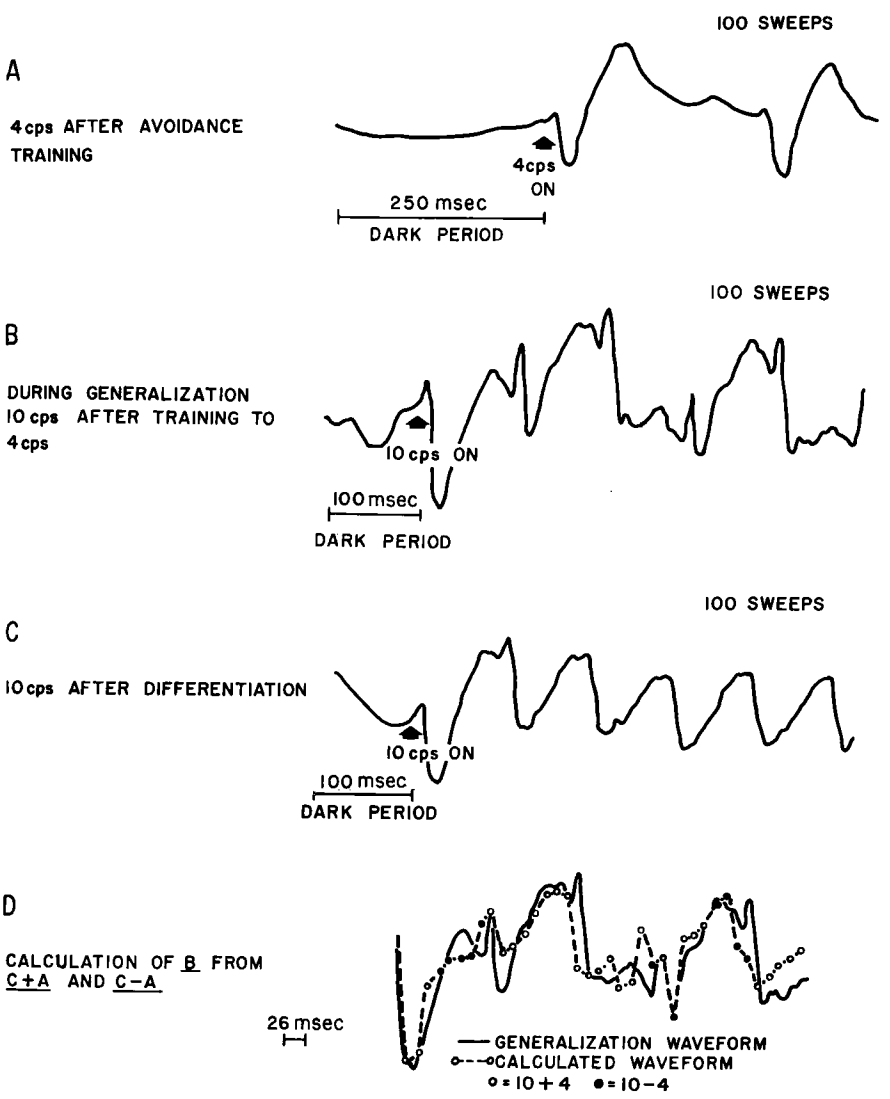

FIGURE 6 Average response computed from visual cortex. A: In response to 4-cps flicker after avoidance training using a 4-cps flicker tracer conditioned stimulus. B: During generalization to 10-cps flicker. C: In response to 10-cps flicker after differentiation training. D: Comparison of generalization waveform with calculated interference pattern. (From M. Weiss, Unpublished data cited in John, Note 28)

Perhaps the correlations observed between the *frequency* of electrical rhythms and *information* about rhythmic stimuli represent only a special case of little general interest or relevance. After all, informationally significant events in natural situations are not characterized by particular frequencies of repetition. To evaluate this possibility, it is useful to examine the actual *waveshape* of the potential evoked by a stimulus. The evoked potential waveshape reflects the temporal pattern of electrical activity in a large neuronal population, following the presentation of a particular stimulus. This potential probably is related to the averaged membrane potentials of the cellular ensemble. Although the evoked potential does not arise directly from summation of the discharges of single neural units, it may be considered to represent the *probability* of coherent activity in the monitored population during that time.

We and other workers, including Galambos, Glivenko, Livanov, and Yoshii, have noticed that the electrical responses of different brain structures seem to become similar as the conditioned response is established. Using high-speed computers for more detailed analysis, we have shown quantitatively that, as a stimulus acquires informational significance during conditioning, the electrical activity evoked in many different brain regions by that stimulus comes to contain markedly similar components.[30] Such common responses are seen in certain regions of cortex, rhinencephalon, diencephalon, mesencephalon—in other words, at most levels of the nervous system. In some instances, the similarity in evoked response waveshape that emerges during conditioning is so striking as to render any quantitative analysis quite superfluous. An example of such data is shown in Figure 7, taken from an animal overtrained for a long period.[16]

This anatomically extensive system displays similarity in evoked potential waveshapes as long as the conditioned stimulus elicits correct behavioral performance. However, if the animal displays erroneous behavior, certain regions of this sytem display idiosyncratic waveshapes, which no longer correspond to the responses observed in other areas. Like the previous data about the appearance of characteristic rhythms, this observation suggests that some of the components of the evoked potential waveshape in such regions may be released from storage rather than evoked by the direct action of the stimulus.

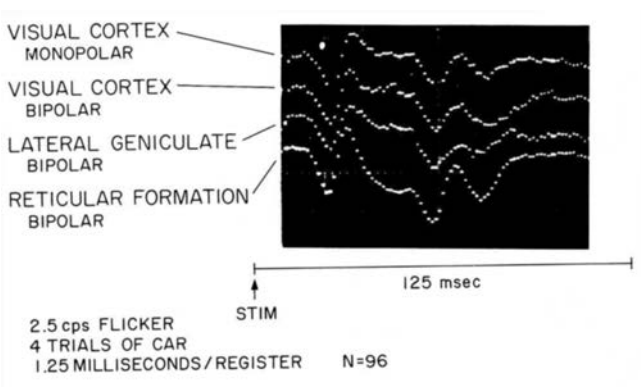

FIGURE 7 Average response waveshapes recorded from different derivations in a trained cat. Note the marked similarity of the potentials in different brain regions. (From John, Note 2)

We have recently carried out an experiment which indicates that the waveshapes of evoked potentials do not merely reflect the morphology of the regions from which the recordings are obtained. Factorial techniques were used to evaluate the similarity of evoked responses elicited in various regions by a single stimulus to some part of the nervous system, as contrasted with the similarity of responses elicited in a given neural region by a variety of central stimuli. The results show clearly that the waveshape of the evoked response recorded from a given region after a specified disturbance is more dependent upon the site of stimulation than on the site of recording.[31] It is as if a disturbance in a given anatomical region possesses a key signature, imbedded in the waveshape of the activity that propagates around the nervous system. One might speculate on whether this phenomenon reflects a process whereby information about nonrandom activity in particular brain regions is distributed to many other regions. Such a process may be involved in the integration of informational aspects of the same event, reflected in different sensory modalities.

Further evidence on the release of certain components of neural response has been obtained from additional studies of generalization.[32] In such studies, we have observed that the evoked potential in many brain regions displays a pronounced second component when a novel stimulus elicits generalization; this component is absent when the behavioral response is not performed.

Figure 8 compares the average response waveshapes appearing in the lateral geniculate and nucleus reticularis under various conditions: evoked potentials elicited in these structures during correct response to the 10-cps conditioned stimulus (flicker) that was used in training; waveshapes obtained during generalization to a 7.7-cps test stimulus; records obtained when presentation of the test stimulus failed to elicit a conditioned response. Two components are clearly evident in the top records, and have been labeled I and II. Component II appears in some structures during the process of learning, as has also been observed by other workers.[33-35]

There is a striking correspondence in waveshape between the upper and middle records. During generaliza-

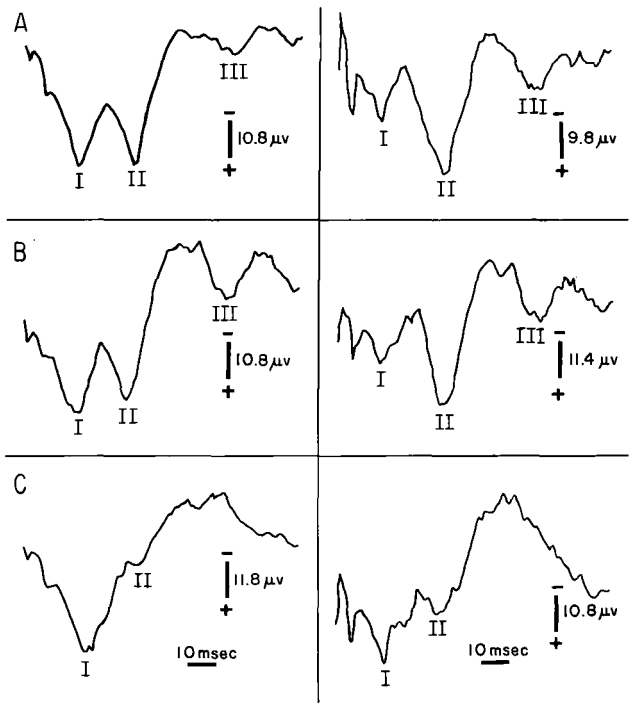

FIGURE 8 Average evoked potentials recorded from lateral geniculate body (left) and nucleus reticularis (right) during behavioral trials that resulted in conditioned response performance (A) to 10-cps flicker, and generalization (B) and no response (C) to 7.7-cps flicker. Generalization and no-response averages are based on 42 evoked potentials, and conditioned response averages on 100 evoked potentials. Analysis epoch is 90 milliseconds. (From Ruchkin and John, Note 36)

tion, the test stimulus causes an electrical response that corresponds closely in form to the potentials evoked in many structures by the conditioned stimulus itself. This has been confirmed by correlational analysis. Comparison of the middle and bottom records shows that the waveshape is radically different when generalization fails to occur. Component II is essentially absent in the bottom record. Statistical evaluations of the difference between those two samples of data have been carried out, and show that the results are significant at better than the 1 per cent level.[36]

We have suggested that Component I reflects the registration of afferent input upon the neural structure, and that Component II is related to the readout of information stored in the region and released in response to the stimulus. It is of interest to examine Component II more closely. We can consider the bottom records of Figure 8 as the effect of input alone, while the middle records show the characteristics of input followed by readout. If we *subtract* average evoked potentials caused by a novel stimulus when no generalization occurs from average evoked potentials produced during generalization, the *difference waveshape* provides a picture of the readout process. Figure 9 shows the results of this operation for a number of anatomical regions.

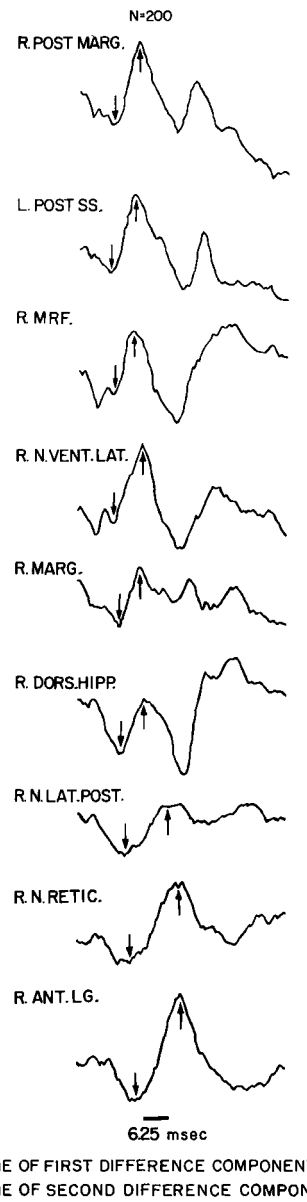

FIGURE 9 Difference waveshapes obtained for a number of regions by subtraction of averaged responses, evoked by a 7.7-cps test stimulus during nonperformance, from averaged responses elicited when generalization occurs. All averages in these computations were based upon 200 evoked potentials distributed among a number of behavioral trials in each category, with a 62.5-millisecond analysis epoch. (From John, et al., Note 32)

It is interesting that the difference waveshape possesses extremely similar form and latency for some regions of cortex, thalamus, and mesencephalon, while showing changes in form and latency for other regions. Numerous examples of this phenomenon have been observed in our laboratories, with no detectable latency differences between some structures within a resolution of one millisecond or less.[2,16] Figure 10 shows a particularly striking example from a highly overtrained cat.

Although essentially identical difference waveshapes have sometimes been derived from raw data that were themselves similar, such results also have been obtained when the raw average responses were of conspicuously different shape. The observed latency differences for those regions where the process first appears are so small that it is unlikely that this electrical process arises in a particular region and propagates to the other structures. Although it is possible that such synchronization is the consequence of the action of a system of "pacemaker" neurons, it also seems necessary to consider the possibility that the process arises independently in these various regions as something like a change of state.

Evidence exists that these released components are not merely related to the production of movements, or to similar unspecific factors. They are not correlated with the appearance of movement in generalization, as has been seen in the study of trials with different response latency. Similar conclusions come from so-called conflict studies.[2,16] In these, we train animals to perform differentiated approach and avoidance responses to two different flicker frequencies, V_1 and V_2. The animals are then taught to perform the same two conditioned responses to auditory stimuli (clicks) at these two frequencies, A_1 and A_2. In conflict, the animal receives two contradictory signals simultaneously, $(A_1 + V_2)$ or $(A_2 + V_1)$. Under these circumstances, it is possible to study the potentials evoked by the two different signals in the same neural regions at the same time. When a signal successfully controls the outcome of a conflict trial, the later components in the evoked potential of some regions are much more pronounced than when that signal fails to determine the conditioned response that is performed. Such findings seem to rule out unspecific origins of these components.

Thus, it appears that the change of potential during time, which is recorded from a neuronal population, may reflect processes of informational significance, and that particular waveshapes can be released from storage in local neural regions. The correlations between the temporal patterns of electrical activity and information are more general than merely the rhythms of electrical waves.

It is interesting that when a differentially trained animal performs one correct and one erroneous conditioned response to the same stimulus, that stimulus elicits two different evoked potential waveshapes in some regions.[37] These various observations suggested that different waveshapes might be elicited by two stimuli that differed in meaning in a way more natural than their frequency of repetition. For this reason, we have turned to the study of the effect of the geometric form of different visual patterns presented at the same frequency upon the evoked potential waveshapes.[38] The recordings in Figures 11 to 14 were obtained from a scalp electrode located 3 centimeters above the inion in man, relative to a reference electrode on the ear lobe. Stimuli with a repetition rate of one per second were presented in blocks arranged in a Latin square design, to provide replications of each observation. All potentials were based on averages of 50 to 200 stimulus presentations. The stimuli were mounted on the wall before the subject and illuminated by a standardized light flash.

Figure 11 shows that the presence of a geometric figure in the visual field changes the evoked potential waveshape. Figure 12 demonstrates that the waveshape elicited by a

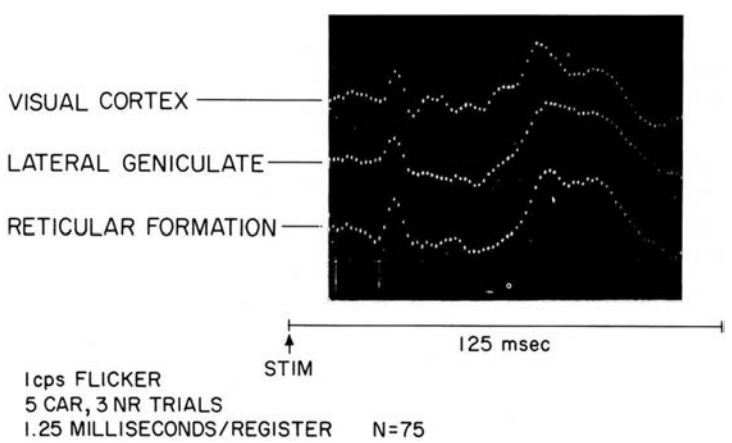

FIGURE 10 Difference waveshapes obtained by subtracting average responses computed during three trials resulting in no performance (NR) from average responses computed during five trials resulting in correct performance of the conditioned avoidance response (CAR). All recordings were bipolar, and 75 evoked potentials were used in each of the constituent averages. Note the correspondence in latency and waveshape of the difference process in these various regions. (From John and Shimokochi, Note 16)

square figure is *different* from that elicited by a circle of the same area.

In Figure 13, the waveshape elicited by a large square is the *same* as that elicited by a small square. The printed word SQUARE (Figure 14) elicits a different waveshape than does the word CIRCLE, although the individual letters and total dimensions of the two have been carefully equated for area.

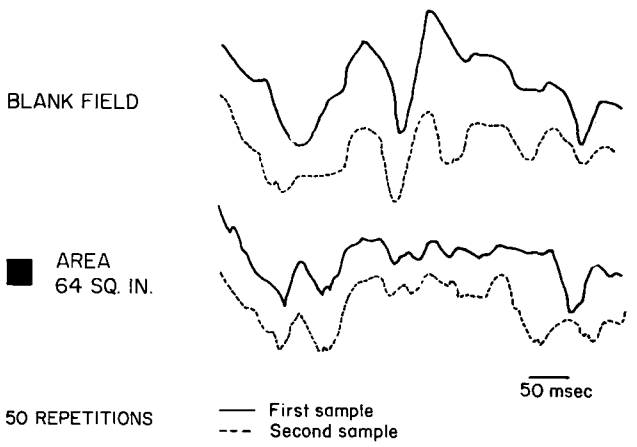

FIGURE 11 Comparison of averaged responses evoked by weak flash illuminating a blank, white visual field or the same field containing a black square, 64 square inches in area. Stimuli were presented in blocks of 25, arranged in a Latin square sequence to provide two replications for each sample. Each replication consisted of 50 repetitions of the stimulus. In this and subsequent figures, the data were obtained from scalp recordings, monopolar derivation, inion versus earlobe reference. Analysis epoch was 500 msec. (From John et al., Note 38)

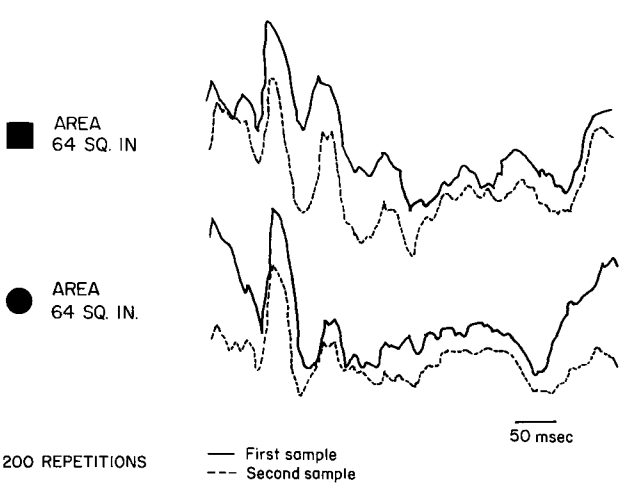

FIGURE 12 Comparison of averaged response evoked by weak flash illuminating a white visual field containing either a black square or a circle, *equated for area.* Averages based on 100 repetitions each. (From John et al., Note 38)

In recent work, Herrington and Schneidau have studied the potentials displayed when a subject imagines a geometric form in his visual field. When an empty visual field was illuminated by a flash of light, the subject imagined that either a square or a circle was in the field. Average response waveshapes computed during this imaginary behavior displayed characteristic waveshapes closely resembling those actually evoked by the presentation of squares or circles in the visual field. The experimenter could consistently identify the thought of the subject from the form of the evoked potential.[39]

In all of the above experiments, pupillary dilation was controlled by homatropine, eye movement monitored by oculograms, and feedback from vocal musculature controlled by requiring the subject to count all stimuli aloud. The observed results must be attributed to processes of central origin, in view of these controls for peripheral effects. The findings suggest that a thought or a percept somehow corresponds to an orderly statistical sequence in a neural network.

The foregoing data show that different waveshapes are

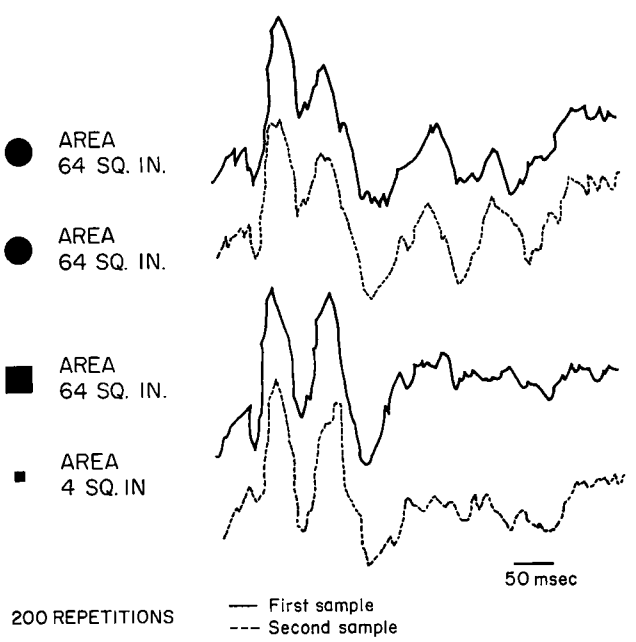

FIGURE 13 Comparison of averaged responses evoked by weak flash illuminating a blank visual field containing either a square or a circle. Stimulus sequence was *large* circle, *large* square, *large* circle, *small* square. Note similarity of response to large and small squares. Each average based upon 200 repetitions. (From John et al., Note 38)

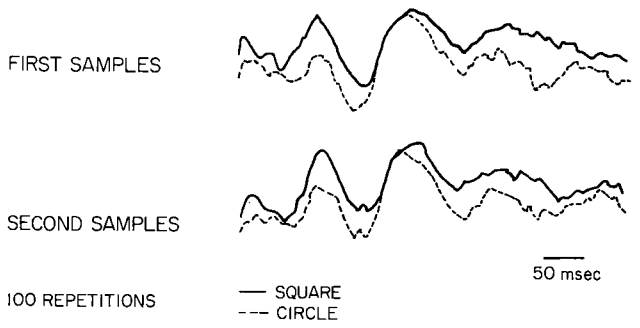

FIGURE 14 Comparison of averaged responses evoked by weak flash illuminating the word SQUARE or CIRCLE, printed in block letters. Total black area of both words was made equal. Note reproducibility of difference between responses to first samples (top), and second samples (bottom); each average based upon 100 repetitions. (From John et al., Note 38)

evoked by presentation of psychophysically equated stimulus patterns that differ in *meaning*. These results demonstrate that the informational relevance of temporal patterns of electrical activity is not limited to the artificial laboratory case of frequency-coded stimuli. Therefore, conclusions obtained from such studies may provide insights generalizable to those neural mechanisms that process, store, and retrieve information in more natural circumstances. Further research will be necessary to clarify the cellular processes responsible for the storage and release of these representational patterns in the brain. However, the electrophysiological data summarized in the latter portion of this chapter do not correspond to what one might expect if the essential process in learning involved the establishment of new pathways between input and output regions. The changes in responsiveness to the CS during learning, as evidenced by alterations in labeled activity and evoked responses, are extremely widespread.

It is true that responses appear in new structures during learning, primarily nonsensory-specific regions of the brain. But the modes of ensemble response to the stimulus change strikingly during learning, and differences between modes of electrical response seem to characterize generalization, as well as to correct an erroneous behavioral performance. The study of difference waveshapes suggests that when readout of memory occurs, a change in the mode of response occurs throughout widespread brain regions. This apparent change of state seems to take place at about the same time and to have basically similar features in widely separated anatomical regions that differ radically in their morphology.

Many aspects of these observations are difficult to reconcile with the notion that memory is a new pathway constructed from a set of cells, which are altered as a result of experience. It does not seem necessary to assume that these macropotential phenomena are manifestations of a selected set of cells whose discharge represents specific past experiences. Admittedly, the evidence presented does not rule out the possibility that information is stored in the brain in such a way that the discharge of a selected set of cells deterministically "stands for" a specific past experience. Yet, single cells are intrinsically noisy, respond to a wide variety of stimuli, and display significant variations of response to a specific stimulus. The informational significance that can be attributed safely to the discharge of particular elements with these unreliable characteristics would appear to be limited.

At this stage in our understanding, it may be worthwhile to consider a statistical alternative. Perhaps the information contained in the activity of a population of neurons is the time course of deviation from the random, or baseline, pattern of activity. Restated, the information content may be represented by the decrease in entropy corresponding to orderly activity in the ensemble. Macropotential phenomena like those which have been described may reflect the temporal organization of neural aggregates. Such a formulation suggests that the memory mechanisms on the level of molecular biology perhaps may specify a temporal sequence of states in an ensemble rather than, or in addition to, the alteration of synaptic efficiency in particular pathways.

Effects of Ablation

K. L. CHOW

THE ABLATION METHOD is the oldest and most widely used technique in the study of brain function. Until very recently, it was virtually the only experimental tool readily available for investigating the functional organization of the nervous system. Among the first studies were those by Flourens in the 1820's and by Hitzig, Ferrier, Munk, Goltz, and others in the 1880's. These workers removed different parts of the central nervous system in pigeons, dogs, monkeys, and other animals, and then showed, by physiological and neurological analysis, that the defects exhibited by these animals could be related to the variously damaged parts of the brain.[1] Later, around 1900, ablation was used to investigate the neural mechanisms of learning and memory. Examples are Starlinger's report in 1895 that bilateral sectioning of the pyramids of the medulla did not affect a trained dog in its ability to lift its forepaw in response to verbal command. Also, Loeb's dog could still walk on its hind legs after bilateral destruction of the cortical leg areas.[2,3]

In the 1920's, Franz and Lashley further refined the method and stated the basic requirements for using it. They applied sophisticated behavioral training procedures and emphasized statistical analyses in order to quantify both the learning process and the memory defects. In addition, they introduced histological controls to verify the brain lesions. The beautiful work of Sperry on the split-brain preparation (this volume) demonstrates the power of the method in its modern form.

The present paper will critically examine the method itself, using examples drawn from experimental animal studies. I hope to give you some general conclusions about learning mechanisms derived from ablation studies and to show how they suggest possible areas of the brain within which the ultimate neural mechanisms must be sought.

The ablation method

Ablation experiments generally follow two different procedures. In the first, a part of the brain, e.g. a cortical area or a subcortical nucleus that one suspects to be involved in a particular learning task, is destroyed; once the animals recover from surgery they are trained to learn this task. Their learning scores are compared with those of normal animals. If the experimental animals learn more slowly than the controls, the damaged part of the brain is considered to be somehow implicated in the acquisition of the task.

Alternatively, animals are first trained and then some part of the brain is destroyed. After recovery, they are retested on the task. If the performance of the animals is impaired, i.e., if they remember the task less well than do the controls, the damaged region is considered to be involved in retention. The distinction between acquisition and retention, or learning and performance, is a useful one, for the neural mechanisms involved in fixation of experiences or read-in of information need not also be involved with storage and retrieval of information.

How the brain tissue is destroyed seems to be unimportant. Various methods have been used. The tissue is dissected off, removed by suction, burned off, or destroyed by chemicals. On the other hand, the same region of both cerebral hemispheres usually must be ablated before the lesion can affect learning and memory. The experimental results I shall discuss were obtained from animals with such lesions made bilaterally and symmetrically in the brain.

Lashley's study on maze-learning in rats is a classical example of how this technique provides significant insight into learning mechanisms.[4] He made lesions, varying in size and location, on the cerebral cortex of rats and then trained them to run in three mazes of increasing difficulty. They are alley mazes with either one, three, or eight blind alleys. Figure 1A shows the lateral view of a rat brain with thalamic areas labeled and Figure 1B shows labeled sensory-motor cortical areas. The rest of the figure includes some examples of the cortical lesions of experimental rats. The main results of this study are summarized in the graph of Figure 2. Evidently the number of errors (i.e., entering a blind alley) made in learning a maze is a function of both the size of the lesion and the difficulty of the maze. The larger the lesion, irrespective of its locus, and the more blind alleys in the maze, the more errors a rat will commit in learning it. The criterion

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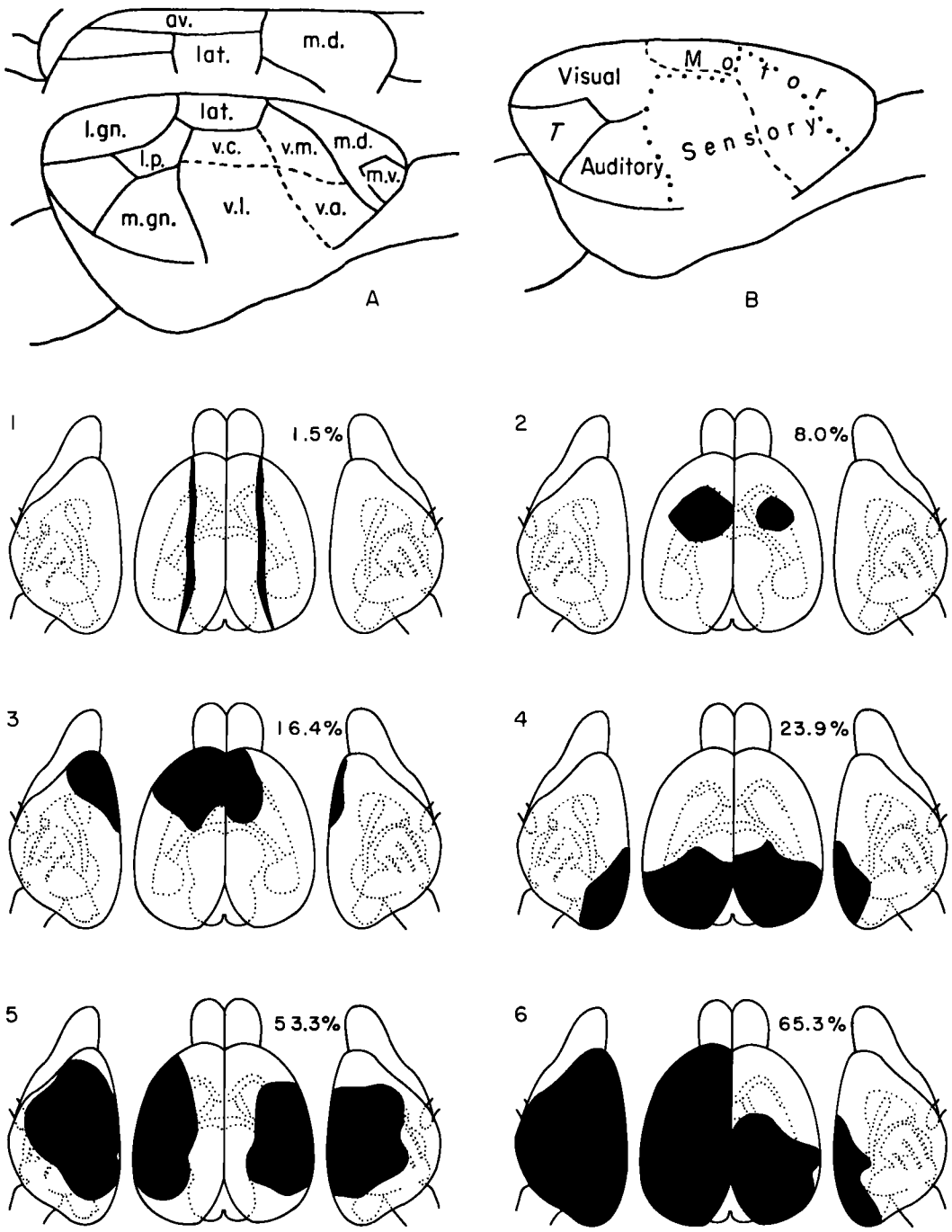

FIGURE 1 A: Lateral and medial view of rat brain with the projection areas of thalamic nuclei labeled. B: Lateral view of rat brain with the sensory and motor areas labeled. Brain maps are examples of the locus and size of cortical lesions (in black); the per cent of lesion size is indicated at the right. Av., anterior ventral nucleus; lat., lateral nucleus; l.gn., lateral geniculate body; l.p., lateral posterior nucleus; m.d., mediodorsal nucleus; m.v., medioventral nucleus; m.gn., medial geniculate body; v.a., ventral anterior nucleus; v.l., ventralateral nucleus. (From Lashley, Note 4)

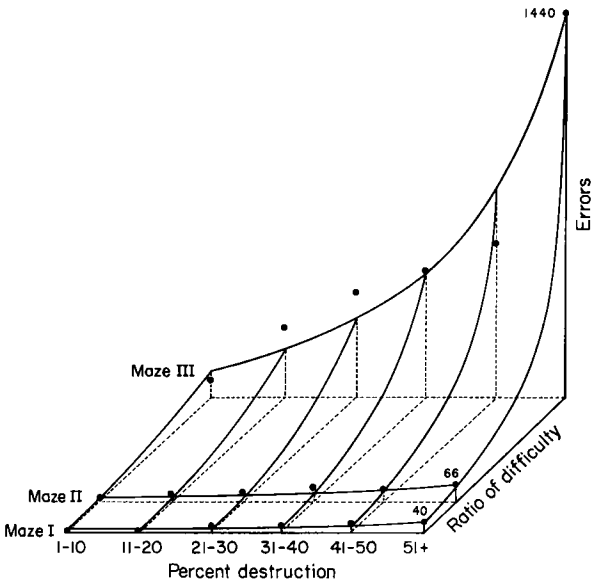

FIGURE 2 Graph to show the learning rate of lesioned rats. The number of errors required to reach criterion is plotted against the size of the cortical lesion and the degree of difficulty of the three mazes. (From Lashley, Note 4)

of learning in this case is ten consecutive errorless trials. The efficiency of learning, or "learning scores," is generally recorded as total numbers of errors or total numbers of trials in relation to criterion. Other measures, such as decreased running time, latency, and so on can also be used.

From these and other results, Lashley proposed two principles: equipotentiality of parts and mass action. By equipotentiality he means "the apparent capacity of any intact part of a functional area to carry out with or without reduction in efficiency the functions which are lost by destruction of the whole." By mass action he means that "the efficiency of performance of an entire complex function may be reduced in proportion to the extent of brain injury within an area whose parts are not more specialized for one component of the function than for another." While some recent reports take exception to these principles, Lashley's conclusions still hold for the effects of cortical lesions on alley-maze performance of rats. His concepts of equipotentiality and mass action help to define the cerebral mechanisms involved in performance of maze tasks by rats.

In contrast to the equipotentiality of rat cortex in maze learning, the monkey brain shows specific loci for different learning tasks. Figure 3 shows the lateral view of a monkey brain with its principal gyri and cortical areas labeled. In a visual discrimination learning task frequently used with monkeys, the animal is required to select one of two arbitrarily determined stimulus-objects to get a food reward. For example, the monkey is shown a red square and a green disk. Food is always put under the red square, and the animal's task is to select it whenever this pair of stimuli is presented. Trials are given until some arbitrary criterion of per cent–correct choices is reached. There are many variations of this procedure. For instance, the two stimuli can be presented in succession instead of simultaneously, the monkey being required to pick the positive stimulus and to refrain from responding to the nonrewarding negative stimulus.[5] Klüver and Bucy reported that monkeys with temporal lobes destroyed bilaterally had difficulty in learning this task, and if they had learned the problem before the operation, they had trouble in remembering it afterwards.[6,7] Numerous subsequent studies on this problem[8-10] show that the critical focus for this task is located in the middle and inferior temporal gyri. This region is not directly involved in the sensory or motor systems, because monkeys without this part of the brain display no detectable sensory or motor defects. Furthermore, only visual learning is affected, for such monkeys learn delayed responses, make tactile discriminations, and so on, postoperatively. This is one of the few instances in which a part of the primate brain outside the sensory motor systems can be shown experimentally to be uniquely involved in learning and memory functions.

Let me turn now from the above two studies that illustrate how ablation is employed in learning experiments to certain difficulties in using this method.

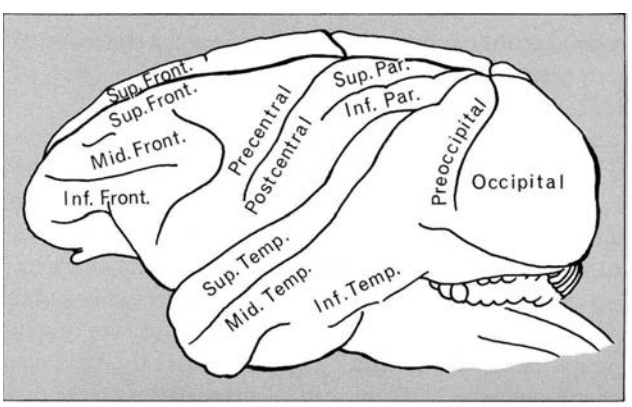

FIGURE 3 Lateral view of monkey's brain with the principal cortical gyri labeled. Front., frontal gyrus; Par., parietal gyrus; Temp., temporal gyrus.

Methodological considerations

The object of the method when it is applied to animals such as rats and cats is to establish a correlation between neural structure and function, i.e., to relate a learned task to a region of central nervous system. It does not matter whether learning eventually involves rearrangement of macromolecules or the growth of synaptic contacts. In the final analysis it will be a behaving organism that will display the learned responses. As this term is generally understood at present, learning refers to a change in the behavior of an organism brought about through practice. Such behavioral changes, however, take many forms, since psychologists have invented many different types of training. At the same time the brain of vertebrates is a large, complex structure and one needs to limit the terrain he explores. Even Lashley's results on maze learning in rats show that functional equipotentiality applies to the cortex only and not to the whole brain. The hope is that a particular learning task will be shown to depend on the integrity of a particular brain region and that this correlation will identify the place where further search for the underlying mechanisms can be conducted.

To establish such a functional localization, the criterion of "double dissociation" is usually required.[11] That is, brain lesion A interferes with learning test "a" and does not influence learning test "b." At the same time, lesion B affects test "b" but not test "a." As I have already suggested, the localization of visual discrimination learning to the monkey's temporal cortex essentially fulfills these conditions.[12] Thus, monkeys with temporal lesions still learn and remember other tasks, such as somatic roughness discrimination, and behave normally otherwise, e.g., they understand the test situation, and so on. Furthermore, lesions in other parts of the cerebral cortex do not affect visual discrimination and yet they reduce the efficiency of performance on other tasks. In this case the criterion of double dissociation is satisfied.

Brain lesions, especially subcortical ones, produce behavioral defects unrelated to the test by rendering the animal incapable of learning. For example, several earlier papers have discussed the role of midbrain reticular formation in sustaining an animal's arousal, consciousness, and attention. In cats, the reticular formation can be completely transected in successive stages without ever totally incapacitating the animal.[13] Figure 4 shows the final extent of such lesions in some cats. Because of the necessarily large area of destruction, such cats display severe motor and sensory deficits, such as partial paralysis, tilted heads, and impaired visual fields. When tested soon after operation, these cats had difficulty learning anything, and if they had been trained on visual discriminations prior to surgery, they failed to retain them. However, when the cats were allowed a long convalescent period, and in addition were helped by the experimenter, they ultimately came to recall, and even to acquire and retain visual discriminations as well as do normal cats. The tutoring seemed to help the animals readjust to the surgically induced perceptual-motor distortions from which they suffered postoperatively. Once such cats overcome these disrupting influences, no true learning defects are evident. Essential controls must therefore be provided for such nonspecific effects as sensory-motor deficits and motivational and emotional changes.[14]

Four additional methodological difficulties inherent in the ablation technique can be pointed out.[15] First, if a brain lesion fails to affect a learning task, it cannot be stated that this part of the brain is unimportant in normal animals. Second, if the lesion does influence performance of the task, it does not necessarily mean that it is the only neural structure involved. Third, the aim of the ablation method is in a way never attainable, for it throws away the object (a region of the brain) one wishes to study. As a result, it reveals only what the remaining, surgically altered system—but not the missing part—can do. Fourth, it is a method of analysis at a gross structural level. Rarely can it be used to study cellular or subcellular mechanisms.

The first two points listed above are easily appreciated when the results of ablation studies are compared with results obtained with other methods. For example, earlier chapters show that widespread alterations of gross electrical potentials occur during conditioned reflex training. The data suggest that learning involves many more brain structures than those that ablation methods suggest are essential. On the second point, lesions of lateral hypothalamus are known to affect an animal's learning of an avoidance response, such as jumping over a hurdle to avoid a foot shock.[16,17] This lesion, however, also causes a significant decrease of serotonin and norepinephrine in many forebrain structures, including those that have no direct connections to lateral hypothalamus.[18,19] So one asks, are the behavioral effects caused by destruction of the hypothalamus or by the secondarily induced changed brain monoamines?

A more direct approach to counter the third point (we throw away what we wish to study) is to use a preparation with all brain regions destroyed except the structure in which one is interested. There are practical limitations to this proposal. However, some attempts to create such a preparation have been made. For instance, I have tried to study visual discrimination after the following surgical procedure. First, one optic tract, together with the corpus callosum, anterior and the posterior commissures, of the

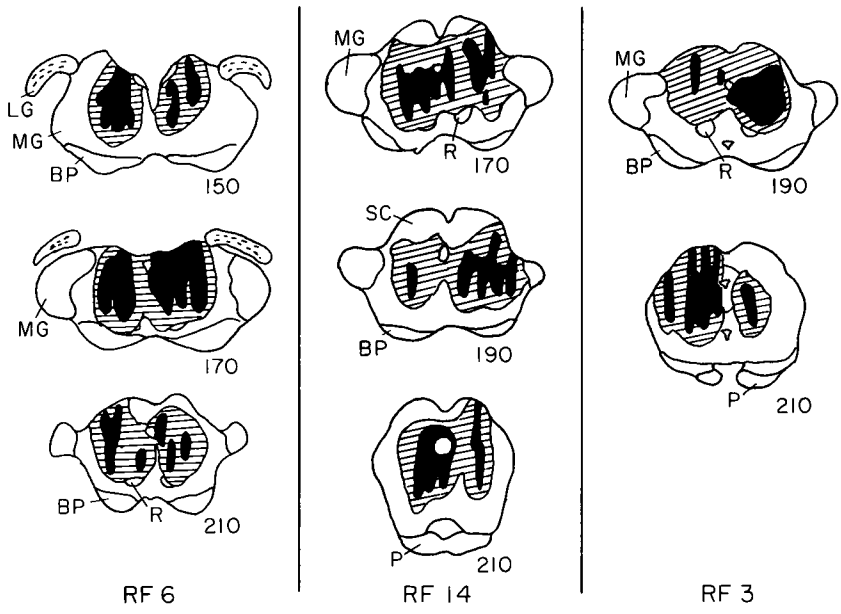

FIGURE 4 Cross-sections of the brain stem of 3 cats with complete transection of the reticular formation. The central necrotic zone is solid black; the surround lesion area with complete destruction of neurons is shown in hatching. The anterioposterior extent of the lesions ranged from 4 to 6 mm. BP, brachium pontis; LG, lateral geniculate body; MG, medial geniculate body; R, red nucleus; SC, superior colliculus; P, pons.

monkey were sectioned. This channels all visual inputs to one hemisphere, at least at the cortical level. Second, the cortex on the side of the visual input was destroyed, except for the visual and temporal cortices. Hence, these monkeys had (more or less) only one visual and one temporal cortex with which to deal with visual information. The remaining hemisphere was left intact so that the animal could sustain its motor and other activities. These monkeys learned visual discriminations at a normal rate, and they displayed the expected visual learning and retention defects when their temporal cortices were additionally ablated. Therefore, these results agree with those obtained when only temporal lesions are made. Furthermore, when such a monkey had survived the final temporal cortical ablation, which left it with only one visual cortex, it could still learn simple visual discriminations.[8]

Organ level of analysis

The methodological limitation that only gross regions are usually studied is shared by most other methods. Evoked potential and electrical brain stimulation methods are also limited to analysis at a gross structural level. However, they attempt to uncover significant trends throughout the learning process rather than simply the end result of the process. Similar statements could be made about many biochemical studies.

In another context, Diamond and I have considered that this organ level of analysis has its place in the organization of biological sciences.[9] The integrated activity of a group of neurons is perhaps more relevant than the action of any single neuron, especially to problems of learning and memory. It may be argued that even when the underlying molecular process is uncovered some day, learning at the behavioral level will not be fully explained until unique properties emergent from that process are considered. Thus, the strategy of research could follow the usual pattern of moving from organisms to organs, then to cells, then to subcellular elements. At each step one studies a phenomenon and hopes to understand it by invoking some mechanisms derived from the next level of analysis. On the other hand, a different approach could be to combine and adapt all methods that are appropriate to solve a behavioral learning problem and to answer a specific question, regardless of the level of analysis.

This structural-functional analysis often has been used to test some neural models of learning, such as the establishment of Pavlovian conditioned reflexes caused by the formation of discrete and specific neural paths. This approach proved to be futile. Lashley's summary of how the engram could not be located illustrated this point elegantly.[20] His own work provided many instances in which the lesion technique was used as a tool to answer questions. For example, the application of mass action to rats' maze learning is not explicable by the increasing sensory motor defects of large cortical lesions, which in turn cause increasing learning and retention deficits. He showed that rats with cerebellar or spinal cord lesions might have to alter their normal mode of locomotion but could still learn and remember the maze by staggering or

rolling through it.[4] Even in blind rats, visual cortical lesions still retard maze learning, indicating that functions other than vision reside in the visual cortex. Therefore, the ablation method should be viewed as but one of several ways of studying parts of the nervous system at the organic level. The generalizations it yields not only give important clues, but also impose constraints for the further search for learning mechanisms at cellular or biochemical levels.

Contributions of the ablation method

Once a functional localization is established, the ablation method could be used to analyze further the neural mechanisms involved in such a correlation. Along with electrophysiological data on the effects of stimulating and recording the brain, ablation results help identify the region to be investigated by biochemical or other methods of analysis. There are several additional facts about these results that may guide the direction of further research.

A Particular Lesion May Be Effective in One Type of Learning Task and Not in Others One of the most common findings is that destruction of a brain structure usually affects only a certain type of learning but not others. Studies on learning in rats, for instance, show the following: (1) For "trial and error," alley-maze learning, the entire cortex functions equipotentially, i.e., destruction of any one part is equally disruptive to any other part.[4] (2) For visual "discrimination learning," only lesions of the visual cortex are effective.[21] (3) For "instrumental conditioning," ablation of either the posterior or the anterior half of the cortex retards the rats' learning, but the posterior cortical lesion has much more effect than the anterior lesion.[22] One way to test this finding is to train rats to respond to a light stimulus by jumping over a hurdle to avoid electrical shocks. (4) For some "classical conditioning," such as conditioned cardiac response, rats with the entire neocortex destroyed will still learn at a normal rate. For this task a shock is paired with a light stimulus. A shock-induced change of heart rate is the response. By repeated pairings of light and shock, the light alone induces the heart-rate alterations.[23]

All the above tasks required the rats to respond appropriately to visual stimuli. (However, cues other than visual ones may also be used in solving the maze problems.) Thus, the results indicate that the locus of visual learning or the site of formation of visual conditioned reflexes can either be distributed equally throughout the cortex, located exclusively in the visual area, dependent more on the posterior than the anterior half of cortex, or not be present at the cortical level at all. Figure 5 summarizes these results.

Similar evidence for multiple representation of learned responses exist. One group of results show that in the monkey visual discrimination learning was affected by temporal cortical lesions, somatic roughness discrimination by posterior parietal lesions,[12,24] and complex voice discrimination by lesions of superior temporal gyri.[25] Each learning task is localized in a specific cortical area. Another group of results show that in cats the effect of the same auditory cortical lesion (including auditory areas I, II, and posterior ectosylvian region) on the response to two tones depends on how the tones are presented. This particular lesion did not affect the learning rate if the animal was required to jump over a hurdle the instant a

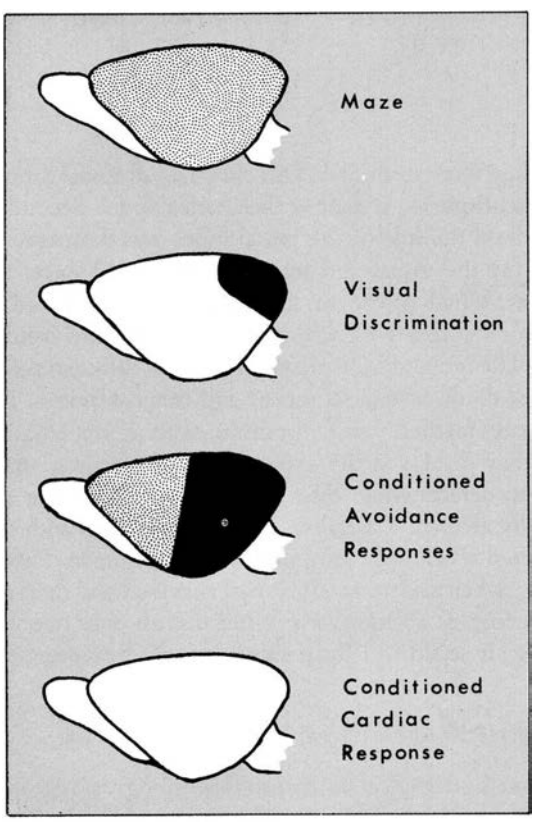

Figure 5 Graph to show differential effects of cortical lesions on various learning tasks in rats. In maze learning, the entire cortex is equipotential. In visual form discrimination, only the visual area is critical. In conditioned avoidance response to visual stimuli the entire cortex is involved, but lesions of the posterior half are more detrimental than lesions of the anterior half. In conditioned cardiac response to visual stimulus, complete decortication does not affect the learning or retention of this task.

low tone (800 cps) was changed into a high tone (1000 cps). However, the lesion would render the cat incapable of learning to respond to a change from a low-high-low (800 cps to 1000 cps to 800 cps) tonal sequence to a high-low-high tonal sequence.[26]

Results such as these raise the question of whether learning is a unitary process for which a universal neuronal or biochemical mechanism should be sought. Perhaps learning is always specific to the behavioral task used to reveal it. There may be different neural substrates for trial-and-error learning, for discrimination learning, for instrumental conditioning, and for Pavlovian conditioning. Furthermore, even though a unitary process might be found, it is possible that some more molar mechanisms will always be needed to render it applicable to behavioral learning situations.

CRITICAL PATHWAYS CAN BE IDENTIFIED Ablation studies suggest that different neural pathways may be essential for each learning task, as the following examples will show. When Lashley found that either deep knife cuts did not interrupt long association fibers of the rat's brain or subcortical lesions did not affect maze learning, he was obliged to conclude that the maze-learning function, distributed equipotentially, must be conducted through short fibers within or immediately below the cortex.[4] On the other hand, in a visual task in cats Sperry has found that cutting these same intracortical fibers in the visual area does not disrupt a learned visual discrimination.[27] This apparent contradiction does not reflect poor observation; both results are correct.

In the monkey, the only known cortico-cortical connections of the visual areas are those to and from the adjacent peristriate region. From there postsynaptic fibers carry visual impulses to other cortical areas. The temporal cortex also has two-way connections with the subcortical nucleus pulvinaris medialis and no others. Therefore, for the temporal cortex to exert its influence on visual function, impulse conduction must travel through either one or both of these two routes. However, large lesions of the pulvinar, or large undercutting of the temporal cortex, designed to interrupt the cortico-subcortical pathways does not affect a monkey's visual learning. On the other hand, cross-hatching the temporal cortex with superficial knife cuts that interrupt cortico-cortical paths has the same detrimental effect on performance as ablation of the temporal cortex. Thus here, as in Lashley's case, it is the cortico-cortical connections that seem primarily involved in subserving the information required in learning.[8,28]

MINIMUM TISSUE VOLUME REQUIRED FOR LEARNING AND MEMORY CAN BE IDENTIFIED The integrating activity of the visual cortex is absolutely essential if a rat is to learn to discriminate forms, i.e., to choose a black square from a white disk. Complete destruction of this area renders the rat incapable of learning the task, and no other cortical lesion affects it. However, if as little as one-sixtieth of the visual area is left intact, the rat learns form discrimination in a normal fashion. If this task was acquired before surgery, the rat remembers it perfectly afterwards.[29] Similar findings were recently reported for cats. In this case, as long as a small per cent of optic nerve fibers remained after surgery, a cat could learn and retain visual discrimination.[30] These results show that any small piece of tissue can take over the function of the whole area, suggesting the importance of following the dynamic aspect of neural organization.

In the visual system the spatial relations of the stimulus are more or less preserved all the way from the retina to cortex. The above results suggest that many similarly organized sets of neural patterns must be transmitted centrally in a parallel fashion by the system. On the other hand, if only one set of patterns is transmitted through the foveal pathway (since a normal animal usually uses its foveal vision only), then this spatial pattern must repeat itself throughout the visual cortex. In either case, specificity of transmitting visual information and its integration into learning functions does not depend on any one set of anatomical pathways. Perhaps at the neuronal level a redundancy of partially overlapping, patterned networks automatically sets up and saturates the entire visual area every time an animal learns a visual discrimination. Or perhaps there is an initial center of excitation, subserving the learning function, that subsequently spreads out through the short axon interconnections peculiar to the visual area. Both these mechanisms could function equally well at any one time and in any one small area. It is even possible that the same neurons could be involved in both processes at different times.

This phenomenon of small remnants substituting for the function of a whole area is not specific to the visual system. After a cat learns a conditioned avoidance response to tonal pattern, such as jumping over a hurdle when a background, low-high-low tonal sequence is shifted to a high-low-high sequence, ablation of auditory areas I, II and EP abolishes this learned auditory habit, and the cat cannot reacquire it. However, if a small piece of auditory area I is left intact, the cat shows good retention.[31]

TASK DIFFICULTY INFLUENCES LESION EFFECTS In many instances, cortical lesions of the same extent cause more impairment of difficult tasks than of easy ones. Here the degree of difficulty is usually defined by the learning

rate, that is, the faster an animal learns a task (in terms of the number of trials or errors to criterion), the easier the task is. Figure 2, showing the effect of cortical ablation on maze learning, illustrates this point. In a rat, cortical lesions of the same size led to greater deficit in an eight-alley maze than in a one-alley maze. This result raises questions about the neuronal organization and biochemical mechanisms involved. Because in this case the locus of lesion is not critical, and if there is a unitary process for learning, the mechanism must be distributed evenly throughout the cortex, be it an alteration of networks or of molecules. Thus it follows that it takes longer to learn a difficult task than an easy one, and that the lesion causes an additional slowing down of the process in the remaining areas. This, in turn, suggests a mutual facilitation in creating this unitary process in the cerebral cortex, which is quantitatively different with different degrees of difficulty.

This differential effect appears not only in the initial acquisition process, but also in the performance of some but not all previously learned tasks. For example, if a monkey was trained to choose the larger of two circles, it would make more errors in discriminating between them when the difference in the size of the circles was small than when the difference was large. Thus the degree of difficulty of this task is correlated with size differences. After temporal cortical ablation, the animal can still discriminate between circles showing large size differences (although with more errors than before), but not between circles showing small size differences.[32]

In this case, both the difficult and easy discrimination task was functionally localized in the temporal cortex. One could suggest the following postulates to account for the result: (1) There are different substrates for the storage of this learning function, each with different degrees of difficulty. They are located in the temporal cortex but not coextensively. The ones for difficult tasks are concentrated at the center of the temporal cortex, but those for easy tasks are spread out in the surround as well. (2) There is a unitary storage mechanism that is outside the temporal region, but there are different substrates for recall within this region. The same lesion in the same animal would abolish selectively, in a graded fashion, the substrates for either storage or recall mechanisms. Here I have assumed that other extraneous factors, such as emotional and attentional effects, are adequately controlled.

AMOUNT OF TRAINING INFLUENCES LESION EFFECTS Suppose two visual discriminations equated for the degree of difficulty were given to a monkey preoperatively. On one of the tasks, training was terminated when the animal attained a criterion of 90 per cent correct response in 30 trials. But with the other task, several hundred additional trials were given. After temporal lesion, the same animal will forget the discrimination learned just to criterion, but remember the one on which additional training was given.[33,34] These results may be comparable to some clinical data. After surgical removal of epileptic foci in the temporal lobe, patients do not recall tests presented to them immediately but remember events in their past.[35,36] The question of why recent memory or tasks that are barely learned are more fragile and more easily disturbed by brain lesions has not been analyzed.

Two possible mechanisms could account for the above monkey study. First, whatever the substrate for the discrimination learning may be, it is formed and stored in the temporal cortex, and with additional training, spreads out to other parts of the brain. A process of self-reduplication of neural networks, such as Lashley proposed to account for the functioning of small remanents, could be applicable here. A second possibility is that an entirely new substrate for the overlearned habit could be stored somewhere else independently during the additional training period. This second view, plus the clinical data discussed above, presumes a variety of qualitatively different neuronal or molecular mechanisms underlying recent or old memories, while the first view suggests a continuous growth of the same substrate.

LEARNING AND MEMORY FUNCTIONS LOST THROUGH BRAIN LESIONS CAN BE RECOVERED AFTER FURTHER TRAINING One common finding of ablation studies is that the lesion effect is usually reversible. Although a monkey with temporal lesions has difficulty in learning a visual discrimination, it usually acquires this task, provided that much more than normal training is given. Also, although a temporal lesion abolishes a monkey's retention of a previously learned discrimination, the animal usually reacquires this task with renewed training. This recovery of function points to the enormous potential of many parts of the brain to take over functions with which it does not normally deal. Experimental results also indicate different degrees of this functional recovery, depending on the learning task used and the species and ages of the animals. An adult monkey or cat will not recover (or only in a very limited way) visual form discrimination after complete destruction of visual cortex. However, when the same tissue is destroyed in kittens they show as adults no difficulty in form-discrimination learning.[37]

In contrast to the recovery of function through retraining, the concept of "diaschises" in clinical literature has been used to describe spontaneous recovery after brain injury without re-education. It refers to a withdrawal of facilitation of some other area caused by the

cortical lesion and the subsequent loss of function of the facilitated area. After a period of time this area receives other sources of facilitation and recovers its function. Whether a learning loss after brain lesion could recover spontaneously has not been studied adequately. There are some indications that after temporal lesions a monkey may recover the retention of a lost visual discrimination if it is trained on some other, new discrimination tasks. In other words, postoperative recovery of a habit may not depend on practicing that specific habit.[38] If this result could be confirmed, the lesion effects indicate not a loss of memory traces but a disturbance of retrieval mechanisms.

In both types of recovery after brain injury, there are only inconclusive results from studies on whether the recovered functions are carried out by some other area than the ablated loci. It seems likely that the recovery depends more on the reorganization of the remaining tissue.[4]

Summary

The ablation method has been the most widely used technique in studying the neural substrates of learning in behaving animals. It contributes to establishing structural-functional relationships between a gross neural structure and a learning task. In spite of its many limitations, this method has revealed some significant generalizations that further analytical studies of neuronal and/or molecular mechanisms must take into account. These are: a particular learning task is usually affected only by lesion of a specific brain region; information transfer of a learning function depends on a particular neural pathway; a small part of remaining cortex can take over the function of the entire area; brain lesions differentially affect easy and difficult tasks or learned and overlearned tasks; most of the learning functions abolished by brain lesions can be recovered by re-education.

These results, together with some theoretical considerations raised by Lashley and Hebb, emphasize the dynamic aspects of neural organization for the types of learning in higher organisms. Such a view "downgrades" the theory of the formation of fixed connections and relies more on the probabilistic outcome of the patterns of neuronal networks in learning situations.

Split-Brain Approach to Learning Problems

R. W. SPERRY

THIS CHAPTER deals with the problems of learning and memory at a level somewhere above that of the brain cell and below that of the whole organism and behavior. The approach might be said to attack learning at the cerebral network level, with an ultimate aim of "breaking the brain code" and understanding the inner "language of the hemispheres." For reasons spelled out elsewhere,[1-3] we have gambled our research efforts largely on the view that the major mysteries in learning and memory, and in the higher functions of the brain in general, lie at this level; i.e., the level of cerebral circuit organization. This view holds that a single brain cell in itself probably does not perceive, think, or emote, nor imagine, decide, reason, nor perform other of the mental functions for which brains are particularly noted, and that a single brain cell probably does not learn or remember anything very elegant. Most or all of these special higher mental or psychic properties of brains are speculatively assumed, in this view,[1-4] to be properties of highly organized circuitry in action, the analysis of which must be tackled accordingly.

To be able to correlate the variables we know in conscious experience with the unknown variables of brain excitation constitutes a primary, though somewhat remote, guiding goal for most of our work in psychobiology. How does the brain code different colors, sounds, flavors, and whole images of the outside world into patterns of cerebral excitation? In brief, it is the problem of the cerebral correlates of subjective experience. As a specific example, take the scene that I can see outside my window. It includes a parked car, a moving taxi, two trees, and a pedestrian. What can we say now about the nature of the inside visual image within the brain? Whether our sample visual image be an initial perception, a particularly vivid visual recall, or exists merely in the form of an illusion, a dream, or a hallucination, there presumably is nothing within the brain that is even remotely similar to the three-dimensional outside scene. The whole scene is somehow re-created, recast, transformed, or coded into the language of the hemispheres. The outside scene, that is, is represented or transformed into a spatio-temporal pattern of cerebral excitation.

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The basis of this cerebral representation or brain code still completely eludes us. We assume, of course, that it probably is constructed or constituted of neuronal and perhaps glial activity; that is, of nerve impulses and associated physiological and biochemical events. But this kind of knowledge is somewhat analogous to knowing the chemical constituents of the ink and paper that have been used to print a particular message in an unknown language. When it comes to the meaningful aspects of the brain process, i.e., the symbols, syntax, and logical design of the code used by the brain to build our sample visual scene and other conscious experience, we are still very much in the dark.

Along with various subsidiary codes in the brain, there is also another basic code that is directly and necessarily involved in learning—the memory trace or engram code. The general brain code proper, by which the outside world is brought inside, consists of active, dynamic excitation patterns having a temporal dimension. By contrast, the engrams or memory traces have a static or spatial organization that makes them independent of the dynamic factors and able to survive periods like those of deep anesthesia, electroconvulsive shock, and cerebral anoxia, in which all dynamic organization fades out or is violently disrupted. The memory traces may lie dormant or inactive for years before the particular experience involved is recalled into activity.

Just as the inside brain process for a visual image differs greatly from the actual outside scene represented, so also must memory traces for reactivating that same brain process differ greatly from the active process itself. Especially, there is reason to think that the engram is highly abbreviated. Presumably a few small, critical changes in the cerebral structure are sufficient to steer the over-all dynamic pattern into a specific form, given an appropriate background or context of cerebral activity. In the calling up of a particular memory, the engram and the active process may be thought of as cofunctions that are mutually dependent in most cases.[5] Some behavior patterns are so thoroughly ingrained and completely supported by engrams that they can be activated in nearly any context. Most memories have particular associative contexts that are necessary for recall.

Accordingly, it would seem a logical necessity that, in

order to analyze the engram code for memory, we must first understand something about the organizational principles or language of those cerebral dynamics in terms of which the engram traces are laid down. It was in this sense that we once described the memory problem as the quest for the secret code of an unknown code for conscious experience, itself a will-o'-the-wisp—a situation with possibilities for confusion, unlimited.[1]

In the problem of learning we must deal, of course, with both of the above two codes, the dynamic and the static. For convenience, we can lump these and related aspects of brain function with which we shall be concerned under the general category of problems in cerebral organization.

Split-brain approach

During the past dozen years or so we have become increasingly convinced that a handy way to approach many aspects of the above problems in cerebral organization is to start by dividing the brain down the middle into its right and left halves.[6,7] From there one may go on to study problems of crossed integration, or integration problems within either of the separated hemispheres, or combinations of these. The vertebrate brain, you recall, is a bilaterally symmetrical organ, and the two hemispheres of the mammalian brain are already fairly separate anatomically in the natural state except for some cross bridges of connecting fibers, the cerebral commissures. It is quite possible to section the commissures surgically either in their entirety or in selected portions, as desired. The complete section of these cross-connecting cables leaves two separate, functional half-brains, each containing for analysis most of the main mental properties of a whole brain. Various experimental advantages are found in working with the two hemispheres separately instead of together as a single organ.[6,8] The bisected preparation provides, for example, superior controls of a quality not obtainable in any other way. There are also a larger variety of circuit combinations for experimental analysis. Further, the possibilities for analysis by surgical elimination of selected centers and cortical areas are greatly extended when the removals can be restricted to a single hemisphere, leaving the corresponding centers intact on the opposite side to maintain background functions.

To help visualize the situation, a schematic view of the bisected monkey brain is shown in Figure 1. Essentially, the commissurotomy leaves two cerebral hemispheres sitting on top of a single brain stem. All direct means of cross communication may be eliminated, or selected parts of the cross-connecting system may be left intact for specific study. Section of the corpus callosum in its entirety plus the anterior commissure eliminates cross communication for the neocortex, which constitutes the great bulk of the cerebral cortex of primates. The optic chiasm is also usually sectioned in experimental studies that involve vision, thus restricting the visual input from each eye to the one hemisphere on the same side of the brain. Each eye thus transmits the contralateral half of the visual field to its respective hemisphere; retinal rivalry and stereoscopic vision are eliminated.

It has become fairly routine to carry out these surgical divisions down through the roof of the midbrain, leaving most of the midbrain tegmentum and all structures below intact, and sparing the decussation of the trochlear nerve, which in the monkey is easily seen and avoided. For completeness, the front tip of the tegmentum may also be divided to section the supramammillary commissure. Brain bisection carried to the extent indicated above (Figure 2) is only mildly incapacitating, at least in terms of laboratory existence. Following recovery, monkeys so operated upon are hardly distinguishable from normal animals in their ordinary laboratory behavior.

Marked functional symptoms begin to appear as brain bisection is extended deeper into the tegmentum, pons, and cerebellum. Division of the cerebellum is easily added to the above, but it may leave the monkey shaky and unsteady on its feet for some months afterward, depending on how symmetric and central the section. We have carried out a few deeper sections, extending down through the pons and below, but only on an exploratory basis thus far, and in studies that were aimed at other problems. The minimal symptoms produced by such clean, deep bisections under optimal conditions remain to be determined. It appears feasible, however, to obtain for study apes, monkeys, and lower forms with the brain completely divided down through the pons. Selective preservation of certain structures like the brachium conjunctivum would be highly desirable in many studies. One can foresee endless experimental possibilities that should go far toward an unraveling of cerebral organization, by use of complete or selected partial midline disconnections such as those mentioned above, in combination with lateralized surgical ablations and electrolytic lesions together with lateralized training and testing techniques, electrode implantations, drug injections, and other methods. The disadvantages of this approach fall mainly under the heading of "cumbersomeness." The surgery sometimes involves a long series of successive sections and ablations, and can get into an extended program in itself. Also, the lateralized training and testing techniques may get involved and lengthy, even with automated programing. In general, an average experiment has required months—up to a year or more—rather than weeks or days.

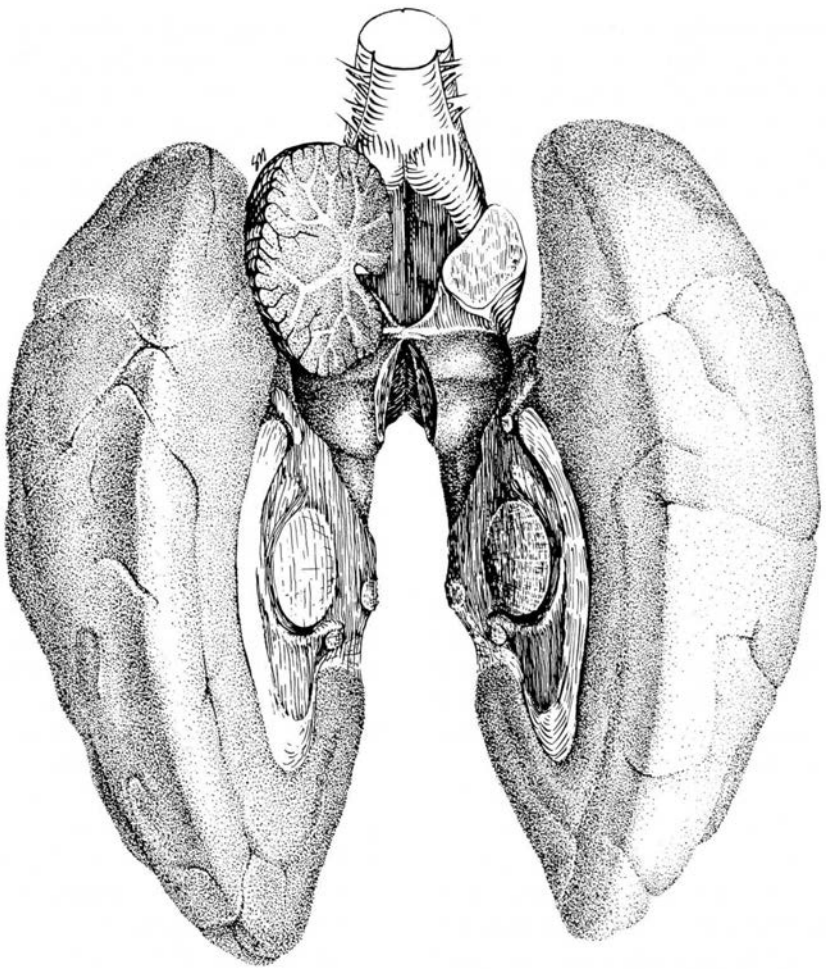

FIGURE 1 View of monkey brain bisected through tectum and cerebellum (schematic).

Complaints about the difficulty of the surgery also continue to be heard, but I don't believe that the surgery in itself need be a major obstacle if one is willing to use proper tools. A good dissecting microscope is essential for cat, monkey, and similar or smaller brains. The microscope should have a light beam set in line with the optics so that it penetrates straight to the bottom of the deep operating field in crevices between the hemispheres. I also use almost continuously the two instruments shown in Figure 3. These are tailormade in different sizes and gauges for specific purposes. The first is a double retractor, Figure 3A, that serves to separate and to hold apart the hemispheres and other deeper structures (Figure 3C). The cutting and other operations are carried out between the blades of these double retractors, using the aspirating needle-knife shown in Figure 3B, which is hollow throughout and connected to a vacuum line. The tips are interchangeable and are made of fine hypodermic needle points in a variety of gauges. Those with tips of gauge No. 26 to No. 30 are used most extensively. In fixed sections of brains that have been divided in this way, one sees very little tissue damage aside from the destruction of the commissures themselves.

BISECTION OF THE LEARNING MECHANISM We turn now to some of the broader features of the findings as they relate in particular to learning and memory. By far the most striking effect of this kind of surgery, speaking very generally, is the establishment of two entirely separate mental domains within the same cranium. Following surgical separation of the hemispheres, things experienced, learned, and remembered by one hemisphere remain quite unknown to the other. The learning experience of the one is inaccessible to, and outside the conscious awareness of, the other hemisphere, almost as much as is the case with two separate brains in separate skulls. As far as we can tell

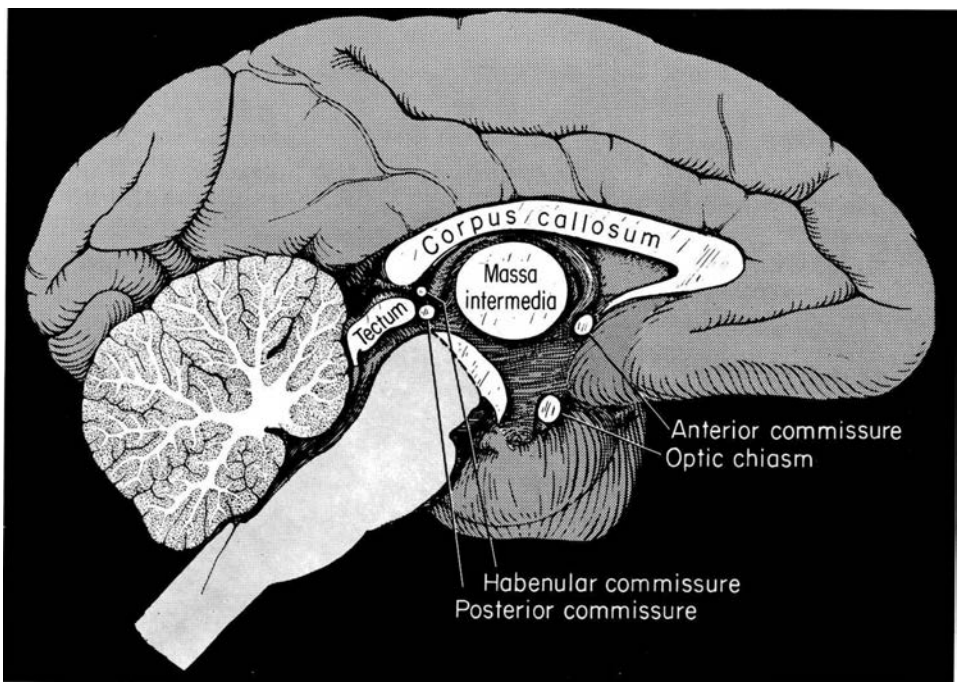

FIGURE 2 The labeled midline structures can be sectioned with only mild effects on ordinary laboratory behavior.

from the evidence to date, it would appear that in the split-brain syndrome, we deal with two separate minds, i.e., two separate realms of conscious awareness, two separate sensing, perceiving, thinking, and remembering systems.

This is shown in many different ways: For example, when the normal individual sees something to the left of his nose and then a little later perceives the same object in the opposite half of the visual field, this same object is, of course, recognized as being the same one seen previously. Not so in the split-brain person, cat, or monkey.[6,9,10] Following disconnection of the hemispheres, things seen in one half-field of vision cannot be recognized in the other half-field. In other words, things seen or remembered through the use of one hemisphere are not recognized when seen through the other hemisphere. Remember that all vision to the left of the vertical midline of the visual field is mediated by the right hemisphere and all vision for the right half of the visual field is mediated by the left hemisphere. Normally, the two half-fields of vision are integrated in the brain into a unified whole, but after section of the commissures each half-field functions separately. Instead of just one inner visual world, therefore, the split-brain animal or man has two inner visual worlds, each quite outside the conscious awareness of the other.

This is demonstrated in human patients who have had cerebral commissurotomy by quick-flash, or tachistoscopic, presentation of visual material to the separate right and left half-fields of vision at 1/10th second or less—too fast, that is, for the subject's eye movements to put the visual stimulus into the wrong or unintended half-field.[10] For example, if a picture of some familiar object such as a pencil, spoon, or cigarette is flashed to the left half-field, the split-brain subject is at a complete loss to try to recognize or identify the same picture when it is flashed to the right half-field. Subsequent memory and recognition are quite normal, on the other hand, when presentation is in the same, or left half-field.

In other words, memory traces laid down in one hemisphere remain confined to that same hemisphere and influence recall within the one hemisphere only. This applies both to short-term and to long-term memory. The only means remaining in the bisected brain for transferring visual memories from one to the other hemisphere are indirect and not unlike those by which one person informs another of something that he has experienced visually.

In commissurotomized animals, hemispheric separation of learning and memory in the visual sphere is demonstrated by cutting the crossed optic fibers in the chiasm so

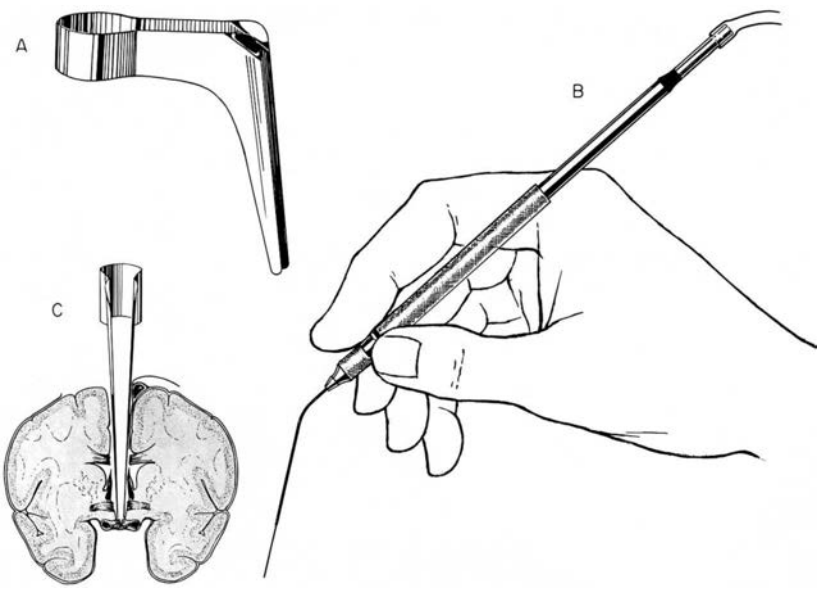

FIGURE 3 Instruments for split-brain surgery. A: Side view of stainless steel cerebral retractor. B: Aspirating needle-knife used between the retractor blades for nearly all the cutting operations carried out between the hemispheres. C: Cerebral retractor in position showing surgical approach to corpus callosum, anterior commissure, and optic chiasm.

that each eye feeds only to the half-field of its own homolateral hemisphere. Independent training and testing of the separate hemisphere can then be carried out by having the animal work through one or the other eye (Figure 4). Monocular vision is obtained by the use of eye covers, contact lenses, peephole arrangements, or differential ocular light filters for polarized or monochromatic light.

Use of special techniques has shown that in animals the separated hemispheres may be trained concurrently and simultaneously to do diametrically opposite tasks. Two mutually contradictory performances may be learned at the same time, something that the normally unified brain, of course, doesn't do. Two sets of polarizing light filters were used by Trevarthen[11] to enable the split-brain monkey to see different things at the same point in space at the same time—again something of which the normal brain is not capable. The filters were arranged so that one hemisphere perceived just the opposite of what the other was perceiving and it was possible to show that with exactly the same set of learning trials, one hemisphere could learn the exact reverse of what the other had been learning with no functional interference evident. Similarly, when different stimuli are flashed simultaneously to left and right half-fields of human patients, separate and conflicting responses are commonly obtained from the two sides, the minor hemisphere expressing itself through the left hand and the major through the right hand speech or writing.[10,12-15]

TWO MENTAL SPHERES FOR MANUAL STEREOGNOSIS

A comparable hemispheric separation has been shown to exist in the somesthetic sphere with reference to things identified by touch with right and left hands or paws.[12,14,16] Recall that the right limbs of a mammal are represented in the left hemisphere and the left limbs in the right hemisphere. In right-handed persons the right hemisphere is usually minor or subordinate to the left. Following surgical disconnection, each hemisphere receives information about the tactual, or stereognostic, activities of its own hand and foot, but has little or no information about the activities of the hand and foot of the opposite side. Deconnection of the hemispheres again seems to create two quite separate realms of inner experience for sensations coming from the right and left extremities. This applies to the right and left halves of the body generally, except that some of the more simple and crude aspects of body sense get bilateral representation in the more axial parts. In the head and neck, bilateral representation is the prevailing rule. In regard to the hands, however, which are the main receptors for stereognosis in man, the cortical representation is more lateralized, and most stereognostic functions

of the two hands are effectively separated by commissurotomy in adult patients.

When an object that has been identified by manipulation with one hand only is then placed in a grab bag out of sight among other objects, a normal individual has no trouble identifying and retrieving the given item by blind touch using *either* hand. A commissurotomized subject, however, can retrieve the item only with the *same* hand, i.e., the hand that was used for the initial identification. An object recognized by use of the right hand cannot be identified with the left hand, and vice versa. In the same way, learned performances developed by tactile training in split-brain animals fail to transfer, as a rule, from one to the other forelimb provided all sensory cues have been carefully confined to the one extremity.[17-20]

INTERMODAL TRANSFER A commissurotomy patient holding a familiar object that has been presented out of sight to the left hand can usually identify the same or a matching object, or a picture of the object, when it is subsequently presented visually. This is the case, however, only if the visual presentation is made in the corresponding half-field of vision, in this case the left half-field. The normal person, on the other hand, can use either or both visual fields for recognizing objects identified by either hand. This also works conversely; that is, if a picture of an ob-

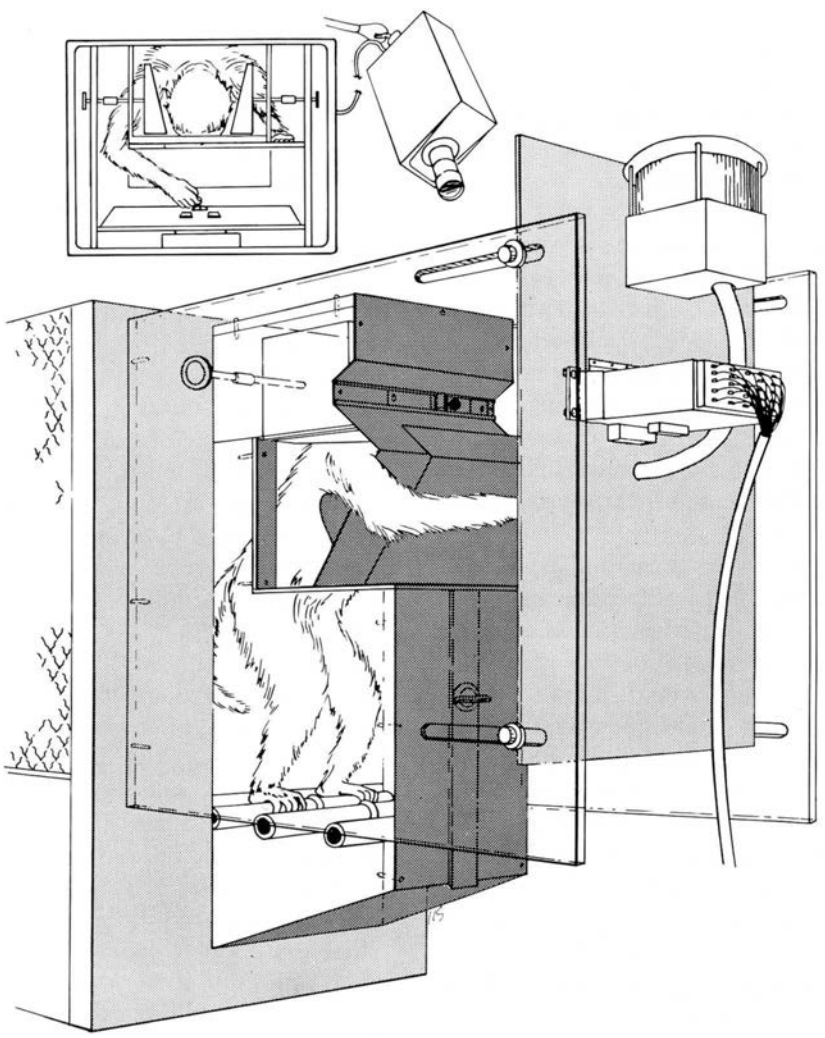

FIGURE 4 Testing unit developed by author for controlling eye and hand use in split-brain monkeys. It may be combined with automated equipment and closed-circuit television as shown, or used with direct manual presentation.

ject is flashed to the left half visual field, a commissurotomy patient can retrieve the corresponding object from a grab bag using blind touch, but only if he uses the hand on the same side. The normal person with commissures intact is able, of course, to use either hand. In summary, cross-modal identification of this sort works readily in these patients within either hemisphere—between the right hand and the right half visual field or between the left hand and the left half visual field—but all contralateral combinations as between the right hand and the left half-field of vision consistently fail. Similar results have been obtained in the monkey.[21] Again we emerge with the conclusion that the conscious experiences and memory of each hemisphere are quite separate and inaccessible one to the other.

LATERALIZATION OF LANGUAGE In performances that involve language, special problems of cerebral organization are encountered that are largely characteristic of the human brain, although they also have some general implications.[14,15] One of the most striking symptoms produced by disconnection of the hemispheres in man is the inability of these patients to describe in speech or writing anything presented to the left hand or the left half visual field. The engrams for speaking and writing seem to be confined almost entirely to the one, the dominant hemisphere, with indications that certain other functions, such as the construction of spatial relations and spatial orientation, are better developed in the right minor hemisphere. Following commissurotomy, the dominant hemisphere talks and writes and communicates generally in a manner hardly distinguishable from normal. Mathematical calculations are also carried out by this hemisphere at approximately the preoperative level.

The minor hemisphere, on the other hand, is rendered almost mute and agraphic. The possibility that a few simple, familiar words can be spoken when they are properly prompted by the examiner cannot be excluded from evidence now at hand, but in general this hemisphere is able to express itself only through simple motor responses like manual pointing, signaling, or drawing. Ordinary tests for perceptual and intellectual capacity that rely on verbal or written expression give an initial impression that the minor hemisphere is generally agnostic, but this clearly is not the case when nonverbal, manual readout is employed. In the two cases most thoroughly tested it seems clear that the minor hemisphere can comprehend both printed and spoken words, but this passive comprehension seems limited to familiar object nouns and perhaps simple adjectives.[13-15] It is uncertain to what extent this language comprehension in the minor hemisphere may have been favored by early brain damage in these epileptic cases or reflects postsurgical learning. In the minor hemisphere, calculations like speech and writing are negligible.

These patients recover the ability to write with the left hand within the first six months after surgery. Tests with lateralized input show that the control, however, comes from the major, not the minor, hemisphere. Writing with the left hand tends to be carried out with the shoulder and upper arm reflecting the greater ipsilateral cortical control over the axial and proximal musculature. The extent to which each hemisphere can control its ipsilateral limbs shows considerable individual variation, correlated largely with the extent of pre-existent brain damage. This suggests that the ipsilateral control mechanisms are delicate and easily disrupted.

We know that the minor, as well as the major, hemisphere is quite capable of learning speech and writing, and does so readily when the dominant hemisphere or the corpus callosum is eliminated in early childhood. In a very small percentage of the population, speech is found to develop bilaterally in the presence of the callosum.[22] Thus, it would seem to follow that an important function of the callosum in the normal human individual is to prevent the bilateralization of learning and memory, especially in the case of language. This seems directly contradictory to the situation in the lower mammals, in which learning and memory tend to be strongly bilateralized in the presence of the callosum and unilateral only after its removal. In split-brain cats a remarkable right–left symmetry has been observed in the learning curves of the two separate hemispheres for both visual and tactual discrimination problems.[16,23] In our split-brain monkeys[24] this kind of right–left similarity was much less evident, and even with the callosum intact, monkeys show some tendency toward unilateral learning.[25] The over-all trend among the higher mammals thus seems to be away from right–left symmetry in hemispheric function.

ROLE OF THE NEOCORTICAL COMMISSURES This brings up the general problem of the basic physiological role of the neocortical commissures. Anatomical and related studies on monkeys and apes have indicated a great predominance of symmetrical cross connections that link mirror foci or homotopic points in the two hemispheres.[26] The anatomical picture has thus appeared to favor a symmetrizing influence of the callosum that would tend to duplicate hemispheric activity and keep the two hemispheres equally advanced in learning and memory. On the other hand, the obvious functional disadvantages that would follow from imposing bilateral symmetry on certain functions suggests that the basic contribution of the callosum may better be conceived as being complemental and supplemental in design rather than symmetrical. Accordingly, it has been inferred that a closer look at the

detailed anatomy of the callosum might disclose much more asymmetry, both local and heterotopic, than initially supposed.[27] From electrical studies on the cat it has appeared that the callosum may mediate a rather faithful transfer of the raw sensory input.[28,28a] On the other hand, the sparseness of cross connections between primary sensory and motor projections as contrasted with the richness of connections between associational cortical areas has long been taken to mean that the commissures must be concerned less with raw sensory and motor information than with the deeper stages of data processing.

The latter, along with the recent experimental evidence that the callosum is directly involved in the intercortical transfer of learning and memory, has led us to believe that a detailed study of the connections and functions of the callosum should furnish a rather direct approach to some of the basic principles of cerebral organization. Microelectrodes implanted in specific functional sectors of the callosum, such as that cross-connecting visual areas 18 and 19, should tap high-level inside crosstalk, and provide a sample of the brain code that might be made interpretable in specific experimental situations. Other speculations have implied that the commissures might be largely inhibitory in nature, their function being to prevent interference from the opposite side when a given process that is centered in one hemisphere is in command. Electrical and lesion studies[28-30] indicate a generalized tonic function for the callosum.

That the commissures mediate interhemispheric transfer of learning and memory has been demonstrated repeatedly. We don't know yet, however, in what form information of the directly trained hemisphere is carried across by the commissures. Would the information carried across by the callosum be sufficient, for example, to enable a hemisphere to see visual images via an optic input that had passed through the other hemisphere? The visual island experiment,[30] in which visual responses formerly present were abolished by section of the callosum, was suggestive in this connection, but not conclusive. It remains possible that only an abbreviated and abstracted part of the original visual input crosses in the callosum.

Within each hemisphere there are local fiber systems that link neighboring and distant points of the cortical visual map, particularly in cortical areas 18 and 19. The callosum might be regarded in part as an extension of these fiber systems across the sagittal midplane serving to integrate the two halves of the visual field. Somewhat similar integration between the hands is involved in bimanual stereognosis. In the case of the hands, however, the basic plan would seem to be more complicated than just a zippering together of the projected hand surfaces along the ulnar or thenar edge.

Callosal function is demonstrated in experiments in which part of the sensory information required for performance enters one hemisphere and the rest enters via the other, as in right–left cross-matching and cross-comparison problems.[10,20,31,32] The callosum has also been regarded as essentially a decussation in the descending efferent path for volitional movements, especially in man when a movement conceived and triggered in the dominant hemisphere is carried out by the subordinate hand.

In summary, we have not as yet been able to conceive in any satisfactory detail the basic contribution of the neocommissures to cerebral integration. As the evidence stands, it appears likely that a great many diverse functions: sensory, motor and associational, inhibitory and excitatory, tonic and phasic, are mediated by the callosum, and that these are further subject to considerable individual and species variation. It also seems clear that the basic functional plan of the neocortical commissures in the primates, at least, is plastic and subject to considerable remodeling in the presence of cerebral lesions and in the face of specific learning situations.

ENGRAM LOCALIZATION The aforementioned findings show that learning and memory can be confined to one hemisphere after section of the neocortical commissures, and has been taken to favor a neocortical locus for the memory trace, or engram. Having narrowed the engram locus to the neocortex of one disconnected hemisphere, it then became possible to localize further the engrams for particular kinds of memory and learning by the cortical ablation method. Ablations and other removals for localizing can be carried out on a much more radical scale in the split preparation than is feasible in the usual bilateral approach. With this method it has been possible to localize the engrams for tactile discrimination habits in cats within a moderately small island of somatic neocortex.[33] Attempts to carry the localization further in the monkey were interrupted when it was found that somesthetic learning with one forelimb was not always confined to one hemisphere, depending on a variety of uncertain conditions that only now are sufficiently understood to permit continuation of the localization project.[20] It appears that bilateral projection in the sensory somesthetic system permits ipsilateral as well as contralateral learning in the disconnected hemispheres for some types of manual discrimination in which cues arise from proximal joints in the arm or where extremely simple or crude cues from the hands are sufficient. However, any moderately complex stereognostic discriminations made on the basis of stimuli arising exclusively from the palm and fingers of the hand seem to be safely confined to the one hemisphere in the monkey.

Attempts similarly to localize visual learning to a small island of occipital cortex turned out otherwise. It was found, not unexpectedly, that the visual cortex by itself was not enough for visual learning; nor even enough, apparently, for visual perception. "Visual island" cats behaved as if they were almost blind, despite good preservation of the afferent pathways, unless other nonvisual cortical areas were preserved along with the visual and visual-association cortex.[30] The relation of visual function to the precentral motor cortex and to the cortical representation of the body schema in this experiment remain uncertain along with other unknowns that were left dangling with need for further clarification.

Related experiments have been aimed at determining the minimum critical cerebral apparatus needed for different types of perceptual learning and also at assessing the nature of the contribution of particular brain structures such as the hippocampus, motor cortex, caudate nucleus, etc. It was found, for example,[34] that unilateral removal of the dorsal hippocampus in split-brain cats produced animals that could do reversal learning in one hemisphere but not in the other. Further reduction in the somatic cortical-island preparation mediating somesthetic learning has included the combined removal of hippocampus, anterior thalamus, and most of the caudate and amygdaloid complex on the side of the cortical island. New tactile discrimination learning was still possible in this radically reduced cerebral system. The various functions of the ablated structures are still present in the opposite hemisphere, of course, and could contribute in an indirect way to sustain the learning process. However, the specific mechanism involved in engram formation and directly related processes were presumably confined to the lesioned hemisphere. Another project along the same lines has been pursued by Voneida[35] in an effort to dissect out the various cerebral components most directly involved in establishing a conditioned response, and particularly to find the locus and nature of the "new connections" formed in conditioned-reflex learning.

PATTERN OF THE ENGRAM The problem of the nature of the engram in all its complexity is well illustrated in the case of language. Fairly distinct language centers have been delineated, but nothing in the lesion data encourages the search for a distinct local engram or a distinct molecule for each word. The meaning of words and their recall clearly depend heavily on the context in which they appear. To understand the contextual dynamics of the recall process it is almost necessary to understand the nature of the engram and vice versa. To discover the basic membrane, cytoplasmic, or biochemical change of which all engrams are made, will not help much in understanding these problems of engram patterning and their filing and selective reactivation.[21]

Motor learning and the engram formation involved would seem at first glance to be rather different from perceptual and cognitive learning. The mechanisms would seem to be of a more primitive and structural design in which the basic pattern of the engram might be better illustrated. The problem of motor learning raises the old question of the nature of the representation of movement or motor response at the cortical level, a question for which we still lack a clear answer. There are reasons for suspecting that a motor response may be organized in the cortex, not in terms of patterns of motor unit discharge or in terms of muscular contractions, or even in terms of over-all limb or body movements, per se, nor the end position of the anticipated movement. It is attractive to think of movements being organized at the cortical level in terms of the expected perceptual effect of the given response. The details of the means of achieving the desired response effect on this scheme would then be left to other mechanisms. The cerebellum, the caudate, and other subcortical mechanisms would then have to translate the desired response effect into the details of muscle coordination needed to bring about a successful match with the perceived end result.

There are some advantages in considering motor learning in these latter terms when it comes to questions concerning the relation of movement and the preparation to respond to perception and to the brain code in general. We suggested earlier[36] that each movement, in addition to the efferent outflow of impulses for muscle contraction, must also include corollary central discharges to prepare the sensorium for the perceptual effects of the movement in order to maintain constancy of the perceived world. This may yet be correct, but if the movement itself at the higher cerebral levels is organized largely in terms of the anticipated perceptual effect, this might allow a simplification of the central machinery by obviating the need for separate corollary discharges for preregistration of the perceptual consequences of movement. On this basis, motor responses could conceivably be organized in the visual as well as in other nonmotor cortical areas. There is some evidence for this[37] in that split-brain animals are able to carry out visually triggered and visually guided responses from a hemisphere in which the motor cortex has been entirely removed.

Postnatal Growth and Differentiation of the Mammalian Brain, with Implications for a Morphological Theory of Memory

JOSEPH ALTMAN

IN THE COURSE of a prolonged period of postnatal maturation, higher vertebrates acquire a rich perceptual repertoire of the varied objects and events of their surroundings, and gradually develop all those motor skills necessary to satisfy their needs and to contend with their environments. These acquired behavioral capacities are most highly developed in mammals and are associated with the evolution of complex, fine-grained sensory analyzers and articulable manipulatory organs as well as with a correlated evolution of certain suprasegmental brain structures. The progressive increase in the mass and complexity of these suprasegmental brain structures is usually attributed to the evolving sensory and motor capacities that necessitate increasingly more complex neural processing and programing circuits, and also an enlarged storage capacity. In this paper I deal with one facet of the problem of neural functioning—how newly acquired information may be preserved or stored by the brain.

A variety of hypotheses have been advanced recently about the way individually acquired information might be stored by the brain; the postulated neural correlate is called the "memory trace" or "engram." In attempting to classify prevailing ideas we may distinguish, first, between functional ("dynamic") and structural ("static") theories of the memory trace. *Functional theories* assume that the storage of new information is made possible by the changed dynamic state or activity produced by new input patterns in a structurally unchanging control system. An example is the suggestion that the memory trace is caused by the establishment of "reverberating circuits," or the reiteration of an initiated electrical activity through closed neuronal loops. There is ample evidence of the existence of recurrent neuronal pathways in the brain, and activity once started in such loops could functionally persevere and thus keep the "memory" of the input for some time. However, it is generally conceded today that such a functional mechanism cannot account for the lasting storage of memory, as procedures that are known to interfere radically with the electrical activity of the brain, such as anesthesia, freezing, coma, electroconvulsive shock, and the like, do not alter or abolish memory traces of long standing, or "long-term memory." However, because the mentioned conditions do interfere with the consolidation of newly acquired information, as indicated by the phenomenon of retrograde amnesia as a sequela of such treatments, such a functional mechanism could conceivably be responsible for a temporary storage of acquired information, or "short-term memory."

Behavioral and psychological investigations indicate that well-learned behavior patterns and memories of significant past experiences have an extremely long life-span. They may be modified or suppressed by other training procedures or experiences, but they do not usually fade away or decay spontaneously with time. This evidence of the stability and durability of long-term memory has been the strongest support of *structural theories* of memory trace, which assume that the formation of engrams is based on some substrate modification, or alteration in the structure of some elements of the brain, when new information is acquired and preserved. Elsewhere I have described three major structural theories of memory, under the terms of intraneuronal, extraneuronal, and interneuronal theories.[1]

The *intraneuronal theories* of memory trace assume that conformation changes in metabolically stable macromolecules within single nerve cells are the structural alterations that are produced in the brain during learning. A well-known instance of an organic storage mechanism of this kind is the coding of genetic information by the nucleotide sequences of chromosomal DNA and its decoding during morphogenesis and daily organic functioning through transfer of this information to nucleolar and cytoplasmic RNA and proteins. An experimentally tested example of the intraneuronal theory is Hydén's idea of memory storage through alterations in the nucleotide sequences of neu-

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ronal RNA, which he describes in detail elsewhere in this volume.

Intraneuronal theories are commonly known as chemical or *molecular theories,* because their underlying assumption is that lasting molecular transformations or macromolecular conformation changes within nerve cells are the structural bases of the preservation of new experiences. In contrast, the extraneuronal and interneuronal theories may be considered *morphological theories,* because they assume cellular or subcellular tissue changes or alterations in the interrelationship of cells during the formation of a memory trace. In this paper we shall be concerned in some detail with these two morphological theories, with emphasis placed on a specific interneuronal theory.

An explicit example of an *extraneuronal theory* is the idea that modifications in the glial matrix of nerve cells subserves the storage of the memory trace.[2] Nerve cells in the central nervous system are embedded in an extraneuronal matrix of glial cells and their ramifications. These glial elements separate nerve cells from one another and are interposed between nerve cells on the one hand, and the capillary, ventricular, and leptomeningeal spaces on the other. Glial processes also surround the axon of neurons, whether myelinated or unmyelinated. Neuroglia, therefore, could have a paramount role in the regulation of exchange of metabolites, electrolytes, and water between the various compartments of the brain and its neuronal elements. Any changes in the glial surrounding of neurons would alter the structural and functional properties of the latter, and it has been postulated that glial changes may mediate in this manner memory functions.

Interneuronal theories are the oldest of all the psychophysiological theories of memory. A variety of interneuronal theories have been described, and their common element is the assumption that the transmission of new information to the brain leads to the establishment of new synaptic relationships among a set of neurons—that is, the formation of new neural circuits. The formation of new conduction paths between the afferent and efferent elements has been attributed to the swelling or shrinking of nerve processes as a result of use or disuse, the outgrowth or withdrawal of new terminal elements, such as dendritic spines and terminal boutons, increase or decrease in synaptic vesicle concentration, membrane alterations at the pre- and postsynaptic juncture, and the like. As an additional possibility, I have recently proposed[3] that the interposition of the postnatally formed, short-axoned nerve cells, or microneurons, between the input and output elements of the nervous system may have a role in the establishment of new connections; this proposition is considered in detail in this chapter.

The interneuronal theory has been in disrepute for some time ever since Lashley,[4] Sperry, et al.,[5] and others demonstrated that severing the interconnections between sensory and motor structures in the cerebral cortex, or disrupting transcortical connections with multiple cuts through its surface (procedures that were designed to interrupt established circuits), produced little or no memory deficits in experimental animals. Modern circuit theories, therefore, either assume more complex cortico-subcortical pathways for such functions or they postulate redundant, multiple circuits for single memory complexes. Various aspects of the circuit theory are discussed in this volume by Dr. Sperry and Dr. Chow.

It is probably correct to state that no one of the structural theories of memory that I have briefly described is adequately supported at present by experimental evidence. Considerable recent experimentation notwithstanding, there is no clear, unequivocal evidence available in support of the theory of the molecular coding of memory, nor is there any compelling evidence available in favor of either one of the morphological theories described. In this paper I shall not attempt to prove that morphological changes occur in the brain as a consequence of learning but will instead only review those observations compatible with such a proposition.

Any theory that proposes morphological alterations in the brain as a consequence of exposure to new environmental conditions necessarily must assume postnatal alterations in the morphological organization of the brain. The evidence we shall review here clearly indicates that the maturing brain undergoes radical structural transformations after birth. Birth is an important psychological as well as physiological event, for it marks the beginning of the individual's exposure to a complex external environment; it is the period when the maturing animal is transferred from a relatively stable, genetically specified organic milieu to an unstable, variable, and genetically unprogramable physical and social environment. The morphological changes displayed in the growing brain after birth undoubtedly represent to a large extent genetically determined, delayed maturational processes. However, the postnatal changes in the organization of the brain could also be the opportunity for plasticity, for externally conditioned and input-contingent reorganization of its "wiring" system. Therefore, it remains to be established experimentally to what extent the changes observed in the anatomical organization of the brain during its postnatal growth and differentiation are correlated with the experiential life history of the animal. We shall present here the results of some pilot studies, which suggest that the morphological organization of the maturing brain is, indeed, sensitive to the conditions of the physical and social environment in which the animal is raised.

Postnatal growth and differentiation of the brain

POSTNATAL INCREASES IN BRAIN WEIGHT The increase in the weight and volume of the brain from birth to maturity is considerable (Figure 1). The mean brain weight of the neonate white rat is 0.33 grams, which increases rapidly to 1.26 grams by the 20th day after birth, reaching an asymptote level of 1.9 grams by the age of 200 days.[6,7] This represents a fivefold increment from birth to maturity. Similar levels of weight increases were also observed in the rabbit and the cat,[8] animals which, like the rat, are somatically and behaviorally immature at birth. In contrast, in animals that are maturer at birth, like the guinea pig (which has its eyes open at birth and can immediately stand and walk), the postnatal increase in brain weight is relatively low—2.5 grams in the neonate and increasing to 4.0 grams in the adult.[8] Man appears to occupy an intermediate position, his brain weight increasing from about 335 grams at birth[9] to an average of 1300 grams in the adult. (The human infant, unlike the neonate rat or kitten, is born with eyes open but, unlike the guinea pig, is somatically and behaviorally immature.)

These weight increases are associated with several morphological changes in brain organization. Following birth there is a considerable increase in the "cell territory" or "dendritic field" of neurons. This is caused by the outgrowth of neuronal processes, with a resulting increase in the neuropil/perikaryon ratio (gray/cell coefficient), or decrease in the packing density of neurons. Functionally, this change represents a progressive growth in the potential connectivity of neurons. Another factor that leads to a decrease in the packing density of neurons, and to an increase in brain weight, is the addition of a large number of new glia cells, whose processes become interdigitated among the neurons. This progressive increase in the glial population of the brain after birth is usually expressed in terms of an increasing glia index, or glia/neuron ratio. Other factors that lead to an increase in brain weight are progressive myelination and vascularization, and, finally, a considerable rate of postnatal cell proliferation. Recent evidence, reviewed here, indicates that, contrary to older views, undifferentiated cells multiply at a high rate after birth in various germinal regions of the growing brain, and a large proportion of these cells become differentiated into short-axoned neurons, or microneurons.

Species	Newborn	Adult	Increment	Source
Guinea pig	2.5 gm	4 gm	60%	Himwich[8]
Man	335 gm	1300 gm	290%	Conel[9]
Cat	5 gm	25 gm	400%	Himwich[8]
Rabbit	2 gm	10.5 gm	425%	Himwich[8]
Rat	0.3 gm	1.9 gm	530%	Brizzee, et al.[7]

FIGURE 1 Increments in brain weight from birth to maturity in a few mammalian species.

Postnatal increases in the connectivity of neurons

Light-microscopic studies, using Nissl-stained or Golgi-impregnated brain sections, have revealed a variety of changes in the morphological organization of the growing brain after birth.

RODENTS In the cortex of the rat the neurons are quite undifferentiated at birth. These neurons appear in Nissl-stained material as small, oval cells with relatively large nuclei and only a very thin surrounding of cytoplasm. The cytoplasm contains little Nissl substance (ribosomes and endoplasmic reticulum). These undifferentiated neurons are packed densely in the cortex (Figure 2).

By the end of the first week of life certain types of neurons, such as the pyramidal cells, begin to assume their typical appearance. A study of Golgi-impregnated material indicates[10] a growth in the dendritic processes of neurons and in the number and caliber of axons reaching the cortex; these result in a decrease in the packing density of neurons. By the end of the third week, Nissl-stained sections of the cerebral cortex of the rat resemble the adult brain, although Golgi-impregnated sections show a continuing growth of neuronal processes (Figure 3), and quantitative studies indicate a further reduction in the packing density of neurons.

Various measures have been employed by anatomists for the quantitative evaluation of relative or absolute changes in the structural composition of the brain during ontogenetic development or phylogenetic evolution. One of these is the gray/cell coefficient,[6,11-14] which is a measure of the relative volume occupied in a selected brain region by the "griseum" (or extraperikaryal space) and the "cell" (or perikaryon of neurons). The gray/cell coefficient, which expresses the packing density of neurons, is usually determined in Nissl-stained material. Other investigators have used Golgi-stained sections to estimate changes in the arborization of neurons, or in the total length or area occupied by axons and by dendritic processes, as measures of changing connectivity among neurons in a population.[10,15-17]

Brizzee and Jacobs[6] measured quantitative changes in the cortex of the white rat from birth to the 20th day postnatally. The volume of the nucleus of neurons increased considerably from birth until the 10th day after birth, and

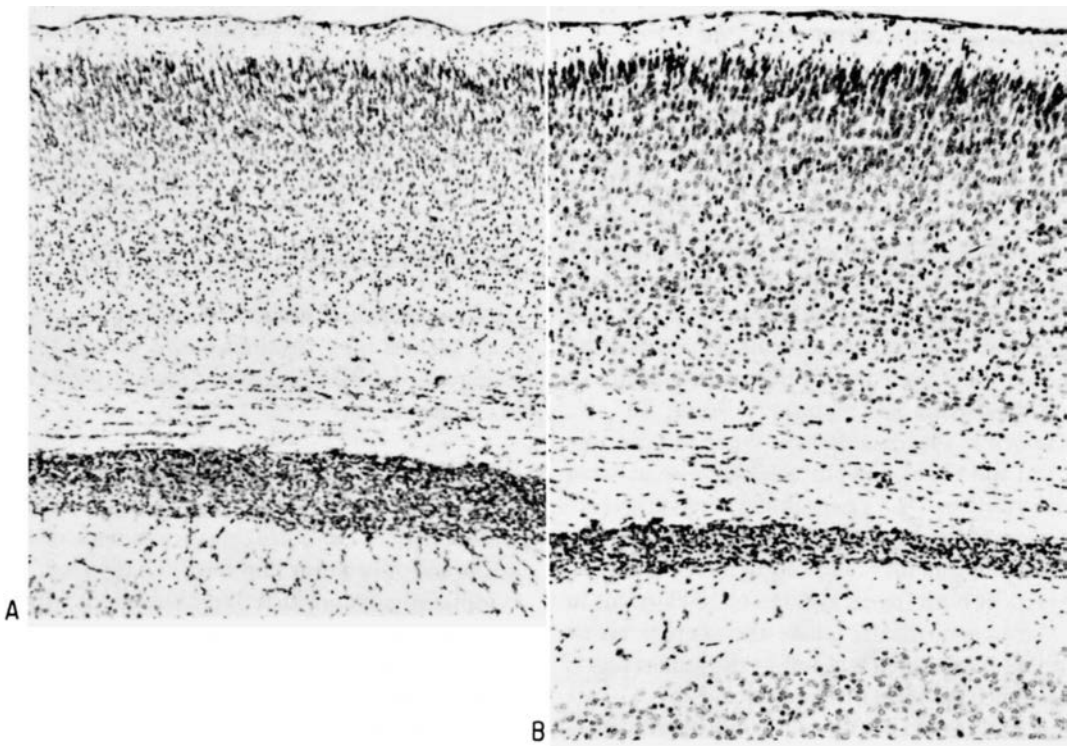
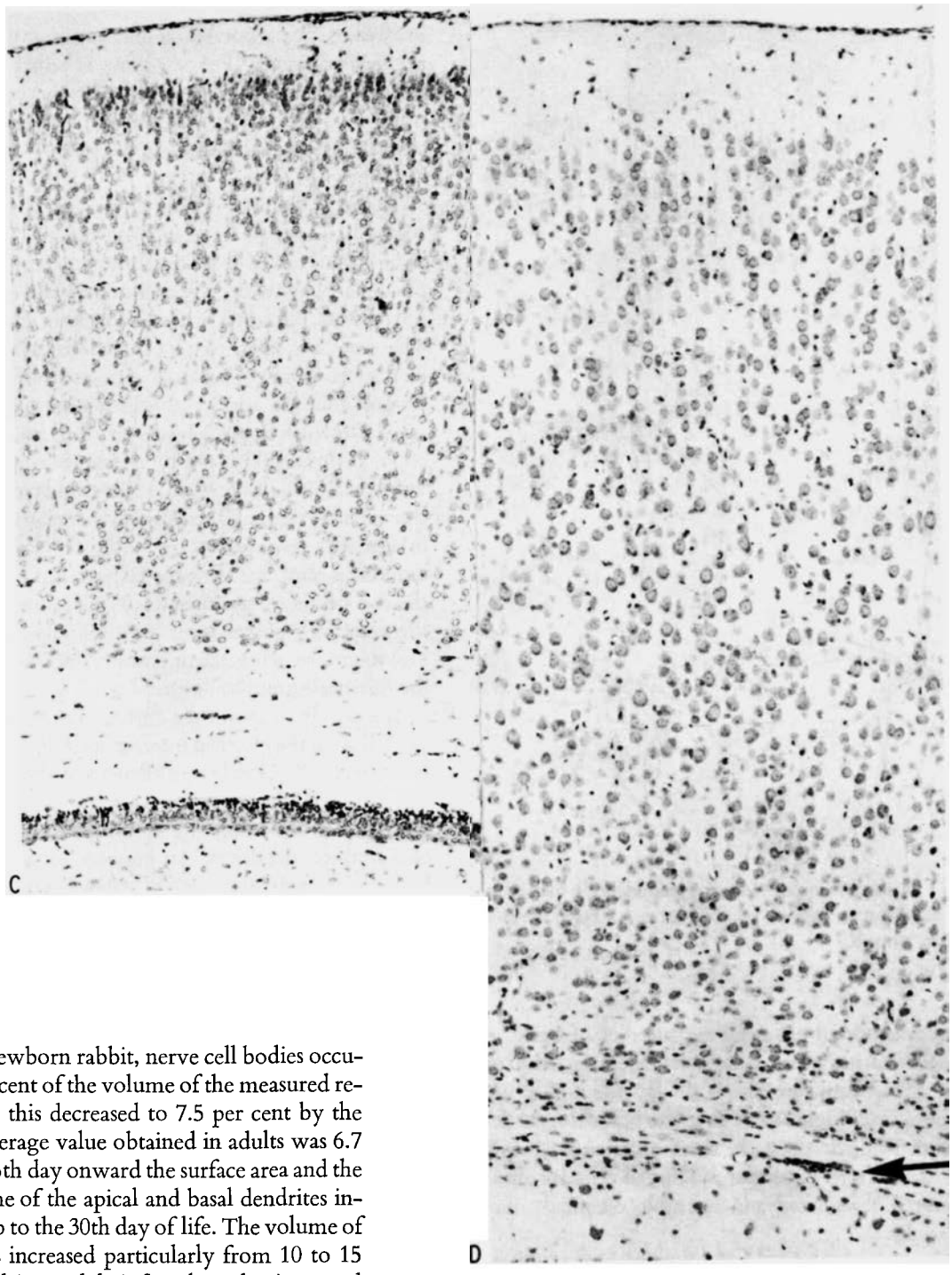


FIGURE 2 Photomicrographs showing the changing packing density of neurons in homologous regions of the sensorimotor cortex in rats of different ages. A, newborn; B, 2 days; (facing page) C, 6 days; D, 20 days. Dark region near the base of the cortex is the subependymal layer of the dorsal aspect of the lateral ventricle. Note inverse relationship between the thickness of this germinal zone (in D reduced to a few scattered cells) and the width of the cortex. All photomicrographs taken at the same magnification, × 101; gallocyanin chromalum stain.

the volume of the perikaryon and of the entire neuron increased continuously to the 20th day after birth. In a subsequent study,[7] with rats available up to two years of age, a decrease in the packing density of neurons was found up to the 100th day (Figure 4). In a study using a silver impregnation technique to visualize axons and the Golgi technique to bring out the silhouette of the entire neuron with its processes, Eayrs and Goodhead[10] also found in the rat cortex a decrease in the packing density of neurons, which was most rapid from birth to 6 days. They also found an increase in the packing density of axons (maximal between 6 and 18 days) and an increase in the packing density of dendrites (maximal between 18 and 24 days). The mean number of dendrites arising directly from the perikaryon reached the adult level by the 12th day, but there was an increase in the branchings at the peripheral dendrites until adulthood, resulting in a continual growth of the dendritic fields of the neurons.

Similar results were obtained by Haddara[18] in mice, using gallocyanin chromalum for staining cell bodies. Greatest decrease in the packing density of cells in the cortex of mice occurs between the 3rd and 7th postnatal day, and an increase in the cell territory and neurons up to the 17th day. In contrast, in the guinea pig, which has a very long intrauterine period of development and is mature behaviorally at birth, the growth of the dendrites after birth is minimal and so is also the reduction in the packing density of neurons.[19] (In a unit area of the cortex, 87 perikarya were counted in the newborn, which was reduced to 77 in the adult.)

RABBITS Schadé and Baxter[17] studied developmental changes in the cortex of rabbits, employing gallocyanin for staining cells and Cajal's method for staining dendrites. In the rabbit cortex, cell volume increased rapidly from birth until the 10th day, there was a decelerated increase until the 21st day, and in the 30-day-old animal, cell volume was similar to that found in adults. In spite of the initial increase in cell volume, from birth on there was a decrease in the proportion of cortical volume occupied by



cell bodies. In the newborn rabbit, nerve cell bodies occupied about 14.2 per cent of the volume of the measured region of the cortex; this decreased to 7.5 per cent by the 5th day, and the average value obtained in adults was 6.7 per cent. From the 5th day onward the surface area and the proportional volume of the apical and basal dendrites increased markedly up to the 30th day of life. The volume of the apical dendrites increased particularly from 10 to 15 days; the apical dendrites and their finer branches increased more than tenfold between 5 and 30 days of age (Figure 5).

CATS The type of growth changes that were observed in rats, mice, and rabbits were also reported to occur in higher mammals, as in carnivores and in primates, man included. During the first three postnatal weeks the cortex in kittens increases from about 0.8 mm to 1.5 mm.[20] In qualitative studies with Golgi staining,[21] the following changes were described in the maturation of the cortical pyramidal cells of kittens from birth to the end of the second month. At birth the pyramidal cells have short apical dendrites, and a few of them have short, unbranched basilar dendrites. By the end of 8 days of age, the apical and

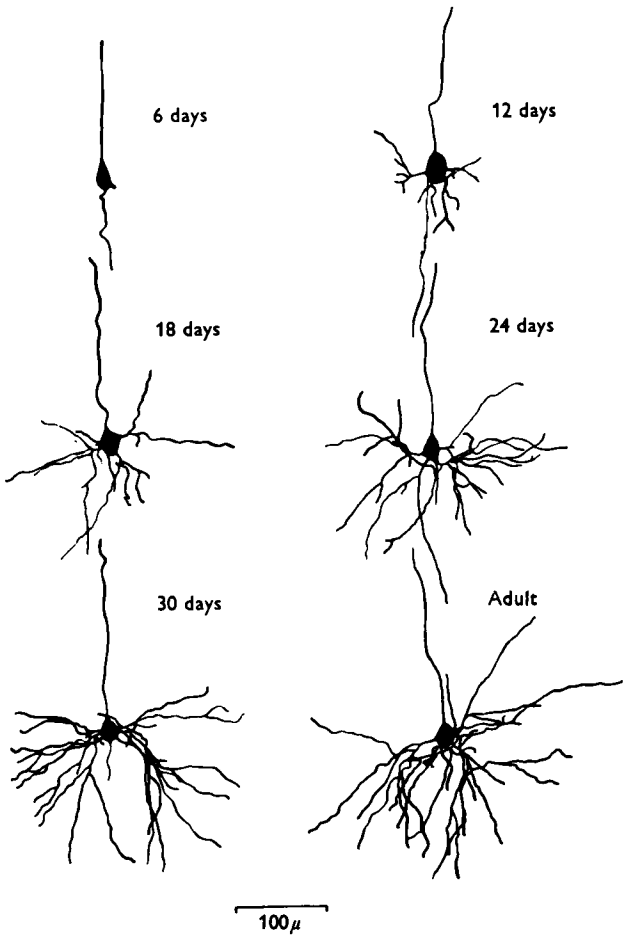


FIGURE 3 Changes in appearance of pyramidal cells of cortex in Golgi-stained sections of rats of different ages. (From Eayrs and Goodhead, Note 10)

basal dendrites acquire a growing number of branches; occasional dendritic spines, which further enhance the connectivity of neurons, are also seen. By the end of two weeks there is additional growth in the branching of dendrites, and dendritic spines become quite abundant. By the end of the third week the pyramidal cells are almost completely differentiated and resemble essentially those seen in adults.

Correlated with these developmental changes in the structure of pyramidal cells, there are simultaneous changes in the development of the thalamic afferents terminating in these regions, and in the elaboration of stellate cells, or short-axoned neurons.[22,23] At birth the thalamo-cortical afferents have simple terminal endings, but by the end of the first week they have rich terminal plexuses engulfing the pyramidal and granule cells on which they terminate. The development of stellate cells is particularly slow and the growth of their arborization may continue until the end of the second month (Figure 6). As Scheibel says: "It may not be overstating the case to suggest that an entirely new neuropil is interposed between the fourth and sixth week of life by the maturation of the Golgi II type axon system." (Scheibel,[22] p. 322.)

The development of the Purkinje cells of the feline cerebellum appears to be even slower.[21] At birth, the Purkinje cell has a well-developed axon that can be traced into the underlying white matter. But the main dendritic trunk of the Purkinje cell is short and lacking in ramifications. The molecular layer of the cerebellum (which is made up of the dendrites of Purkinje cells and the cells and fiber systems that are associated with them) is quite undeveloped. Between 8 and 12 days the Purkinje cell dendrites expand considerably, and by this time most of them have well-developed primary, secondary, and tertiary branches. Between the third and sixth week there is a further growth in dendritic arborization and in the development of spiny branchlets, and the Purkinje cells are fully developed by the end of the second month. By this time all the cells of the external granular layer have migrated inward, either into the molecular layer or past the Purkinje cells and into the (internal) granular layer.

The development of the cortex in kittens has also been studied with the electron microscope.[24] In the neonate kitten, clusters of closely spaced neurons, with oval nuclei and a thin rim of cytoplasm, are separated by groups of large dendrites. There are few interposed elements between these dendrites, or between dendrites and cell bodies. Synapses were seen in contact with dendrites but not with the soma of neurons, and dendritic spines were not encountered. In the one-week-old kitten, well-developed cell bodies were seen, and in those regions where cell processes arose the cytoplasm was enlarged. Large, as well as very fine, dendritic processes were encountered at this age, as were axo-dendritic and axo-somatic synapses. In the two-week-old kitten, the cell body and processes of neurons were frequently surrounded by the processes of another cell type, possibly glial cells. The conclusion arrived at in this study was that the dense packing of neurons in the neonate kitten cortex is caused by the absence of neuronal processes and of interdigitating neuroglial processes. With the eventual growth of these elements, the cell bodies become segregated from one another, until individual neurons become completely invested by elements of the neuropil.

MAN As we mentioned earlier, a nearly fourfold increase occurs in the brain weight of man from birth to adulthood (from about 335 grams to 1300 grams). This weight increase is partly the result of an increase in the volume of

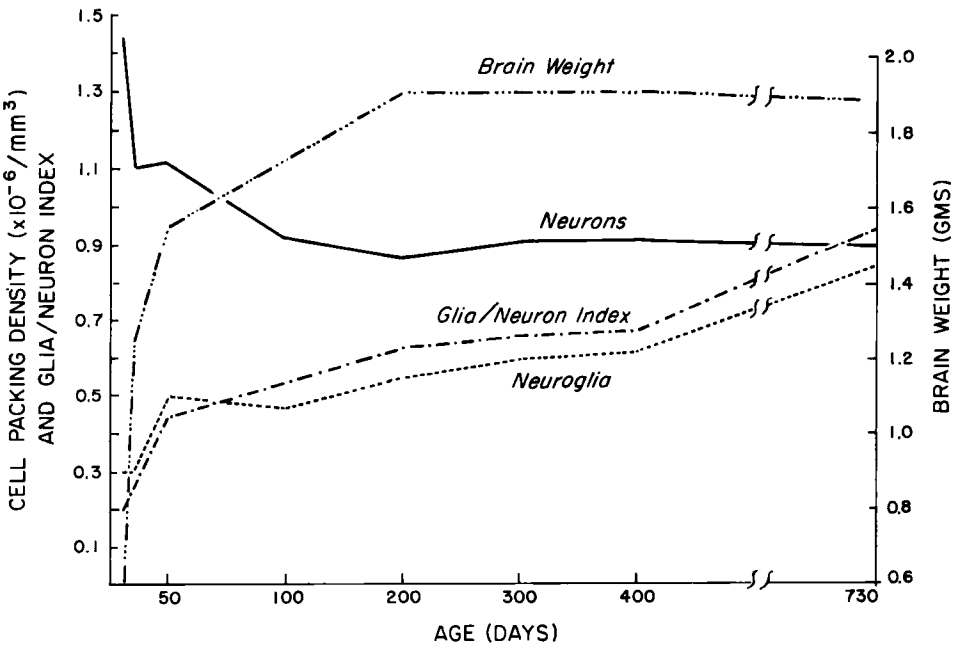


FIGURE 4 Changes in brain weight, neuron packing density, glial packing density and glia/neuron index in cortex of rats of different ages. (From Brizzee, et al., Note 7)

neurons and in the extraperikaryal space, as shown by the quantitative studies of Schadé, et al.[25] In a selected region of the cortex, a considerable increase in the volume of pyramidal cells was observed from birth until one year of age. This increase was first rapid, then slowed in layers III and V, and it was gradual throughout the period in layer IV. Changes in the packing density of pyramidal cells during development were considerable in all layers, but were most pronounced in layer IV, where the gray/cell coefficient changed fivefold during this period. The radical increase that occurs in the human brain from birth to four years of age in the outgrowth of neuronal processes is most strikingly shown in the Golgi-stained preparations of matched brain regions collected and prepared over several decades by Conel (1939–1963).[9] While in the brain of the newborn the apical dendrites of pyramidal cells, for instance, are bare shafts with very few branches, there is a gradual growth during development in the arborization of the apical and basal dendrites and in the number of spines on the apical dendrites (Figure 7). Of all species, the development of the cortex is most prolonged in man.

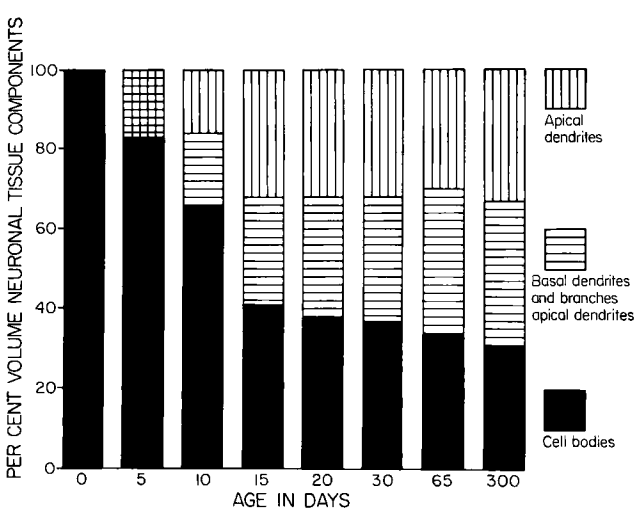


FIGURE 5 Changes in percentage distribution in volume of neuron cell bodies, apical and basal dendrites in the rabbit cortex as a function of age. (From Schadé, et al., Note 25)

PHYLOGENETIC CONSIDERATIONS Toward the end of the last century, Nissl[26] pointed out that neurons are less densely packed in the human brain than in the brains of mole and dog, and he suggested that the development of the "intercellular gray" is an index of phylogenetic evolution. This concept was further advanced later by von Economo,[11] who pioneered in the quantitative determination of the gray/cell coefficient in the cortex of man. He held that the high coefficient in man is the result of an unusual development of the dendritic processes of neurons, permitting a higher degree of functional association among

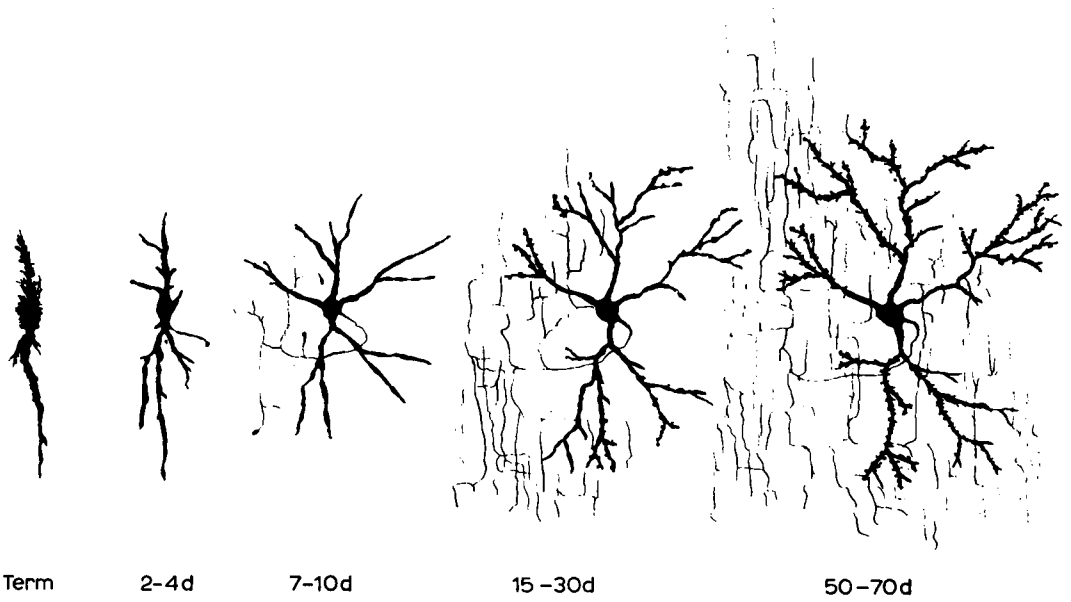


FIGURE 6 Development of the dendritic processes of granule cells (Golgi type II neurons) as seen in Golgi sections from the cortex of kittens of different ages. (From Scheibel and Scheibel, Note 23)

them and contributing to man's intellectual capacity. Later studies[12-14,27,28] have essentially confirmed this evolutionary hypothesis. According to recent studies by Haug,[14,28] the volume of cortex increases during phylogeny to a greater extent than the number of nerve cells, with a concomitant growth in the gray/cell coefficient (Figure 8). This indicates an evolutionary selective pressure on the enhanced development of the neuropil. Phylogenetic and ontogenetic studies, accordingly, support the idea that the growth of the neuropil is an important aspect of the evolution and the morphogenetic development of neural capacity in animals, including man.

Postnatal increase in glia cells, and progressive vascularization and myelination

Brizzee and his collaborators[6,7] studied the changing proportion of glia cells and neurons in the postnatal development of the nervous system in rats and cats. Unlike the neuron packing density, which decreases systematically after birth, the packing density of glia cells increases. This change is often expressed in terms of a glial index[29] or glia/neuron coefficient.[14] Brizzee and his collaborators found, in conventional histological preparations, that the glia/neuron index increased in the rat from 0.2 at 10 days to 0.95 at two years (see Figure 4). Using a different technique of estimating glia/neuron ratio, they obtained an estimate of an even greater increase. In the cat the glia/neuron index increased from 0.83 at approximately 6 months of age to 1.48 in the adult. As the neuron packing density did not change from 6 months onward, the 50 per cent gain in brain weight observed during this period must have been at least partly attributable to the considerable increase in glia cells. Our own autoradiographic results, which are described below, show that in various mammalian species there is a considerable increase in the number of new glia cells during the early phases of postnatal development and, also, that this process continues at a lower rate in adults.[30-32]

It is highly probable that the increase in the dendritic outgrowth of cells contributes, at least potentially, to the enhanced connectivity of neurons and, hence, to their functional capacity. However, the role of the increase in the number of glia cells is not as clear, because the function of glia cells is itself not well established. Some glia cells, such as astrocytes, appear to be intermediaries between nerve cells and blood capillaries, presumably playing a role in the transportation of nutrients and waste products. It is possible that the increase of astrocytes is related to the growing metabolic demands of the maturing brain. Correlated with this is the considerable vascularization, or growth in capillary density, in the cortex of rat from infancy, as was shown by Craigie[33,34] and Horstmann.[35] Another class of glia cells, the oligodendroglia, seem to be responsible for the myelination of axons in the central nervous system, and their increase may very well be re-

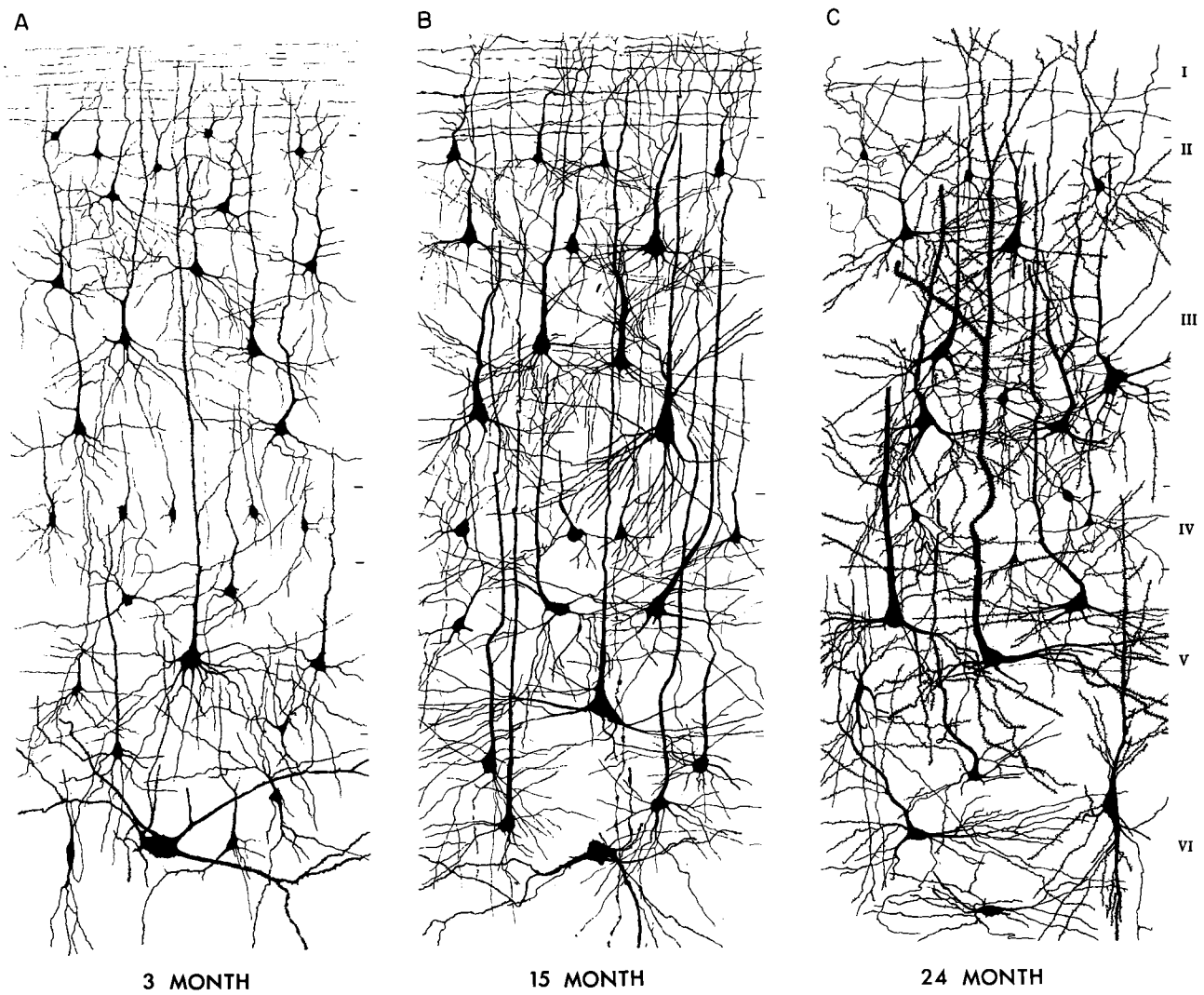


FIGURE 7 Tracings from Golgi-impregnated sections from the region of the gyrus temporalis superior in human children aged 3, 15 and 24 months. Note increased apparent arborization of neurons, and density of dendritic spines, with increasing age. (From the work of Conel. [A] Conel, 1947; [B] Conel, 1955; [C] Conel, 1959, Note 9)

lated to the progressive myelination of the brain after birth.

MYELINATION The myelogenesis of the growing human brain was extensively investigated in the latter part of the last century by Flechsig (see Note 36) and subsequently by Kaes in 1907.[37] Myelination occurs in various tracts of the spinal cord before birth, whereas the myelination of other fibrous tracts, in particular those of the forebrain, is a postnatal phenomenon that takes place during early or late infancy.[38,39,40] The myelination of the cortical gray matter continues beyond puberty, possibly into adulthood. According to Kaes, the myelination in any given brain region occurs in an inside-out sequence, the lower layers acquiring their myelin sheath before the upper layers. And, according to Flechsig, there is considerable difference in the rate of myelination in different cortical regions. On the basis of his studies, Flechsig was able to construct a myelogenetic map of the human cerebral cortex; it indicated that the sensory projection areas and the motor area myelinate much earlier than the intercalated, phylogenetically more recent brain regions. The latter were called by Flechsig "association areas," or regions presumed to be responsible for higher psychic functions. Because the myelination of axons alters their conductile properties, this progressive myelogenesis can be considered, more conser-

	Volume of Cortex in cm^3	Total Cells in Billions	Volume/Cell Coefficient
Man	230	6.9	13.6
Chimpanzee	96	5.5	6.0
Cercopithecus	20	2.5	3.1
Hapale	2	.27	2.8
Tarsius	1	.31	1.0

FIGURE 8 Volume of cortex, estimated number of cells, and volume/cell coefficient in several primate species. (After Shariff, Note 13, and Haug, Note 14)

vatively, to reflect the recruitment of more and more conductile elements into the circuitry of the brain. The interrelationship between myelogenesis and behavioral capacity has been studied by only a few workers[41] and it certainly deserves further investigation.

PHYLOGENETIC CONSIDERATIONS It has often been argued that the increase in glial packing density may be another evolutionary index of brain development. Thus, Friede[29] reported that the glia/neuron ratio in the cortex increases in the following order: frog, chicken, mouse, rabbit, pig, cow, and horse, and is highest in man. However, Hawkins and Olszewski[42] have subsequently shown that the glia index is highest in the brain of whales, and have suggested, therefore, that high glial ratio may be related to the greater length of axons in animals with great bulk. This theory subsequently received experimental support in the observations of Friede and Van Houten,[43] who found that the number of perineuronal glia cells around neurons in the bovine spinal cord increases with the length of the axon of the neurons with which they are associated. A recent quantitative study by Haug[28] indicates that there is no relationship in vertebrates between glia/neuron index and phylogenetic position. Glial density may be more intimately associated with the metabolic demands of neurons.

The postnatal origin of microneurons

Mitotic neurons are seldom seen in the brains of mammals after birth; this gave rise to the generally held view that neurogenesis—the formation of new neurons—is an embryonic, prenatal phenomenon. This conclusion has occasionally been questioned by some investigators, as direct and indirect evidence has been available for postnatal neurogenesis in some brain regions. It is well known, for instance, that the pronounced postnatal growth of the cerebellar cortex is dependent on the continued multiplication of neuroblasts in the germinal external granular layer and on their migration inward into the molecular and (internal) granular layers.[44,45] Similarly, good quantitative evidence was obtained about half a century ago[46] of an increase in the total number of neurons in the cerebral cortex of rats up to the 20th day after birth. Is it possible that, although differentiated neurons cannot divide, undifferentiated cells (neuroblasts) can multiply in the brain after birth and that they subsequently become differentiated into neurons? That a mitotic germinal zone is present after birth around some parts of the forebrain ventricles in animals and man has long been known,[47-49] and the recent introduction of a radioactive tracer technique for tagging proliferating cells—thymidine-H^3–autoradiography—made it possible to inquire into the fate of these multiplying cells.

Thymidine is a specific precursor of DNA and is incorporated into the chromosomes of nuclei when the DNA strands are duplicated by cells preparing for multiplication. When animals are injected with thymidine-H^3, the cells preparing for division tend to incorporate the administered radiochemical and thus become labeled. These labeled cells can then be identified with autoradiography. By killing animals one to six hours after injection, the sites and regional rates of cell proliferation in the brain can easily be established. By killing animals after varying periods of survival following the injection, the fate of the originally labeled cells, such as the kinetics of their remultiplication, their migrations, and their modes of differentiation over time can also be investigated.

By using this technique, it has been established[31,50,51] that cell proliferation in the rat remains very high for some time after birth in the ependymal and subependymal layers (Figures 9 and 10) of the forebrain ventricles (the olfactory ventricle and certain regions of the lateral and third ventricle) and in certain subpial zones (as the external granular layer of the cerebellar cortex). A significant, although lower, rate of cell proliferation was also found in the white and gray matter in various brain regions. The rate of cell multiplication was found to be a function of age after birth, and in young adult rats, cell multiplication becomes very low in most brain regions although it does not cease altogether. The cells multiplying in the brain give rise to a variety of cell types, including neuroglia cells and short-axoned neurons (called stellate cells, granule cells, or microneurons). We have paid particular attention to the genesis of short-axoned neurons in brain regions where they form homogeneous, discrete layers, because where they occur intermingled with glia cells, they cannot be distinguished with any certainty in Nissl-stained material from astrocytes.

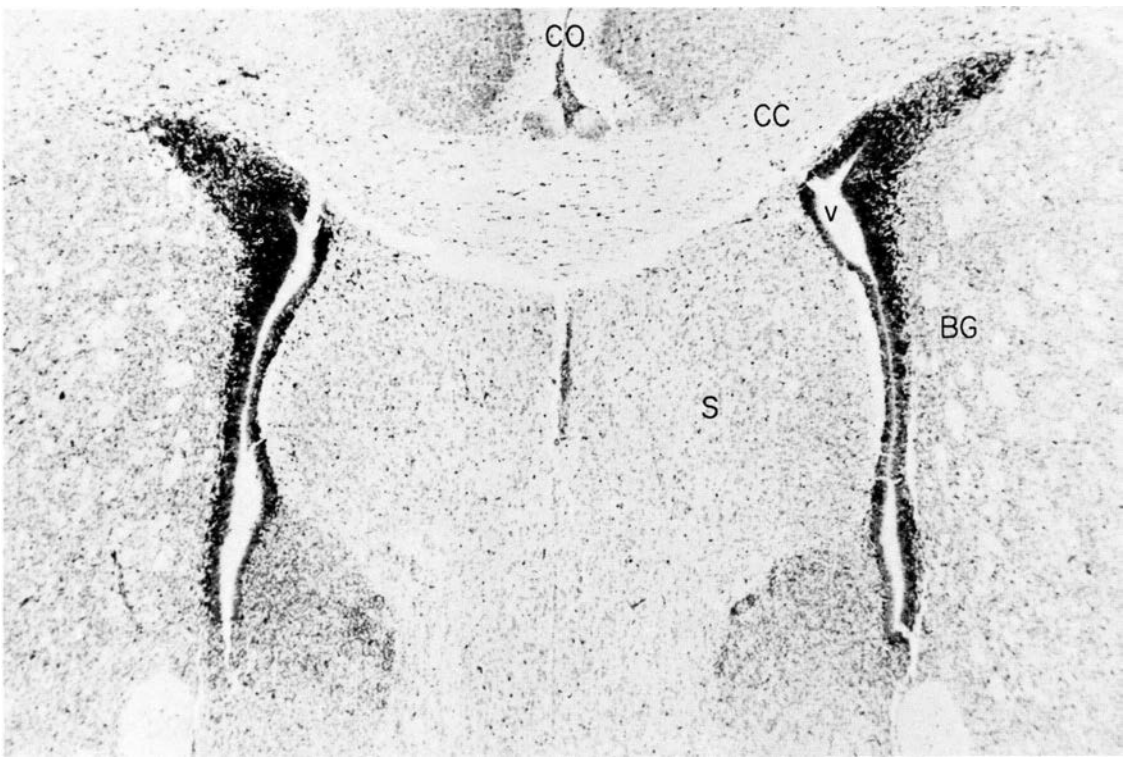


FIGURE 9 Autoradiogram showing labeled cells in the ependymal and subependymal walls of the lateral ventricle in a coronal brain section from the level of the septum. The rat was injected intraperitoneally with thymidine-H^3 on the 5th, 6th, 7th and 8th postnatal day, and killed on the 9th day. BG, basal ganglia; CC, corpus callosum; CO, cortex; S, septum; V, lateral ventricle. Gallocyanin chromalum, X 40.

NEUROGENESIS IN SENSORY RELAY NUCLEI In these studies it was established that the cells multiplying at a high rate around the wall of the olfactory ventricle migrate outward, at a speed of 50 microns per day, into the laminated olfactory bulb. Here the labeled cells become differentiated into granule cells, forming the granular layers and also contributing short-axoned neurons to the internal plexiform and mitral cell layers. The rate of cell multiplication declines in this region by the end of the second week, but a low rate of proliferation is indicated at least until the end of the second month of life. These granule cells of the olfactory bulb represent a neuronal system strategically situated to affect the transmission of sensory messages from first-order neurons (olfactory nerve fibers) to second-order neurons (olfactory tract fibers). Another system of this type is represented by the granule cells of the cochlear nucleus; in the rat these are also formed largely after birth.

NEUROGENESIS IN THE CEREBELLUM In conformity with previous observations, a high rate of cell proliferation was established with the autoradiographic technique in the external granular layer of the cerebellar cortex, and, in young rats, in the internal granular layer as well (Figures 11 and 12). Cells from the external granular layer could be traced migrating through the molecular layer, past the Purkinje cells, and into the internal granular layer, at a minimum speed of about 60 to 70 microns per day. Cell multiplication slows down considerably in the internal granular layer by the 13th day after birth, and in the external granular layer by the 16th day (see Figure 13). However, the large complement of newly formed cells does not disappear from the external granular layer until the 21st day, by which time the external granular layer disappears altogether. It has been established that the bulk of the microneurons of the cerebellar cortex (the basket cells and stellate cells of the molecular layer and the granule cells of the internal granular layer) are derived from the postnatally arising and differentiating cells of the external granular layer.

In this context, it is interesting to note the intimate relationship that exists between the duration of postnatal

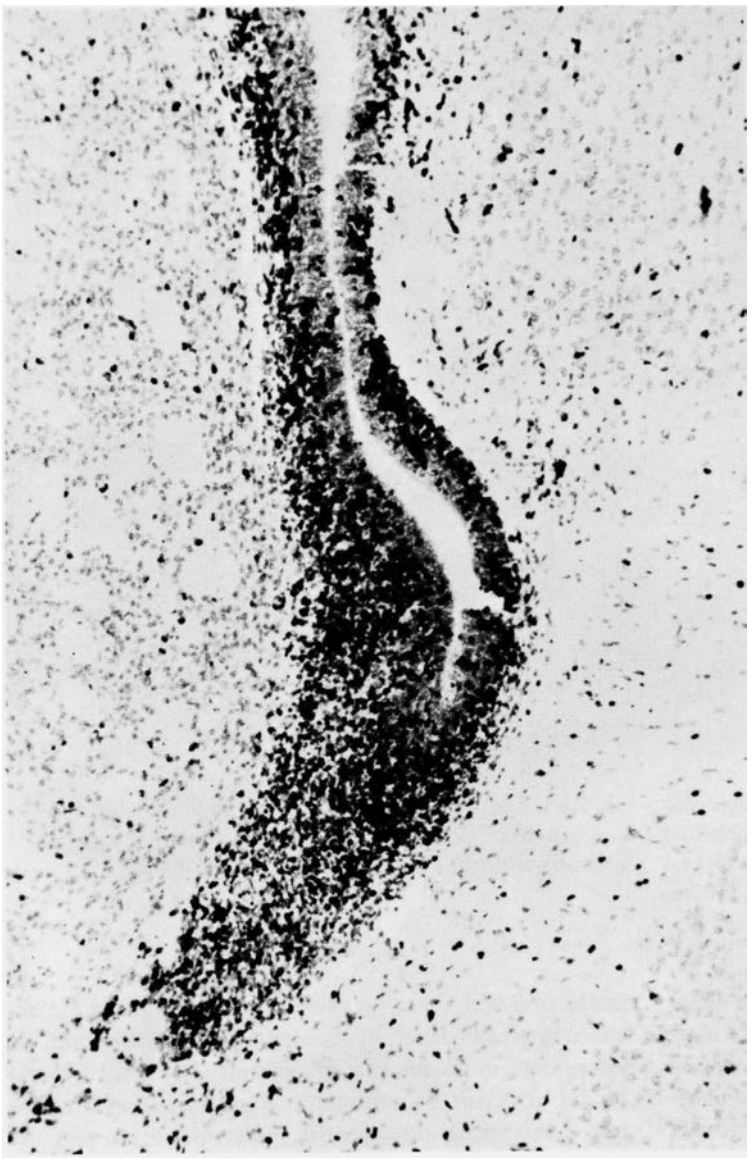


FIGURE 10 Autoradiogram from a rat injected with thymidine-H^3, as described in the previous illustration. Note that the majority of the cells of the subependymal layer are labeled, indicating a very high rate of cell proliferation in this region. Gallocyanin chromalum, X 101.

cerebellar neurogenesis in different species and the degree and temporal course of the development of motor coordination in those species. We can consider as a major turning point in cerebellar neurogenesis the dissolution of the external granular layer; this occurs a few days after birth in guinea pigs, at the end of three weeks in rats, at the end of two months in cats, and during the second year of life in man. The guinea pig can walk and run soon after birth, and its locomotor repertoire appears to undergo relatively little further development. The newborn rat cannot efficiently use its limbs for standing and walking for several days after birth, but it improves gradually, and by the end of the third week it can jump and climb (these are motor abilities quite beyond the capacity of the guinea pig). The locomotor development of the kitten is still slower, but by the end of about 6 weeks the growing kitten begins to display the smooth and graceful locomotor and manipulatory skills that characterize adult cats. Motor capacity is very highly developed in man, who slowly learns to utilize his hindlimbs for bipedal locomotion and who, like so many primates, can efficiently use his hands and fingers in skilled manipulatory tasks. The acquisition of erect posture, locomotion, and the skillful use of the fingers is roughly correlated with the cessation of cerebellar neurogenesis, as expressed by the final dissolution of an external granular layer during the second year. These

correlations between cerebellar development and the development of coordinated motor skills are in agreement with physiological and pathological studies which indicate that the cerebellum has a major role in the smoothing and error-correcting of skeleto-muscular motor acts.

NEUROGENESIS IN THE HIPPOCAMPUS Cell multiplication is also high in the neonate and infant rat in the ependymal and subependymal wall of the lateral ventricle, and labeled cells can be traced migrating by way of the fibrous tracts of the forebrain to various forebrain regions. Thus, cells moving by way of the fimbria of the hippocampus were traced migrating past the pyramidal cells of Ammon's horn into the basal polymorph cell layer of the dentate gyrus. The newly formed cells at the basal polymorph cell layer appeared to retain their proliferative capacity to a considerable extent, and they could be traced migrating toward the granular layer of the dentate gyrus, forming a distinct zone made up of small, darkly staining undifferentiated neuroblasts (Figure 14). The cells located in this zone have a reduced proliferative capacity, and within about two to three weeks after arrival at this site they become differentiated into typical granule cells (Figure 15). The proliferation of neuroblasts in the dentate gyrus and their differentiation into granule cells continue late into adulthood in rats.

As the presence of the external granular layer in the cerebellar cortex can be taken as an index of neurogenesis, so also the presence of a discrete zone of small, darkly staining cells at the base of the granular layer can serve as an index of hippocampal neurogenesis. As a result, we found in a recent study (to be published) that in guinea pigs injected with thymidine-H^3 at one or six days of age, and killed 6 hours afterwards, many cells were intensely labeled in the polymorph cell layer and at the base of the granular layer. In animals surviving for 12 days after injection, there was a considerable increase in the number of labeled cells, combined with label dilution within cells, in-

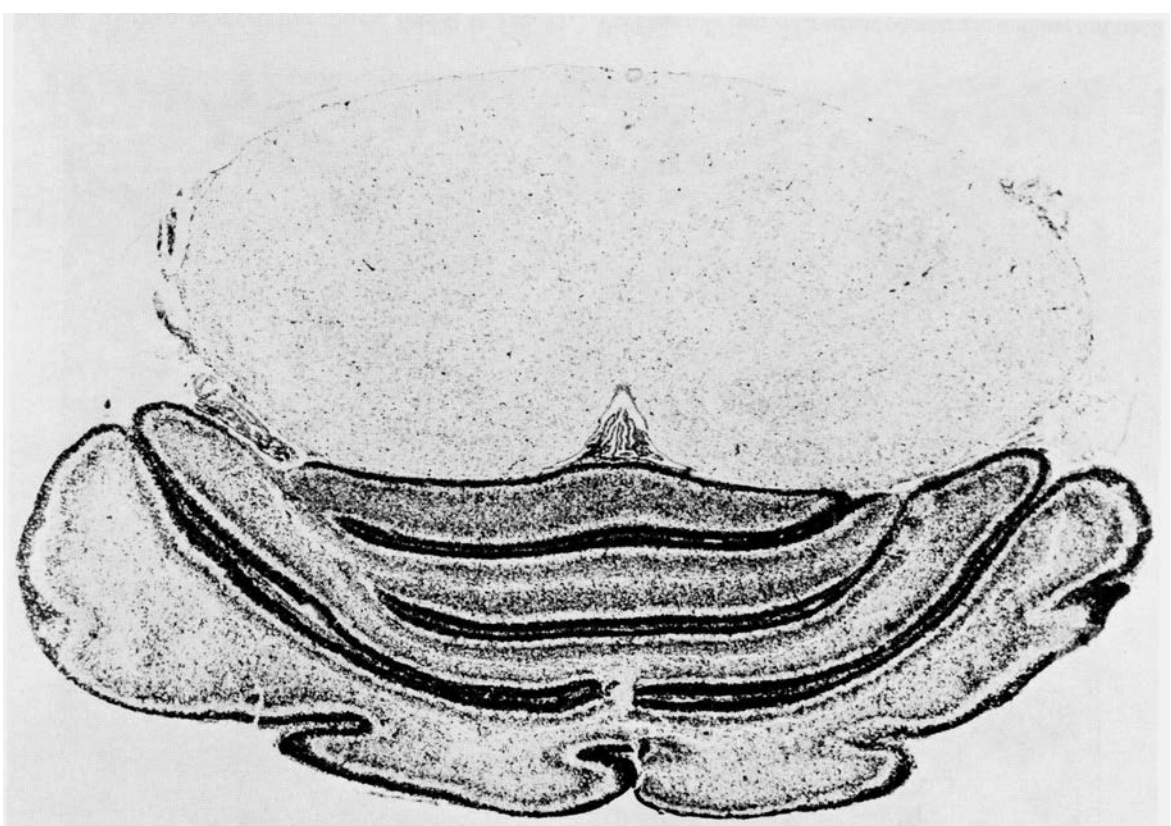


FIGURE 11 Autoradiogram of the medulla (lower half of picture) and cerebellum from a rat injected with thymidine-H^3, as described in Fig. 9. The cells of the external granular layer forming the external wall of the folia of the cerebellar cortex are labeled. Gallocyanin chromalum, X 20.

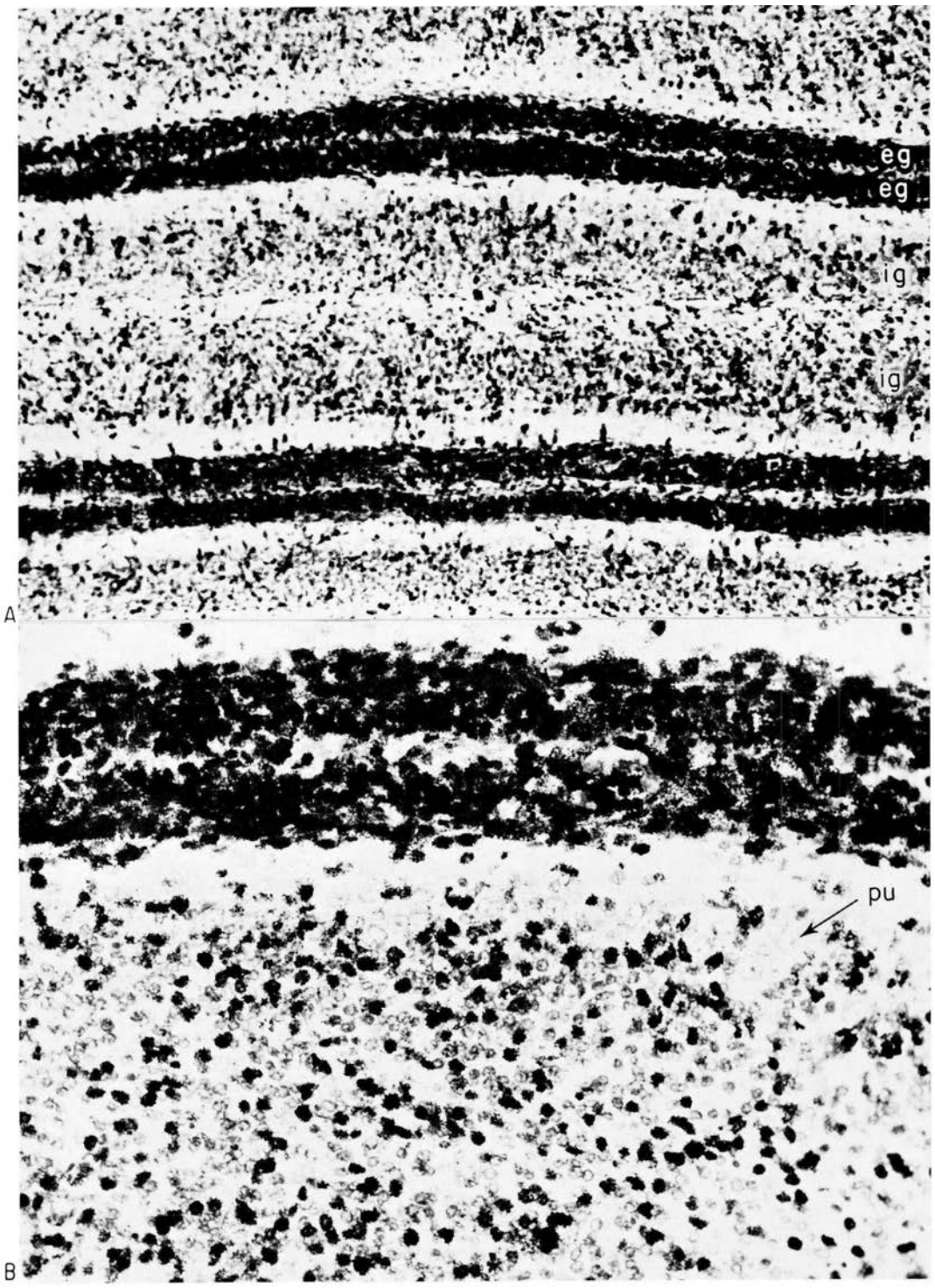


FIGURE 12 Portion of cerebellar cortex, shown in Fig. 11, with apposed external granular layers (eg) and internal granular layers (ig). A, X 101; B, X 256. Note that practically all the cells of the external granular layer, and a considerable proportion of those of the internal granular layer, are labeled. Purkinje cells (pu, with arrow pointing to one) are not labeled. At this age the poorly differentiated Purkinje cells form a several-cell-thick zone and the molecular layer above them is poorly developed.

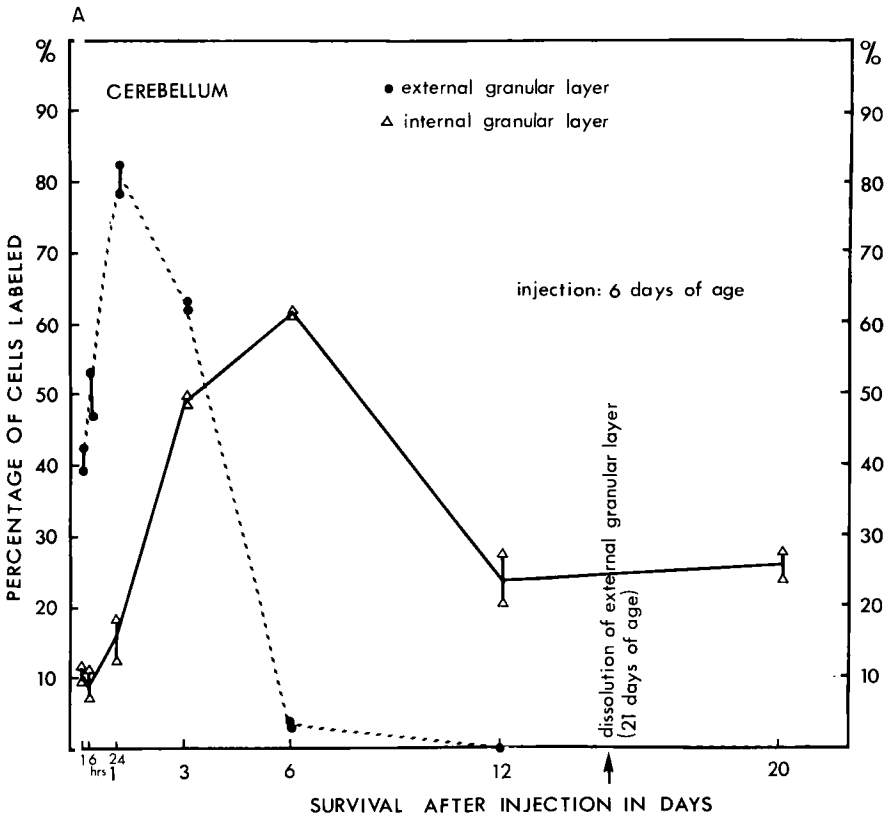
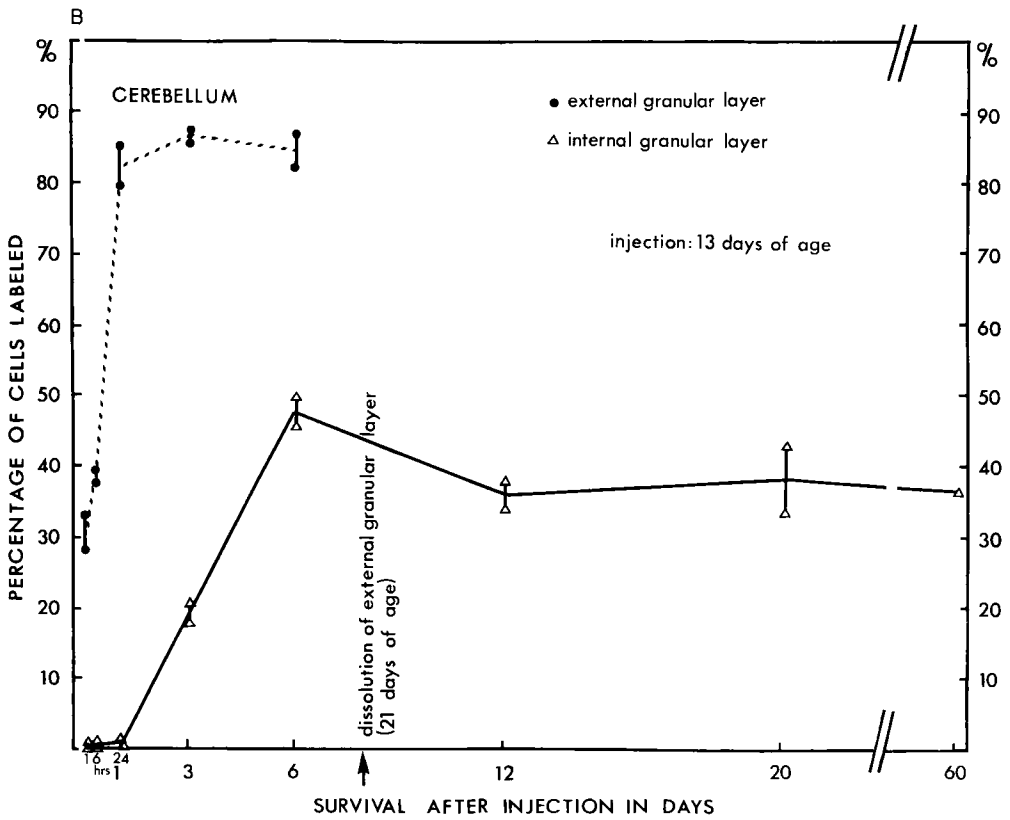


FIGURE 13 A,B Percentage of labeled cells in the external and internal granular layers of the cerebellar cortex in rats that were injected at 6 and 13 days of age and survived for different periods after injection. In the external granular layer cell multiplication ceases apparently after 3-day survival (about 16 days of age) and the cells formed here migrate into the internal granular layer; in the latter, local cell multiplication is minimal at 13 days of age.



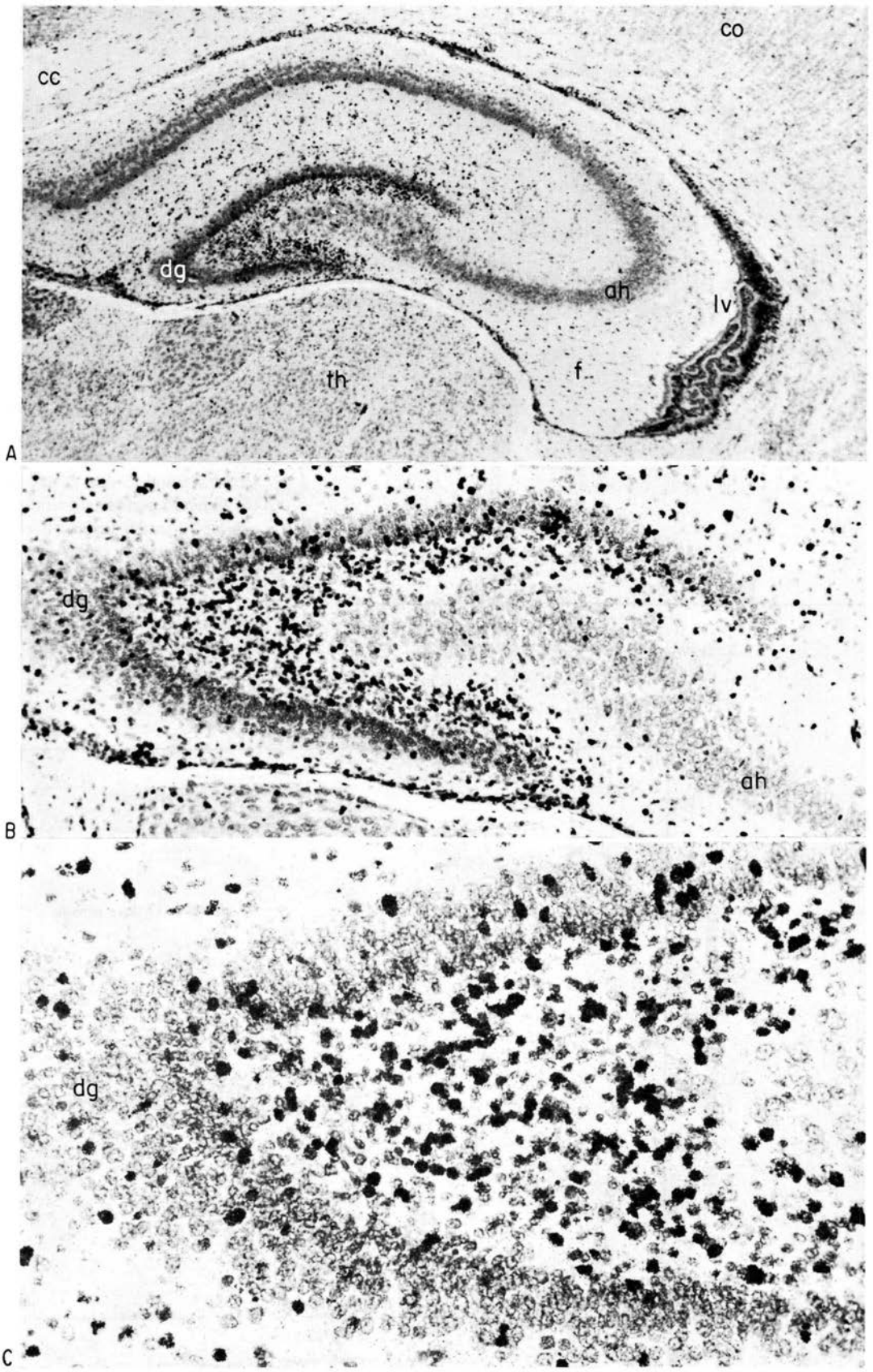

A
cc
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FIGURE 14 Autoradiogram of the hippocampal region from a rat injected with thymidine-H^3, as described in Fig. 9. ah, pyramidal cell layer of Ammon's horn; cc, corpus callosum; co, cortex; dg, granular layer of hippocampal dentate gyrus; f, fimbria of hippocampus; lv, lateral ventricle; th, thalamus. A, X 40; B, X 101; C, X 256. Note that labeled cells are most numerous within the arms of the granular layer, in the layer of polymorph cells. From this region the multiplying cells migrate into the granular layer.

dicating continuing cell multiplication. In the latter animals the bulk of the labeled cells could be identified as the small, darkly staining neuroblasts, situated at the base of the granular layer, and a good proportion of this type of cell was labeled. In animals that survived for 30 days after injection, the small cells at the base of the granular layer were mostly unlabeled, and the labeled cells could now be identified as differentiated granule cells situated within the granular layer. Thus, unlike the cerebellar cortex of guinea pigs, where neurogenesis ceases soon after birth, the hippocampal neurogenesis of the precocious guinea pig continues for some time postnatally. (The germinal zone below the granular layer is quite clearly discernible even in the sexually mature, young adult guinea pig.)

Gauging hippocampal neurogenesis by the presence of this germinal zone below the granular layer, we may say that hippocampal neurogenesis continues for a long time after birth in all mammals. In the cat (unpublished data) there is an unusually rich germinal zone below the dentate gyrus at 30 and 60 days of age (Figure 16). The significance of the persisting neurogenesis of the hippocampus remains to be established.

In conclusion we may say, therefore, that microneurons, wherever they can be identified, are predominantly of postnatal origin in mammals that are immature at birth. In some brain regions, as the cerebellum, this neurogenesis is correlated with the development of the behavioral function subserved by them; in other structures, such as the hippocampus, the functional significance of the postnatal origin of microneurons is unknown. We should also add that information is not available currently about the time of origin of the microneurons of the cerebral cortex.

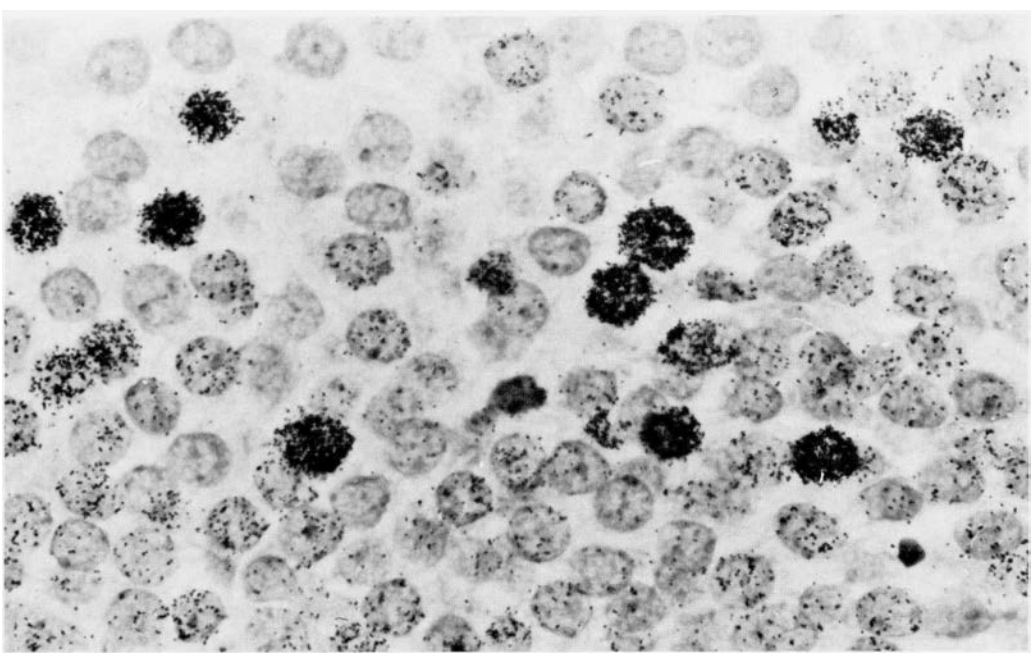

FIGURE 15 Autoradiogram of labeled granule nerve cells in the hippocampal dentate gyrus of a rat injected with a single dose of thymidine-H^3 at 2 days of age and killed 60 days later. Note the large number of intensely and lightly labeled neurons. Gallocyanin chromalum, X 640.

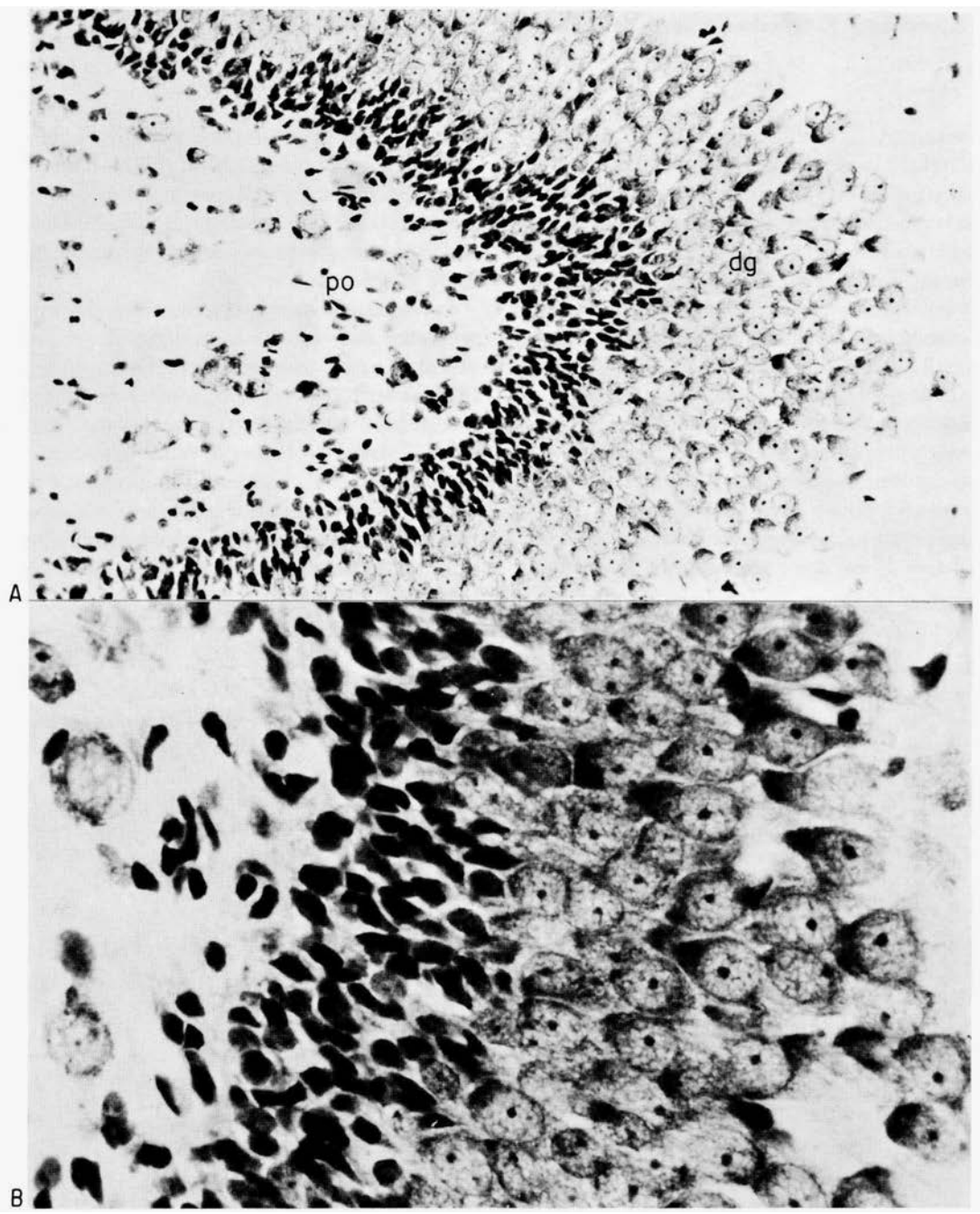

FIGURE 16 Histological section of the hippocampal dentate gyrus of a 30-day-old (uninjected) kitten. The granular layer (dg) is composed of an outer zone of differentiated neurons (large, pale cells) and an inner zone of undifferentiated neuroblasts (small, dark cells); po, polymorph layer. A, X 256; B, X 640. Cresyl violet.

Behavioral influences on the postnatal differentiation of the brain

The foregoing results show clearly that radical changes occur in the structural constitution and organization of the mammalian brain after birth. These changes result primarily from the acquisition of a large population of new glia cells and microneurons by various suprasegmental brain structures and the correlated growth of the dendritic plexuses of macroneurons associated with them. The question that arises is whether these postnatal changes represent a late phase in the maturation of the brain—one that is fixed or is determined strictly by morphogenetic mechanisms—or, alternatively, whether they represent the plastic phase in the structural organization of the brain—one that is contingent on sensory input and feedback obtained while the animal is interacting with its external environment. Is the proliferation of the precursors of microneurons delayed in altricial mammals until birth because their function is a postnatal one—that of adjusting the genetically specified programs of behavior in accordance with information about specific environmental conditions as it is acquired by the growing animal?

At present, only a few studies are available that are concerned with the effects of differential rearing of developing animals on the growth and differentiation of their brains. In this context, we do not wish to deal with studies in which radical physiological or behavioral manipulations were used for this end. For instance, removal of the thyroid gland in neonate rats leads to retarded development of the brain. This includes a deceleration in the normal increase of the gray/cell coefficient in the cortex, and these changes are associated with behavioral deficiency.[52] Conversely, administration of pituitary growth hormone to the mother leads to an acceleration of brain development in the young, both in terms of greater dendritic growth[53] and in terms of neuron number,[54] and this is correlated with improved learning capacity.[55] Such treatments do not represent a manipulation of sensory input and feedback to the behaving animal and, therefore, demonstrate merely a correlation between altered brain development and behavioral capacity.

In a series of experiments, Krech, Rosenzweig, and their collaborators (summarized in Bennett, et al.,[56]) studied the effects on cholinesterase concentration in the brains of rats reared in "enriched" and "restricted" environments. The "enriched" animals are raised in a larger cage, in which they can move about freely and play with various objects and with each other, whereas the "restricted" animals are reared singly in small isolation cages and are thus deprived of both sensory stimulation and the opportunity for motor exercise. In the course of the studies, these investigators[57] found that the cortical gray matter was thicker in the enriched animals (although their body weight tended to be lower) than in the restricted animals. The increase was most prominent in layers II and III of the visual cortex, and this change was attributed partly to a growth in dendrite processes and partly to an increase in the total number of glia cells. In a comparable study,[58] we investigated the effects on cellular proliferation in the brains of rats reared in enriched and restricted environments. We obtained histological evidence of an increase in the area of the cortex in the enriched animals, and autoradiographic evidence of an enhanced rate of cellular proliferation in the mature enriched animals.

Because the highest increase in labeled cells was seen in the white matter of the cortex, we have referred to this change as an increase in the glial population. We should point out that newer studies from our laboratory have cast doubt on this interpretation. We have established[32] that a considerable proportion of cells present in the white matter of young rats are not perifascicular glia cells, but rather undifferentiated migrating elements coursing here on their way from the subependymal germinal zone of the lateral ventricle to the gray matter of the cortex. Therefore, it is possible that the increase in new cells in the white matter, which we have seen in enriched adult rats with a one-week survival period after injection, may represent an increase in the proliferation and migration of undifferentiated cells. The fate of these calls is now being investigated in our laboratory.

Preliminary results from our laboratory[58b] indicate that other behavioral variables, such as handling rats during infancy, can radically alter the development of the brain, in particular cell proliferation in such structures as the cerebellar cortex, the hippocampal dentate gyrus, and the neocortex.

Microneurons: their properties and their possible role in the storage of the "memory trace"

Microneurons may be distinguished from the "typical" neurons, or macroneurons, of the mammalian brain by four sets of criteria: morphological, physiological, phylogenetic, and ontogenetic.

Morphological Characteristics of Microneurons

Microneurons consist of three types of relatively small nerve cells. First are those believed to be devoid of axons, such as the amacrine cells of the retina and some of the granule cells of the olfactory bulb. (Presumably such nerve cells cannot conduct action potentials.) It is interesting to note that electron microscopists recently have identified two-way synaptic junctures in these microneurons, both

of which have presynaptic and postsynaptic membrane surfaces with respect to an adjacent neuronal element.[59,60] Second are small neurons with unmyelinated short axons that terminate within the area of the dendritic field of the neuron. These are the Golgi type II neurons, or stellate cells, which are particularly abundant in the neocortex. Third are nerve cells with comparatively long, unmyelinated axons, which, however, do not interconnect different brain regions but, instead, terminate in a different component or layer of the very same structure in which the cell body and dendrites are located. One example is the granule cells of the granular layer of the cerebellar cortex whose axons (known as parallel fibers) terminate in the molecular layer of the same structure. Another example is the granule cells of the hippocampal dentate gyrus, whose axons (known as mossy fibers) establish synaptic relationship with polymorph and pyramidal cells in Ammon's horn of the hippocampus.

SOME FUNCTIONAL CHARACTERISTICS OF MICRONEURONS These three types of microneurons share one common functional property: they have a restricted, local output. In this respect they differ from macroneurons, which typically have long axons by means of which information is transmitted over long distances—in afferent neurons from the sensory surfaces of the body to the central nervous system; in relay and commissural neurons from one brain region to another; and in efferent neurons from the central nervous system to the motor organs of the body. The majority of macroneurons (although certainly not all) have myelinated axons and conduct digital (all-or-none) messages at a relatively high velocity in an obligatory (nondecremental) fashion.

Because the output of microneurons is local by definition, they must be regarded as interneurons whose role is not the transmission of sensory messages or motor commands, but rather the modulation of the interaction among the afferent, efferent, and higher-order macroneurons. That is, microneurons must be associative, switching, or regulatory elements of the brain. An example is the role played by microneurons in the cerebellar cortex, a problem that has recently been investigated by Eccles and his collaborators.[61] Three types of microneurons exist in the cerebellar cortex—the granule cells, basket cells, and stellate cells (the classification of the Golgi cells is ambiguous) —and these directly or indirectly modulate the activity of the Purkinje cells, whose axons represent the only output line from the cerebellar cortex. The interaction among these elements is a complex one; some (the granule cells) exert an excitatory influence on the Purkinje cells, others (stellate cells and basket cells) an inhibitory one. Of some interest in this context is that the microneurons located in sensory relay structures are recipients of centrifugal, feedback fibers from higher brain regions. Thus, the amacrine cells of the retina receive the centrifugal fibers of the optic tract,[62] the granule cells of the cochlear nucleus receive the centrifugal fibers of the olivo-cochlear bundle,[63] and the granule cells of the olfactory bulb receive centrifugal efferents coursing in the olfactory tract.[64,65]

ASPECTS OF THE PHYLOGENY OF MICRONEURONS The theory that the microneurons are associative elements of the nervous system is attributed to Cajal, who pointed out the ascending role of short-axoned interneurons in phylogeny. It has been repeatedly stressed by some anatomists that the number of stellate cells increases in the cortex of higher animal species and is most pronounced in man.[66-69] Similar changes were also reported in the number of granule cells of the hippocampal dentate gyrus.[70-72] Unfortunately, there are virtually no quantitative studies on this issue. In a recent study, Mitra[73] reported that the proportion of stellate cells in the visual cortex of the rabbit is 31 per cent, in the cat it is 35 per cent, and in the monkey 45 per cent, and he related this to visual capacity increasing in the same order. This observation was paralleled by the ontogenetic development of the visual cortex in the rabbit. At 10 days of age, 13 per cent of all the cells were stellate cells; this increased to 27 per cent in the 17-day-old rabbit and to 31 per cent in the adult.

ASPECTS OF THE ONTOGENY OF MICRONEURONS The autoradiographic results described earlier regarding the postnatal development of microneurons showed that the short-axoned neurons of the nervous system represent its last-developing component. In this context, it should be pointed out that in the same brain regions in which the bulk of the microneurons develop postnatally, the macroneurons associated with them are already in or near their final location at birth. Thus the Purkinje cells of the cerebellar cortex are exclusively of prenatal origin, and so also are the mitral cells of the olfactory bulb and the pyramidal cells of the hippocampus. Interestingly, the precursors of microneurons migrate through the dendritic field of the macroneurons with which they will be associated. The cells of the external granular layer of the cerebellar cortex move through the molecular layer, in which the Purkinje cell dendrites are located, and then move past the Purkinje cells. In so doing, they apparently leave their axons (the parallel fibers) behind in the molecular layer. Similarly, the precursors of the granule cells of the hippocampal dentate gyrus migrate through the dendritic field of the polymorph and pyramidal cells and presumably leave their axons (the mossy fibers) behind to establish their synaptic contacts. It is tempting to speculate a mutual relationship

between the outgrowth of the Purkinje cell dendrites, for instance, and the initiation of differentiation and migration of the precursors of granule cells. As we have shown,[74] cell proliferation in the external granular layer occurs primarily in the outer, or subpial, zone of this layer, whereas the cells of the inner, or juxtamolecular, zone, which are spindle-shaped, represent the migratory elements. The studies of Purpura, et al.,[21] indicate that in the cerebellar cortex of the developing kitten the Purkinje cell dendrites terminate at the lower boundary of the external granular layer and that the destruction of the external granular layer by X-irradiation is associated with a failure in the development in the dendritic processes of the Purkinje cells.

The previous description of the migratory pattern of microneuroblasts also implies that axonal growth in microneurons may be radically different from axonal growth in macroneurons. The axons of macroneurons appear to be endowed with "specificity" (of unknown nature), which makes it possible for them to grow unerringly toward their genetically determined terminal sites. Thus, the regenerating axons of the retinal ganglion cells (optic tract fibers) of fish and amphibians[75,76] will grow accurately from a specific quadrant of the retina to the retinotopic representation of that quadrant in the distant optic lobe. In microneurons, by contrast, it is not the axon that searches for its terminal site but rather the soma of the neuron that migrates away with its dendritic pole pointed forward in the direction of its migratory route; the dragging axon is left behind, where it establishes synaptic contacts. Does this mode of axonal growth reflect the circumstance that the connectivities of microneurons are not "specified" by the same mechanisms as those of macroneurons?

ARE MICRONEURONS RESPONSIBLE FOR THE STORAGE OF MEMORY? The possibility that the short-axoned neurons of the brain are its associative and storage elements has been repeatedly raised by writers in this area ever since Cajal made the suggestion on the basis of logical, phylogenetic, and ontogenetic considerations.[1,3,23,67,77,78] The logical argument is that because microneurons have a restricted local input and output, they can have only an associative, possibly reiterative, function in the channeling of neuronal information. The phylogenetic argument is based on the apparent relative and absolute increase in the number of microneurons as we pass from lower to higher invertebrates and vertebrates. Finally, the ontogenetic argument points out that the microneurons are among the last elements of the brain to mature during the development of the individual. The increasing number of interneurons, interposed among the afferent, relay, commissural, and efferent neurons, obviously must raise the possible number of synaptic connections among diverse neuronal elements, and it presumably also increases the computing power and storage capacity of the nervous system. The considerable increase in the relative size of the neuropil in higher forms of species, and in the course of ontogenetic development (as indicated by the rising gray/cell coefficient), reflects further the increasing importance of growing connectivity, which is made possible by the outgrowth of the dendritic fields of microneurons and associated macroneurons.

But how is order and specificity of function established in this complex system with its growing number of functional elements and growing number of interconnections? Do most of these newer components of the nervous system represent merely potential connections or reserve elements, which must be induced to grow and are recruited into functional components by organizational forces exerted through specific patterns of peripheral input? Is it interaction with the environment (or "experience") that induces the development of these elements and specifies the mode of interconnection among them? These and related questions cannot be answered at present, but some of the experimental methods at our disposal today allow us to begin to tackle these problems.

The Enhancement of Learning by Drugs and the Transfer of Learning by Macromolecules

GARDNER C. QUARTON

THIS CHAPTER REVIEWS TWO TYPES OF RESEARCH that have as their goal the clarification of the chemical processes necessary for learning and memory. No one can doubt that such processes are important determinants of these phenomena, both because molecules make up the structure of the components involved in all functions of the brain and because changes associated with function in these components must obey the same chemical laws found in the rest of biology. However, recognition of this highly general role of chemical events in learning and memory is not particularly helpful, except as a reminder that if the chemical processes in the brain are interfered with, in any of a number of ways, learning and memory will probably be impaired or prevented nonspecifically.

Current investigators would like to specify more precise roles for chemical events and frame definite hypotheses that can be disconfirmed. Customarily, there have been attempts to narrow the possible role of those chemical events in learning and memory. Untested but currently fashionable theories of memory have suggested many answers to such questions as: Are there identifiable and specific chemical events, occurring at particular times and particular places, that are critical in determining if an episode of learning occurs at all or that limit rates or levels of learning efficiency, learning consolidation, forgetting, or retrieval? Are there chemical processes that, in some sense, constitute the store of learned material in a coded form?

The distinction between these two questions may disappear as we analyze the problem more carefully. It is, for example, a somewhat arbitrary decision in computer data processing whether to consider a block of past input as existing in the computer as a transform of the original data. This is because past input, current input, and programming instructions are all coded in similar "machine language" and combine to determine the sequential states of the machine. It is probably unwise, at this point, to stretch the analogy between brain and computer and it is, in any case, convenient to break up the problem into a number of separate issues.

Research on the biology of learning and memory can be conveniently divided into two major categories. The first category attempts to combine psychological studies of humans or animals that are learning or demonstrating memory with biological studies of brain function. The second ignores, for the time being, the behaving animal and searches for mechanisms that could plausibly account for behavior. Many of the earlier reports in this volume make a contribution to the second category. Here I will cover two kinds of studies in the first category—those which claim that drugs enhance learning or memory, and those which suggest that the learning process itself can be bypassed and the learned material placed in the brain in a coded molecular form, from which it can be elicited by usual methods.

The two kinds of studies presented here are combined, not only because they are both currently of interest to investigators, but because it is just possible that an apparent transfer of learning is, in fact, an instance of learning enhancement. An understanding of the logic behind both types of investigation seems to be necessary for an interpretation of either.

However, there are serious methodological objections to all combined behavioral–biological studies of cognitive functions. The systems being investigated are big and poorly isolated. There are too many uncontrolled variables, and it is difficult to talk about such investigations without inventing and reifying hypothetical functions and states that may not exist. It is almost certain that these combined behavioral–biological studies will at best supply us only with plausible hypotheses that will require amplification and verification at more basic levels of neuroscience.

This report is made at a very critical time in the history of this science. We cannot yet report the results of conclusive investigations. We are forced to substitute an evaluation of current strategies of investigation and, to add weight to one or another plausible hypothesis, we

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must use those tentative findings on which there is agreement. In spite of the tentative nature of our current answers and the methodological reservations mentioned previously, these investigations are encouraging because they indicate the beginning of serious interest in the biological basis of learning and memory.

The logic of enhancement studies

The processes involved in learning and memory are clearly complex. For instance, an introduced drug that demonstrably improves performance can act at multiple loci in the brain, can act at different stages in the learning and memory processes, and presumably can act on different cooperating mechanisms. But the initial step of any enhancement study is to demonstrate that the drug in question actually does improve performance; this leads automatically to the second step, which is to try to demonstrate where and at what time the drug acts and how it influences performance.

Experiments have shown that the following factors contribute to the variability of drug effects: (1) the type of drug administered; (2) the timing of drug administration in the sequence of learning stages; (3) dosage of the drug and mode of administration; (4) delivery of the drug to the whole brain or to some part of the brain that is isolated successfully; (5) the type of task involved in learning and in retrieval; and (6) such organism variables as age, sex, species, and strain within species.

Because of the problems introduced by the complexity of experimental design, it is usually not feasible, in the same experiment, to investigate the effects of varying all these factors that interact in a complex and nonlinear fashion. However, they all deserve critical attention and analysis, because their interactions often explain results that would otherwise appear paradoxical.

The point in the behavior sequence at which the drug is administered is very important because it defines types of experiments requiring different interpretations. For instance, it can be demonstrated that a group of animals specifically treated with some of the drugs just before learning shows better performance during or after learning than does a comparable group that has not received the drug.

In a second type of experiment, the drug is administered before learning, but a period of time intervenes between the last learning, or acquisition, trials and the later test trials, which demonstrate retrieval of information. Some drugs appear to prevent or delay the loss of learned behavior that ordinarily would occur between the last learning trial and the first test trial. Although all experiments of this class differ somewhat from the simple experiment that tests the effect of a drug on learning rate, it is obviously difficult to differentiate effects on learning from effects on storage, because the improved storage could be caused by a more efficient learning.

In a third type of experiment, the drug is administered after learning trials and before a period of no learning, followed by a period of standard testing. If the drug enhances learning, it is alleged to do so not by influencing learning efficiency but by influencing some process that intervenes between learning and test trials. This type of experiment has been used to investigate two major hypotheses. In the first, the assumption is that some stored trace of memory decays after the completion of the learning trials and that the addition of the enhancing agent slows the rate of decay or changes the type of storage to a more stable form. It is assumed in the second hypothesis that the stored information does not decay, but is displaced by material of a similar kind learned in the intervening period. A drug that prevents exposure to the interfering learning task improves performance of the originally learned task.

In a fourth type of experiment, a drug is administered twice—once before learning and once before test trials. In this kind of enhancement, learning that occurs under the influence of a particular drug is more effective in test trials if the organism is again placed under the influence of that drug. When learning has occurred without a drug, demonstration of effective learning in test trials also may depend on the drug's absence. This phenomenon has been called state-dependent learning. It is also sometimes called dissociated learning, because learning and retrieval of one task can be dissociated from other kinds of learning and/or retrieval by the presence or absence of the drug. This phenomenon is included here because it is obvious that what may appear to be drug-enhanced performance of a particular type of learned behavior may, in fact, be merely the demonstration of state dependence. During the last few years this phenomenon has been studied in detail by a number of investigators. Overton has demonstrated that tasks learned under a barbiturate are best recovered under barbiturate.[1] In a reversal learning experiment he has been able to show that if an animal is taught to go to the left when not under a drug and to go to the right when under the drug, it will subsequently go to the left in test trials when *not* drugged and to the right when drugged. This selective type of "enhancement" has been explained by saying that the drug acts as part of the stimulus set the animal uses as a cue to the appropriate response. An alternative is that the internal connections in the brain necessary to elicit a particular type of learning behavior are shifted by the presence of the drug, and in their shifted state they may

facilitate the learning of some second stimulus–response pairing. The available data do not permit a choice between these hypotheses.

Review of experimental studies of enhancement

There is now a large literature on the enhancement of learning and memory by drugs. This has recently been reviewed by McGaugh[2] and by Jarvik[3] and there is an annotated bibliography on the subject.[4] Only a small number of experiments will be considered here. They have been selected either to illustrate the interaction of the factors discussed above or to suggest the action mechanism of an enhancing drug.

STRYCHNINE In 1917, Lashley reported the facilitation of maze learning by the use of strychnine.[5] Many studies have been done since then, with considerable attention being paid to the dosage, the type of learning task, and other factors. McGaugh and Petrinovich have carried out a series of interrelated experiments that demonstrate the enhancing effect of strychnine and certain similar agents and also show with particular clarity that the effect of the drug depends on an interaction with other factors.[6-10] Large doses of strychnine interfere with learning, whereas small doses clearly facilitate it. The effect of a given dose can vary even with the strain of mouse used in the study. Differences in the effect of the drug are attributable to the sex and age of the animals. Other studies also illustrate that an enhancing drug may be effective when administered just after the learning trials, suggesting both that the drug facilitates some type of storage phenomenon independent of the effect on learning and that there is an optimal time interval during which the drug can act effectively.

There is now evidence that strychnine acts to block inhibitory processes at inhibitory synapses in the central nervous system, or may even cause reversal from inhibition to excitation.[11,12] This suggests that if strychnine improves learning or prevents the loss of stored information, it does so by removing some inhibitory influence on certain systems of neurons in the central nervous system. To date, no experiment gives any information that helps to explain when in the learning process or where in the brain the enhancement occurs.

AMPHETAMINE Other drugs that probably facilitate synaptic transmission in the central nervous system have also been studied extensively. Early studies with amphetamine gave conflicting and paradoxical results.[13-16] It is now believed by many investigators that the drug's facilitating effect is, like strychnine, dose dependent. Large doses may disrupt the learning process while small doses, carefully adjusted, may enhance it. Leaf[17] has carried out studies that suggest amphetamine may disrupt learning in which many trials are massed closely together early in the experimental period. On the other hand, the same dose of the same drug may enhance learning when the trials are more widely spaced over a longer period. Possibly an early effect of amphetamine is disruptive because the drug produces early somatic sensations that upset the animal and mask any facilitating effect the drug may have. Later, the animal may become used to the somatic sensations, so that the enhancing effect, possibly caused by heightened arousal, is unmasked. However, it is not at all certain that the effect of amphetamine is mediated through an effect on arousal. Stein[18] has shown that amphetamine increases the response rate in animals that press a level to self-stimulate certain areas of their brains. He interprets this as a lowering of the "reward" threshold. It is possible that amphetamine alters the threshold of reinforcement and motivation mechanisms.

SCOPOLAMINE Scopolamine is known to have an antagonistic effect to acetylcholine; and, it acts at cholinergic synapses by a so-called anti-muscarinic effect. It demonstrates a competitive or surmountable antagonism to acetylcholine rather than a blocking action by destruction of the transmitter Scopolamine has been of particular interest to those investigating memory because it has been used for years to produce increased memory loss and the so-called twilight sleep phenomenon in childbirth. It is discussed here because Leaf[17] has recently shown that under some conditions this drug may facilitate certain types of learning (free operant avoidance) if it is given in small doses during a task that has early massed learning trials. In other words, it acts in a fashion exactly opposite to that of amphetamine, and demonstrates again that the effect of the drug depends on an interaction between dose level and the nature of the task.

POTASSIUM ION The three drugs discussed so far all appear to act at synapses, and either enhance or inhibit the effectiveness of synaptic transmission at specialized nerve endings. Ions constitute another class of agents that may enhance learning. In 1962, Sachs demonstrated that when given intraventricular injections of potassium ion, in doses of 25 to 37.5 microequivalents, rats improve their performance of conditioned avoidance tasks. In contrast, calcium injections produced less effective performance in animals than did uninjected controls. It is not known how potassium acts to produce this effect, but Sachs suggests that the state-dependent learning phenomenon holds for potassium.[19]

RIBONUCLEIC ACID Because of the belief that macromolecules, particularly nucleotides, might have some bearing on the rate of learning or of forgetting, a number of investigators have administered ribonucleic acid during learning and memory experiments. For instance, Cameron and Solyom[20] administered yeast RNA to several groups of human subjects with memory difficulties in an effort to see if their performance on standard tasks could be improved. These investigators interpret their data as demonstrating a facilitation of memory by yeast RNA. Work in other laboratories have failed to confirm these findings.[21]

Because human experiments are extremely complex and a number of factors enter into the performance of the tasks, it is often useful to complement controversial studies with humans by investigations of simpler animals. In 1963, Cook[22] demonstrated that if rats are given 160 milligrams of yeast RNA per kilogram of animal weight, an improvement in the rate of learning is produced; in addition, when it is administered before learning, a decrease in the amount of learning loss can be demonstrated over time. These experiments support the hypothesis that RNA has an effect on learning and prevents extinction. Replication of this study in a somewhat modified form has been reported by Goren.[23] There is at the present time no real explanation for the mechanisms of RNA action in this situation. It has also been proposed that exogenous RNA uses up available ribonuclease and thus leads to an increase of endogenous RNA, which might be coded with particular engrams. This possibility, too, remains a speculation at the present time because the details of the process have not been worked out.

TRICYANOAMINOPROPENE Tricyanoaminopropene (TRIAP) is a biologically active component of aqueous solutions of malononitrile. Both TRIAP and these solutions are believed to increase RNA synthesis in brain under certain conditions. Hydén has shown that TRIAP decreases RNA synthesis in glia but increases the amount found in neurons.[24] This drug has been investigated for its effect on learning and memory. Chamberlain and colleagues[25] showed that under special conditions it enhanced performance of avoidance conditioning in rats and increased the rate of consolidation of learning in an unusual experiment on fixation of performance in spinal cord. In this study, they demonstrated that if the posture of the animal is made asymmetric by a unilateral cerebellar lesion, this posture will become fixed if sufficient time elapses between the lesion and a transection of the cord. By this method, a minimum consolidation time was determined to be approximately 45 minutes. If TRIAP was administered to the rats, the consolidation time was shortened to approximately 30 minutes.

MAGNESIUM PEMOLINE In 1965, Glasky and Simon reported that magnesium pemoline, a 2-imino-5-phenyl-4-oxyazolidinone and magnesium hydroxide, enhanced nucleic acid synthesis by increasing the activity of true RNA polymerase in vivo and in vitro.[26] This drug, under the trade name of Cylert®, has been used in Europe as a stimulant. Plotnikoff[27] reported a series of learning and memory tests in which rats were given doses of 5, 10, and 20 milligrams per kilogram of body weight. Under all these dosages, the time interval required for a rat to learn to jump out of a box to avoid shock was shortened. In similar studies of retention, controls showed a loss of the response, but all three dosage groups showed continued evidence of learning after many trials.

NITROUS OXIDE I have mentioned that memory mechanisms might be facilitated if the tendency of environmental stimuli to interfere with consolidation could be blocked. In 1957, Summerfield and Steinberg[28] reported that in human subjects nitrous oxide administered after learning led to greater retention than occurred after a control period during which subjects breathed air. This experiment is analogous to Jenkins and Dallenbach's classic 1924 experiment.[29] He showed that human subjects who learned nonsense syllables and then went to sleep for a period of eight hours performed better on waking than they did when they learned other nonsense syllables and then stayed awake for eight hours. He assumed that during sleep the human subjects were not exposed to the effects of other interfering tasks.

Conclusions on enhancement

1) Although learning appears to be an extremely complex process in which multiple factors contribute to successful performance, certain chemicals can act as facilitating agents. Yet dosage size is vital, as certain doses enhance and others impair performance. Enhancement may not be demonstrable for all learning tasks, and it may be possible to show enhancement for one species or for one strain within a species when it cannot be demonstrated for all animals. Only a series of experiments to determine the role of organism factors, of learning task type, and of the time of drug administration can show what part of the learning process is facilitated by a drug.

2) Drugs that enhance learning fall into distinct classes. Probably some of them act at the synapse, changing the threshold of a behavioral response by altering chemical transmission; the actual synapses involved are as yet unidentified. The other drugs are less well understood. One group of substances modifies behavior by altering the quantity of available ions in nervous tissue. These may act

through a very large number of possible ionic mechanisms, which are difficult to separate experimentally. RNA introduced from sources outside the animal has been reported to enhance learning, as have drugs which increase RNA synthesis in neurons. These findings are difficult to interpret without more detailed investigations.

3) Although it is not known in detail how any of these agents that have been demonstrated to enhance learning actually accomplish this feat, it is quite possible that they act by affecting single rate-limiting chemical steps. This would mean that they do not determine the message that is learned, stored, or retrieved. They may influence only the rate of information processing, and this influence may be extremely indirect and nonspecific.

The logic of transfer studies

Only in the last decade has it been believed that memories might be stored in macromolecules—a hypothesis first proposed by Katz and Halstead in 1950.[30] This hypothesis led to the more radical one that learning may be transferred from one animal to another by means of a brain extract. The logic is based on two points. First is the analogy with the transformation principle as it has been developed for the genetic code. In 1928, it was discovered that heat-killed pneumococci could make nonpathogenic material pathogenic, if the pathogenic but dead bacteria were added to the living nonpathogenic bacteria. It was later learned that DNA was the message carrier and that it entered the chromosomes of the host animal by some sort of genetic recombination. Once in the cell it could direct protein synthesis in the host animal. A crucial part of the argument is that the message transmitted from the donor to the recipient was specific.

The second argument for macromolecular storage is based on the enormous capacity of large chain molecules to store information in permutations of their parts. Hydén has estimated that a chain of naturally occurring RNA base sequences can encode 10^{15} bits.[30a]

These arguments led to experiments in which a brain extract, believed to contain macromolecules containing memory information, was injected into recipient animals. When first tried, these experiments were wild gambles; it is still not possible to say whether they have payed off, because they have proved difficult to replicate and therefore the scientific interpretation is still very much in doubt.

Review of experiments on the transfer of learning by brain extract

It is convenient to divide learning experiments into those with invertebrates (particularly planarians) and those with vertebrates (particularly mammals).

Planarian learning is discussed in the chapter by Eisenstein (this volume) and a number of recent reviews and bibliographies are available,[31,32] so I shall discuss only two controversial issues. Can it be demonstrated that planarians learn? Is this learning transferable from one animal to another?

Planarians are probably capable of learning, in some sense, although their behavioral repertoire is not large and there is a wide range in the judgment of human observers as to the presence or absence of certain types of responses. A large number of experimental conditions must be carefully controlled if the animals are to perform as expected. Jacobson has recently published a convincing report of conditioning of planarians.[33]

In 1962, McConnell reported that the learning in planarians survived if the animal was cut up and subsequently allowed to regenerate; in addition, the learning seemed to be transferable to an untrained animal by cannibalistic ingestion.[34,35] There have also been reports that ribonuclease may remove the conditioning in regenerating planarians, suggesting that RNA is necessary for retention of "conditioned" behavior.[36]

Recently, Jacobson has reported what appears to be a carefully designed study in which RNA was extracted from trained planarians and introduced into untrained animals. The latter's subsequent responsiveness to light cannot be accounted for by nutrition, handling, or stimulation received by the donor group. The necessary condition for the effect appears to be the pairing of light and shock in the training procedure.[33]

In this study, three groups of planarians were used for donor groups. One group of two hundred animals received trials of light paired with shock; a second received an equal number of light and shock stimuli that were not paired; the third received no light or shock stimuli. The RNA extraction was done in such a way that the experimenters did not know the identity of any group. An experimental group of twenty-five planarians was given extract prepared from donors that had received light paired with shock. Control groups received injections from the other two donor groups. The injections and testing were "blind," and the test trials did not include shock, so that no new learning occurred. The experimental group was clearly differentiated from both control groups. These results are impressive if they can be repeated in other laboratories.

Like other animals in a conditioning situation, planarians appear to demonstrate an extinction of the conditioned response if the tests continue after criterion has been reached, but without the pairing of the conditioned and unconditioned stimuli. Jacobson[37] has shown that the effects on naive recipients of "RNA" from planarians after

extinction cannot be differentiated from the effects produced by "RNA" from conditioned planarians. This interesting phenomenon is difficult to interpret.

The studies of the transfer of learning by brain extract in mammals are conveniently classified according to a scheme demonstrated in Figure 1. The figure is essentially a matrix in which the columns are chosen on a principle of partition based on the origin and destination of brain extract—from one individual back into that same individual; from one individual to another of the same species; or from one individual to another of a different species. The rows of this matrix describe different learning tasks increasing in complexity from habituation to discrimination learning. Studies have been done on other types of learning, but they are not included in this selective review.[38]

It is obvious, by analogy with the enhancement experiments, that many factors combine to produce effective performance. We must be concerned with the mode of preparation of the extract and the possible chemical components that may convey the information. We must also be concerned with the mode of injection and the possible pathway by which the macromolecules arrive at their action site. Such experiments may be effective in one species or in one strain and not in others, and it is quite likely that the specific type of learning task is critical.

In 1966, Albert reported a series of studies investigating the transfer of learned experience by brain extracts within the same individual rat.[39] His experiment was based on models developed by Bureš[40] and by Russell and Ochs,[41] in which learning is confined to one hemisphere of the brain. In these studies, one half of a rat's brain is made reversibly nonfunctional by producing spreading depression by injecting potassium chloride through a cannula. The animal is then taught a task and the suppressed side is allowed to recover; then the side in which learning presumably occurred is itself suppressed in the same manner. Under these conditions, the animal will not demonstrate learning. If, on the other hand, the animal is given one or more trials in which both halves of the brain are used, and if the side originally learning the task is then suppressed, the animal appears to have transferred the learning from one half to the other half of the brain, and now performs perfectly well.

On day one, Albert depressed side A with a 12 per cent potassium injection and trained the animal in an avoidance task; on day two, he removed the cortex from the medial

	Transfer of brain extract from one side of an animal's brain into the same animal.	Transfer of brain extract from the brain of one animal to another animal of the same species.	Transfer of brain extract from the brain of one animal to another animal of different species.
Habituation			Ungar (47)
Approach Learning		Jacobson (42)	Jacobson (48)
Avoidance Learning	Albert (39)	Rosenblatt (38)	
Discrimination	Albert (39)	Jacobson (46)	

FIGURE 1 Illustrative experiments in the transfer of learning in mammals.

portion of side B and injected it intraperitoneally into the same animal. On the third day, he depressed side B and tested the animal to see if it could learn the avoidance task more rapidly than a control group. (The medial portion of the cortex was selected because in preliminary experiments Albert had shown that this portion must be intact for memory.[39])

The medial cortex was removed by suction and placed in 5 milliliters of a 0.9 per cent solution of ice-cold sodium chloride. It was broken into small pieces by manipulating it by hand in a homogenizer and was injected into the peritoneal space with an 18-gauge needle. Both preinjection and postinjection training tasks took place in an avoidance box 10 by 36 by 18 inches deep. The animal was placed midway in the box between a white and a black end. It received a shock if it remained in the white end but could avoid shock by returning to the black portion of the box. The results demonstrated that animals which had received extracts from the trained side performed demonstrably better at their learning task than did control animals which had received no injection. Albert also did experiments to determine the length of time it took for memories to consolidate in the donor animal. He found that if the brain fragment was removed immediately after learning, the transfer phenomenon could not be demonstrated. He concluded that the donor animal must live for approximately eight hours before learning achieves a form in which it can be transferred by injection.

Albert also did an experiment in which he transferred learning from an animal to itself by using a "discrimination" learning task. The donor animals received training in an avoidance box with side A of the brain depressed by potassium chloride. They were all trained to go to the white end of the box to avoid a shock. The trained medial cortex of the experimental animals was injected into the peritoneal cavities; the B side, which had previously learned the task, was then suppressed. They could avoid the shock either by going to the white end or by going in the opposite direction to the black end. The experimental animals that had received extract from their own brains after training tended to go to the white end of the box, whereas the control animals went in either direction.

The standard experiment for "within-species transfer" can be illustrated by the study performed by Jacobson.[42] Donor rats in a standard Grayson–Stadler box were given preliminary training to approach the food cup when it made a clicking sound. The animals were deprived of food for 48 hours and their behavior was slowly shaped. First, two pellets were placed in the cup; then the cup was operated with pellets in place and the click occurred when the animals were farther and farther away from the box. They were not rewarded when they merely investigated the cup. They then received 200 food-reinforced clicks for four days and 100 food-reinforced clicks on the fifth day. The animals were then killed and approximately one gram of brain was removed and extracted by a procedure (to be discussed below) that was alleged to have produced an "RNA" extract. This extract was injected into the experimental animals. The experimental animals and controls were studied in the same learning situation, and the injected animals showed a much greater tendency to go to the food box when the click occurred. Jacobson and his group have since improved and standardized their experimental procedure.

Experiments differing in detail but with somewhat similar results have been reported by Fjerdingstaad, Nissen, and Roigaard-Petersen[43,44] and by Rosenblatt et al.[38,45] In many laboratories efforts have been made to replicate the Jacobson study. The degree of similarity between studies is difficult to determine.

Transfer in an avoidance task can be demonstrated by studies reported by Rosenblatt.[38] He used an automatically recording shuttle box with an electrified grid floor. His donor animals were trained to avoid a shock by crossing the box within two seconds of a buzzer. A brain extract from donor animals was prepared in a rather special manner that will not be discussed here. The 14 experimental animals received extract from buzzer-trained animals, intraperitoneally, while the 14 control animals received extract from untrained donors. Although the scoring was done by a complicated procedure that is difficult to evaluate, the experimental group appears to have done significantly better than controls.

I shall return to Jacobson's investigations to select an example of "standard" experiments on transfer effects in a discrimination learning task, where the extract is taken from one animal and given to another of the same species.[46] This experiment, usually called the "click-light experiment," was similar to Jacobson's work previously described, except that in addition to the donor rats trained to go to the food cup on a click, another group was trained to go to the food cup on a light cue. The brains of the click-trained and light-trained groups were prepared in the fashion already described and injected into donor animals. The click-trained recipients tended to respond to the click and not to the light and the light-trained recipients tended to respond to the light and not to the click.

Jacobson and his group have also reported a similar study in which animals performed in a Y-maze. Donor animals trained to discriminate a brightness level could be used to prepare the extract for animals that would show considerable savings in using this same cue in their learned response. Rosenblatt and his group have also reported experiments in which specific discriminated cues were use-

ful to the recipient animal when injected from specifically trained donors.[38] Fjerdingstad et al.,[43] reported studies apparently demonstrating transfer of a discrimination task, but the results are based on small samples and are difficult to interpret. Mention of them is made here because the recipient animals seemed to show a preference for conditions *exactly opposite* those of the donor animals from which they received extract.

The only experiment on habituation in mammals reported at this date is that by Ungar.[47] In addition, his work is a demonstration of transfer from one species to another. In this experiment, rats were trained to habituate to the sound of a steel hammer on a plate. The donor rat was exposed to the hammer sound every five seconds for two one-hour periods daily, plus 100 test trials, which were used to measure the degree of habituation. The startle response slowly disappeared with habituation. Complete habituation appeared to occur in 9 ± 4.5 days. Donor rat brains were removed and homogenated in saline and injected into mice. Brains from animals that were not habituated to the hammer sound were similarly prepared. Ungar reports that those mice receiving sound-habituated rat-brain habituated themselves to a 50 per cent criterion in 2.1 ± 2.5 days. On the other hand, those receiving control brain did not habituate to a similar criterion until 12.0 ± 1.5 days.

The simple approach-to-click learning task also has been used by Babich, Jacobson, and Bubash to demonstrate transfer of learning across species.[48]

EVIDENCE FOR THE NATURE OF THE MOLECULE In Jacobson's initial experiments, he attempted to purify the brain extract and believed that he was making a preparation rich in brain RNA. In his basic procedure the animal was killed by ether, the whole brain was removed, and the anterior and posterior portions were rejected. The remaining brain weighed about one gram. It was placed in a cold mortar with five milliliters of 90 per cent cold phenol and five milliliters of isotonic saline, and ground in a mortar with pure sand for about five minutes. It was centrifuged at 18,000 revolutions a minute for 30 minutes at 0° C. In an effort to avoid phenol contamination, the aqueous portion was carefully removed and brought up to 0.1 molar with magnesium chloride. The "RNA" was precipitated with two volumes of cold ethanol for an average of 15 minutes, centrifuged at 6000 revolutions per minute for 15 minutes, and the supernatant poured off. The ethanol was evaporated in air and the pellet fraction was dissolved in one to one-and-a-half milliliters of isotonic saline preparation.

This procedure has been used in other laboratories, but it has been repeatedly argued by chemists that the method does not insure that RNA is the only active macromolecule injected into the recipient animals. A number of other experimenters have begun by using a crude brain extract.

The argument that RNA is the substance containing the effective component of the brain in transfer experiments has been supported by Jacobson, et al., Nissen, et al., and by Albert. Rosenblatt and his group and Ungar, et al., believe that the effective substance is a polypeptide. The argument for RNA is based on the apparent transfer obtained by those using an RNA extraction procedure as outlined above. Successful results have also been claimed by Jacobson, et al., with still further purified substances prepared on sephadex columns. They say that in at least some studies the effective preparations were proved negative for protein by a biuret test.

The argument against RNA and in favor of polypeptide has been made in detail by Rosenblatt.[45] He argues that because the transfer was obtained from an intraperitoneal injection, RNA would be broken down in getting to the brain. He also argues that the activity of the preparation persisted after ribonuclease incubation. Ungar also found this, but Albert believed that the activity of his preparation was lost after such incubation. Both Rosenblatt and Ungar believe that the molecular weight of the effective substances was under 3000, and maintain that the transfer effect is abolished by chymotrypsin. They say that the effective substance appears to be soluble in phenol and insoluble in acetone. Rosenblatt showed by the Folin-Ciocalteu test that peptide was present.

There is still further evidence against the role of RNA in this transfer. In 1966, Luttges and colleagues attempted to measure the amount of labeled RNA which can enter and be detected in brain.[49] Brain RNA was labeled in vivo by injecting P^{32} orthophosphate directly into the intraventricular cavity. Additional labeled P^{32} RNA was prepared in mouse L-cells propagated in tissue culture. In both instances, the RNA was extracted by the cold phenol alcohol-precipitation method. It was reprecipitated ten times in the presence of 0.001 molar ethylenediaminetetra acetate (EDTA) to remove the bulk of the ethanol-soluble P^{32}. Rats were injected intraperitoneally with 10^6 counts per minute labeled RNA, were killed at various times, and the radioactivity was measured in peritoneal fluid, blood, and brain. Definite amounts of radioactive phosphorous were found in peritoneal fluid and blood, although it rapidly disappeared and was discovered in the urine. Negligible amounts of RNA were detectable in brain.

These studies taken together suggest that the substance that accounts for the alleged transfer of learning is not likely to be RNA—unless extremely small quantities are effective. Much more systematic studies of brain extracts

are clearly required before any conclusions can be made.

Evidence for and against transfer

The basic experiments demonstrating transfer have now been carried out in one form or another in at least six laboratories, with positive results. Naturally, these have created great interest in the scientific community. In a number of laboratories, carefully designed studies utilizing the same methods as those apparently employed in the original experiments have failed to replicate the original results.[49,50] Some of these negative results have been published in a joint letter.[51] Other experiments with slight modifications in procedures have also been negative.

This conflict in experimental results has led to a standard problem. On the one hand, it is always possible that the hypothesis is wrong and that the original results were obtained by accidents of sampling. On the other hand, it is possible that the experiments were correct and demonstrate a valid phenomenon. The failure to replicate perhaps can be explained because some of the subtle conditions existing in the original experiments were not duplicated in the later attempts or because some aspect of the study was not carried out in precisely the same way as in the original studies.

At the present time, it is impossible to determine the ultimate scientific opinion on this matter. Many investigations were carried out using an RNA extraction method, so it is obvious that a greater effort should be made to use polypeptide extracts and that a considerably greater effort should be made to purify the substances used. It is also possible that these experiments are valid when they are carried out "within an individual" as they are in the Albert studies, but not across individuals or across species or genera. Therefore, replications of the Albert type may clarify the situation. We are left with a scientific puzzle that can be solved only by careful systematic studies in many laboratories. Negative studies should not be used as an argument to kill further research on the transfer problem. It has been noted by a number of workers that in many areas of research that began with similar vague exploratory experiments, original efforts to repeat the early positive studies were unsuccessful, and it was only after the phenomenon had been considerably clarified that the reasons for the failure to replicate became clear.

Implications of experiments on the transfer of learning

SHOULD INCONCLUSIVE FINDINGS BE ANALYZED? As the evidence for the transfer of learning has been seriously challenged because of difficulty in replication, an argument can be made to postpone any analysis of the implications of these studies until the evidence for or against the phenomenon is more certain. I shall defend a contrary position. If these studies ultimately turn out to be unacceptable, the controversy over the possibility of the transfer of learning will still have served to force clarification of our hypotheses concerning the biological mechanisms of learning and memory. If, on the contrary, more and more evidence emerges in support of the transfer concept, a careful analysis of the implications of specific experiments may serve to guide research toward further, crucial experiments that will select among equally plausible alternatives.

EXPERIMENTAL RESULTS DO NOT SUPPORT ONE SIMPLE HYPOTHESIS Those transfer experiments already completed make up an assortment of studies that do not support any one simple hypothesis. This forces us to consider the different types separately. It is quite possible that some experiments will be duplicated and that others will not. Some experiments, if replicable, will turn out to have many alternate interpretations, many of them not particularly radical and possibly not even critical in selecting among alternate and incompatible theories of memory. Other experiments, also called "transfer of learning experiments" in the jargon of the laboratory, will turn out to be highly crucial in forcing a choice among alternative explanations. Following this line of reasoning, I shall draw tentative inferences from categories of transfer experiments, but not from the whole set.

EVALUATION OF EXPERIMENTS ON HABITUATION Two types of reservations must be proposed in evaluating Ungar's experiments on habituation. First, even if we accept the experimental results at face value, we do not need to conclude that any message was transferred from one animal to another. Habituation in a donor animal can lead to the production of some molecular species that would speed habituation in the recipient by relatively nonspecific mechanisms. In other words, the mechanism by which habituating rate was altered in the recipient animal may more nearly resemble "enhancement" than it does the transfer of specific learned patterns.

Second, Ungar uses the definition proposed by Thorpe, which states that habituation is the loss of an innate response to a stimulus repeated without reinforcement.[52] Thorpe himself assumes that the reader will differentiate habituation from such a process as sensory adaptation, which also can cause a diminution in response. There is a more useful definition of habituation, used by many experimenters at the present time (see Eisenstein's chapter). This describes habituation as the decrease in the magnitude

of a specific response to a specific class of discrete stimuli when these stimuli are repeatedly presented, *if the decrease in response magnitude can be shown not to be caused by sensory adaptation, fatigue, or some similar process;* this is shown by a return of the original response magnitude following a minor change in some stimulus parameter. Ungar accepts the simpler definition and does not demonstrate a return of the initial response by any alteration of the stimulus, so we cannot be sure whether he has speeded up habituation or some other process, such as fatigue, that could account for the same apparent change. Also, we do not know if the habituation-speeding factor produces effects specific to the response he studied or if there is a class of responses, not elicited by experimentally presented stimuli, that were also reduced in magnitude by the injection of brain extract from another animal.

Thus, the studies of "transfer of habituation" are inconclusive in several respects. Similar reservations can be made concerning planarian habituation studies.

EVALUATION OF EXPERIMENTS IN WHICH ANIMALS APPROACH A FOOD CUP WHEN A CUE IS PRESENTED These experiments have been subjected to some criticism by psychologists. Jacobson and the other experimenters who have used this procedure are themselves aware of limitations in it and of the reservations that must be made in interpreting the results.[53,54] Later experiments have met some difficulties based on the need for a clear definition of the reinforced response, and on the need for studies that equalize exposure to clicks, adaptation to the box, handling, etc., between experimental animals and controls.

The most important problem in interpretation, however, lies elsewhere. An approach to the food cup by animals that have received brain extract from trained animals may be a nonspecific effect rather than a sign of the transfer of associative learning. The increased incidence of the critical response may be elicited by stimuli other than the specific cues identified in the description of the experiments. It may be produced by some nutritional, motivational, emotional, or activity-level effect of the injection of brain extract. It may be a consequence of some nonspecific effect of the quantity of stimulation.

Difficulties of interpretation of this type are usually met by changing the nature of the learning task in such a way that one can demonstrate that the animal must recognize specific features of the eliciting stimulus if the increased incidence of the preselected response is to occur. Jacobson and other groups working on the problem have done this by designing experiments of the type I have included under discrimination studies (Figure 1), but these deserve separate evaluation.

It is probably useful to point out that the effect produced in these approach-to-food-cup studies could not be a simple enhancement of learning because, unlike the habituation studies I have discussed, the recipient animals are not in a learning situation; their trips to the food cup are unrewarded.

EVALUATION OF EXPERIMENTS ON AVOIDANCE CONDITIONING These experiments are particularly difficult to evaluate because of many differences in specific features of procedures.

If we are concerned with the implications of these experiments for possible transfer of specific information about the learning situation, we must evaluate the Albert experiments separately from the Rosenblatt studies. Albert does not appear to have used any cue for the animal other than placing it in the part of the box in which a shock was possible. As a result, it is difficult to be sure of the specificity of information used by the animals to avoid being shocked. Also, Albert used a savings method of testing his recipient animals. Hence, even if we accept his positive results at face value, the improved performance in recipient rats could be a nonspecific enhancement of learning in the experimental animals brought about in some way by the extract from trained brain.

Rosenblatt, on the other hand, used a shock-avoidance design in which both light and buzzer could have been avoidance cues, or one or the other could have been a control. As he did not use a savings method for demonstrating transfer, the effects produced cannot be explained by mere enhancement of learning in the recipient animals. Rosenblatt does not describe his procedure in detail, and he investigated many parameters at the same time. However, if avoidance experiments of this type could be duplicated it would suggest that information associating a specific stimulus and some punishing consequence could be transferred in brain extracts.

EVALUATION OF EXPERIMENTS ON DISCRIMINATION The discrimination studies also vary greatly in details, making it difficult to evaluate them as a group. However, they all have the common requirement that a donor animal learn to discriminate one cue from at least one other cue and that the response be associated only with the stimulus selected by the experimenter and discriminated by the animal. If responses to this specific stimulus occur above the level of chance in recipient animals that have had no training permitting association of this stimulus with specific reward or punishment, the experiments support the argument that the recipient animals have been supplied a patterned template that couples a transform of the discriminated stimulus to a patterned performance. Such experiments do *not* suggest how all this is accomplished.

This conclusion seems to me to be the most important statement that can be made in a cautious review of this type. The supporting logic must be scrutinized for defects, and the empirical data that is gathered to demonstrate this type of phenomenon must be based on easily replicable methods.

WHAT FORM COULD THE PATTERNED TEMPLATE TAKE IN THE MOLECULAR SOUP OF THE EXTRACT? If transfer of discrimination learning could be effectively demonstrated, it would still be necessary to find the molecular code that made it possible. At the present time, arguments favor either RNA or proteins. The experimental data are still clearly inconclusive, even if there were no doubt about the behavioral aspects of the experiments.

There are, however, added problems that deserve at least passing consideration. It would require multiple bits to code the unique features of the stimulus, the unique features of the response, the effectiveness of stimulus–response coupling, the temporal relationships, and perhaps the consequences of obeying or ignoring the stimulus. These multiple bits of information must be stored in a relatively stable form in the brain extract, and cannot be coded by the presence or absence of a particular molecular subspecies that differs from another molecule by only one building block.

If it develops that transfer of discrimination learning requires serious consideration, we shall be forced to evaluate the merits and plausibility of a number of different types of codes, including the following:

1) Multiple recognition sites on one molecular subspecies. The quantity of the subspecies merely determines the amplification of the effect.

2) A macromolecular subspecies, with information stored in its primary sequence, but which directs the synthesis of a number of separate molecules in the recipient animal.

3) A macromolecular subspecies capable of higher conformational changes that are stable under the conditions of use and store the multiple bits required.

4) An assembly of molecules, so combined that the assembly has multiple-bit recognition properties.

5) A set of separate, but different, molecules that together, in spite of disordered arrangements of individual molecules, transfer the multiple bits.

6) A profile of different quantities of different molecular subspecies.

Almost all these possibilities pose major problems of formation, stability of information storage, mechanism of amplification, and so forth. It is especially difficult to see how the particular molecular code is assigned to a particular learning pattern.

HOW IS THE PATTERNED MULTIPLE-BIT STORE GENERATED IN THE DONOR BRAIN? A successful experiment of the discrimination type would imply that the multiple-bit information templates in a stable molecular form must have originated in the donor animal's brain and that the unique information pattern must have resulted from some consequence of learning and consolidation. These consequences must maintain a rather large amount of information invariant through a series of transforms. Timing, discriminated cues associated with the stimulus, and perhaps other specific information must enter the brain as patterns of nerve impulses, arriving over a pattern of input channels, patterned in time. The mechanisms that could produce such a transformation are at present totally unknown, but they must be necessary if learning can create or select molecules capable of producing the effects of learning in an animal that has not learned.

HOW DOES THE PATTERNED MULTIPLE-BIT STORE REACH THE RECIPIENT BRAIN (AND POSSIBLY A SPECIFIC SITE IN THE RECIPIENT BRAIN) WHEN IT IS PLACED IN THE RECIPIENT ANIMAL AT SOME DISTANT LOCUS (SAY THE PERITONEAL CAVITY)? A successful experiment of the discrimination type would indicate that a multiple-bit template stored in a stable molecular form can enter the bloodstream from the peritoneal cavity. It can then reach and enter the brain through the blood-brain barrier, and can find its way—either because it goes everywhere within the brain or because of specific recognition mechanisms—and arrive at a site or sites at which its information can be transformed into patterned behavior mediated by patterned neuronal activity.

It is not easy to dismiss the evidence that very little labeled RNA can be found in brain after it is injected in the peritoneal cavity. It certainly suggests that the molecule is not RNA, or that very few molecules are involved in the information transfer.

HOW DO THESE PATTERNED TEMPLATES FROM OUTSIDE THE BODY SUCCESSFULLY SUBSTITUTE FOR THE SIMILAR TEMPLATES WHICH, UNDER ORDINARY CIRCUMSTANCES OF LEARNING, ARE ASSUMED TO BE PRODUCED IN THE RECIPIENT ANIMAL? A successful experiment of the discrimination type could suggest that free substitutability is possible between templates from outside the cell and those made within the cell. Why is there no competition between foreign imports and domestic products?

HOW IS SOME DEGREE OF PERMANENCE ACHIEVED BY THE FOREIGN IMPORT MOLECULE? A successful experiment of the discrimination type also requires that the

template introduced into the body must either last long enough to provide the pattern that influences the elicited but unlearned behavior, or that it have some mechanism for replicating the relevant part of the message.

THE PROBLEM OF COMMON INTERINDIVIDUAL VOCABULARY AND SYNTAX If Albert is correct in concluding that transfer is possible only when donor and recipient are the same animal, we will be saved some perplexing problems. It is at least plausible that a complex molecule with multiple recognition sites would find its way both to an effective set of neurons within one brain and to a special site on or in organelles, where it could, in some unknown fashion, determine a pattern of behavior. However, if the vocabulary of such multiple-bit patterns is identical in different individuals or even in different species, an additional assumption must be made—that the same meaning is assigned to a message vehicle in all animals of the category, and that therefore this assignment is innate and independent of the experience of individual animals. Under the circumstances, the effect of experience would be to select and use an assigned meaning-message vehicle coupling, rather than to create it. This innate assignment does not seem plausible.

It should be stressed that the serious problems of a cross-species vocabulary do not arise unless the learning pattern requires considerable specificity of the template. If the transfer across species can be interpreted as a type of "enhancement," it is not difficult to imagine mechanisms that could be effective in a number of different species.

Summary

The data reported here under the headings "enhancement" and "transfer of learning" must be considered in conjunction with other experiments of the behavioral–biological type, particularly those described in this volume by Drs. Agranoff and Hydén. Taken together, there is some evidence for the involvement of macromolecules (RNA and/or protein) in some aspects of the learning-consolidation-memory functions. In my opinion, we cannot at this point argue that anything is stored in multiple bits in large molecules. The data (with the possible exception of that derived from the transfer of discrimination learning) is consistent with a wide range of biological theories of learning.

A survey of this type tends to put the emphasis on the inconclusiveness of most of the experiments so far completed. It seems obvious that our most important need is not necessarily to investigate how complex psychological processes are handled by biological mechanisms, but to find ways of identifying simple instances of these processes that have not lost their critical features in the simplification.

Agents That Block Memory

B. W. AGRANOFF

OUR LABORATORY is engaged in studies of the effects of selective blocking agents on behavior. Compared with the precise anatomical, electrophysiological, and analytical chemical techniques described in this volume, this frontal attack might be considered crude and perhaps foolhardy. Direct approaches are deceptive and most biological phenomena are not simple. Nevertheless, the macro experiment often brings new techniques and insights. This chapter includes a description of our work[1] in the context of related experiments in other laboratories, compares where possible, the various experiments, and finally restates the problem we are studying in the light of our present knowledge.

Selective blocking agents

These blocking agents are for the most part microbial antimetabolites, characteristic tools of this decade in biochemistry. Some of them—the antibiotics and antitumor agents—have revolutionized modern medicine. To the biochemist, they are highly unlikely-looking synthetic organic molecules that function as metabolic monkey wrenches. Hundreds of such agents have been screened for possible use. Often the structure of an isolated compound is unknown and the mechanism of selective toxic action has not been clarified. Generally, they have a molecular weight below 1000 and they block enzymes that constitute key steps in cell growth, such as cell wall synthesis, DNA replication, and RNA transcription and translation.[2] Selective blocking agents from plants and animals can act on animal cells to block mitochondrial oxidative phosphorylation, sodium transport, and other specialized functions. In the past few years, selective blocking agents have been used in studies on learning and memory. That a selective blocking agent affects a specific physiological process does not prove a causal relationship. However, inferences drawn from large numbers of such experiments may well clarify the underlying biochemical mechanisms.

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What are the properties of memory?

We will deal primarily with studies on long-term memory. Higher animals, most commonly vertebrates, can be subjected to sensory inputs for a short period of time (seconds to minutes) and subsequently demonstrate motor outputs that would probably not have followed the inputs before stimuli were applied. The new behavioral sensory-motor sequence is stored for long periods of time—months, years, often the lifetime of an animal. A general scheme for such a sequence is seen in Figure 1. Experimental animals receive training trials until they reach a preset criterion—such as nine out of ten correct consecutive responses—or until they have completed a specified number of trials. For example, animals must move to the correct arm of a Y-maze in order to avoid a mild electrical shock. Because they can go right or left, they begin with a 0.5 probability of making a correct response, and they soon improve. When tested days later, they make fewer errors than they did the first day. They can be injected or treated with a disrupting agent during the pretraining or post-training period. A characteristic of long-term memory is that it is susceptible to disruptive agents immediately following training and that it ceases to be susceptible within minutes or hours. Thus, animals treated immediately after trials lose memory of the training, while animals injected later do not.[3] The period of time during which memory becomes insusceptible or fixed has been termed consolidation. It was proposed 60 years ago[4] to explain amnesia following external interference in humans. The rapid acquisition of new behavior, its fixation or consolidation shortly after training, and its long-term storage and retrieval are the operational characteristics that for us define long-term memory.

Long-term memory has been studied almost exclusively in vertebrates. The demonstration of an experimentally reliable long-term memory in simpler animals could prove extremely useful. Out of blind faith in the universality of biological mechanisms, we suppose that long-term memory formation is similar in different species. We further assume that the process whereby memory is fixed in a long-term form involves covalent bonds and can be studied with conventional biochemical tools. As we will see, there is evidence for at least one form of short-lived memory.

This type of memory may not involve covalent bond formation. It is possible that in organisms with short lifetimes, short-term memory is present and the long-term component is absent.

Over the past 30 years, electroconvulsive shock studies in man and animals have confirmed the existence of a post-trial consolidation period, as had been predicted.[5] Of particular interest was the report[6] that cooling and the resultant apparent cessation of electrical activity in the brain of a hamster did not destroy memory, but did prolong the consolidation period as determined by susceptibility to electroconvulsive shock.

THE USE OF SELECTIVE BLOCKING AGENTS IN BEHAVIORAL STUDIES The antimetabolite-behavior experiment was introduced in 1963.[7] They reported the effects of the purine antagonist, 8-azaguanine, a substance known to block RNA synthesis. The purine analog was injected intracisternally into rats previously trained in a swimming maze. The drug did not reportedly interfere with performance of a previously learned maze or with the rate of swimming, but it did block the acquisition of a new maze in animals tested thirty minutes after injection. The authors did not distinguish a defect in learning from other complicating factors, such as changes in motivation, which might differentially affect performance in a new maze compared to one previously learned.

Chamberlain, Rothschild, and Gerard[8] measured the effect of intraperitoneally injected 8-azaguanine in rats learning a Hebb-Williams maze and observed no effects. The drug did affect the fixation time of hind limb asymmetry following a cerebellar lesion. If the spinal cord was transected less than forty-five minutes after development

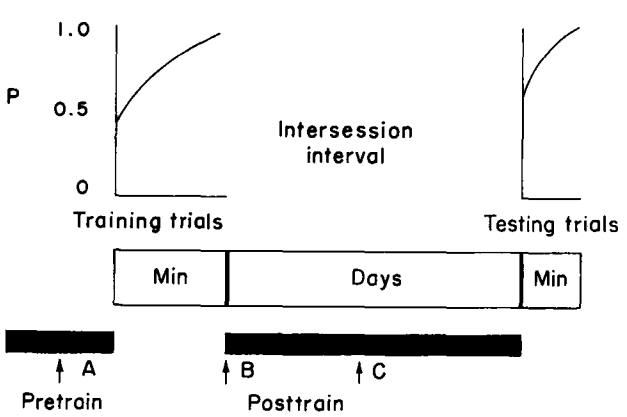


FIGURE 1 Representation of schedules for experiments on long-term memory formation (see text).

of the centrally created asymmetry, the effect did not persist. If 8-azaguanine was injected intraperitoneally about five hours before production of the asymmetry, the fixation time was extended to seventy minutes. While this preparation was only a model for memory formation, there did appear to be a surgically disruptible fixation period.

In 1963, Flexner, Flexner, and Stellar[9] reported that puromycin, the antibiotic antimetabolite, blocked memory of training in a maze when injected bilaterally into temporal sites in the mouse brain. Mice were placed in the stem of a Y-maze and were trained to move into the correct arm (right or left) within five seconds in order to avoid a shock via floor grids. The shock was continued until the mice found the correct arm. Training to a prescribed level of probability of correct responding (criterion) took about twenty minutes.[10] Good memory of the training could be demonstrated five weeks later. Table I is based on the findings of that study. In uninjected control animals (Group A) memory was retained for at least five weeks. Bilateral temporal injections of puromycin blocked memory formation. Five days after training, however, such injections were ineffective. Bitemporal injections obliterated memory only if administered within three days. Frontal or ventricular injections had no effect. If combined temporal, ventricular, and frontal injections were given as long as forty-three days after training, memory was reportedly lost. The earliest injection time was twenty-four hours after training. There was no report on the effect of the drug if it was injected in the hours immediately after the trials. Thus, the usual type of consolidation or memory fixation was not investigated. The intracerebral injections were performed under anesthesia by means of a stereotaxic apparatus. The combined effects of anesthesia, drug, and the intracerebral lesion made by the needle precluded experiments in which animals were injected immediately before training.

In an additional experiment, animals were trained to go to one arm of the Y-maze and three weeks later were trained to go to the opposite arm. If untreated and then retested they demonstrated the most recent training. However, animals injected in bitemporal sites a day after the reversal training and tested three days later had no memory of the recent training. They showed preference for the arm to which they had been trained originally. Interest in these experiments was heightened by the knowledge that puromycin selectively and reversibly blocks protein synthesis both in vitro and in vivo. Puromycin (Figure 2) is a nucleoside-amino acid and resembles structurally the aminoacyl terminus of tRNA.[11] The growing peptide chain attaches to the free amino group of puromycin and is prematurely released from the polysome as peptidyl puromycin.[12-14] On

TABLE I

Effect of intracerebral puromycin on memory in mice

	Intersession Interval (Days)		Injection Site	Memory
a	1–35		O	Retained
	Train → Inject → Test			
b	1–3	3	T	Lost
c	1	3	F; V; V + F	Retained
d	6–35	3	T	Retained
e	18–43	3	T + V + F	Lost
f	28–43	3	V + F; V + T; T + F	Retained

T = temporal sites V = ventricular sites F = frontal sites

the basis of this and further studies on the effect of puromycin on protein synthesis in the mouse brain, the University of Pennsylvania group tentatively concluded that if protein synthesis in the entorhinal cortex and hippocampus is blocked for eight to ten hours between twenty-four and seventy-two hours following training, permanent memory formation is blocked.[15-18] Further, the fact that memory could be disrupted weeks after training suggested that there existed a maintenance phase of long-term memory that was also susceptible to puromycin and presumably required protein synthesis. Memory block was not achieved with chloramphenicol or with subcutaneous injections of puromycin, and this was attributed to insufficient inhibition of brain protein synthesis.[15] These proposals have since been revised.

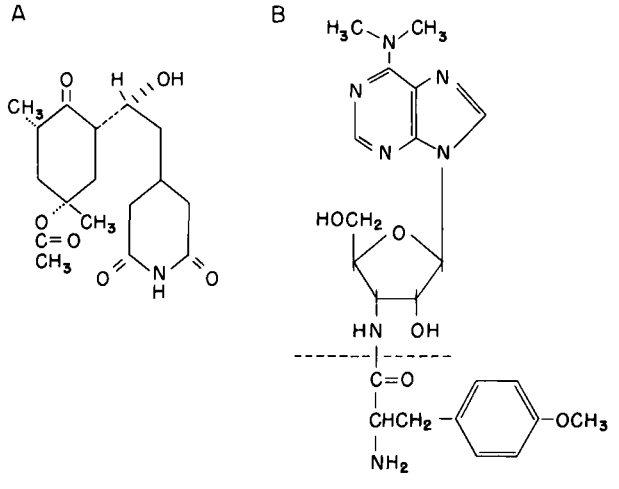


FIGURE 2 A: Acetoxycycloheximide, a highly potent inhibitor of protein synthesis. Replacement of the CH_3COO-group with H yields cycloheximide (Actidione), a shorter acting inhibitor. B: Puromycin. A nucleotide and an amino acid are joined (at the dotted line) in a stable amide linkage to form an inhibitor of protein synthesis.

STUDIES ON THE GOLDFISH In 1963, our laboratory became interested in the goldfish as an experimental animal for the study of behavior. The goldfish appeared to us to have several advantages. (1) Large numbers can be accommodated in a laboratory in relative convenience. (2) It appears to learn quickly and to retain what it has learned for long periods of time. We were impressed with the ingenious experiments on goldfish made in the laboratory of M. E. Bitterman.[19,20] (3) The optic tracts are completely crossed; thus the animal is a visual "split-brain" preparation.[21] (4) It is poikilothermic, permitting study of temperature effects. (5) Regeneration has been demonstrated in the goldfish nervous system, making possible interesting combined lesion-behavior studies.[22,23] (6) The small brain is suitable for high-resolution autoradiography in which heavy labeling is desired. (7) Above all, it seemed that a relatively primitive brain devoid of a cerebral cortex would constitute a simple system for the study of memory.

We then began the investigation of the effect of puromycin on goldfish memory. Solutions were injected into hand-held fish, which were returned to their individual plastic home tanks within seconds. We found that small volumes (about 10 microliters of solution) could be injected intracranially into goldfish with a Hamilton syringe and a 30-gauge needle fitted with a mechanical stop.[24] The needle tip overlies the tecta and does not touch the brain, and with practice large numbers of fish can be injected rapidly without apparent brain damage. An apparatus[24,25] similar to that described by Horner, Longo, and Bitterman[19] was employed for training (Figure 3). Goldfish were placed in individual shuttle boxes in which light was paired with repetitive electric shock. To avoid shock, fish learned to swim over a hurdle from the light to the dark end of the box. A trial cycle consisted of twenty seconds of light alone, twenty seconds of light paired with shock, and twenty seconds of darkness. A correct response was scored when a fish swam over the hurdle before the onset of shock. Initially, fish were placed in the shuttle boxes and allowed to remain in the dark for five minutes. During the subsequent five minutes, five trials were administered. The sequence was repeated four times on the first day of an experiment and twice on the fourth day. Thus fish were given twenty trials in a forty-minute session on day 1 of an experiment, returned to their home tanks, and given ten trials in a twenty-minute session on day 4. A diagram-

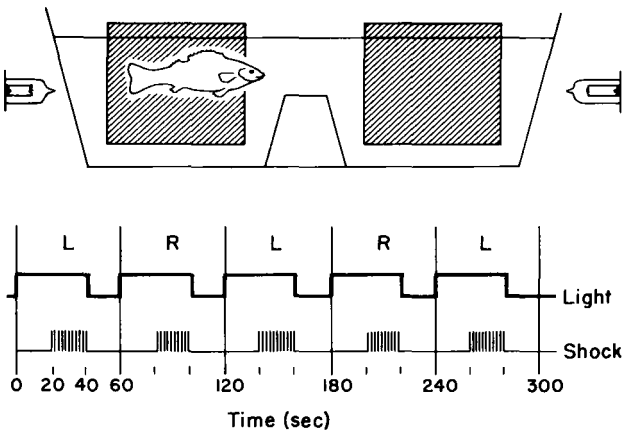


FIGURE 3 Shuttle-box and schedule for training shock avoidance. A block of five trials is outlined (see text). (From Agranoff, Davis and Brink, Note 35)

matic representation of the schedule and results with uninjected fish is seen in Figure 4A.

The task and training schedule employed resulted in extremely stable memory. Under the conditions of partial learning used, training proceeded during a fixed time interval. Naive fish, not previously exposed to the apparatus, avoided the shock for up to 30 per cent of the first ten trials and continued to improve in subsequent trials. If they were allowed to perform the task day after day, the learning curve flattened out at about 80 per cent correct response. When the interval between trial 20 and 21 was varied from one minute to as long as one month, there was no significant memory loss. Thus, the fish retained completely the memory of their training. In initial experiments, results from the first ten trials on day 1 were compared to the ten trials on day 4. We found[24] that 90 micrograms of puromycin injected intracranially immediately after training produced a significant memory deficit that was seen on day 4.

In subsequent experiments, we introduced a regression analysis.[25] On the basis of large numbers of uninjected fish, we could predict for each fish a day 4 score from the day 1 performance. The use of a regression permitted us to normalize differences in individual levels of performance. Some of the variation may have resulted from differences in the methods of handling our weekly shipments of goldfish. Seasonal variation in performance is also minimized by the regression. Even with this analytical method, fish are less satisfactory subjects during the summer months. Some key experiments[26] are summarized in Table II. The predicted score based on the trials in day 1 is subtracted from the score actually achieved. The memory is represented by A minus P, termed the retention score, and its significance is established by a t-test of A vs. P. One hundred and seventy micrograms of puromycin injected immediately following trial 20 resulted in a complete memory deficit. Moreover, the same dose of drug injected sixty minutes after the animals were returned to their home tanks produced no observable effect on memory.

This latter experiment ruled out nonspecific actions of puromycin as the cause of the lowered performance on day 4. Also, puromycin appeared to obliterate all of the memory of the training. During the forty minutes in the training apparatus the animals rested in the dark for twenty minutes, yet had not fixed any memory by the end of the trials. The results are represented diagrammatically in Figure 4B. These experiments are directly comparable to the previous literature on consolidation[5] and confirm the concept of a disruptible period immediately following training.[27] A fixation curve for 170 micrograms of puromycin and a dose-response curve for various doses of the drug injected immediately following trials are shown in Figure 5. In other experiments we have shown that electroconvulsive shock produces memory loss in goldfish.[25] The consolidation curve appeared to be slightly longer than that seen with puromycin. By cooling animals ten degrees during the posttrial period, we prolonged the disruptible period of memory formation. Thus, by using a poikilothermic organism, we obtained the results predicted from earlier experiments.[6]

Short-term memory

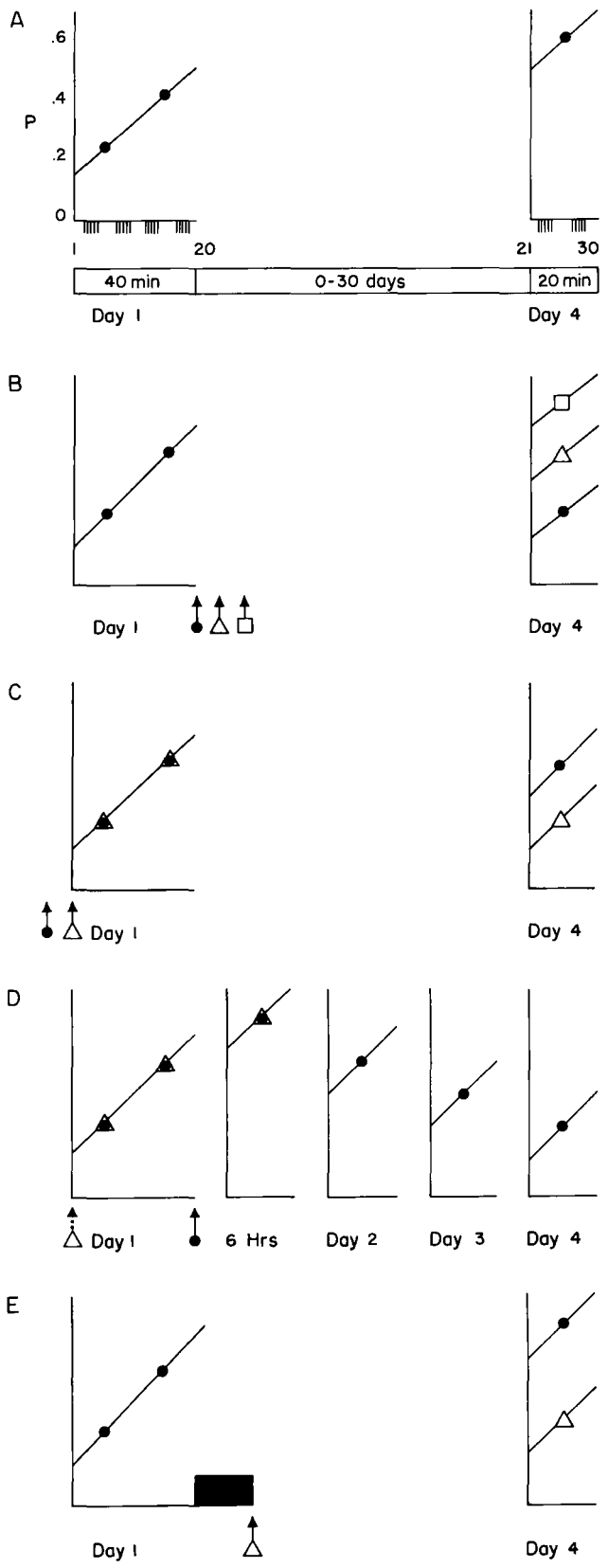
Fish injected with puromycin showed no postural or locomotor impairment, which encouraged us to try pretrial injection experiments. The results are shown diagrammatically in Figure 4C. Puromycin injected immediately

TABLE II

Effect of puromycin on memory

	Trials Day 1			Trials Day 4		
N	1–10	11–20	Treatment	21–30 A	21–30 P	Ret. Score (A-P)
72	2.3	3.4	Uninjected	5.3	5.3	0
36	2.5	3.8	Puromycin dihydrochloride 170 μg, Immediate	2.7	5.4	–2.7
35	2.5	4.6	Puromycin dihydrochloride 170 μg, 60 min delay	5.5	5.6	–0.1

The retention score (A-P) is an index of memory. See text.



pretrial had no significant effect on learning.[26] On day 4, however, the animals had a memory deficit. They were able to learn but not to fix their memory of the training under the action of puromycin. Yet, fish trained an hour or more before injection showed perfect retention. The deficit is reminiscent of Korsakoff's syndrome. Treated goldfish can demonstrate old memory and they can acquire new tasks, but they cannot retain memory of the new task.[28] The experiment suggests that the memory manifested in the performance improvement during training on day 1 is not puromycin-susceptible. In other words, the memory would not have been susceptible if we had waited for an hour following training before injecting puromycin. Hence, in goldfish only the formation of long-term memory is susceptible to the drug.

Also shown in Figure 4C is an experiment in which puromycin injected twenty minutes before training did not appear to affect learning.[26] This additional experiment indicates that puromycin does not have a delayed effect on behavior, which would have explained the lack of effect of the injections immediately before learning trials. Animals injected twenty minutes before trials had partial memory of training on day 4. This means that the effect of puromycin on memory diminishes one to two hours after its injection.

FURTHER EVIDENCE FOR SHORT-TERM MEMORY In additional experiments, groups of fish were trained, injected immediately after trial 20 with puromycin, and then retested at various times.[29] Previously we had demonstrated that under these conditions long-term memory could not form, and in confirmation, the performance of that memory gradually decayed over the next two days (Figure 4D).

FIGURE 4 Diagrammatic representation of memory of shock avoidance and effects of 170 μg of puromycin. A: Uninjected goldfish. Improvement in performance is seen in each block of 10 trials. No significant loss of memory is seen even 30 days following initial training. B: The effect of posttrial injection. Puromycin blocks memory formation when injected immediately following trials but not an hour later. C: The effect of pretrial injections. Acquisition appears unaffected, but memory is disrupted by injections immediately pretrial. Injection 20 minutes pretrial has little effect on memory fixation. D: Effect of pre- or posttrial puromycin on performance at various times following training. Performance is unimpaired at early times but decreases over a 2-day period. E: The effect of the training environment. Fish allowed to remain in the training apparatus do not begin the puromycin-susceptible phase of fixation until they are returned to their home tanks.

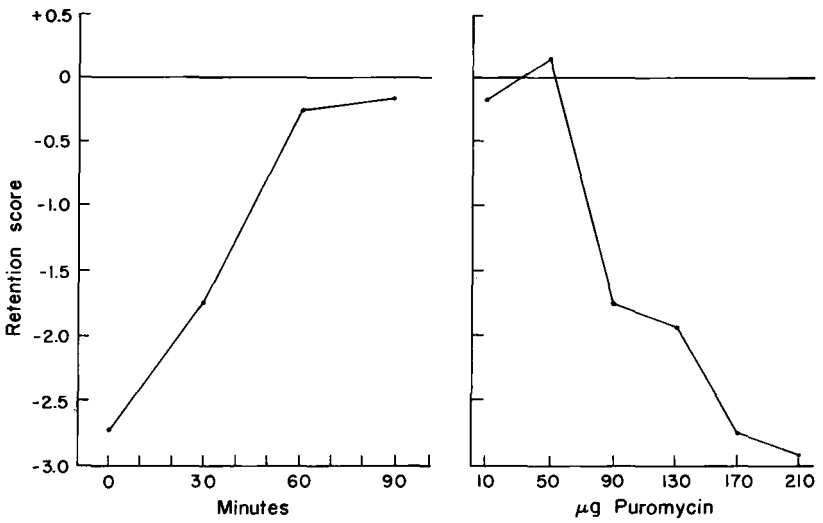


FIGURE 5 A: Effect on retention score of the time of injection of 170 μg of puromycin following trial 20. Minutes indicates interval between last learning trial and injection. B: Effect on the retention score of the dose of puromycin injected immediately after trial 20. (From Agranoff, Davis, and Brink, Note 35)

Our selection of a three-day intersession interval was fortunate. Had we chosen a shorter interval, we would not have been able to observe obliteration of memory with puromycin.[30]

Inasmuch as long-term memory forms during the hour following training, one might have expected a reciprocal curve for the decay of short-term memory, so that the sum of short- and long-term memory would produce the constant level of performance seen experimentally for various times following trial 20. This was not the case. Several possible explanations may be offered. Short-term and long-term memory might not affect performance additively. They might even arise independently. If, as suggested previously, only short-term memory is seen in short-lived animals, long-term memory may have arisen phylogenetically in addition to, but not in place of, short-term memory in animals with a long generation time.[31] One might predict that if the two are independent, it should be possible to disrupt short-term memory selectively and then find long-term memory appearing after a stage of unretrievability. Such studies have not been reported. In some preliminary experiments with rats, it was shown[32] that bitemporal injection of difluoroisopropylphosphate blocks memory when administered at short and at long periods after trials, but not at intermediate times. These deficits in performance may prove to be in retrieval rather than in fixation or storage. Recently, Barondes and Cohen[33] performed experiments on short-term memory decay in the mouse. They used the bitemporal injections of puromycin and the Y-maze described by Flexner. However, they injected untrained animals, allowed them to recover for five hours, then trained them and retested them at specified intervals up to three hours later. They found a rapid decay of the memory. Thus, the short-term memory decay in mice corresponds roughly to a complement of the fixation of long-term memory in fish, while a consolidation or fixation curve for mice injected with puromycin has not yet been demonstrated. This study is also interesting in that puromycin is able to block memory five hours after its injection, while it appears to be ineffective in the fish two hours after injection.

THE EFFECT OF THE TRAINING ENVIRONMENT Fish do not appear to fix memory during the training session. Some experiments[29] have added significance to this observation. Fish were trained and then allowed to remain in the shuttle box for one or more hours after trial 20 (Figure 4E). If they were then returned to their home tanks they showed normal memory on day 4. If, however, they were injected with puromycin and then returned immediately to home tanks, there was a highly significant memory deficit seen on day 4. In other words, fish allowed to remain in the shuttle box do not fix the memory, but do so after they are returned to the home tanks. This and related experiments suggest that the removal from the training environment serves as a trigger to initiate the fixation process. By manipulating the environment, we can initiate or retard the onset of the puromycin-sensitive step.

How does puromycin block long-term memory formation?

With these studies in mind, I should like to discuss the mechanism by which puromycin blocks memory. Although puromycin is principally an inhibitor of protein synthesis, large doses can cause other effects. Experiments were performed with puromycin aminonucleoside and with O-methyltyrosine, two moieties of puromycin.

Neither of these substances blocks protein synthesis[34] or affects memory.[35] The intact puromycin molecule is necessary for both behavioral- and protein-blocking activity.

The time course and dose effect of puromycin on the incorporation of leucine into goldfish brain protein was investigated. Puromycin was injected intracranially and at varying times thereafter, and leucine-H^3 was injected intraperitoneally. The animals were killed thirty minutes after the leucine-H^3 injection. In an earlier study[26] we found that the depth and duration of inhibition of leucine incorporation into goldfish brain was a function of the amount of puromycin injected intracranially. These experiments were performed with groups of five fish. The ratio of radioactivity in the trichloroacetic acid (TCA) precipitate to that in the supernatant fraction was compared to that of controls. In subsequent experiments,[36] we employed several groups of 10 pooled brains for each experimental point. One hundred and seventy micrograms of puromycin produces an almost immediate depression in protein synthesis; that depression remains maximal for at least eight hours. This time period is contrasted with the behavioral finding that puromycin injected twenty minutes pretrial has begun to lose its effect. This comparison suggests that events early in the inhibition, rather than the depth of inhibition itself, blocks memory formation. Also, an immediate depression in protein synthesis now can be seen with 50 and 90 micrograms of puromycin—doses that produce a moderate effect and no effect, respectively, on memory. However, experiments with modified shuttle boxes and different training paradigms indicate that 50 micrograms of puromycin can produce a marked memory loss under different training conditions.

It is perhaps unwise to make a comparison of depth or duration of protein inhibition with behavioral results from any one training task. Other possible difficulties in interpreting the pertinent inhibition data should be mentioned. First, we measured the rate of incorporation of an amino acid into protein during a 30-minute interval. The supernatant fraction was measured at the end of the thirty minutes. We had no way of knowing the average amount of radioactivity in the supernatant fraction during the thirty minutes that protein was being incorporated. Therefore, the ratio of counts in the TCA precipitate to counts in the supernatant fraction is not a precise measure of the efficacy of incorporation. Also, we have no measure of the amount of radioactivity in the blood. Changes in the soluble fraction of brain could be obscured by high blood levels of labeled leucine. A further problem is introduced by the possibility that under actual training conditions, the rate of leucine incorporation into brain protein is different from that in the resting animal.[37] Hence, puromycin might have a different effect on protein synthesis under different conditions of training. We should also remember that we are measuring total brain protein. The incorporation pattern in an area, a cell, or an organelle in the brain, or a single protein species with its specific turnover number different from the average, might reflect more truly a puromycin-susceptible step in memory formation. All of the above qualifications militate against the search for a direct correlation as a means of assessing the relation of protein synthesis to the memory block.

Studies in mice and in goldfish with cycloheximides

Perhaps the strongest suggestion we have at present that the protein-blocking action of puromycin mediates the memory block is that acetoxycycloheximide (AXM) (Figure 2A), a potent inhibitor of protein synthesis in vivo, blocks both protein synthesis[36] and memory formation[35] in goldfish. We have established that intracranial injections of AXM in the goldfish are several hundredfold more potent than puromycin in both respects. One-tenth microgram of AXM produces a memory deficit as well as a protein synthesis block. The mechanism of cycloheximide antibiotics is not known. They are glutarimide derivatives that block the transfer of aminoacyl RNA to the polysome, but apparently do not damage the ribosomes.[38-40] Disaggregation of the polysomes by various agents and their eventual reaggregation are both blocked by AXM. We have not proved a causal relationship, but we feel our present findings are consonant with a requirement of protein synthesis in the formation of long-term memory in the goldfish.

In contrast to our behavioral results, Flexner[41] finds that large amounts of AXM block mouse brain protein synthesis without affecting memory formation. Furthermore, when puromycin is injected together with AXM, memory in the mouse is not blocked, even though a profound deficit in protein synthesis is seen. Some differences between experiments with goldfish and mice are summarized in Table III. As a consequence of their experiments, Flexner, et al., no longer feel that protein synthesis is directly involved in memory formation. They suggest it could better be related to a stable messenger RNA that codes for a protein that serves in turn as the inducer of its messenger. Puromycin could block formation of the protein and allow its messenger to decay. AXM, on the other hand, would preserve the messenger on the polysome. Protein is ultimately needed for behavioral expression. A temporary memory loss with AXM was recently observed in Flexner's laboratory[42] and is used as further evidence for the hypothesis. An alternative explanation of the mouse experiments is that peptidyl puromycin is the agent that blocks memory formation. Because AXM blocks peptide

TABLE III

Comparison of effects of puromycin and acetoxycycloheximide

	Body Wt.	Brain Wt.	PURO	AXM	PURO + AXM
Mouse*	30g	440mg	90–180 μg Memory ↓ Pr. Syn ↓	120–240 μg Memory O Pr. Syn ↓	Memory O P. Syn ↓
Fish†	9g	85mg	170 μg Memory ↓ Pr. Syn ↓	0.1 μg Memory ↓ Pr. Syn ↓	Memory ↓ Pr. Syn ↓

*Bitemporal sites—Intracerebral
†Intracranial

assembly, it should also block the formation of peptidyl puromycin. While an explanation for the antagonistic effects of AXM and puromycin on mouse memory awaits further experimental clarification, it is significant that the only known interaction between these two antimetabolites is in their effects on protein synthesis. Thus, the effect of AXM on puromycin in the mouse is, in a sense, supportive evidence that the puromycin effect on memory *is* related to the block of protein synthesis.

In recent experiments, Barondes[43] has shown that pretrial injections of cycloheximide blocks protein synthesis without affecting memory. It also antagonizes the puromycin effect, in analogy to Flexner's posttrial experiments with the related drug, AXM.[41] Actinomycin D,[44] an RNA-blocking agent, does not block acquisition or memory formation at concentrations that block brain RNA synthesis. The latter finding casts doubt on the recent hypothesis of Flexner et al.,[41] because the presence of actinomycin D should lead to the depletion of messenger RNA. Experiments on the combined effects of AXM and actinomycin D in the mouse have not been reported, but would be of interest. Appel[45] has suggested that antibiotics might produce a "chemical ablation." Thus, drugs might produce temporary lesions in areas of the brain related to a specific function. Anatomical and electrical studies on the puromycin effects in the mouse might permit the detection of a correlative effect with puromycin not seen with a combination of puromycin and AXM.

Summary and present views on memory formation

Our findings on shock avoidance in the goldfish are consistent with the concept that acquisition of a new behavior is a special case of differentiation. That is, all possible input and response patterns are preprogrammed and are selectively evoked by experience. While seemingly deterministic, this model, as suggested by Edelman,[46] permits the brain the same freedom as that given a composer who must write for an existing musical instrument. As in antibody formation, a selection mechanism is required. The "instructions" an organism receives from the outside world are in the form of natural selection and mutation, and are inherited in the form of neural interconnections.

Our work with goldfish emphasizes the difference between the selection process and the fixation process (Figure 6). For selection, a mechanism is necessary that increases the probability that a specific response will be made. That is, if an animal initially has an equal probability of making several responses, there must be a means of specifying the appropriateness or significance of the response he will retain after it has been performed. The selection process may involve direct interaction with an unconditioned stimulus or some other psychological mechanism, such as "reinforcement," "drive," etc., the physiological basis of which is not understood. The experimental animal is in an excited state during acquisition and we know from other studies that the rate of protein synthesis in the brain is generally elevated.[37] The training process results in short-term memory, evidenced by improvement in performance from trial to trial. It may last hours to days. The studies by Barondes, et al., of mice and by us of the goldfish agree that pretrial injections of blocking agents do not affect learning. The selection process is insensitive to puromycin, the cycloheximides, and probably actinomycin D. It is significant that differentiation of the sea urchin proceeds to the blastula stage in the absence of RNA synthesis following treatment with actinomycin D.[47] For further differentiation, RNA and protein synthesis must occur.

The fixation of memory into a long-term form occurs after the training trials have been completed. Long-term memory formation is blocked by puromycin, and in the fish is also blocked by AXM. The fixation process occurs

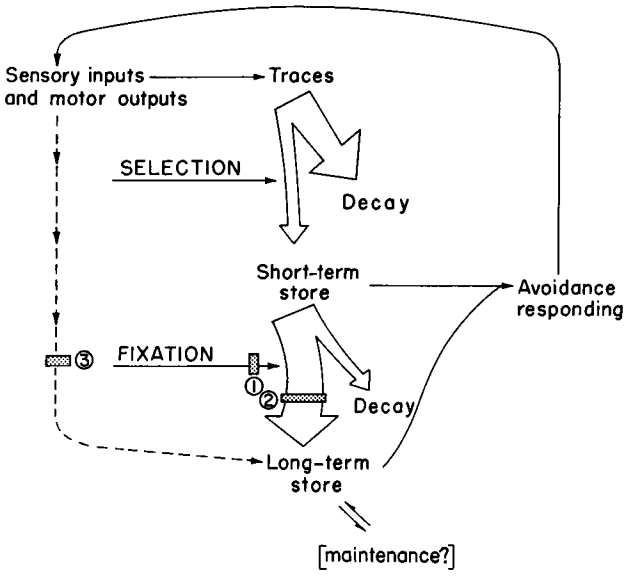


FIGURE 6 Hypothetical steps in memory formation of shock avoidance in the goldfish. Selection occurs during training. Selected traces are converted to short-term memory. It is not affected by agents which block protein synthesis. Fixation occurs shortly after training and is blocked by puromycin and by acetoxycycloheximide. The drugs could block release of a "now print" message (1); the fixation process itself (2); or some process of memory formation which is unrelated to short-term memory (3). There is no evidence for a maintenance phase of long-term memory in the goldfish.

at a time when the general level of excitement is decreasing. The environmental effect in the goldfish experiments suggests that a decrease in the general level of excitement may be necessary to allow the fixation process to begin.[48] If a specific protein is synthesized during fixation, as might be inferred from our studies, it is forming when amino acid incorporation into protein is generally decreasing. The fixation of long-term memory corresponds to the "now print" mechanism suggested by Livingston.[49] Considerable evidence for an anatomically distinct locus for the generation of the now print mechanism in man is found in neurosurgical cases.[28] While selection from the diverse preprogrammed possibilities in the brain is seen as trial-to-trial improvement in performance, the now print message fixes the experience of an entire session. We do not know whether the blocking agents prevent such a message from being initiated or whether they prevent the message from being carried out at the brain loci that contain the nascent behavioral information, presumably specific synapses. It is convenient to think that the behavioral information initially present as a temporarily altered synaptic state is directly converted during fixation to a permanently altered state. It is possible, however, that long-term memory arises independently of short-term memory, and may even be present in another part of the brain.

If puromycin and AXM block protein synthesis at the synapse, some interesting problems are posed. For instance, ribosomes are apparently absent presynaptically.[50] Campbell et al.[51] have recently shown a high rate of synthesis in brain mitochondria that are present in large numbers in the vicinity of the synapse. In the mouse, however, chloramphenicol does not block memory,[16] although it is a specific inhibitor of mitochondrial protein synthesis.[52] Cycloheximide has also been shown to block mitochondrial protein synthesis in the rat brain[51] to about the extent that it blocks ribosomal protein synthesis. From these results, it is difficult to implicate mitochondrial protein synthesis in memory fixation.

We have suggested that the diversity of behavioral responses is determined by natural selection and mutation. Let us consider the economies that may have given rise to the brain. Because biologists often think of mutation in terms of organisms grown in pure culture in the laboratory, one may readily suppose that there is survival value in regulatory mechanisms that prevent them from consuming substrates too rapidly or wastefully from the standpoint of chemical energy conservation. In nature, however, various organisms compete for the same substrate, and those species survive that can take up nutrients most rapidly and against the steepest concentration gradient. Reactions that are metabolically economical are perhaps not as biologically important for survival as are fast, less reversible ones. Glycogen and nucleic acids are formed by mechanisms energetically more expensive than those at first proposed.[53]

A second concept that deserves emphasis is the inexpensiveness of storing biological information. Each diploid somatic cell of a higher organism contains approximately the amount of DNA necessary for the synthesis of an entirely new organism. One epithelial cell, for example, is believed to contain the specifications for all of the billions of cells of the body, including the brain, and for all of the antibodies its lymphoid cells can manufacture. The two meters or so of DNA in a diploid nucleus appear to be sufficient for this purpose. The reason for reduplication is obscure, particularly because somatic cells do not serve to make up a new organism. We are led to the suggestion that this DNA might be present simply because there is no selection pressure for its loss (with rare exceptions, such as in the erythrocytes of some higher species). Similarly, the complex microstructure of the brain may also reflect much fortuitous storage in addition to the necessary instructions for survival. In natural selection, time is expensive and information storage is cheap—hence the brain.

Biochemical Changes Accompanying Learning

HOLGER HYDÉN

IN MY EARLIER CHAPTER, which dealt with RNA and proteins of neurons and glia, one of the main conclusions was that available biochemical data point to the neuron plus its surrounding glia as constituting a functional unit of the central nervous system.[1]

A considerable synthesis of macromolecules is characteristic of this unit. No other somatic cell can compete with the neuron in RNA production. Increased functional demands by physiological stimuli can induce RNA synthesis, resulting in a 10 to 100 per cent increase of neuronal RNA content. Another way to express this is to say that increases in RNA content in the cortex are associated with availability of sensory information.[2]

Physiological stimulation has shown that RNA newly synthesized in the neurons has the same base ratio characteristics as has the bulk of RNA already present in the cells.[3] The main part of neuronal RNA consists of cytoplasmic RNA with ribosomal base ratio characteristics and a low turnover.[4-7]

An increased amount of neuronal RNA has also been found during learning experiments.[8-10] This finding may in itself be an *unspecific* sign of increased activity, not correlated to a specific learning process.

Therefore, a series of questions can be asked. Does present knowledge—based on experimental data—allow the conclusion that a macromolecular mechanism serves the process of learning and long-term storage of information? At which level would such a mechanism operate? Do macromolecular changes occur in those brain cells that are specific for learning, but not as a result of other types of physiological stimulation that cause increased neural function? Are such molecular changes transient or permanent? As vehicles for phenotypic expression in the highly differentiated brain cells, do RNA molecules furnish neurons and glia with proteins that will lead functionally to a further differentiation as a result of experience? Are the proteins the executive molecules of neural function, both at synapses and at cell membranes?

In animal experiments, learning means the capacity of a system to react in a new or modified way as a result of experience. Memory can be defined as the capacity to store new information that subsequently can be retrieved in order to steer the particular function correlated with the information. At present, three experimental approaches have been used. One has been to interfere with the synthesis of RNA and proteins in the brain and to observe the effect on behavior (Dr. Agranoff, this volume[11]). A second approach has been to establish a new behavior and to observe if specific biochemical changes occur in the brain cells in the relevant areas. At our laboratory we have followed the second line. Eventually, a combination of the two approaches could be decisive. A third approach has been to extract macromolecules from brains of conditioned animals and to observe the effect on the behavior of unconditioned animals into which the macromolecules are introduced, or to use drugs to enhance learning and increase retention.

Before proceeding further, I would like to stress that no data support the view that the brain cells contain mechanistically taping memory molecules that store information. In current literature, however, such views are discussed and debated, doing little more than add to the anatomy of confusion. In the recently cultivated field of molecular neurology, more than in other fields, apparent discoveries may turn out to be delusions. It may be appropriate to quote Polanyi[12] about the content of empirical statements: "It relies on clues which are largely unspecificable, integrates them by principles which are undefinable, and speaks of a reality which is inexhaustible."

The discussion to follow considers some experimental data against the background that a molecular mechanism must have access to the genome in order to acquire, store, and retrieve information. It is possible that experience stimulates gene areas first to synthesize primary gene products and, eventually, protein end products. This mechanism, together with that for the generation of electrical impulses, is probably an integrative part of the executive side of the system. The first requirement for such a mechanism is that it operate at high speed. The time constants are considerably smaller than those of the nerve impulses, which are 10^{-3} seconds. Conformational changes of macromolecules can be assumed to occur at about the rate of 10^{-6} to 10^{-8} seconds.

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Three sets of data are presented: results of an analysis of the synthesis of mouse brain RNA during avoidance learning; changes found in fish brain RNA composition during instrumental learning; and an analysis of cortical nerve cell and glial RNA in two learning experiments on rats.

1) The first example is an experiment by Zemp and his collaborators on the incorporation of uridine into mouse brain RNA during short-term training.[13] It was a double-labeling experiment, which has many advantages. Pairs of 6- to 8-weeks-old mice were used, one as a test animal, the other as control. Ten microliters of H^3–uridine was injected into each side of the midline of the test mouse's brain. Another mouse received the same amount of C^{14}–uridine. Thirty minutes after the injection the test mouse was trained for 15 minutes.

Three types of controls were used: (1) yoke controls, which received light, buzzer, and electric shock in a random manner; (2) quiet controls, kept in home cages; and (3) shock controls, which were shocked 30 times during the 15-minute period. The training apparatus consisted of a box with two compartments, one for a control, the other for the training animal. There was an escape shelf around the walls for the training animal, but not for the control. A buzzer and light were presented for three seconds as the conditioned stimulus. As the unconditioned stimulus, an electric shock was delivered through the grid and remained until the mouse jumped to the shelf. Then the mouse was replaced on the floor for the next trial. A single trial averaged 25 seconds. The animal was considered to have made an avoidance response if it jumped to the shelf before onset of shock. Mice kept in home cages and mice shocked 30 times during 15 minutes were also used as controls. The mice were killed immediately after training, and ribosomal and nuclear RNA were prepared from whole brains. As an indicator of the relative amounts of isotope that had penetrated into the brain, the ratios of the labeled uridylic and cytidylic acids were determined in the supernatant and used as a reference. This labeling of two brains provides a good possibility for determining incorporation of precursors into RNA and to judge differences between trained and control mice by the ratio of the radioactive labels.

Immediately after the 15 minutes of training, the RNA of the brain ribosomes and the nuclear RNA of the trained mice contained a greater amount of radioactivity than did the RNA of the controls. The incorporation was also studied in kidney and liver RNA, but no such differences were found. Figure 1 shows the sedimentation patterns on sucrose gradients of nuclear and ribosomal RNA. The RNA from the trained mouse had a greater radioactivity than did the RNA from the control brain. The electric

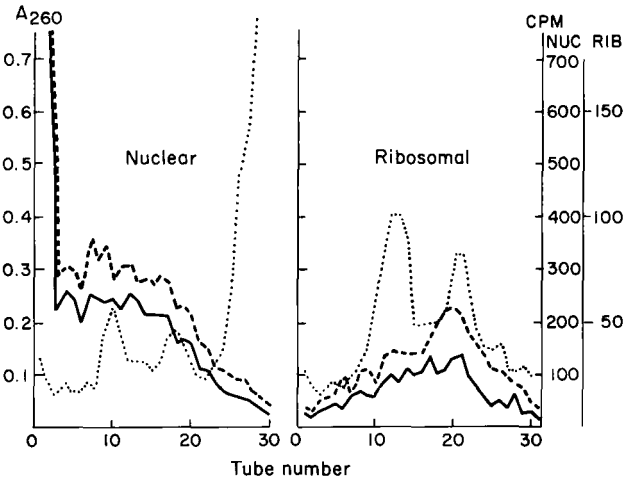

FIGURE 1 Sucrose gradient centrifugation of RNA from brains of trained and yoke control mice sacrificed immediately after training. One mouse was injected intracranially and intraperitoneally with H^3-uridine, and another mouse similarly with C^{14}-uridine. After 30 min, the former mouse was trained while the latter served as the yoke control. The training experience lasted 15 min, after which the brains were quickly removed and homogenized together. Ribosomes and nuclei were isolated and RNA was extracted from each fraction. This RNA was centrifuged in a 5–15% sucrose gradient in 30 ml in the Spinco SW 25 rotor for 18 hr at 25,000 rpm, and 1-ml fractions were collected from the bottom of the tube. Absorbance at 260 mμ, C^{14}, and H^3 were determined for each fraction. The results were corrected for the ratio of H^3:C^{14} in the UMP pool (see Table 1). *Solid line* = C^{14}, *dashed line* = H^3, *dotted line* = absorbance at 260 mμ. (From Zemp, et al., Note 13)

shock administered to the training animals did not by itself lead to an increased label of RNA over that in the controls. The conclusion of the experimenters is that the increase in labeling observed in trained animals represents an increase in RNA synthesis. They are otherwise cautious in the interpretation. An increase in brain-cell RNA synthesis has been observed, however, when mammals or fish have been subjected to physiological, sensory, or motor stimulation. In all cases analyzed, it seems to be an unspecific reaction by which an increased neural function is sustained by a synthesis of RNA and proteins.

It is not clear that the physiological stimulation, behavioral activity, and motivational contexts were equated in control versus experimental groups. Until this problem has been surmounted, it seems difficult to conclude that such synthesis of brain RNA can be considered a specific correlated to learning.

2) A more specific result has been obtained by Shashoua.[14] These are current experiments and, with the con-

sent of Dr. Shashoua, I will briefly present a significant and reproducible result. He has used goldfish and "challenged the entire CNS" by causing both sensory and motor parts to function in a learning experiment as simple as it was effective. He attached 0.5 grams of polystyrene foam below the jaw region of the fish, turning the ventral side up and lifting the head above the water.

In one hour the fish had learned how to swim dorsal side up, but at a 45° angle. In three to four hours it had adjusted its behavior to normal posture and swimming. Shashoua injected the fish with H^3-orotic acid above the tectum, and after the experiment the whole brain was homogenized. RNA was extracted and the uracil/cytosine ratio of the newly synthesized RNA was determined after alkaline hydrolysis. The significant and reproducible result was an increase of the uracil/cytosine ratio from 3:1 brain RNA in the control to 6:1 in the trained fishes. This agrees well with the base ratio changes of cortical nerve cells that we found in rat learning experiments.[15] In the latter case the uracil/cytosine ratio increased even more—about 5 times.

In both types of experiments, the protein coded by such an RNA can be assumed to be unusual and highly specific.

3) Two types of learning experiments were performed on rats.[8,16,17] (For methods, see my earlier chapter in this volume.) Neurons in the portions of the cortex and brain stem that were involved in learned behavior were analyzed. In contrast to the results after physiological and chemical stimulation, the RNA formed in the neurons during learning had in one case high adenine-uracil values, and in the other case was asymmetric and had high adenine values.

The adenine-uracil-rich RNA fraction had a low G + C/A + U ratio of 0.70 to 0.90. It could not be extracted by cold perchloric acid, acetic acid, or water. The same was the case with the asymmetric, adenine-rich RNA. It is likely, therefore, that this fraction consists of polymer RNA and not of small molecules.

Nuclear and cytoplasmic RNA fractions that stimulated the incorporation of amino acids and were rapidly labeled have been described from mammalian cells. They had a low G + C/A + U ratio of 0.9 to 1.0 and an asymmetric base ratio composition with adenine values higher than uracil values.[18,19] Protein synthesis in mammalian cells and its dependence on messenger RNA with a slow turnover or on rapidly synthesized RNA in the nucleus or cytoplasm is, however, still far from understood.

Work now in progress on hybridization and template activity may elucidate other characteristics of the different RNA fractions of neurons and glia.

In the first of the experiments mentioned above, normally right-handed rats were induced to use the left hand in retrieving food from far down a narrow glass tube.[17] Two training periods of 25 minutes each were given daily. Neurons in both sides of the cortex were analyzed, as were neurons from those areas whose destruction prohibited transfer of handedness. These control centers are situated bilaterally in the sensory motor cortex and comprise about 1 mm^3 of the cortex. Layers 5 to 6 are the most important. There the neurons have a large nucleus in comparison with the amount of cytoplasm. Therefore, the analytical result will reflect mainly nuclear RNA. Eighty-eight rats were used for the analysis of 14,000 cortical neurons. Although other control experiments were also performed, the learning experiment described has an advantage in that the controls are present in the same brain. Therefore, a paired t-analysis could be performed on the results from the neurons of both sides.

We found significant increase in the amount of RNA per cell from the learning side of the cortex. In an extension of the work published,[15] the amount of RNA was found to have increased from 220 $\mu\mu$g of RNA per ten nerve cells to 310 $\mu\mu$g. When the base ratios of the neuronal RNA of the control side were compared with that of the learning side, it was found that the ratio G + C/A + U had decreased significantly from 1.72 to 1.51.

We furthermore performed a detailed analysis on whether the extent and type of RNA change could be correlated with the learning process in individual cases.[15] For the original RNA base ratio data, the reader is referred to Hydén and Egyházi.[17]

The data are divided into two groups (Table I). The cell material from the cortex of animals 1 and 2 was taken on the rising part of the learning curve on the third to the fifth day, i.e., during an early part of the learning period. The material from the other two rats was taken on the asymptotic part of the curve on the ninth to the tenth day. In this case, the animals had reached the maximal number of successful performances per training period on the sixth to seventh day. The increase of the RNA content per neuron of the animals examined on the third and fifth day is from 25 to 30 per cent. Qualitatively, the RNA formed in the neurons, i.e. the Δ RNA, is characterized by a DNA-like base ratio composition with adenine and uracil values around 26. (Rat DNA has the following base composition: A 28.6, G 21.4, C 21.5, and T 28.4.) The cytosine values were remarkably low. The results were statistically significant, and were not the same as those obtained from the animals that had trained for nine or ten days and performed with a maximal number of "reaches" for food, i.e., 70 to 80 reaches per period of 25 minutes. The RNA result deviated both quantitatively and qualitatively from that of group one. The relative RNA increase per neuron in the learning cortex was 60

TABLE I

RNA composition of neurons correlated to performance

Animal	Training periods 2 × 25 min/day (days)	Number of successful reaches	Relative increase of RNA per neuron, %	RNA composition of control neurons	ΔRNA composition
1	3	107	33	*A* 19.0 ± 0.4	*A* 25.5 ± 3.0
				G 24.9 ± 0.5	*G* 36.1 ± 4.8
				C 38.5 ± 0.8	*C* 9.7 ± 10.7
				U 17.6 ± 0.4	*U* 28.7 ± 4.2
2	5	163	23	*A* 18.2 ± 0.4	*A* 24.5 ± 3.2
				G 25.5 ± 0.5	*G* 35.7 ± 4.3
				C 36.3 ± 0.7	*C* 11.7 ± 7.9
				U 20.0 ± 0.4	*U* 28.1 ± 3.4
3	8	625	57	*A* 16.6 ± 0.3	*A* 26.2 ± 2.7
				G 26.8 ± 0.5	*G* 34.9 ± 2.7
				C 39.1 ± 0.8	*C* 16.1 ± 6.5
				U 17.5 ± 0.4	*U* 22.8 ± 1.8
4	9	1041	105	*A* 18.2 ± 0.4	*A* 21.0 ± 0.9
				G 28.6 ± 0.6	*G* 35.2 ± 1.6
				C 33.6 ± 0.7	*C* 24.0 ± 1.9
				U 18.8 ± 0.4	*U* 19.8 ± 0.9

to 100 per cent. The base ratio composition of the RNA formed (Δ RNA) was similar to that of ribosomal RNA.

As a conclusion to this part of the discussion, I should point out that when the nerve cells within the learning part of the cortex were taken during the early and acute period of the learning process, the relative RNA increase per neuron was small. The nuclear RNA formed, however, had a DNA-like base ratio composition. Thus, a stimulation of the genome seems to occur early in a learning situation that the animal has not previously encountered. By contrast, when the animals performed well according to criterion, the main type of RNA synthesized had high guanine-cytosine values. At this stage of learning, the relative increase of Δ RNA was greater than during the first part of the learning period. Thus, a differentiated formation of RNA occurs in the neurons engaged during a learning period, and the beginning of that period seems to be characterized by a genic stimulation, judging by the character of the RNA that is formed.

The quantitative, and especially the qualitative, RNA changes in the neurons raise the pertinent question of whether new species of proteins also are formed during the establishment of a new behavior. Although the determinations of the bilateral biosynthesis of protein in the control centers of rat handedness are not completed, a preliminary result may be of interest. After separation of proteins from the control center for handedness on the learning side of the cortex, we found four protein bands that had no correspondents on the control side. Whether these reflect a protein synthesis cannot be determined, however, until incorporation studies have been made.

In the second learning experiment, before young rats could eat they had to learn how to balance on a thin, one-meter-long, steel wire strung at a 45° angle between the floor and a small platform containing food.[8,16] Training periods took place for 45 minutes per day. Seventy-eight rats were used for the analysis of 12,000 nerve cells. Vestibular Deiters' nerve cells, clearly involved in this balance experiment, were analyzed. These nerve cells have a comparatively large cytoplasm and a small nucleus. Therefore, if a whole cell is analyzed, characteristics of nuclear RNA will drown in the bulk of the cytoplasmic RNA, as the ratio of nuclear to cytoplasmic RNA is 1:50. No base ratio changes in the cytoplasmic RNA were detected during learning, although the increase from 680 to 750 micromicrograms of RNA was determined. The nuclei were isolated and the base ratios of the nuclear RNA investigated. Then a clear increase in the ratio A/U of the nuclear RNA was found (1.06 to 1.32), but there was no significant change of the ratio G + C/A + U.

Because no control neurons can be obtained from the same brain in such an experiment, four different types of control experiments were performed using physiological stimulation and stress involving the vestibular pathways.

We found no significant changes in the A/U ratio in these controls, although a significant increase of RNA per neuron could be determined. This indicates that the increase in adenine and the decrease of uracil was specific for the learning experiment. What may be the significance of the synthesis of a nuclear RNA fraction with such high adenine values? RNA has been extracted from defined parts of chromosomes of the fly *Chironomus* and has been found to have an asymmetric composition with high adenine values.[22] This type of nuclear RNA-stimulating amino acid incorporation has also been found in the freshwater flagellate *Euglena*,[23] and in starfish oöcytes.[24] The conclusion, therefore, is that nuclear RNA with a high A/U ratio, found in the second type of learning experiment in rats, was chromosomal RNA.

Table II presents earlier and new data obtained from both nerve cell nuclei and glia in this learning experiment. The increase of the total amount of neuronal nuclear RNA was estimated to be about 20 per cent, as was the increase in glial RNA. The table shows that the Δ RNA fraction of the nerve cell nuclei has a high adenine and a low uracil content. It may also be noted that the G + C/A + U ratio of 1.25 does not differ significantly from the corresponding control value of 1.38. The enormous increase of the A/U ratio from 1.02 to 4.9 is striking.

However, this A/U value of 4.9 has a great error as the result of two errors in uracil in control and learning. There is a significant change in A/U between control and learning (1.06 and 1.32, respectively), and a significant increase and decrease of adenine and uracil, respectively, in the Δ RNA fraction, so the A/U value of Δ RNA must be high. In order to obtain a more reliable picture of this latter A/U value, a greater number of measurements would have been needed.

The A/U ratio found in Δ RNA synthesized in the neurons may be compared to the RNA produced in the Balbiani rings in *Chironomus* chromosomes; it is found to be about 2.[22] Control experiments in the rat learning experiments demonstrated the significance of the increased adenine and decreased uracil values of the newly formed nuclear Δ RNA, so it seems justifiable to assume that it is of chromosomal origin.

The neuronal glia reacted in the following way during learning (Table II). The adenine content of the Δ RNA fraction increases by 70 per cent, and the cytosine content is markedly decreased in comparison to that of controls. The glial Δ RNA is similar to the adenine-rich, asymmetric RNA being formed in the neurons during learning, and has a G + C/A + U ratio of 0.7. The high A/U glial ratio of 2.70 indicates that the Δ RNA being formed in the glia during learning is of chromosomal origin, analogous to the Δ RNA formed in the neuron.

Therefore, we may conclude that factors in the environment (the new learning situation not previously encountered by the animal) stimulate the genome of the glia and the neurons engaged and that this response can be characterized in biochemical terms. Analysis of the RNA in two learning situations has thus shown that the re-

TABLE II

Microelectrophoretic analyses of the composition of the nuclear RNA in the neurons and the glial RNA of the Deiters nucleus in a learning experiment in rats. Purine and pyrimidine bases in molar proportions in percentages of the sum.

	Nucleus			Glia		
	Control	Learning	Δ RNA fraction	Control	Learning	Δ RNA fraction
Adenine	21.4 ± 0.44	24.1 ± 0.39	38.1 ± 3.25	25.3 ± 0.16	28.3 ± 0.45	43.0 ± 2.83
Guanine	26.2 ± 0.45	26.7 ± 0.87	28.8 ± 5.75	29.0 ± 0.24	28.8 ± 0.31	27.0 ± 2.24
Cytosine	31.9 ± 0.77	31.0 ± 0.96	26.7 ± 6.95	26.5 ± 0.43	24.3 ± 0.36	14.0 ± 3.12
Uracil	20.5 ± 1.01	18.2 ± 1.11	6.4 ± 8.08	19.2 ± 0.27	18.6 ± 0.21	16.0 ± 1.88
G + C/A + U	1.38 ± 0.042	1.37 ± 0.049	1.25 ± 0.218	1.25 ± 0.030	1.13 ± 0.028	0.69 ± 0.076
A/U	1.06 ± 0.056	1.32 ± 0.084	5.9 ± 7.5	1.32 ± 0.020	1.52 ± 0.030	2.70 ± 0.364
No. of animals:	5	8		5	6	
No. of cells: (From HYDÉN-EGYHÁZI)	500	900				
No. glia samples:				33	42	

Naturwissenschaften, 1966

sponse of neurons and glia engaged in the behavior to be established differs from the response in physiological and chemical stimulation with respect to two important parameters. First, during learning the content of RNA increases in both neurons and glia. Under physiological and chemical stimulation, the RNA changes were inverse. Second, adenine–uracil-rich, asymmetric RNA is formed in both glia and neurons during learning. During stimulation, the RNA being formed has ribosomal RNA characteristics with respect to base ratios. By inference, the adenine–uracil-rich, asymmetric RNA formed in learning is assumed to be of chromosomal type.

A preliminary study of the duration of neuronal RNA with the high A/U value gave the following results.[8] Twenty-four hours after the experiment was stopped and the animals were returned to their home cages, no such RNA fraction could be found. When the training was resumed for 45 minutes and the nuclei analyzed, the RNA fraction with high A/U ratio was again found. The disappearance of the RNA fraction does not mean that the fraction had ceased being synthesized. It probably means only that it is present in such small amounts as to be inaccessible by present methods of analysis.

Based upon experiments in our laboratory, we concluded that learning and memory are dependent on neuronal and glial synthesis of RNA, which eventually gives rise to protein.[8,33] This is supported by experiments in which inhibitors were used.

Flexner and coworkers[20] found that inhibition of protein synthesis by puromycin injected into the temporal cortex and the hippocampus destroys memory (maze learning) in mice. In analyzing the mechanism of this phenomenon they used an antibiotic (acetoxycyclohexiimide), which inhibited protein synthesis by inhibiting transfer of amino acid from sRNA to the polypeptide, but did not interfere with the messenger RNA. The heximide was without effect on both short- and long-term memory and, furthermore, protected memory against the destructive effect of puromycin. The investigators then drew the conclusion, in conformity with the view expressed from our laboratory, that the initial macromolecular change underlying maintenance of memory involves a change in the quantity of one or more species of messenger RNA.

In further experiments on rats, Flexner and collaborators[21] used acetoxycycloheximide and observed an initial period of memory consolidation that was independent of protein synthesis. During an intermediate period, memory could not be expressed when protein synthesis was inhibited more than 90 per cent. Memory returned, however, at least 20 hours after protein synthesis had been restored to normal rates. Flexner's group concluded that protein concentration in the brain can fall to low levels but the loss of memory is only temporary, provided that certain species of RNA are conserved to direct the resumed protein synthesis when inhibition has disappeared.

In behavioral experiments, Agranoff and collaborators used goldfish into which puromycin was injected intracranially. When the drug suppressed 50 to 80 per cent of the protein synthesis in the brain, memory fixation was temporarily suppressed.[34,35] The *formation* of long-term memory required protein synthesis in the brain; the *maintenance* of long-term memory did not appear to depend on such synthesis.

A working hypothesis

It seems that long-term memory is not the result of a pattern of circulating currents, because information can be retrieved even if the animal has been subjected to such low temperatures that the electrical activity of the brain is stopped. Nevertheless, a link can be assumed to exist between such electrical activity and the synthesis of macromolecules in brain cells. There are several alternative ways to consider how synthesis of RNA may lead to a specification of neuronal nets, over which the impulse activity becomes facilitated. The evidence I have presented indicates that a first stage of learning exists, during which activation of gene areas leads to a synthesis of the RNA specific for such areas. Based on the data, it can be assumed that the protein coded by this RNA is unusual and highly specific. At a response, the protein forms a specific executive substance of all neurons through a net. The protein formation leads to an increased differentiation, a modulation of the protein pattern of the cells. The pattern or part of it decides if the neuron responds to a certain process.

On the other hand, increased RNA synthesis could sustain an increased rate of protein synthesis at the synapses over a polyneuronal net. An increased rate of enzyme activities might lead to an increased impulse activity.[25] It should be remembered that in the rat handedness experiment there is a stage of RNA synthesis following that of the high adenine-uracil RNA. This second stage is interpreted as one in which increased synthesis of guanine–cytosine-rich RNA sustains the augmented function of the neurons, as a result of physiological and chemical stimulation. In other words, it is a reaction reflecting quantitative but not qualitative responses. Considering, then, the great number of synapses on each neuron, one is faced with the question of time sequence following stimulation of neurons of the net.

In the working hypothesis outlined below, the role of macromolecules in neurons and glia is discussed as a "thought mechanism" for acquisition and storage of information in brain cells at three levels.

LEARNING AT THE FIRST LEVEL—THE GENERAL CASE Phenomenologically, this is exemplified by the newborn child demonstrating its stepping reflexes—co-ordinated neuromuscular events, triggered by pressure against the foot pad and the posture (Teitelbaum,[26] this volume). Still more complex events are found in insects. For example, the capricorn beetle larva bores a chamber twice as big as itself to fit the adult beetle into which it will eventually develop. The larva lies with its head towards the entrance of the chamber because otherwise, as an adult insect, it would not have room to turn around (Fabre[27]).

These complicated behavioral patterns are assumed to be the results of integrative effects of the functional units in the nervous system, the neuron plus its glia, differentiated through an evolutionary and ontogenetic time sequence and genetically steered. These units govern the neural activities, from motor reflexes to genetically programmed activities, such as in insects. We presented evidence (in this volume) that the neuron and its surrounding glia constitute a metabolic and functional unit in which the glia seem to be the metabolic stabilizers of the sensitive neurons. The evidence also indicated that protein synthesis in the neuron may be programmed by transfer of RNA from the glia. Another example may be that acidic protein, specific for the brain, is mainly localized in the cell bodies of the glia, and only in the nuclei of the neurons.[28] This distribution may be a result of the glial programming of the neuron. The acidic nuclear protein could steer gene activities by blocking histones, and in this way controlling the controllers.

Neurons and glia from different areas differ in their RNA composition. This indicates a protein specificity of the neuron–glia unit and leads to the view that all neurons that share the characteristic proteins, both in the cell bodies and at the synapses, respond in an integrative way when the equilibrium is changed, biochemically and with respect to the impulse activity.

LEARNING AT THE SECOND LEVEL If the brain cells and their genome are considered, learning at the second level can be taken as a *selective mechanism*. This is demonstrated when an animal is placed in a new situation that requires learning; the handedness experiment is an example.

In that case, a highly specific RNA was produced in both the neurons and glia. This was interpreted to mean that when gene areas were activated RNA was produced. At this point, one could ask what is known about the activity of DNA in highly differentiated cells. Only a small percentage of the DNA in such cells seems active. Furthermore, external substances—hormones, for example—are known to be able to penetrate to the genes and, through RNA, to exert their effect on the specialized target cells. Additional parts of the genome of brain cells could be activated by a mechanism similar to that operating in hormonally influenced cells. It may be noticed, however, that hormones cannot have a direct influence on the genetic content of all cells.[29]

In considering neurons and glia, it is tempting to suggest a bridge between electrical field strength and RNA synthesis in brain cells. RNA synthesis may be induced by different mechanisms. Katchalsky and Oplatka[30] have shown that in solution RNA exhibits hysteresis phenomena related to changes in *p*H. Hysteresis cycles are time independent, but are dependent on the history of the system, and the changes in free energy are low during phase transitions of nucleotides. On the other hand, energy values gave an effective signal that differ from thermal noise. Conformational changes of RNA and DNA by electrical field strength, or a change in the zeta potential,[31] are possible ways to activate gene areas for the synthesis of new species of RNA and, eventually, specific proteins in brain cells.

How would such a mechanism operate? Each time the same, meaningful signal came, a specific part of the protein pattern would be activated by the same electrical pattern that had induced its synthesis; in its turn, the protein would activate the transmitter. Placing a protein that was responsive to electrical patterns at a step before the transmitter would also constitute a mechanism for the required time sequence.

One could assume that such specific proteins were stored in a more permanent form and suggest membranes. The brain tissue contains numerous membraneous structures, from the neuronal membrane, the cytoplasmic membranes, to the abundant glial membranes. Such an arrangement would allow a time-sequence response if the protein pattern of the membrane was differentiated over the surface.

In the thought model, the specificity of neurons is shown in the modulation of their protein pattern and is placed one step before or after the transmitter.

LEARNING AT THE THIRD LEVEL This level of learning merits only a few words. It would encompass an instructive mechanism, a permanent change of information-rich macromolecules during the life cycle. At a higher level, this would be the mechanism for insight learning, for problem solving by pursuing gradients of deepening coherence. No experiments have yet been reported.

When examined historically, few hypotheses reveal themselves as new. Yet, new data may make a problem appear in a more revealing shape. Bragg once said that the essence of science lies not in discovering facts, but in discovering new ways of thinking about them.[32]

Brain Mechanisms and Memory

P. G. NELSON

DR. IRWIN KOPIN, Dr. Eric Kandel, and I were asked at the Boulder meetings to put together our thoughts on memory and the different ideas we feel emerged from that month of lectures and discussions. This chapter is the result.

One classification of the hypotheses concerning the mechanisms underlying learning and memory is shown in Table I. The hypothesized mechanisms are divided into those in which specific macromolecules serve to encode information, those of various field effects, and those emphasizing the importance of connections or synapses between nerve cells. The connectionistic category, in turn, is subdivided into specific and nonspecific connectionistic models. I would like to expand on these various categories and then to consider in slightly greater detail one aspect of the specific connectionistic category. Obviously these categories are not mutually exclusive; for instance, macromolecular function is going to affect all the other categories. Dr. Hydén has invoked electrical fields as possible determinants of nucleic acid and protein production; fields may affect a connectionistic system, and so on.

Previous discussions have dealt in a comprehensive fashion with the relationship of nucleic acids and proteins to learning and memory. The macromolecular hypothesis is posed in its most extreme form by the transfer experiments that Dr. Quarton analyzed so lucidly. Information is considered to be stored in the specific configuration of large molecules (notably RNA and/or polypeptides), and these molecules are themselves sufficient to produce a more or less specific change in the behavioral repertoire of an organism. The experimental basis for this theory is at present controversial, but the macromolecular hypothesis has great appeal because the macromolecules can serve as an efficient information-storage mechanism, as has been shown so clearly with regard to genetic information. The steps by which the specific experiences of an animal result in a comparably specific alteration in RNA and protein seem very obscure at this point, and hypotheses as to how the macromolecules may produce specific behavioral changes are also lacking. Dingman and Sporn,[1] have pointed up the necessity of fitting the molecular machinery into the complex anatomy of the neuron. The question of how a variable readout of the highly stable information in DNA might be affected by physiological processes is a critical one that is discussed elsewhere in this volume; however, few answers are currently available.

Experiments with antimetabolites that block protein synthesis have shown that disruption of protein synthetic pathways can block the consolidation or retention of learned behavior (Agranoff, this volume). These experiments would seem to support macromolecular theories of memory. The remarkable increase in the detailed knowledge of the process of protein synthesis makes these experiments very attractive, but the results of such experiments with antimetabolites have become extremely complex and, at present, their interpretation is not at all clear cut. For instance, the time course of suppression of protein synthesis does not parallel the effect of the antimetabolites on consolidation of memory. Furthermore, it seems questionable to assign a specific role to the brain proteins in the process of memory or information storage because proteins participate in such a wide variety of cell functions. Disruption of protein metabolism might well be accompanied by a general malfunctioning of the brain. Some controls for the nonspecificity of the protein blocking effects have been found, but these results do not as yet seem conclusive.

Turning to the second category—the field or nonconnectionistic concept of brain function—there is some direct data indicating that such processes can be of importance in the central nervous system. An example is given by spreading depression in which a wave of activity can be made to propagate slowly across the retina or the cerebral cortex. Spreading depression manifests itself as a paroxysmal burst of neuronal activity associated with depression of activity, direct-current potential shifts, and large movements of ions and metabolites.[2] Caffeine and strychnine waves are other sorts of massive activity not solely dependent on neural connections, for this activity will propagate across cuts in neural tissue. Steady polarizing currents applied to the brain markedly alter the behavior of the tissue as has been amply demonstrated by Purpura (this volume). Such currents undoubtedly alter

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TABLE I

Hypotheses as to the brain mechanisms underlying memory

I. Macromolecular

Information stored as specific molecular configuration

II. Field

A. Electrical potential

B. Chemical concentration (ionic, hormonal)

C. Anatomical (extracellular space)

Information stored in specific field configurations

III. Connectionistic

A. Systems with essential equivalence of connections between the neurons in an assembly

Information in the assembly is represented by activity of the entire assembly. Activity of any single cell carries little information.

B. Systems in which specific connections are important.

Information is stored as the effectiveness of particular pathways which will converge on particular cells to activate them.

thresholds and synaptic properties of neurons in the polarized tissue, but whether all of the effects of polarizing currents can be explained on these grounds is an open question. At any rate, the experiments of many workers certainly show that electrical fields can produce dramatic changes in neural behavior. There has been considerable doubt whether adequate potential gradients occur under physiological conditions in the nervous system to produce functionally significant effects, and the effects mentioned above (spreading depression, strychnine wave, etc.) are pathologic processes. In the spinal cord, it has been possible to demonstrate interaction between afferent fibers[3] and between motoneurons.[4,5] This interaction is probably the result of the electrical currents generated by active elements influencing their neighbors. This interaction is of short duration (one millisecond or so), and occurs despite the apparent absence of any specialized areas of membrane apposition (such as tight junctions) between motoneurons. The voltage gradient throughout most of the field around an active motoneuron is small (about one millivolt per millimeter), although substantially greater focal gradients do occur. More orderly arrays of cells than occur in the spinal cord might be expected to generate much more substantial fields with activity. For instance, quite large field potentials are found in the hippocampus. If the fields accompanying spike activity in some cells are effective in modifying the activity of their neighbors, this would serve as a synchronizing mechanism. It seems to me that this would act as a modulating influence on synaptically produced patterns of activity rather than as a primary determinant of activity.

Dr. Adey and his coworkers[6,7] have emphasized the usefulness of studying slow-wave activity and brain impedance changes as concomitants of learning. Slow-wave activity has been suggested as an aspect of neural activity that may be primary to or independent of individual spike activity. Adey has interpreted observations on the composition of the extracellular space as indicating that this space would probably have properties that strongly affect brain function, and that variations in ionic and other chemical gradients would also be of importance. An important question regarding these field-concept phenomena is to what extent they are to be regarded as operating independently of the synaptic connections of the nervous system. If field potentials, for instance, serve primarily as modulators of the basic, synaptically organized structure of the brain, a natural framework exists by which the field effects can be understood and interpreted. If, however, the field phenomena are taken as the primary mechanisms by which the brain performs its function, a large gap exists at present between the experimental and conceptual basis for the field concepts and a large body of neurophysiological observations. At present, the specificity and complexity that have been demonstrated for cellular neuronal integrative mechanisms represent a much more highly developed approach to brain function than does the field approach.

With these field theories, information is represented by the specific configuration of the electrical, chemical, or structural "fields," and learning and memory would consist of alteration and preservation of the alteration, in such a configuration. A detailed and comprehensive theoretical treatment of these postulated field effects in terms of information storage is still lacking and the experimental work is in a relatively inconclusive state.

In the numerous discussions put forward in this book, it has been clear that some sort of distinction should be made between two different kinds of connectionist theories of how the brain works and how memory might be represented. The extreme statements of the two viewpoints might be put as follows: (1) Some sort of equivalence of connections between cells exists in certain parts of the brain, and the behavior of an entire assembly or ensemble of cells must be considered as representing or encoding information; the behavior of any given cell is relatively unimportant. (2) The second viewpoint attaches great significance to the particular connections of each cell, and specific information is represented by the firing of a particular cell.

Now, everyone would agree that there is excellent

evidence for the importance of highly specific and reliable connections in the nervous system. The sensory systems[8] and the various reflex arcs of the spinal cord are clear examples of this specificity. It is equally clear, however, that synaptic transmission partakes of a statistical and, in some regards, indeterminate quality. Work on the quantal nature of transmission at the neuromuscular junction has indicated this, and statistical properties appear characteristic of central synapses, as well. The monosynaptic connections between group Ia afferent fibers and spinal motoneurons have been shown to partake of this statistical property.[9-11] The amplitude of the synaptic potential resulting from activation of a single group Ia fiber, for instance, may vary from zero to over three millivolts in a random fashion (R. E. Burke, personal communication). It should be emphasized that this statistical process is entirely compatible with a highly specific, highly reliable connectionistic mechanism as represented by spinal-reflex pathways. Such a probabilistic feature of a system, therefore, does not indicate that it is nonspecific in its connections.

A more serious problem with the specific connectionistic concept is posed by the increasing multiplicity of inputs to neurons at higher levels of the central nervous system and the increasing dependency of the response of one cell to one input upon the state of activation of other inputs to the cell. One may ask whether, in the highest integrative or associative regions of the brain, this multiplicity is so great that specific connections have relatively little to do with the system's operation (or, alternatively, that the activity of any given neuron may be highly ambiguous in its significance, and represents essentially zero information). The system may approach the randomly connected nerve networks, such as those that have been analyzed in simulation studies by a number of workers.[12,13] These systems can be shown to exhibit a number of interesting properties, such as rhythmic activity occurring spontaneously or in response to transient or steady stimuli. Their behavior can be changed in a number of ways independent of specific connections, and the changes may exhibit some degree of stability. Such parameters as threshold, refractory period, and average connectivity will determine the behavior of the system as a whole.

So far, little has been done in biological experiments toward the demonstration of this sort of mechanism and, in fact, such a theory is hard to come to grips with experimentally. Furthermore, I believe most of the consequences of this kind of model can be duplicated by connection-specific models. For instance, the high degree of frequency-selective activity at different brain sites, as shown by Dr. John in his conditioning experiments, makes one think of the old hypothesis of resonant circuits in the brain. That is, variable-length, synaptically linked chains of neurons could be selectively tuned to inputs of different frequencies. Such sharp frequency-selective features have been seen in interneuronal populations in the spinal cord and thalamus.

The specific connectionistic framework has the appeal of being experimentally approachable in a number of ways. Neurophysiological and biochemical techniques have revealed a good deal about synaptic function. Various candidates for synaptic transmitter chemicals have been identified, and the mechanisms of transmission have been explored extensively. It should be possible to answer in some detail such questions as: Does neural activity at a synapse alter the effectiveness of that synapse? For how long a time period may it be altered and how easily? What are the cellular mechanisms underlying any change that may occur? To what extent are the effects of increased activity dependent on the temporal patterning of that activity?

Possibilities for synaptic changes might be divided into presynaptic and postsynaptic mechanisms. The presynaptic changes are of several sorts and time courses. Posttetanic potentiation,[14] low-frequency depression or habituation,[15] and heterosynaptic facilitation or inhibition[16,17] may be related to the membrane potential of the presynaptic fiber and a process called transmitter mobilization.[18] These processes characteristically last from seconds to many minutes. Repeated high-frequency activation of a synapse may produce potentiation that lasts for an hour or two.[19,20]

A number of ionic species move during neuronal activity and changes in sodium, potassium, chloride, and calcium concentrations must occur. These ionic changes might well be involved in inducing alterations in transmitter metabolism and, in fact, sodium ions are necessary for the resynthesis of acetylcholine in cholinergic presynaptic terminals.[21] However, evidence for long-term ionic effects as a function of nerve activity are lacking.

Dr. Kopin has made a number of points in regard to metabolism at the adrenergic synapse. With activation of these synapses, turnover and production of transmitter is increased to compensate for its increased output. This seems to be the result of a feedback mechanism of regulation (with decreased transmitter resulting in greater activity of existing enzyme), rather than induction of more enzyme. No postactivation overshoot of transmitter concentration or persistent increase of enzyme activity have been shown beyond the period of nerve stimulation, with stimulation periods of one hour. All the components necessary for the production of transmitter are present in the nerve terminals. In addition to the amount of trans-

mitter formed presynaptically, its incorporation into vesicles and the release of these vesicles from the terminals are also important steps determining the effectiveness of the synapses. These steps may also be functions of the ionic milieu in the synaptic region. At the cholinergic synapse the amount of transmitter per vesicle or quantum can be varied by at least one procedure—poisoning by hemicholinium.[22] Again, long-lasting effects of neuronal activity on these aspects of presynaptic function have not been demonstrated.

Receptor sensitivity is an important postsynaptic determinant of synaptic efficacy, and this has been shown to change dramatically under conditions of denervation and re-innervation, especially in the neuromuscular system.[23,24] Evidence for change with use or disuse are somewhat equivocal and contradictory. Two types of experiments have been done in this connection. If acetylcholine is applied to a receptor membrane, a desensitization phenomenon can readily be demonstrated.[25] It is still not known, however, if naturally (that is, neurally) applied acetylcholine produces desensitization. Two similar experiments give contradictory results.[26,27] If chronic underactivity of the nerves innervating a muscle is produced, one might expect a change in receptor sensitivity. It appears that some such change may well occur, but it is not nearly so marked as is the case with denervation.[28]

A number of procedures have been used to alter the activation state of the monosynaptic reflex arc of the spinal cord. Changes in reflexes accompany muscle tenotomy or denervation of synergic muscles,[29,30] but the functional consequences of the experimental procedures are complex and not entirely worked out.[31] The precise nature of the relationship between use and synaptic efficacy is, therefore, not revealed by these experiments.

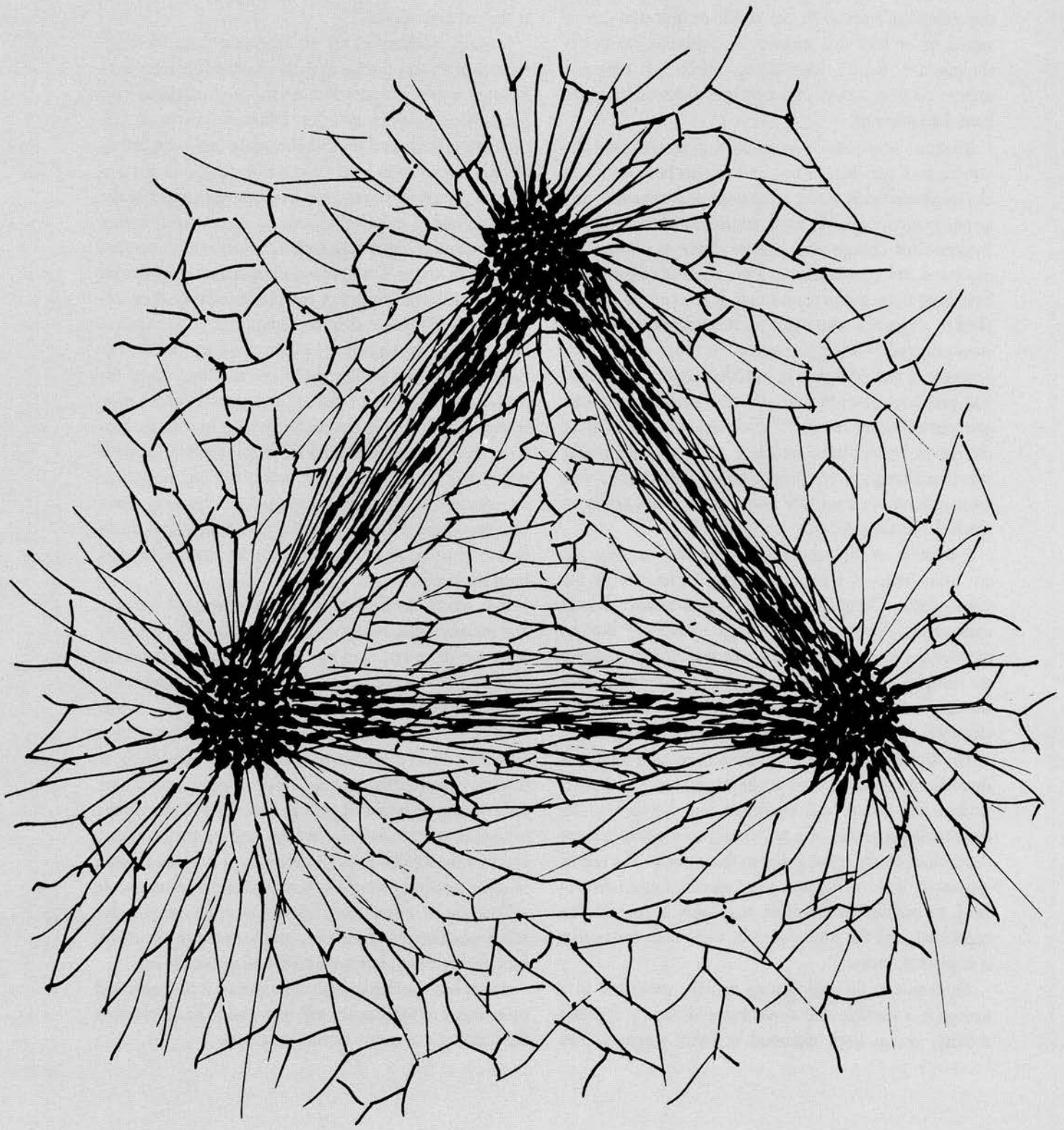
The trophic relationship between nerve and muscle is dramatically demonstrated in the experiments of Buller, Eccles, and Eccles, and of Buller and Lewis.[32,33] The contractile properties of a muscle are to a certain extent determined by the nerve going to that muscle. The rate of activation of the nerve and other specific factors not related to impulse propagation may play a role in this regulation, and the importance of these different factors is at present unclear.[33]

Alteration in the endogenous activity generated in a neuron is a variable that could be modified by synaptic activity, as has been discussed in other chapters. The circadian rhythms shown by Strumwasser indicate that long-term changes in spontaneous activity may occur. It may be relevant that in the neuromuscular system where denervation results in an increase in receptor sensitivity, it may also result in the occurrence of pacemaker activity at the synaptic region.[34]

Growth mechanisms are an appealing form of long-term change, and change in dendritic structure (or receptor area) would be a powerful mechanism for determining connections between neurons. Dramatic effects on cell structure in the lateral geniculate nucleus under conditions of light deprivation have been demonstrated in the developing nervous system, but changes were slight at the cortical level. Functional alterations in the visual cortex as a function of light deprivation were found to be very marked, however. Comparison of unilateral and bilateral light deprivation illustrates the complexity of these results.[35] Dr. Altman's chapter demonstrates the intricate nature of the growth of the postnatal brain, and the experiments on changes in cortical size as a function of the experiential richness of the environment have exciting implications.[36] Activation has long been known to produce changes in protein and RNA production in nerve cells,[37] and Dr. Hydén's work in this volume shows that changes in RNA production specific to a learning situation may occur.[38] The relationship of this class of phenomena to synaptic physiology has not, to my knowledge, been explored.

It is crucial to the connectionistic theory of learning and memory that synaptic efficacy be alterable as a consequence of neural activity. A considerable number of experiments have been done in the area of synaptic function as related to use and disuse of the synapse. While positive effects have been reported, I think it is impressive how little direct quantitative evidence now exists to show that use improves the function of the synapse over a time scale of days or weeks. It is to be hoped that further physiological and biochemical studies will be forthcoming, and that the fundamental mechanisms of synaptic change in some relatively simple systems will be worked out. It will clearly be only a small, first step in unraveling the detailed mechanisms of memory and learning in the intact functioning brain, but it may well be a crucial step.

As has been pointed out several times, it is probable that more than one mechanism will be found to be involved in the learning and remembering processes.



INTERDISCIPLINARY TOPICS

"Dynamically created forms, if somehow consolidated, become molds for the course of further activity." WEISS, PAGE 804.
This sketch shows fibers of cells between centers of growing clumps forming a structural triangle that guides cell emigration, PAGE 814.

INTRODUCTION

SCIENCE IS CONSCIOUSLY directed toward achieving knowledge that is explicitly formulated, systematically ordered, dependable, and general in scope. The neurosciences face problems of great complexity, yet the separate disciplines are, to some extent, concerned with issues disconnected and isolated from one another. This fragmentation is caused in part by the habits of thinking of scientists and in part by the degree to which investigation is restricted by specific concepts and technology.

Interdisciplinary efforts within the neurosciences should try to clarify the generality of specific hypotheses and to increase the systematic order in the entire field. Accordingly, this study program concludes with a section that supplements the introductory essays and the surveys of current research. By raising issues of method, emphasis, interpretation, and strategy, or by focusing on hypotheses that cut across many fields, the following papers discuss problems broader than those usually considered within the separate disciplines.

Chemical Evolution of Life and Sensibility

MELVIN CALVIN

DURING THE PAST TEN YEARS or so I have been concerned with the evolution of living things from the nonliving world. Once I would have begun this article by discussing a solid earth formed roughly five billion years ago. I would have talked about its primitive atmosphere, subjected to a variety of energy inputs, generally in the form of cosmic radiation, ultraviolet radiation from the sun, heat that gave rise to electrostatic discharge, and possibly the radioactive ionizing energies in the earth's rocks. The combination of these energy sources produced a series of changes, and I have tried to identify them. That is, I have devised a chain of events from the primitive atmosphere to the first living cell, and tested the hypothesis of that chain whenever it was susceptible to one or another form of testing.

Such a discussion would have terminated with the living cell, that remarkable system of molecules enclosed within a boundary (membrane) and capable both of reproducing itself and of transforming energy in a directed way. In this paper, however, I will look at the subject more broadly, and consider the cell as simply a stage in a much longer sequence of biological events.

One stage beyond the cell, which is separated by its membrane from the rest of the world, is a system for transmitting information from the environment into that cell, so it, in turn, can react to the environment. I would like to discuss, then, the evolution of that information-gathering, information-transmitting, information-processing, information-storing function that we now know exists in the nervous system of even primitive animals. Such a discussion must of necessity be speculative. We know much less about the real mechanism by which the neuronal system works than we do about the energy-transforming system that was part of the earth's evolutionary pattern.

Primitive (prebiotic) chemistry

Let us go back to earth in its initial form, as we understand it today. Its geological history is depicted in Figure 1. The atmosphere that formed shortly after earth took shape probably contained the simplest molecules, atoms of the first row of the periodic table, generally in their most reduced form. These were carbon attached to hydrogen (methane), oxygen attached to hydrogen (water), nitrogen attached to hydrogen (ammonia), and hydrogen itself, as well as some partly oxidized carbon, such as CO (Figure 2). The period of chemical evolution that led to the single-celled organism was presumably early in earth's history, and the complexity of organic chemical organization increased during that time. After organic evolution began—that is, after living things appeared—the complexity of the inanimate organic environment decreased. This cross-over point between chemical evolution and organic evolution (after the single cell appeared) is now believed to have taken place about three billion years ago, or perhaps even earlier.

When the highly specialized nervous system began is totally unknown. Presumably it arose somewhere around two billion years ago; at least the information-transmitting cells may have appeared then. That figure is still purely guesswork, but it seems fairly certain that some recently discovered fossils may be of forms one billion years old. Single-celled organisms probably appeared as long as two billion years ago, and by 500 million years ago, multicellular forms of life, well known to us today in their fossil forms, had appeared. By that time they must have included organisms that had neuronal systems resembling those of their counterparts today. However, a paleobiochemical identification of the nervous system is still to be made.

Some of the "molecular" fossils of some three billion years ago have been found, on analysis, to be hydrocarbons—not random ones, but highly specialized hydrocarbon chains and rings related to the steroids of today's living organisms. That these molecular forms were present so early in earth's history tells us that complex metabolic machinery was then in existence, so the single cell must have come into being before then.

Chemical evolution can also be examined by hypothesizing a series of events that led up to the point at which the earliest biomolecular forms appeared; starting with the simple molecules of the primitive atmosphere (Figure 2), we can try to reproduce some of the chemical transformations. Roughly fifteen years ago I carried out some of the first experiments of this type, starting with carbon dioxide, water, and hydrogen, and was able to show that reduced carbon could be made with ionizing radiation. In

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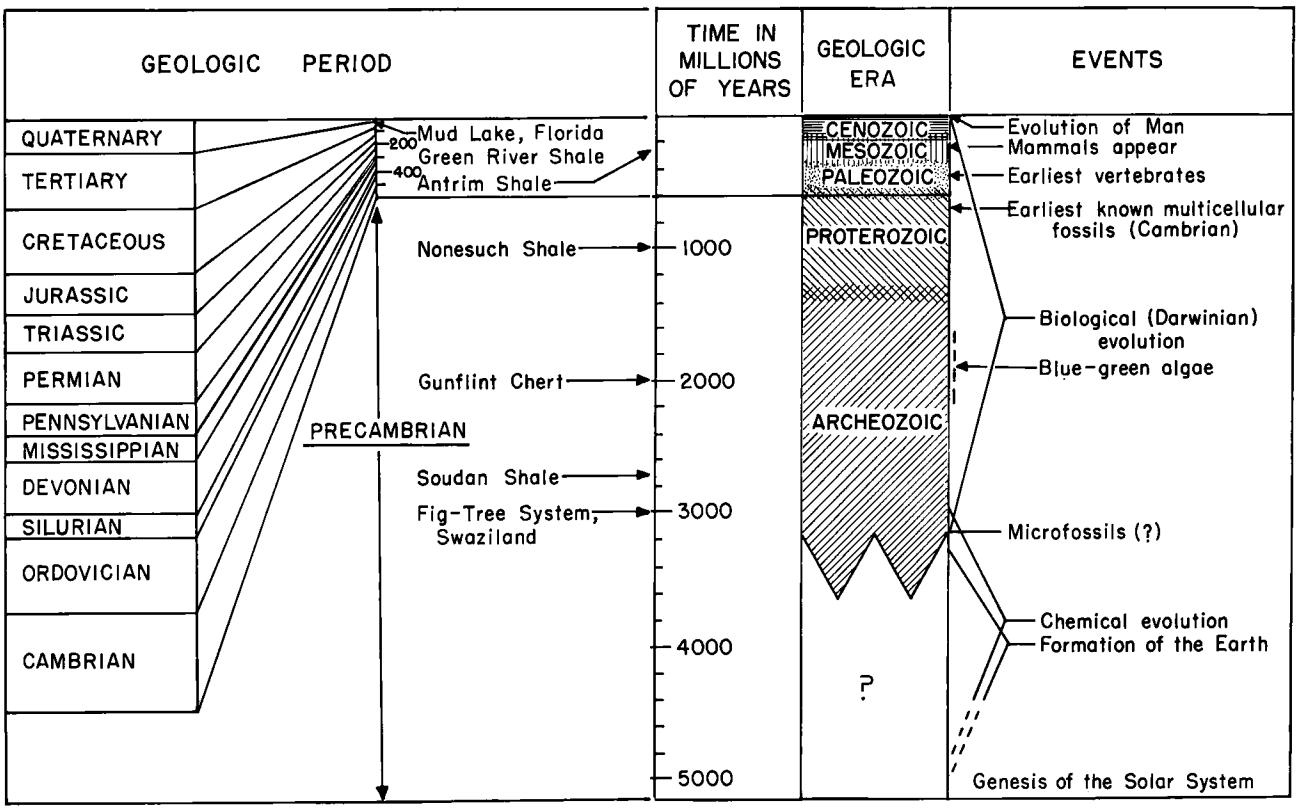

FIGURE 1 Geological time scale for evolution of the earth. (S. W. Palay, 1958 *Exp. Cell Res., Suppl.* 5)

fact, after the ionization and breaking of the water and hydrogen molecules and the construction of reduced carbon, carbon-to-carbon links could be created. Shortly after these studies, Miller added ammonia to the primitive reducing system and was able to show the presence of even more complex compounds. A whole series of experiments has examined products formed when high energy (or ionizing) radiation in the form of ultraviolet energy, electrical discharge, etc., is introduced into an atmosphere presumed to be equivalent to that of the early earth. Figure 2 shows the simple primordial molecules, the compounds obtained by ionizing radiations, and the molecules of the modern cell, including the amino acids. There really should be a fourth row in this illustration to show even more complex molecules—the heterocyclic purine and pyrimidine rings, for instance, which are the basic units for

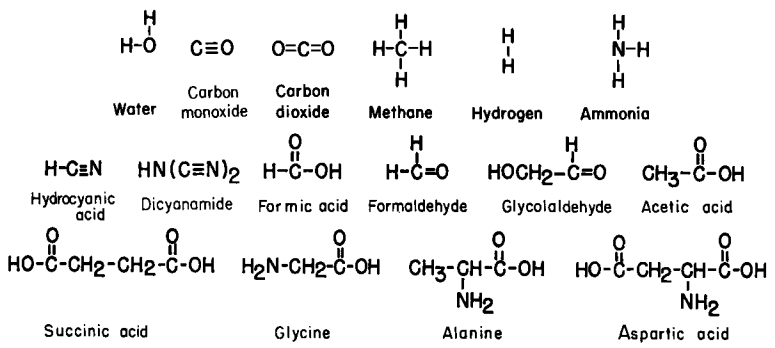

FIGURE 2 Simple primordial molecules are in top row; in the second and third rows are the compounds obtained when ionizing radiations are introduced into the gaseous system shown in top row. Molecules in the second and third rows are those of which the living cell is constructed today, and upon which it operates. In bottom row are the amino acids typical of those that build proteins.

the construction of nucleic acids. These biopolymers are of major importance in the reproduction of living things, just as the biopolymers of the amino acids—polypeptide and protein molecules—are essential for the construction and function of living organisms.

However, dissociating energy alone did not give rise to the biopolymer(s) or to the selective, energy-converting apparatus that we now know in the living cell; nor did it give rise to the limiting membrane of that cell.

This brings us to the next stage in chemical evolution, that leading to the biopolymers. Common to all the chemical reactions required to produce the essential biopolymers (polypeptides, nucleic acids, polysaccharides, and lipids) is dehydration condensation—the single reaction characteristic of all the construction reaction for the biopolymers that are formed from the simple molecules. In the polymerization reactions leading to proteins, a water molecule between the carboxylic acid end and the amino end of an amino acid is eliminated (Figure 3). This gives rise to a peptide linkage, and produces a dimeric molecule with the corresponding original functions at each end—an acid group at one end and an amino group at the other. The reaction can continue by adding units at either end. For the construction of the polysaccharides—the biopolymers we recognize as the structural elements of the cell wall in plants as well as one of the energy storage units in both plants and animals—a dehydration condensation also takes place (the reaction characteristic of all biopolymers formed from simple molecules). It occurs between a semiacetal hydroxyl group and some other hydroxyl from a carbohydrate molecule, giving a linkage that also involves the elimination of water. Thus, a disaccharide is made that has the semiacetal hydroxyl and the alcoholic hydroxyl at either end, and that, as a result, can grow onto the polymer. Lipids, the fat-type molecules, show the same type of reaction. Water is eliminated between an acid hydroxyl group and an alcoholic hydroxyl, to create the ester bond. This, however, does not give rise to a potentially indefinitely long polymer, but can give rise to large molecules. With the polysaccharides and proteins, however, polymers can be created that can grow indefinitely, and because of the variety of individual units can make an enormous variety of molecular structures.

The last great class of biopolymers (Figure 4) includes three different kinds of dehydration reactions. In the first stage of chemical evolution a purine ring (adenine) can form. A dehydration condensation, or polymerization reaction, between one of the NH groups in the molecule and the semiacetal hydroxyl of a five-carbon sugar is now possible, giving rise to one type of linkage, adenosine (riboside). Another type of dehydration condensation can take place between a phosphoric acid molecule and the primary alcohol group (5′) at the end of the sugar molecule, giving rise to a phosphate ester, adenylic acid (ribotide). And, finally, a second phosphate ester group can be formed between another hydroxyl group of the phosphoric acid and the secondary alcohol (3′) on another sugar molecule.

These three dehydration condensations have now produced a unit capable of growing indefinitely, much like polypeptides. An additional hydroxyl on the first sugar molecule (a secondary hydroxyl) can also undergo con-

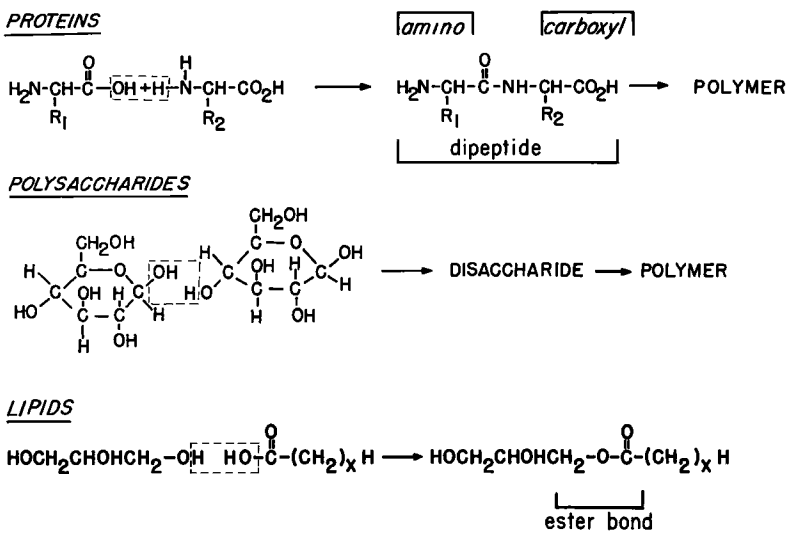

FIGURE 3 Dehydration condensation of amino acids, sugars, and lipids.

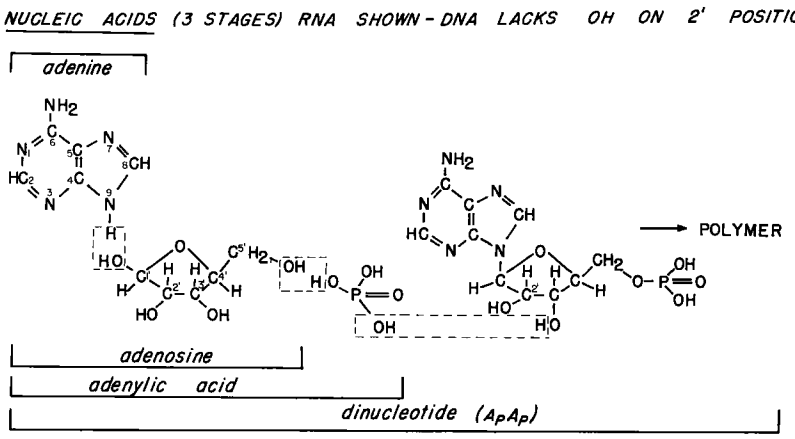

FIGURE 4 Dehydration condensation of nucleic acids.

densation. Therefore, we have a unit with two functional groups (hydroxyl and phosphoric acid) that can condense with each other at either end, and thus grow indefinitely to make long linear arrays.

Now, today's living organisms use phosphoric anhydride linkages in order to induce dehydration condensations in aqueous solution. But how could dehydration condensation have taken place *in the absence* of a living organism? Can any kind of system be devised in the laboratory that will permit such an occurrence, using only those materials available from the initial energy input on the methane-ammonia-hydrogen-water system? The formation of phosphoric anhydride linkages in a nonliving system can indeed be achieved, but only in special circumstances. It may very well be that these special circumstances obtained at a very early stage in the earth's history. However, I find it satisfying to achieve the linkages by using molecules that are made when high-energy radiation is introduced into the initial chemical milieu of the primitive reducing atmosphere.

A series of those interesting molecules is represented by the cyanamides. HCN itself, potentially capable of inducing dehydration, is formed in the primitive atmosphere, and two molecules of HCN reacting with ammonia can form dicyanamide, an ammonia molecule in which two cyano groups have replaced two of the hydrogen atoms. These dicyanamides, in turn, are now energy-rich materials, as they have a carbon-nitrogen linkage with two or three bonds between the carbon and the nitrogen atoms. This multiple bonding can be used to accomplish the dehydration condensation. The bond between the carbon and the nitrogen is unsaturated—that is, it can take up water, among other things, in a specific manner. We have used dicyanamide as a condensing agent—and the only energy source—to induce *all* of the several forms of dehydration condensation in dilute aqueous solutions. The technique is useful because the dicyanamide groups react with phosphoric acid, with carboxylic acid, and with the alcohols more rapidly than with water itself. We can therefore use (di)cyanamide in water solution.

The reaction series by which a peptide is formed by dehydration condensation is demonstrated in Figure 5, in which dicyanamide is shown in its various tautomeric forms. Notice that one of the forms can be written as a carbodiimide and thus corresponds to the well-known reagent that has been used most frequently in a nonaqueous medium for the same reaction series. An amino acid is added to the carbodiimide linkage, and once the oxygen atom is attached to the carbon it stays there, and the carboxyl carbon is subject to nucleophilic attack by another amino group; the nitrogen attacks the carbonyl carbon and forms the peptide link. Notice also that the original cyano group of the carbodiimide has become urea. A water molecule has been added to the carbon-nitrogen triple bond (carbodiimide), inducing the dehydration condensation to the dipeptide. The same kind of reaction can be used to form phosphatic esters, glycosidic linkages, fatty acid esters, and the like, and all this has been performed in the laboratory.

We have reached the stage of biopolymers dissolved in water. The question now is: can some higher degree of order be obtained?

Generation of higher degrees of molecular order

Let us examine what happens when a polypeptide becomes eight or ten units long; that is, when it begins to have factors built into it that may give rise to a secondary

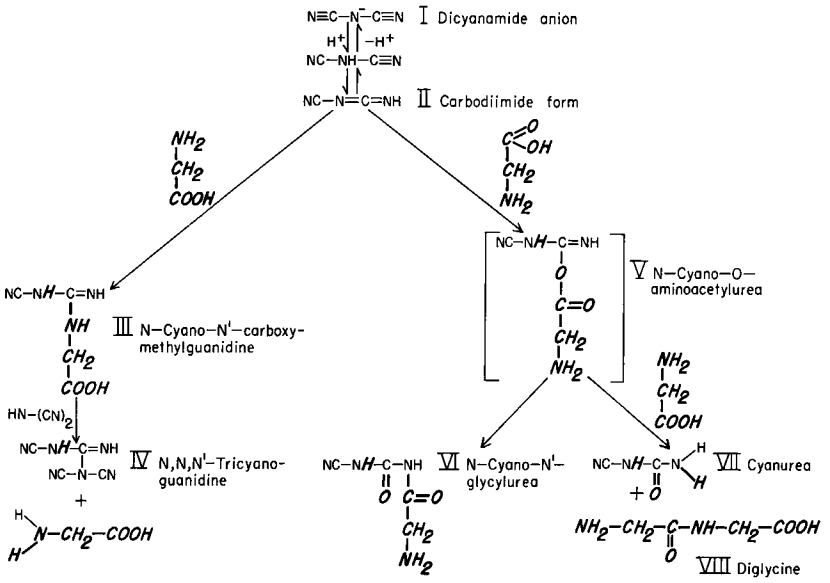

FIGURE 5 Series of reactions by which a peptide is formed by dehydration condensation reactions. Dicyanamide is shown in its various tautomeric forms. Notice that one of the forms can be written as a carbodiimide and thus corresponds to the well-known reagent that has been used most frequently in a nonaqueous medium for the same reaction series. An amino acid is added to the carbodiimide linkage, and once the oxygen atom is attached to the carbon, it stays attached. The carboxyl carbon is subject to nucleophilic attack by another amino group; the nitrogen attacks that carbon and forms the peptide link. Notice that the original cyano group of the carbodiimide has become urea. A water molecule has been added to the carbodiimide, producing the dehydration condensation to the dipeptide.

order. The primary order is the hooked-together sequence of amino acids; the secondary order grows from that. Figure 6 shows the nature of some of the amino acids (some of the available R-group varieties) that give rise to the primary polypeptide (or protein) structure—the R_1, R_2, R_3, etc., sequence. The secondary structure of the polypeptide (Figure 7) is helical and coiled; its nature is built into the peptide structure by the carbonyl groups of amide linkages. These links form bonds with the hydrogen of the link three or four amides removed from it

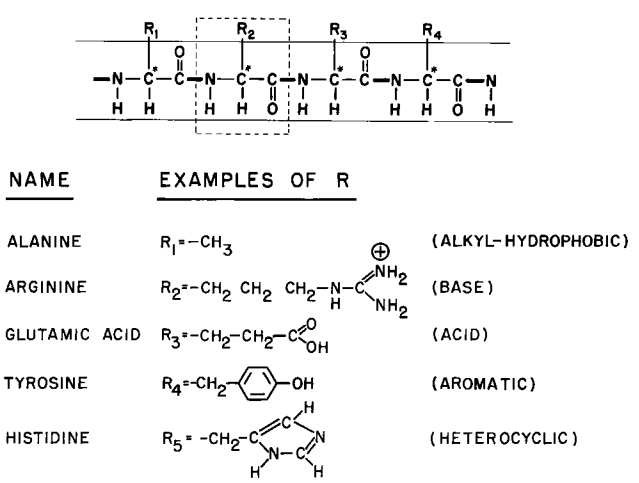

FIGURE 6 Primary protein structure.

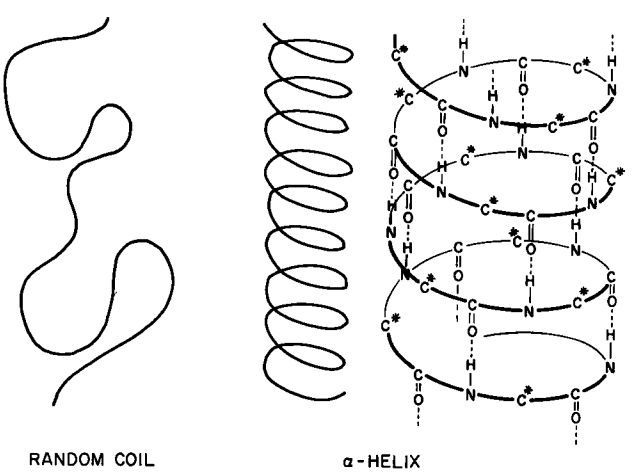

FIGURE 7 Secondary protein structure.

down the chain. The helix is the secondary structure of the linear array, and it is thermodynamically stable under suitable conditions. This characteristic is demonstrated in Figure 8, which gives the results of an experiment in which conditions were arranged for melting out the helix, and producing a random coil; the conditions were then reversed, causing the secondary structure to return. By adjustment of the *p*H, the structure can be changed from a random coil at alkaline *p*H to an alpha helix at *p*H 4.9; this is completely reversible. Apparently, then, the secondary structure is built into the linear array and does not have to be "wound up by hand, "so to speak.

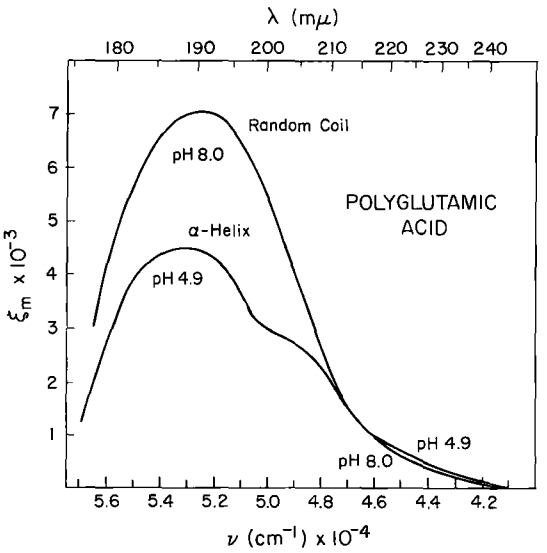

FIGURE 8 Absorption spectrum of polyglutamic acid in both helical and random coil forms. By adjustment of the *p*H, the structure can be changed from a random coil at alkaline *p*H to an alpha helix at *p*H 4.9, and this is reversible. Thus, apparently, the secondary structure is built into the linear array and does not have to be "wound up by hand." (Tinoco, Halpern, and Simpson, 1962. *In* Polyamino acids, polypeptides and proteins, University of Wisconsin Press, Madison)

The folding of the helical component is also built in and can be reversibly removed. This tertiary structure is built into the secondary structure, which, in turn, is a function of the primary structure. This can be shown by melting out, or destroying, the tertiary structure by raising the temperature; it re-forms upon cooling. Figure 9 shows the folded helix melting out of chymotrypsin as the temperature is raised. At *p*H 3.5, the folded helix is not melted out until a temperature of about 50° C is reached, and it reforms upon cooling, returning to the folded structure, such as that for myoglobin, seen in Figure 10. An aggregation and de-aggregation from what might be called quaternary structures is also reversible. Figure 10 shows how four chains of a protein (hemoglobin), folded into a tertiary structure, are packed together in a quaternary structure.

Thus far we have traced a degree of order from the amino acids, which are randomly formed, to the polypeptides, to the helix, to the folded helix, to the packed, aggregated folded helices; in other words, from primary to secondary to tertiary to quaternary structures.

The ordering of the polynucleotides is shown in Figure 11, which indicates how they organize into a secondary helical structure in much the same way as a polypeptide organized itself in a secondary structure. Two nucleic acid chains are here involved in the formation of the secondary helical structure of the nucleic acids, the reversibility of which is shown in Figure 12, which demonstrates a tem-

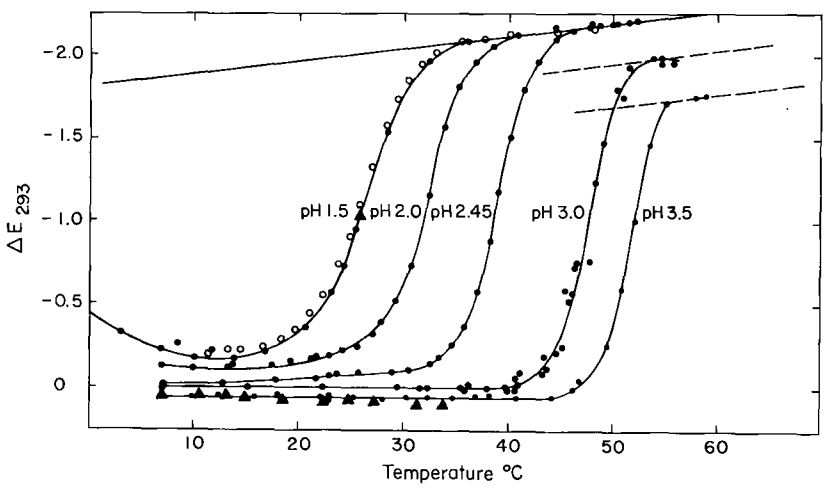

FIGURE 9 Change in extinction coefficient as a function of temperature for alpha-chymotrypsin at various *p*H. At *p*H 3.5, the folded helix (tertiary structure) is not melted out until a temperature of about 50° C is reached, and it re-forms upon cooling, returning to the folded structure, such as the one shown for myoglobin in Figure 10. The tertiary structure, not the alpha helix itself, is being melted out. (Biltonen, 1965, private communication)

Myoglobin (after Kendrew) Hemoglobin (after Perutz)

FIGURE 10 Structures of myoglobin and hemoglobin. (Kendrew, 1963. *Science,* Vol. 139, p. 1259, and Perutz, 1963. *Science,* Vol. 140, p. 863)

perature melting-out process. By raising the temperature from 22° to 99°, the double helix of the nucleic acid units melts into a random coil, and when it is cooled down again to 22° it reconvenes into the helical structure. This is the second degree of structure corresponding to the helix-coil transition in the proteins. The second order structural feature is built into the linear array of the nucleic acids.

Autocatalysis

The next step we must take in our examination of evolutionary developments is to introduce the notion that once certain kinds of structures appear, they, in turn, can in some way control, or induce, their self-formation from their component units. Chemists call this autocatalysis.

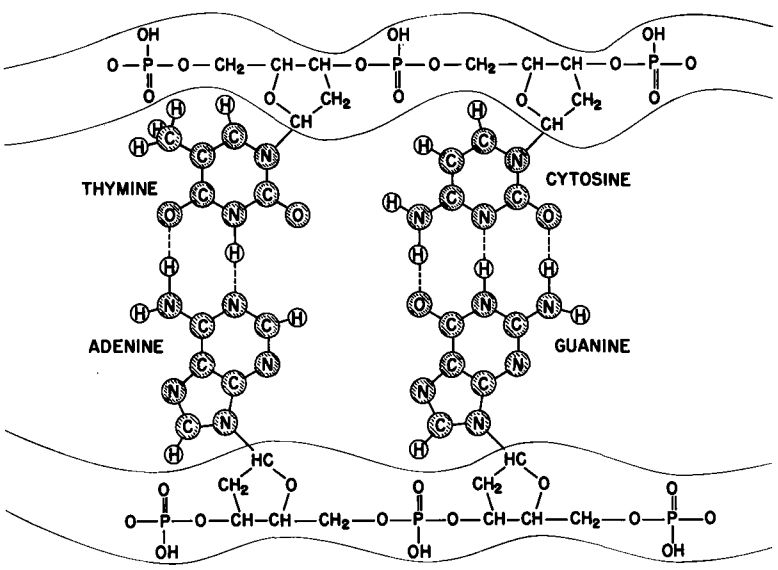

FIGURE 11 Molecular drawing of components of DNA.

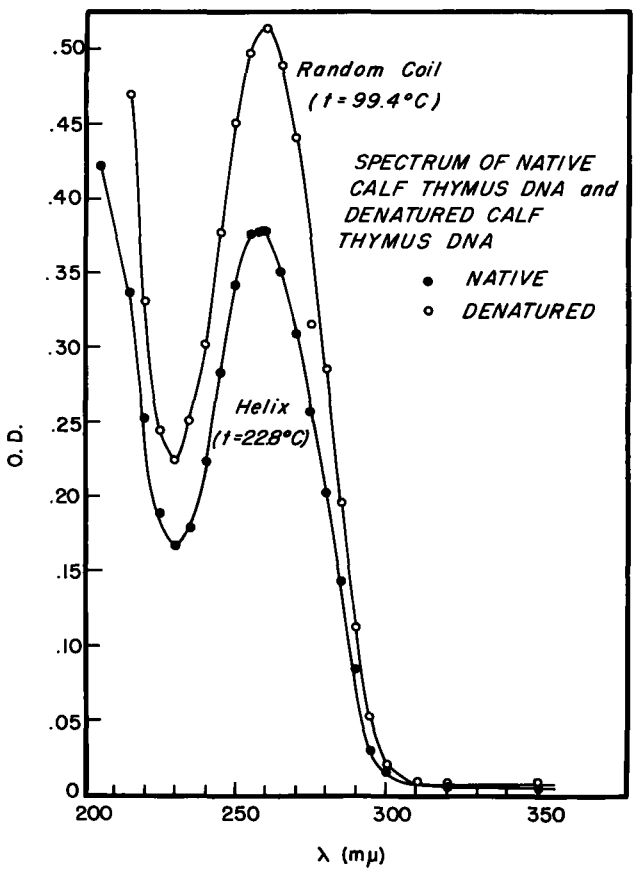

FIGURE 12 Hyperchroism on nucleic acid. (Tinoco, Halpern, and Simpson, 1962. *In* Polyamino acids, polypeptides and proteins, University of Wisconsin Press, Madison)

Figure 13 shows diagramatically the results of a number of observations. If a dehydrating condensing agent such as carbodiimide is used in an attempt to induce a hexamer of thymine to undergo a dehydration condensation between a hydroxyl group on one end of the hexamer and the phosphate on another one, the attempt will fail. Instead, a variety of other events occurs, and the yield of condensation products is extremely small. However, if a polyadenylic acid, which contains the complementary base of thymine, is added to the reaction mixture, the condensation reaction is much more efficient, with about a ten per cent yield of the condensation product—that is, the one obtained by hooking the two hexamer units together. Here the polymer is catalytic for its own complementary polymer formation. In the same way, polythymidylic acid catalyzes the condensation of adenylic acid to polyadenylic acid. Here is a cross-autocatalysis that is the essential feature of the self-reproducing genetic system, and it can be demonstrated in a simple, nonliving, laboratory system. This autocatalytic quality is an essential feature of the chemical evolutionary scheme.

Appearance of linear structure—linear array

We must now go to a higher degree of organization and order, and get to a fifth stage, which brings us into the region of visible structure. Figure 14 shows the example of taking apart the protein material of collagen and reassembling it into visible collagen fibrils. By readjusting the salt concentration of the solution, the collagen fibrils, after being dismantled, reaggregate into visible units that are

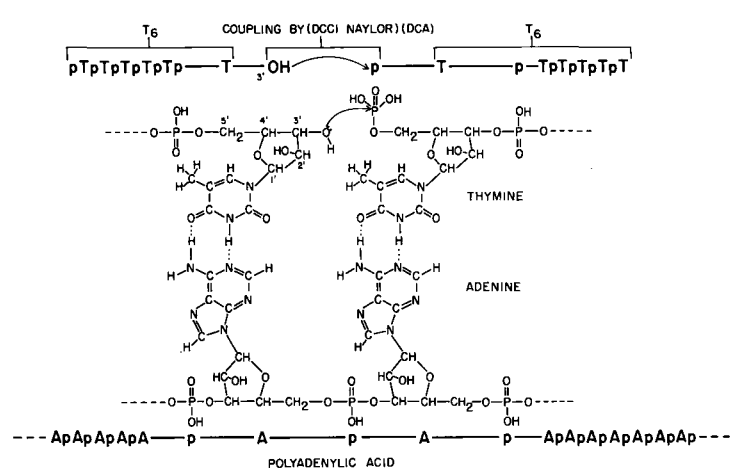

FIGURE 13 Autocatalytic reactions in chemical evolution. Hexamer of thymine is shown at top. (Naylor and Gilham, 1966, private communication)

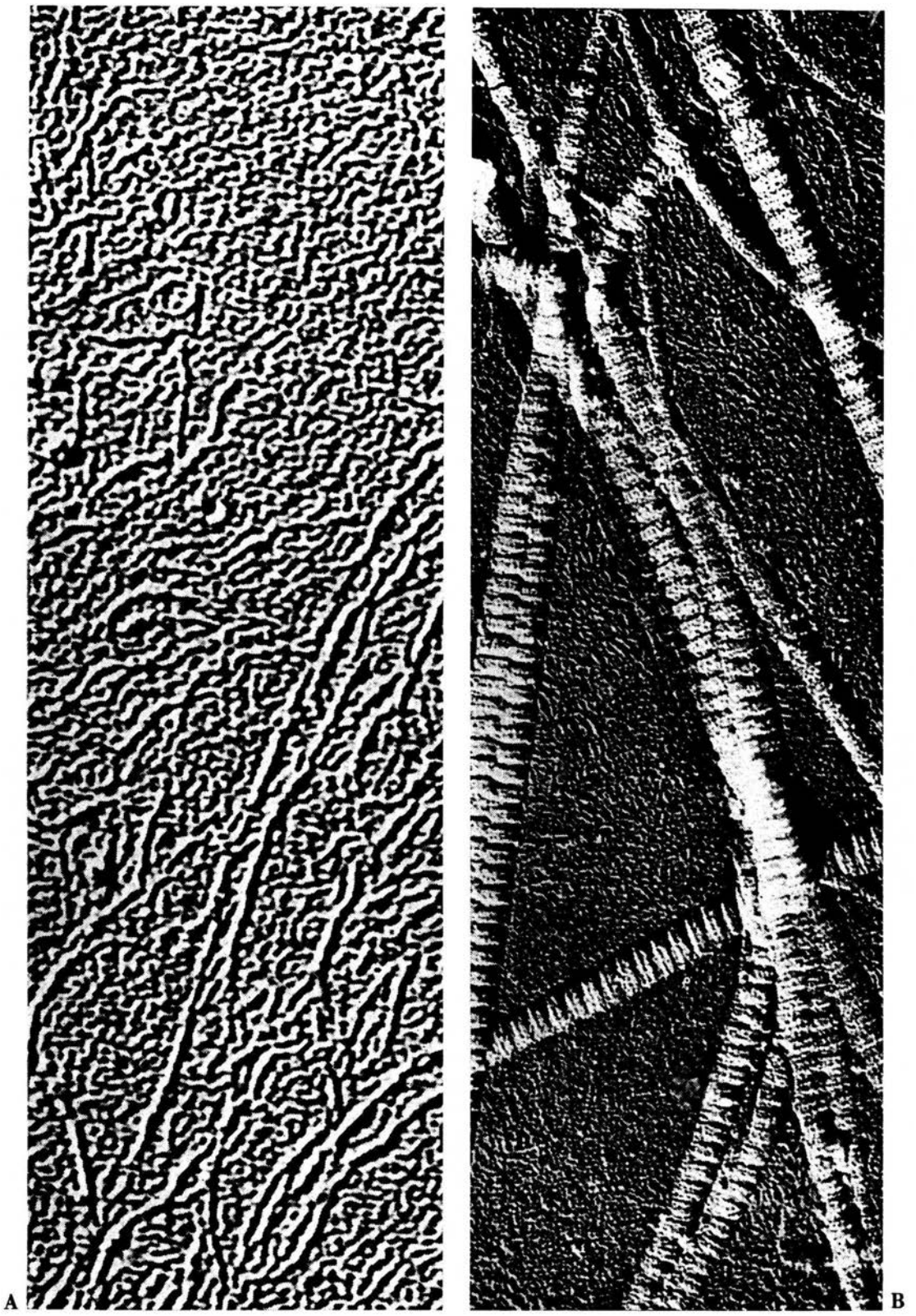

FIGURE 14 A: Filaments of collagen, a protein which is usually found in long fibrils, were dispersed by placing them in dilute acetic acid. This electron micrograph, which enlarges the filaments × 75,000, was made by Jerome Gross of the Harvard Medical School. B: Fibrils of collagen formed spontaneously out of filaments such as those shown above when 1 per cent of sodium chloride was added to the dilute acetic acid. These long fibrils are identical in appearance with those of collagen before dispersion.

identical with the naturally occurring collagen fibrils. We have now reached the level of visible structure that is contained implicitly within the units of the molecules themselves. We have traveled all the way from methane-ammonia-water-hydrogen in a non-living world to visible structures of the living organism—and without the introduction of Maxwell's demon.

The next step is to reconstitute linear living structures from molecules. One example is the reconstruction of the tobacco mosaic virus (TMV) particle. The native TMV particle (Figure 15) displays a definite diameter and length. The reconstituted TMV protein without RNA is seen in Figure 16. If the particle is taken apart and dissolved, nucleic acid is removed and the protein molecules reaggregated, but at random lengths. If, however, the separated protein molecules and the separated nucleic acid molecules are combined and reaggregated in the presence of each other, the reconstitution is more precise (Figure 17). Here we have reconstitution in a linear array. The length is determined by the nucleic acid; the protein tends to aggregate, but its length is indeterminate.

A still higher order of reconstitution is that of four different types of bacteria flagella (Figure 18). Three of the bacteria have long, wavy flagella, and the fourth type is shorter and curly. It is possible to separate the flagella from the bacteria, and dissolve them into molecular solution, with a protein molecular weight of about 40,000. These can then be reconstituted (Figure 19). If the monomer from III is seeded with fragments of III, a type III flagellum results. However, if a monomer from IV (the so-called "curly" type) is seeded with fragments of III, the curly type predominates. This shows that the protein structure of IV is different from that of III, and that the flagellar growth pattern is determined by the protein molecular structure and not by the seed.

Much more complex spontaneous reassembly processes have been demonstrated. An example is the enzyme complex of alpha-keto acid oxidases. Here, a complex made of three different proteins (decarboxylase, acyltransferase, and flavoprotein) is reassembled into a specific, catalytically active complex, using the component molecules in the ratio 12:1:6. This is the elegant work of Reed (see his chapter this volume).

The flagella (and viruses) I have been describing are essentially linear arrays. A two-dimensional array is the next stage in the development, and thus we are on the way to a film, or membrane structure. Such a membrane surrounds the energy-conversion (enzyme complex) and structure-determining apparatuses (genetic material).

Structure of a membrane

An electron micrograph (Figure 20) shows an individual membrane within the chloroplast (a model for a membrane, if you like), and you can see that the membrane is not smooth, but is composed of granular units of protein. These are probably hooked together by properties of their own structure and then covered by some kind of lipid layer. This same type of structure is shown in greater detail in Figure 21, and the cracking of the lamellar structures can be seen at various levels. The structures are composed of granules of different sizes at different levels, with alternating protein and lipid layers.

We have not yet reconstructed an energy-converting membrane such as the chloroplast lamellae. We have taken it apart, but have not learned how to put it back together. A few efforts have been made, and I fully expect that in the not-too-distant future some reconstructions will occur.

However, an effort has been made to reconstruct another interesting membrane by using methods as yet rather crude. (This is the first mention I have seen in the literature of an attempt to reconstruct a biologically active membrane.) This is the reconstruction of a membrane of *Mycoplasma,* a slime mold (Figure 22), and is a step toward the reconstruction of the two-dimensional, asymmetric membranes essential for the containment of the functioning structures inside the living cell.

This is but a bare beginning. We must probe much more deeply into the constitution, construction, and reconstruction of the various types of biological membranes. The basic physics and chemistry of synthetic, and partially synthetic, model systems demand that we develop the as yet extremely rudimentary synthetic and theoretical principles.

Development of the nervous system

The next step in our evolutionary travels is the development of the nervous system. We have repeatedly heard that the factor which selects for the survival of a system—whether it be a simple chemical system or a single living organism—is the environment in which it resides. If one particular array of physical-chemical systems has survival advantage over another in a particular environment, it is clear that the former has a better chance of reproducing itself and fully occupying the environment than will the latter. This only means that the physical-chemical system with some reproductive survival advantage in a particular environment will eventually use up all the raw material of

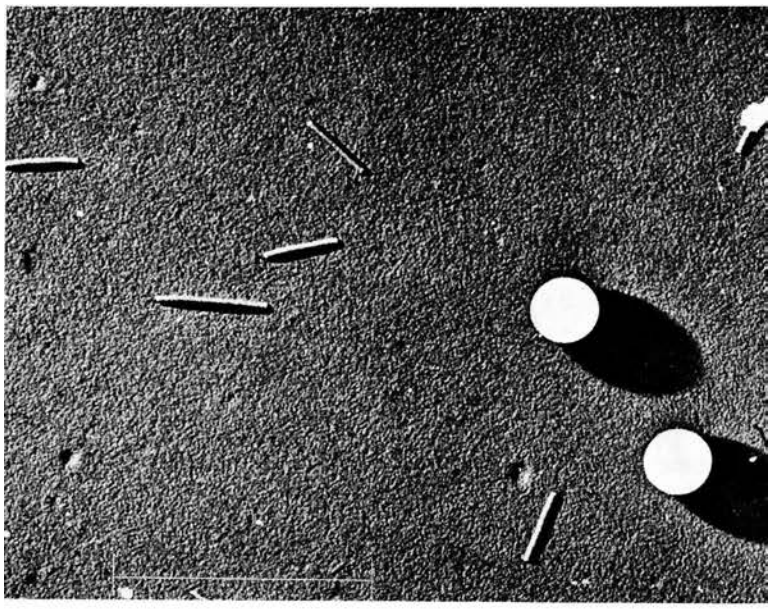

FIGURE 15 Native tobacco mosaic virus (TMV). (Williams, Virus Laboratory, University of California)

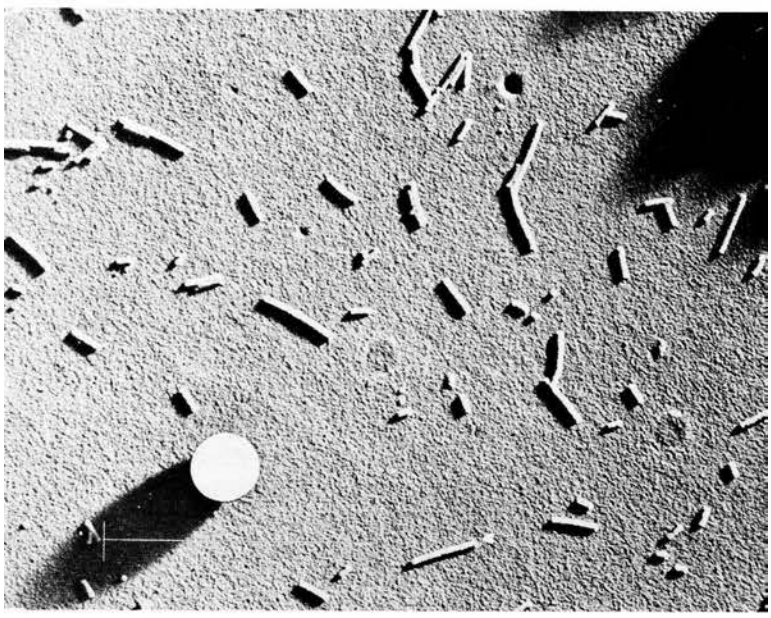

FIGURE 16 Reconstituted TMV protein without RNA. (Ibid.)

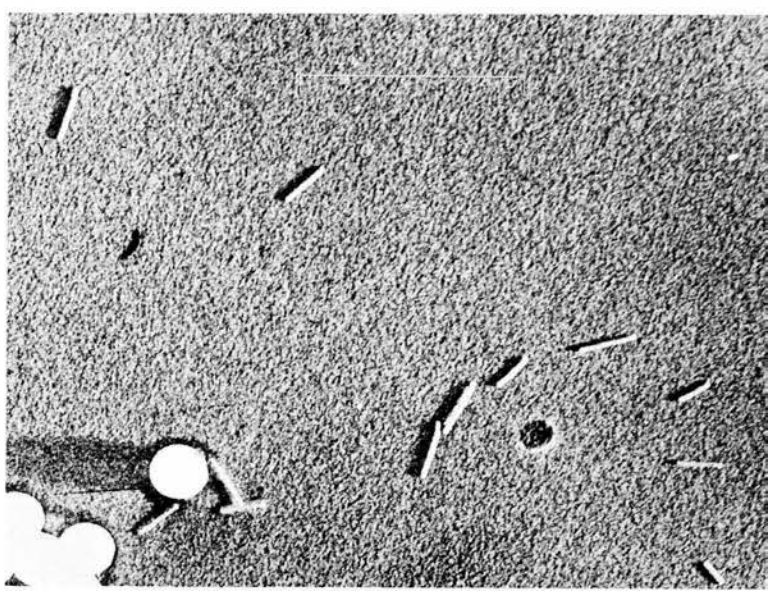

FIGURE 17 Reaggregation of separated TMV protein and nucleic acid molecules. (Ibid.)

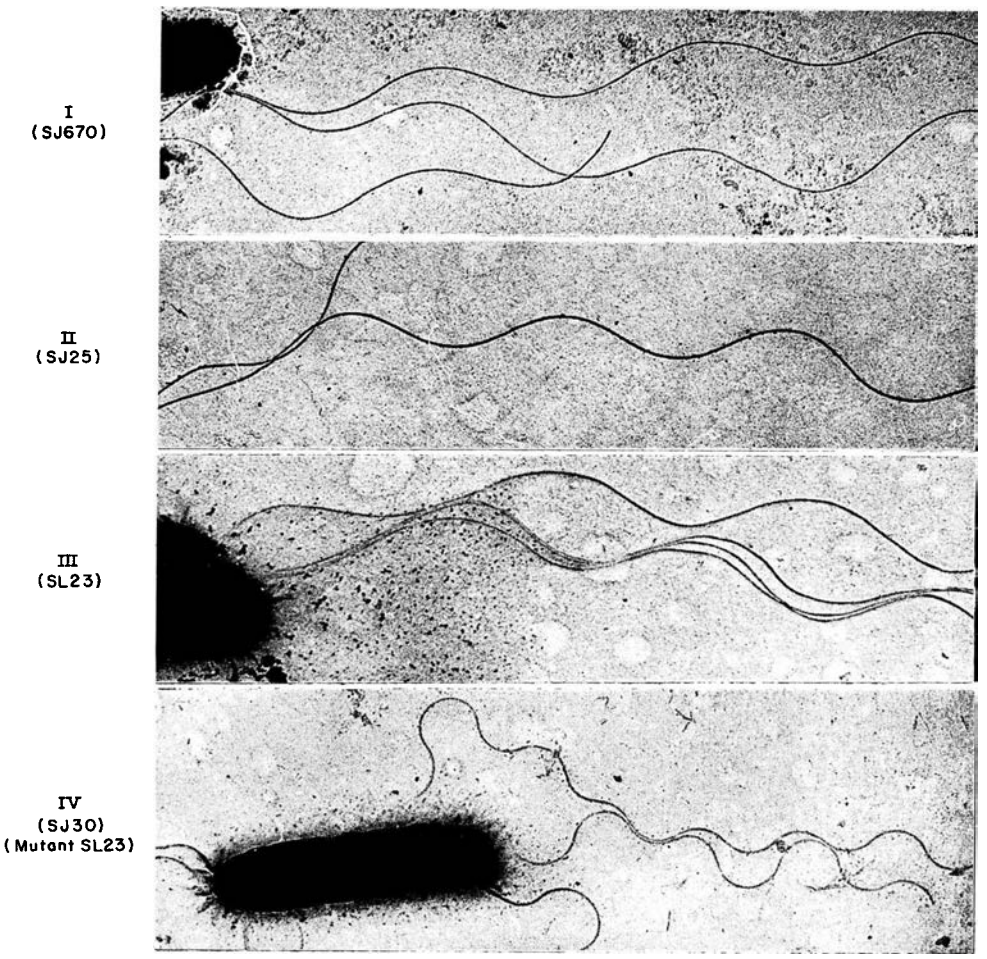

FIGURE 18 Four kinds of *Salmonella* flagella. Top three are long and wavy; bottom one is shorter and curly. (Asakura, Eguchi, and Iino, 1966. *J. Mol. Biol.*, Vol. 6, p. 302)

that environment to the exclusion of other systems that require the same materials.

This basic process has been taken for granted in all of the evolutionary steps we have discussed, and brings us to the evolution of a single entity surrounded by a membrane that keeps it separated from its outside environment—in other words, a cell. Most of the responses to the environment made by that cell, that single entity, are responses to chemical stimuli. A chemical stimulus can be some change in the chemistry of the environment, and the organism responds to that change by a physico-chemical reaction of its own, which is dependent upon the concentration of some chemical in its environment. Thus, a cell reacts to a gradient of the concentration of the chemical. Initially, there is no "directionality" to that kind of sensing, other than the gradient itself, but it works—so long as the organism is single-celled.

Even when cells aggregate linearly, each one can sense the environment directly and does not need any help. Examples of such a linear array are *Nostoc,* a unicellular alga, and the aquatic plant *Nitella.* Every single cell is exposed to the outside environment in at least two dimensions, so the linear aggregation does not require any kind of specialized information-gathering or information-transferring equipment. However, if the cells aggregate in two dimensions and the sheet is allowed to close and form a hollow sphere, or tube, there are cells inside that do not have direct access to the outside environment. Those, then, obviously need help from some information-transferring apparatus. Each cell already has a membrane that is asymmetric because the inside is different from the outside. Some of the cells that lie in double or triple layers on the outside of the tube, or sphere, could extend their membranes through the layers and find a way of transmitting

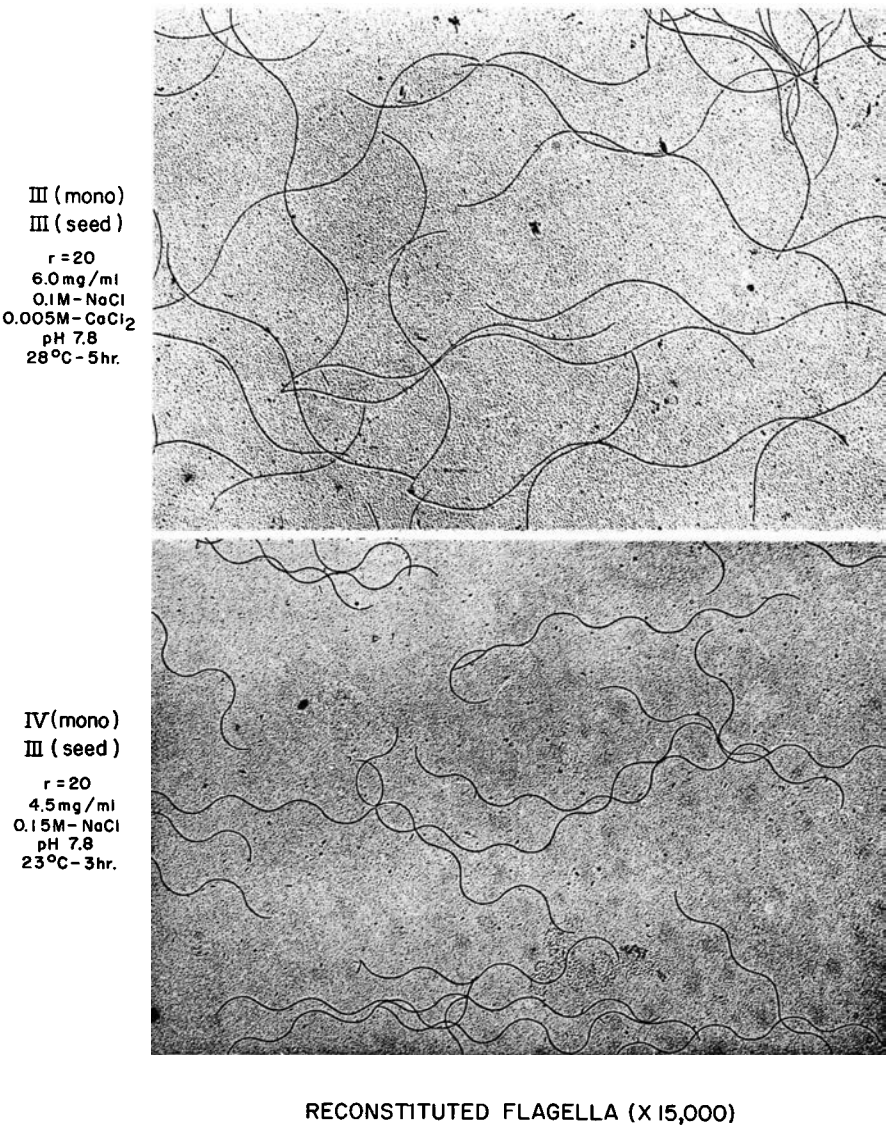

FIGURE 19 Reconstitution of flagella. At top, monomer from III is seeded with fragments of III, and a III-type flagellum results. At bottom, IV-type (curly) monomer is seeded with III, producing predominately IV-types. (Ibid.)

information about the environment to the inside cells. The organism (system of cells) that devises such a mechanism, with a minimum loss of information in the transfer, is the one that can react to changes in the environment with the least loss to itself and its offspring.

The evolution of a transmitting system is the first step in the evolution of the nervous system. Presumably the sensing device on the outside is still chemical (it could be physical if it were responding to light, for example). The sensing device, the initial transducer, can remain unchanged, but the transmitting system must, in some way, be improved beyond that of the single cell.

We already have an asymmetric membrane that evolved in response to the requirement for initial encasement of the cell. Depending on where the organism is located, the membrane is able to keep salts and certain organic molecules in or out, as indicated. Thus will arise an electrically asymmetric membrane. For example, in order to move into fresh water from salt water it must devise ways of retaining inside the salt it needs for its functioning in the presence of the osmotic gradient. Thus there evolved—almost simultaneously with the initial cellular enclosure—semi-permeable membranes and salt pumps. We do not yet know the mechanisms of semi-permeability or selectivity with respect to salt, nor do we know the mechanisms of the pumping apparatus—that is, ways in which

salts, or metabolites, are pumped from a low concentration back up to the high concentration needed inside the organism. A typical salt concentration is of the order of 500 millimolar. If the cell is put into fresh water it must have, first, a mechanism to prevent the NaCl from leaking out and, second, a mechanism for pumping back the material that does leak out, as no permeability barrier is perfect. Such a mechanism did indeed evolve, even for single-celled organisms.

What is needed now is the evolution of a device for transmitting without decrement any chemically or physically sensed environmental changes. One might speculate that only a confining tubule, down which the concentration gradient could move, would be necessary. However, as a concentration gradient moves down even this narrow passageway, it transmits information content with a logarithmic decrement. This is not a very satisfactory method, and is the crux of the next stage in the evolution of information transmittal.

Obviously, if repeating stations with periodic energy outputs were built into the mechanism, the signal could be rebuilt up over the noise. The electrically active asymmetric membrane is used by the living cell in just this fashion. The essence of the idea is as follows: the nerve cell does not generally transmit an impulse by a gradient transmission, but by a frequency code.

Before examining the mechanisms of transmission, let us look at the neuron, the physical apparatus which the specialized information-transferring cell has become. Figure 23 is a drawing by Valverde of a highly developed neuron. The cell body was originally a little "blob" surrounded by a membrane. Now the membrane has extended processes, the dendrites, in many directions, and most of them take messages from other cells. One, the axon, sends its own messages. A single cell may receive information from at least 500 other cells, and the message it sends out is synthesized from all the messages it receives. The transmitting end of one cell and the receiving end of another form a synapse (Figure 24). The message comes down the presynaptic tube to a gap between the two cells. As the electrical message comes down the cell wall, something happens at the gap (and I mean just "something";

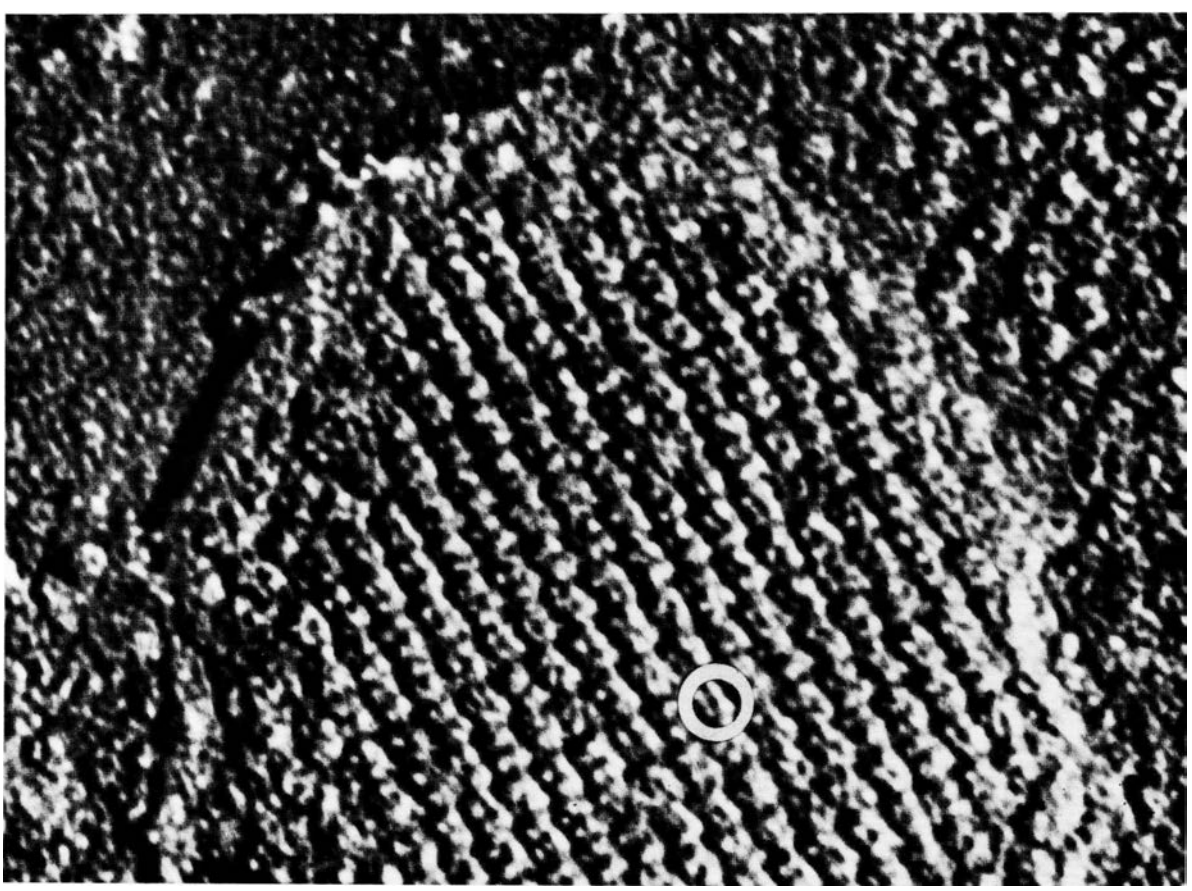

FIGURE 20 Lamellar structure of chloroplast.

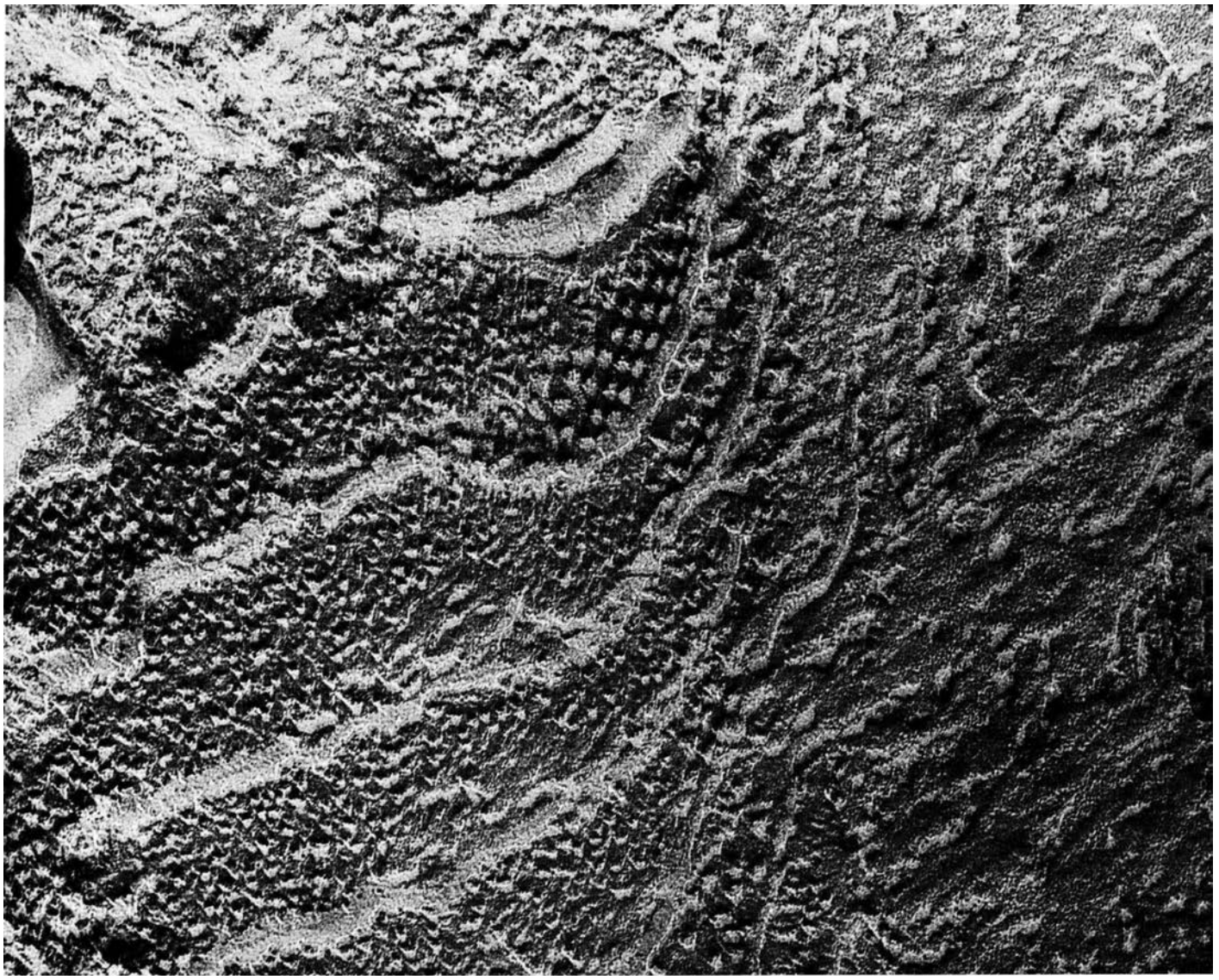

FIGURE 21 Same type of structure at higher magnification, showing cracking at various levels. Structures are granular, and granules are at different sizes at different levels, with lipid and protein layers alternating.

we don't know exactly what). It lets a few vesicles, which presumably contain a chemical, loose into the gap between the two cells. The chemical is released, travels across the gap, and affects the postsynaptic surface in a way that starts the message down the next cell. These are the two important components of nerve transmission: the axonic transmission, or, I should say, the nerve impulse itself; and the synaptic transmission from one cell to the other. We must find ways and means of understanding these two essential features in molecular terms in order to devise evolutionary schemes for them.

This is the basic apparatus. Figure 25 shows the frequency response in terms of light intensities on *Limulus* eyes and the resultant signals from the nerve connected to it. Although there are a few signals at low intensity, and they increase as intensity increases, the signal that passes down the nerve is always the same size. This is because of the "repeating station" mechanism. Figure 26 is a higher magnification of one of these signals, showing more clearly the voltage pulse as it passes a particular point on the nerve.

What causes that voltage pulse in the nerve and how is it controlled? It seems to be the result of a salt concentration change of some sort, and this notion has already been introduced by the general nature of membranes. According to the Hodgkin-Huxley theory of nerve impulse conduction, the current flows inward at a particular point and then outward (Figure 27). By controlling the polarization

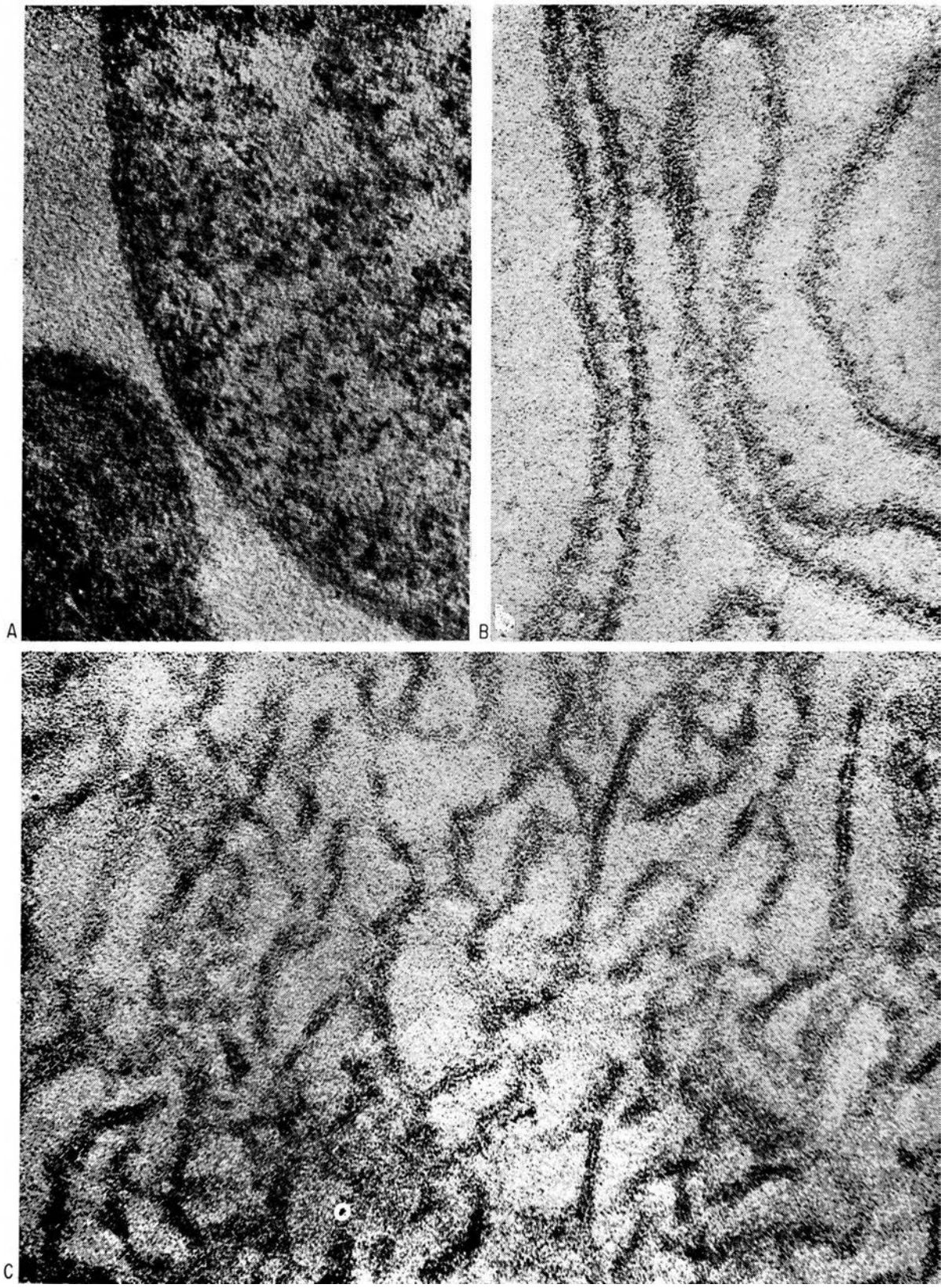

FIGURE 22 Membrane of slime mold *Mycoplasma* at × 163,000. A: Cells of *Mycoplasma laidlawii*. B: Isolated membranes. C: Reaggregated membrane material. After the lipid and protein are redissolved the membrane is reconstituted, and here appears similar to the original, intact membrane. (Razin, et al., 1965. *Proc. Natl. Acad. Sci. U.S.*, Vol. 54, p. 219)

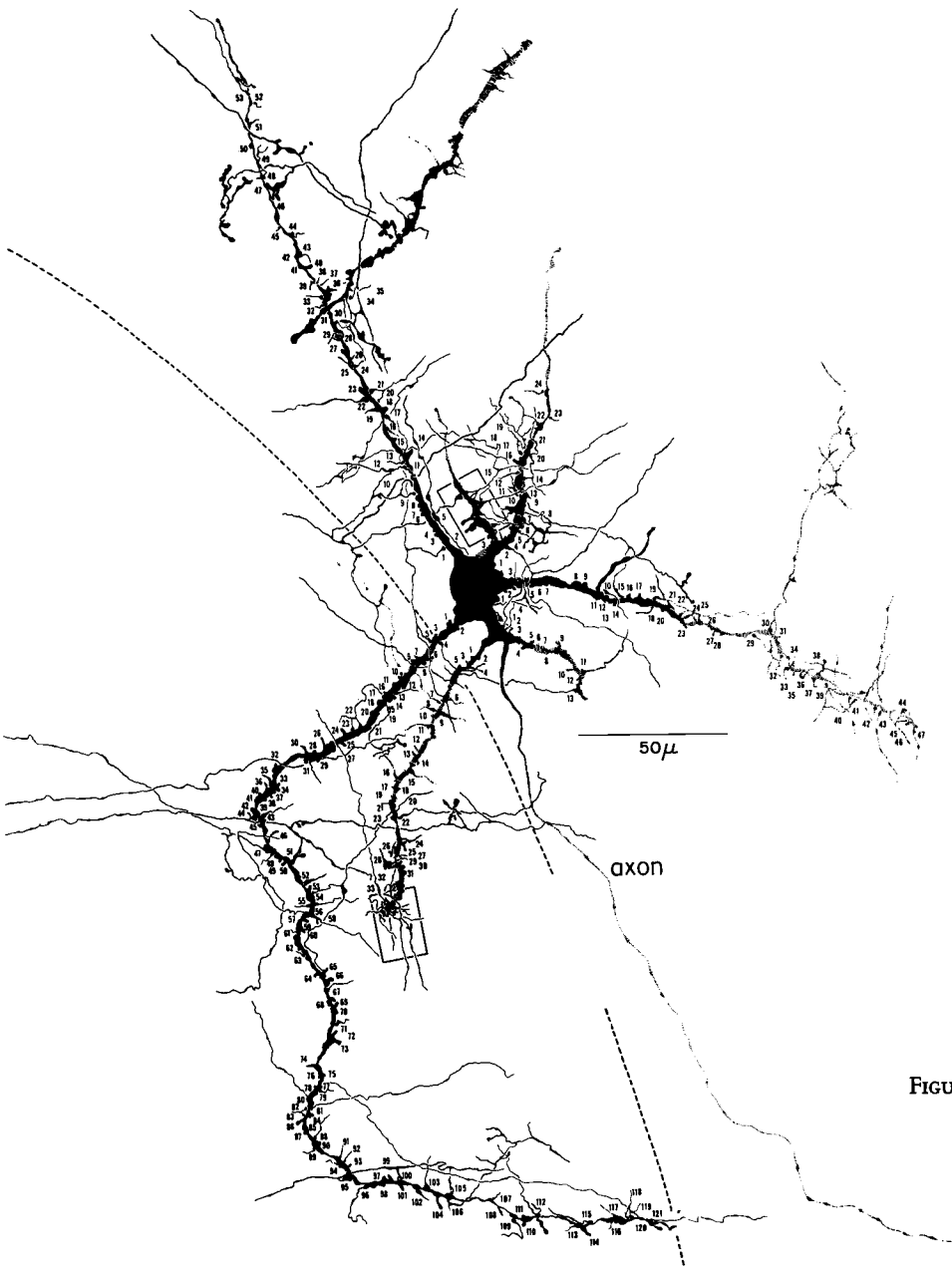

FIGURE 23 Highly developed neuron. (Valverde, *J. Neurol.*, in press)

of the membrane and the ions on both the inside and outside of the membrane, it was possible to show that the current was made up of two components—sodium ions flowing inward and potassium ions flowing outward. To impose a particular potential difference, the sodium ion current going inward grows rapidly and then decays; the potassium ion current going outward starts more slowly and then remains constant for that particular polarization (+56 millivolts). These currents are interpreted in terms of changes in the selective conductivity of the membranes for Na and K ions, which can be measured separately, as shown in Figure 28. When a voltage is impressed from the outside, the sodium ion conductivity rises very rapidly as the sodium leaks into the membrane, thus reducing the negative potential on the inside. The potassium then gradually begins to leak out. The sodium conductivity increases rapidly and then decreases; the potassium conductivity increases more slowly and stays more constant, until the impressed voltage is reduced.

If these two conductivity changes are put into a suitable equation for corresponding voltage changes, the voltage being determined first electrochemically by the potassium

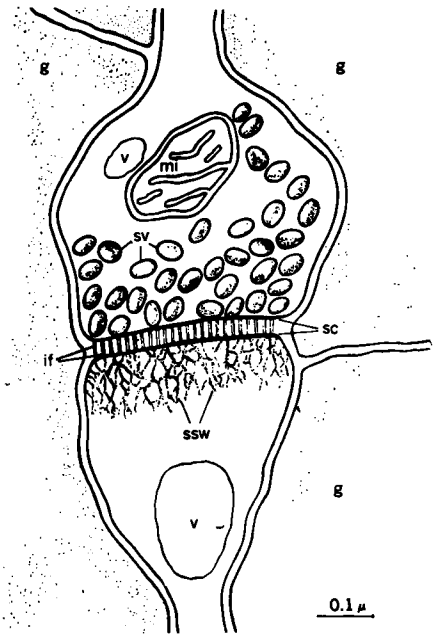

FIGURE 24 Typical cortical cell. A common form of synapse in the mammalian brain. The axonal (presynaptic) side above; the dendritic (postsynaptic) side below. g, glia; if, intersynaptic filaments; mi, mitochondria; sc, synaptic cleft; ssw, subsynaptic web; sv, synaptic vesicles; v, vesiculate body. (After de Robertis, 1962)

and then by the sodium, the passage of the nerve impulse becomes apparent (Figure 29). When the potential across the membrane is changed, something happens to it to make it quickly permeable to sodium, to allow a quick recovery of sodium resistance, and to make it slowly permeable to potassium. As the potassium ion leaks out, the voltage is returned to its original value, and as this takes place the potassium current is gradually shut off. The voltage signal travels down the nerve by the mechanism shown in Figure 30. The voltage is negative on the inside of the nerve cell and positive on the outside. Then at some point a reverse voltage is impressed. Sodium ions flow from the outside in, pulling sodium ions from the neighboring part of the membrane; potassium ions flow from the inside out, but more slowly. The sodium ion flows

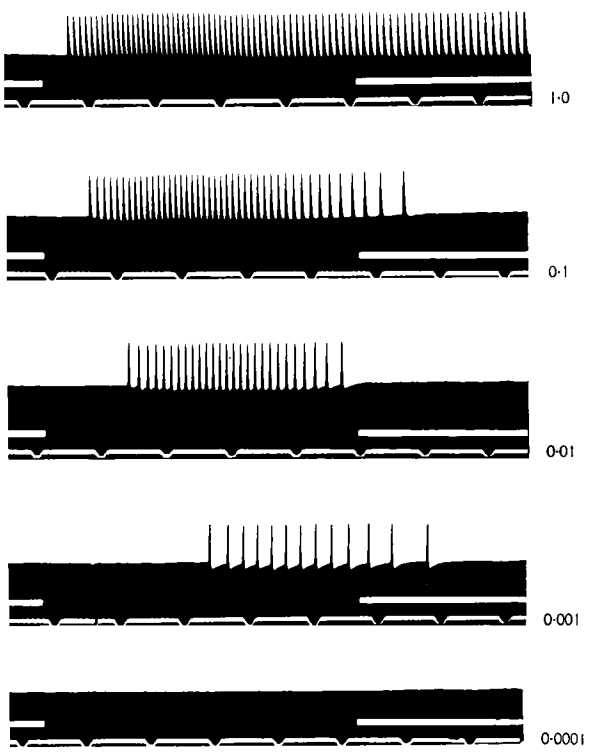

FIGURE 25 Pulses are signals that come out of a nerve. At very low intensity there are few signals; as the intensity is increased the frequency increases, but the signal that passes down the nerve is always the same size. This is the result of the "repeating station" mechanism. (From A. L. Hodgkin, Note B5)

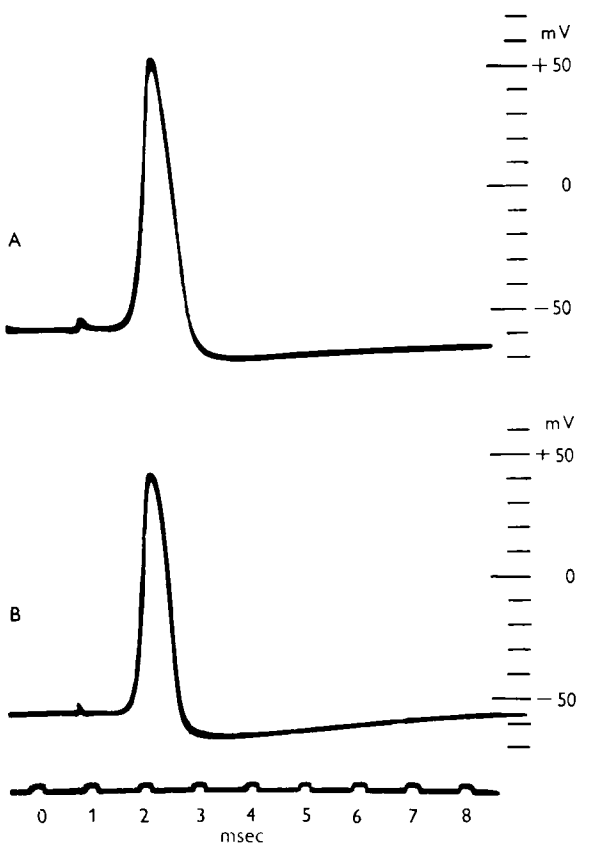

FIGURE 26 Higher magnification of action potential recorded with internal electrode. (Ibid.)

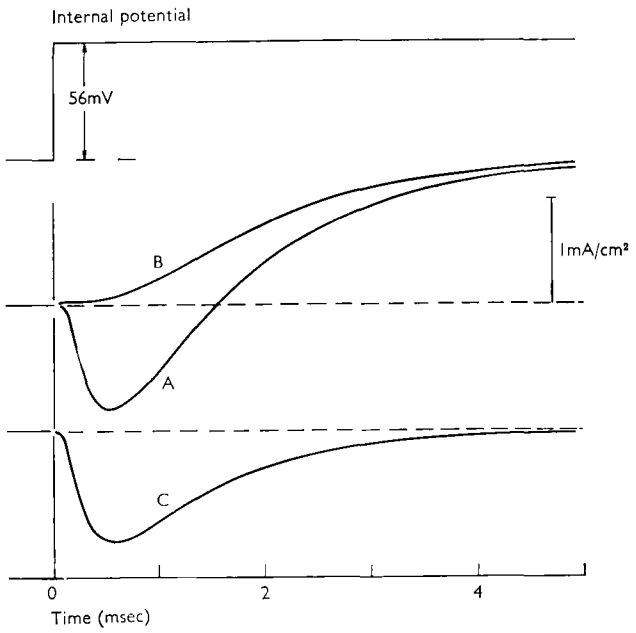

FIGURE 27 Separation of membrane current into components carried by Na and K; outward current upwards. A: Current with axon in sea water = $I_{Na} + I_K$. B: Current with most of external Na replaced by choline = I_K. C: Difference between A and B = I_{Na}. Temperature, 8.5° C. (From Hodgkin, 1964, based on Hodgkin and Huxley, 1952)

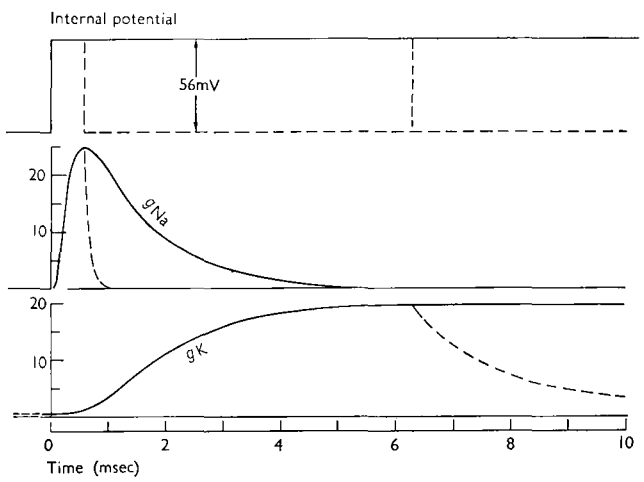

FIGURE 28 Time course of sodium conductance (g_{Na}) and potassium conductance (g_{Na}) associated with depolarization of 56m V; vertical scale in mmho/cm². The continuous curves, which were derived from those in Figure 27, are for a maintained depolarization; broken curves give the effect of repolarizing the membrane after 0.6 or 6.3 msec. (From Hodgkin, 1964, based on Hodgkin, 1958, and Hodgkin and Huxley, 1952)

from one part of the cell to the other, closing the voltage gap. The first unit is depolarized and becomes permeable to the sodium ion. Some macromolecular change occurs in the polymer membrane itself, so that it immediately becomes conductive for the sodium ion. This causes the migration of the depolarization wave down the nerve.

Where does the energy for this depolarization come from? It derives from the salt concentrations on the two sides of the cell. Inside the membrane there is a sodium concentration of 50 millimolar and a potassium concentration of about 400 millimolar; outside the membrane the situation is reversed, with 20 millimolar for potassium and 400 for sodium. That electrochemical concentration cell stores the energy for driving and regenerating the depolarization wave as it travels down the nerve. The amount of sodium and potassium that exchanges per square centimeter of membrane is very small (about four picomoles per pulse). Remembering that this is per square centimeter of nerve membrane and remembering the millimolar concentration as well, it can be seen that many pulses can be passed before the amount of sodium and potassium on either side of the membrane is appreciably changed. But a pump is required. It gradually pumps the potassium back in from the outside, and pushes the sodium out from the inside. It is an ion pump coupled to metabolic energy, which, in some way, burning sugar, eventually pumps ions against their gradient.

There is at least one place at which electrochemistry and polymer chemistry can come together to seek an answer to the prime question: what conceivably could be the mechanism of the permeability change induced by changing the membrane polarization from −50 mv to +50 mv? How is something done suddenly to the permeability of sodium and potassium—a change that is gradually reversed by potassium leaking out of the cell and sodium into the cell, thus returning the permeability and polarization to their original values? To trigger the event, the polarization change across the membrane must be about 50 millivolts. Keep in mind that kT is about 25 millivolts. How big a dipole would be necessary to permit it to turn in this field gradient against the 25 millivolts of thermal energy? A dipole of 50 Debyes will turn completely in a 50 millivolt field over 100 Å, the approximate thickness of the cell membrane. By making that simple kind of energy calculation, you can see that the protein molecules, for example, which have unsymmetrical charge distributions, could very easily be involved in a switch that opens or closes the membrane to the sodium or potassium ions.

In fact, a closer comparison of the theoretically obtained action potential (Figure 29) and that actually observed (Figure 25) reveals that the observed action potential rises

more sharply than the theoretical and has a slightly convex, or more rapid, fall as well. An examination of the original charging rate data gives an indication that there is a sudden change in dielectric constant of the membrane when its potential is displaced −25 to −50 millivolts from the rising value. (This re-examination and calculation was performed by Mr. Donald E. Phelps of the Laboratory of Chemical Biodynamics, University of California, Berkeley.)

The polarization is maintained by the salts (the K ion concentration); the resting potential of the nerve membrane is maintained primarily by a potassium ion electrochemical concentration cell. This, in turn, keeps that protein-lipid dipole, whatever it is, oriented in such a way that sodium (and to a lesser extent potassium) cannot go through. The moment the potential across the membrane is reduced, the protein-lipid-carbohydrate complex re-

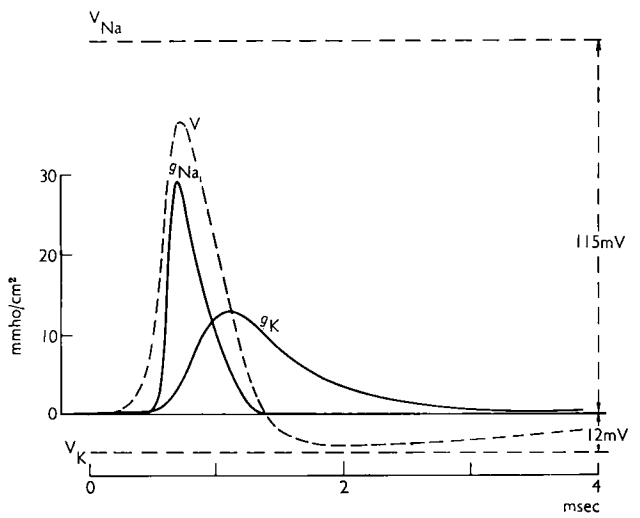

FIGURE 29 Theoretical solution for propagated action potential and conductances at 18.5° C. Total entry of sodium = 4.33 pmole/cm²; total exit of potassium = 4.26 pmole/cm². (Ibid.)

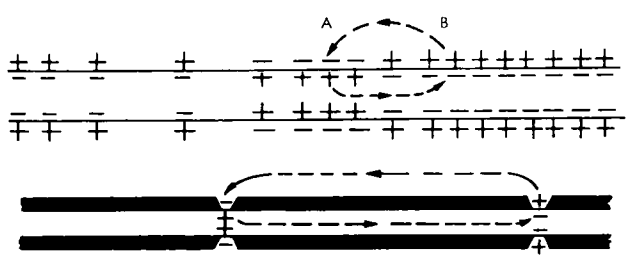

FIGURE 30 Local circuit theory of nerve conduction. The upper sketch represents an unmyelinated nerve fiber; the lower a myelinated nerve fiber. (From Hodgkin, 1964)

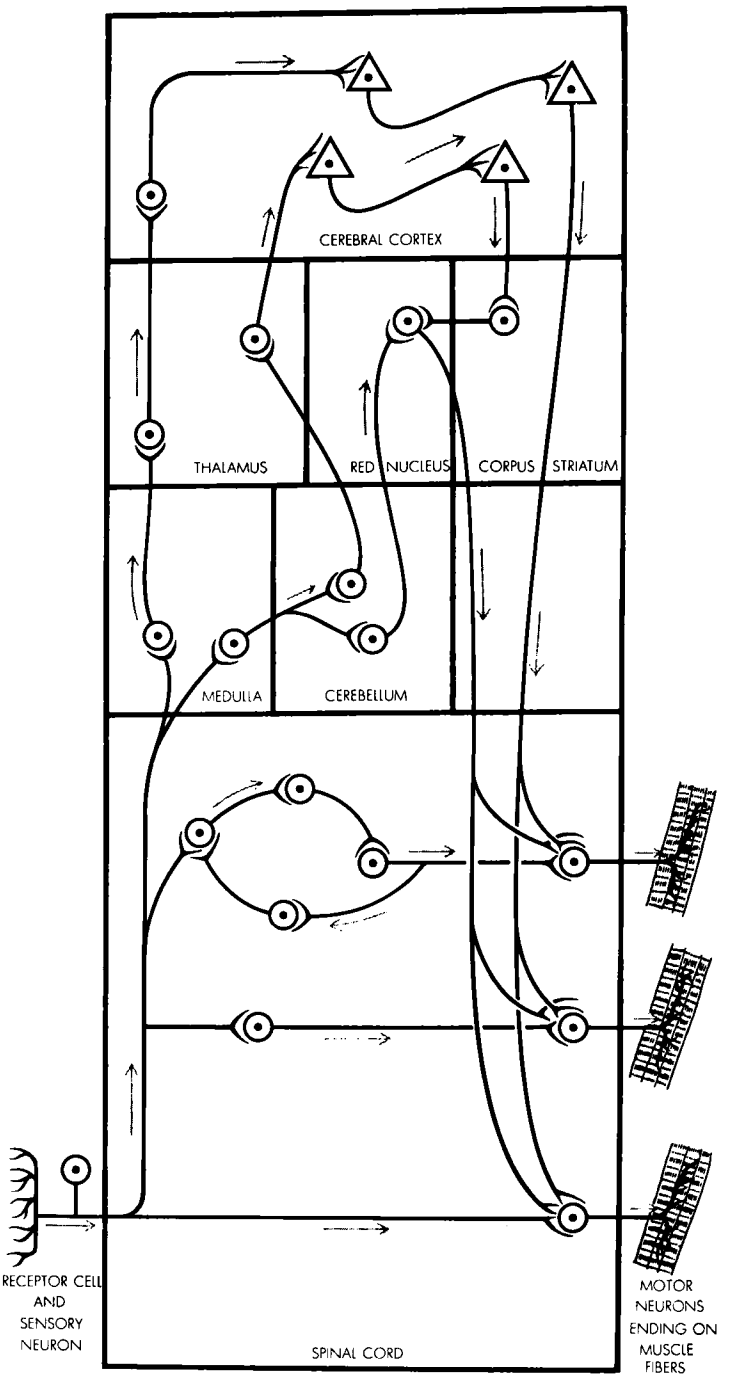

FIGURE 31 Simplified flow diagram of the nervous system in higher animals. Receptor cell detects environmental change and starts the signal, which travels from cell to cell, with various kinds of circuits at different levels. Direct input-output system is at bottom, on muscle fibers, with no processing. Stages and types of filtration and processing exist, however, at other levels—from the spinal cord (with feedback), up into the cerebral cortex. (Katz, *Scientific American*, Sept. 1961)

laxes, and the sodium-potassium can go through. As the potassium builds back up to the ten-fold concentration difference, the potential is gradually restored, protein configuration is turned back, and the permeability of sodium and potassium is cut off.

It is astonishing that one should come up with the right size of dipolar separation, corresponding roughly to the 100 Å dimension of the membrane, for the critical voltage that switches it open and shut. This is significant, for it suggests that the mechanism is probably not extraordinarily complex. Certainly, it provides a model that perhaps we can reconstruct by synthetic systems.

Synaptic transmission, the other essential component of the information transferring system, can now be conceived as a further development of membrane sensitivity. It is no far cry from the turning dipole to its possible susceptibility to ions (acetylcholine, catecholamines, Ca^{++}, and other neurologically active ions [curare]) or potential ions (LSD), as well as the dipolar ions, gamma-amino-butyric acid, phosphocholine, etc. Certainly the interaction energy may be expected to be large, for the membrane must have a highly polar permeability-determining center for such highly charged materials. A degree of specificity in these transmitter substances may also be expected. Finally, through changes in secondary, and even primary, protein structure under RNA and DNA control, a variability (plasticity) of membrane structure can be visualized in the electrical input to a cell or groups of cells. The organization of such a total system in higher animals is shown in Figure 31. There is a receptor cell that detects the environmental change and starts the signal traveling from cell to cell, with various kinds of circuits at different levels. At the bottom is a direct input-output system on the muscle fibers, with no processing. You can also see, however, that there are all kinds of levels of processing—in the spinal cord (with feedback), in the cerebellum, the red nucleus, and, finally, up into the cerebral cortex, with all stages of filtration and processing in between.

At this point I can go no further than to suppose that in some way the facilitation or inhibition of the various transmissions makes up the information-processing and storage device. However, we must remember that these facilitations and inhibitions are ultimately recorded in molecular structure.

Summary and conclusion

Cellular aggregation and specialization arose from the selection pressure for efficient use of available raw materials in the environment. Such aggregation, in turn, demanded the development of devices for transmitting and processing information about the environment from sites where it could be detected to sites that could respond to it appropriately, lest the aggregate system (sensibility) disappear. Such devices, insofar as we understand them, are extensions and ramifications of the molecular organizations that evolved from the initial response of the atoms in the earth's primeval atmosphere to a succession of energy fluxes—fluxes that are still going on today.

1 + 1 ≠ 2 (One Plus One Does Not Equal Two)

PAUL WEISS

IN SCHOOL we learned that one apple plus one apple makes two apples. One apple and one pear is just one apple and one pear. If we choose to ignore "appleness" and "pearness," being concerned only with weights and numbers, as, for instance, in counting and weighing parcels for postal shipment, apples and pears would, of course, be reduced to just so many items, to be tallied by sheer summation. In equating such items, we gain as well as lose. We gain an easy way of measurement, but lose what nowadays would be called "information content." Apples and pears do not become alike; we simply discount their differences for a particular purpose.

But can we ever retrieve information about distinctive features once we have tossed it out? If not, can science, as man's striving for as complete and rational a picture of the universe as is obtainable to him by observation, experiment, and logic, stoop to trading loss of information content for the simplicity, convenience and, yes, true elegance, of blotting out distinctiveness based on disparity; for instance, between pears and apples? In fact, in nature, even two apples cannot be equated, if one lies rotting on the ground while the other, still growing, hangs on the tree.

In short, all algebra applied to nature implies abstraction. Sheer adding up always leaves out some relevant information. Whether such omission is passable depends on our purpose, and that, in turn, depends entirely upon our special interest. And since interest, by definition, connotes biased self-limitation, the information thus gained remains incomplete, short of the comprehensiveness to which science in its professed universality aspires. So, how sure can we be that sheer analysis alone—the physical or mental dissolution of a complex into a shambles of measurable but disconnected units—does not irretrievably destroy highly relevant data about nature? Is Phoenix, rising from its ashes, a true image of nature or just a myth?

In our day, the answers to such questions have become a matter of faith. The success story of learning more and more about less and less, which in the present context means about ever smaller fragments of nature, has grooved our faith in nature as an assembly plant of microevents. No doubt faith in the omnipotence of analytical decomposition has opened the mainsprings for the stream of scientific progress. What we are apt to overlook in our enthusiasm is that there are other sources which could powerfully augment that stream were they not left to dissipate and dry up for doctrinary reasons. Doctrine has barred them from joining the mainstream by artificially erected walls, by conceptual injunctions against admixtures from sources suspected as contaminated because they failed to pass the orthodox test of purity, namely, that *one* plus *one* must be made to equal *two*.

The unorthodox dissenters usually phrased their argument in the age-old adage that "the whole is *more* than the sum of its parts." Look at this phrasing and you will discover the root of the distrust, and indeed, outright rejection, of the valid principle behind it. What did they mean by stating that "an organism is *more* than the sum of its cells and humors"; that "a cell is *more* than its content of molecules"; that "brain function is *more* than the aggregate of activities of its constituent neurons"; and so on? As the term "more" unquestionably connotes some tangible addition, an algebraic plus, one naturally had to ask: "More of what? Dimensions, mass, electric charges?" Surely none of those. Then what? Perhaps something unfathomable, weightless, chargeless, nonmaterial? All sorts of agents have indeed been invoked in that capacity—entelechy, *élan vital*, formative drive, vital principle—all idle words, unpalatable to most scientists for being just fancy names for an unknown X.

Unfortunately, in their aversion to the supernatural, the scientific purists poured out the baby with the intellectually soiled bath water by repudiating the very aspects of wholeness in nature that had conjured up those cover terms for ignorance. And as a prophylactic against their resurgence, they fostered a militantly doctrinaire "reductionism," which axiomatically prescribed that all the relevant macroinformation about nature must, and eventually will, be derived completely from adding up and piecing together the microinformations about the smallest sample units. Never mind that physics had to give up that claim gradually as Boltzmann's thermodynamics, Planck's quantum theory, and Heisenberg's uncertainty principle came on the scene. The life sciences have failed to follow suit and break out of the strait jacket of a doctrine for which their own subject matter furnished the most telling disproof. They might have come around more readily, though, if they had realized that systems with aspects of wholeness are by no means confined to living nature, but

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are of universal occurrence. In fact, their very universality should clear them of the stigma of vitalism.

Let me take a further step toward destigmatization by pointing to a veiled source of confusion that seems to have confounded past dealings with the problem—the failure to distinguish between a natural phenomenon as such and the symbols of language we have to use in order to describe it. A phenomenon to which we ascribe wholeness is certainly not *more* in algebraic terms than the sum of elementary phenomena composing it. It just is *different*. The difference is that between matter and structure. If there is a "more" involved, it lies in the terms of our description. It is we who, as describers, feel compelled to add extra terms of information for the sake of making the description of the integral phenomenon complete and pertinent.

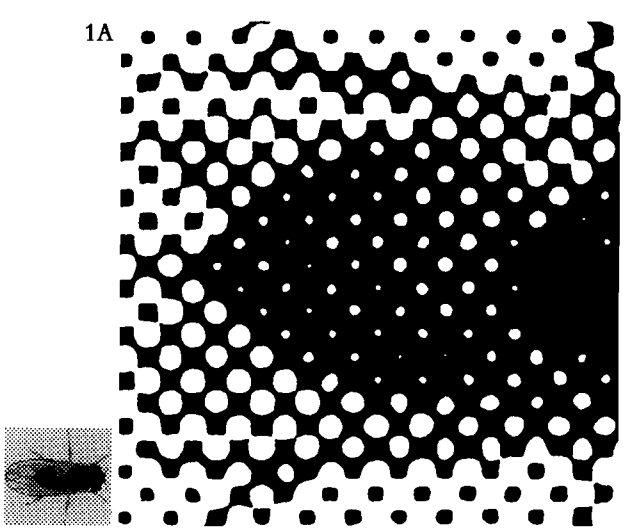
This neutral and philosophically noncommittal characterization of the problem tries to allay, or if you prefer, circumvent, the present warfare of dogmas. It should soothe the apprehensions of those who have built faith in absolute reductionism as bulwark against onslaughts on their sense of intellectual security, and it should assure those others who felt disenfranchised because of their holist faith, their day in court. There is a current fad to present the subject matter of the life sciences in terms of a dogmatic schism—an antithesis of "molecular" and "organismic" biology, professing a reductionist and a holistic philosophy, respectively. The former is respected for its "rugged naturalism," the latter suspected of flirting romantically with the supernatural. What I shall try to show is that exclusive commitment to either thesis is unnatural. The molecular and the organismic are but two different vantage points from which to look at living systems, neither of them granting a monopoly to insight. They are complementary and co-equal. To document this proposition is the main object of the following discourse; I hope that it will serve as an object lesson.

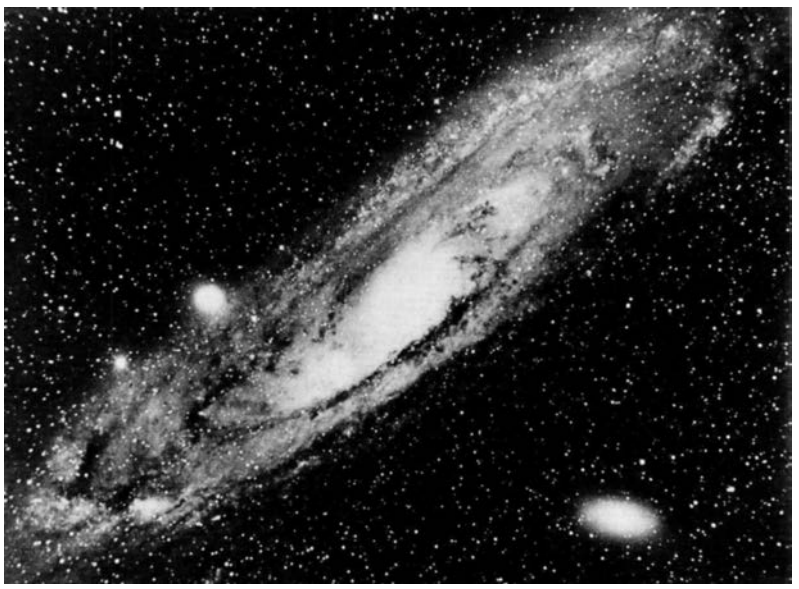
Specifically, these are the points I aim to prove: (1) that as our brain scans features of the universe we shift range and focus back and forth between telescopic and microscopic vision, as it were; (2) that as we move downward on this scale, we mostly gain precision and lose perspective; (3) that as we move upward, new and relevant features, formerly unrecognizable and unsuspected, come into view; (4) that this emerging novelty pertains to macrosamples of nature—that is, that it reflects properties of *collectives*—of groups, assemblies, systems, and populations, composed of microsamples; and (5) that the required additional terms to characterize such collectives must come from rigorous scientific procedure rather than from anthropomorphic translocutions and allegorical allusions to mythology.

And now I turn from these somewhat pedantic generalities, which to some presumably will seem commonplace, to practical examples by which the validity of those five points can be tested. A brief glossary of our terms of reference may serve as introduction. Let us ask first: Of what do we deprive a system when we dismember it and isolate its component parts, whether bodily or just in our mind? Plainly, of the *interrelations* that had existed among the parts while they were still united. So, in trying to reconstruct the system from the fragments, whether bodily like Humpty-Dumpty, or symbolically in our imagination, we must make up for the deprivation by adding a proper term that specifies the lost relations. This may simply amount to adding vectors to algebraic terms. The requirements for added specifications will vary with the different degrees of order emerging from the union (or reunion) of elements combined in groups. The simplest case involves only a loose and widely variable relation, such as "togetherness"; it displays novelty, but little order. If, besides novelty, the collective shows regularities of pattern which recur with a high degree of invariance, we confer upon it the designation "organized."

But here again we would do well to make a further distinction between true and merely simulated organization. We must distinguish between the genuine order, such as emerges within a group by virtue of its intrinsic dynamics, and a mere semblance of order, such as an aggregate of unrelated units acquires by imposition or imputation from without. Examples of the latter are puppets, or the proverbial camel our fantasy projects into a cloud, or, in fact, any effigy of a natural system, as in the following instance.

Figure 1 shows a meaningless array of dots in inert coexistence, with nothing recognizably in common besides





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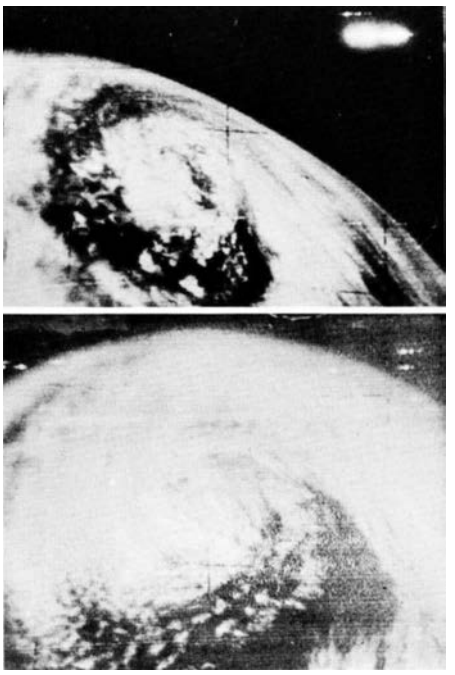

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the paper they were printed on. Yet, from a greater distance or, what is the same, at lower magnification (inset), we recognize them as the component bits of information about a continuous, well-structured image. That image, of course, is dead; the dots of printer's ink composing it are physically as unrelated as fly specks. What gives the picture its meaningful integration, are we, the viewers, with our eyes and brain. The dots do not "add up." We add to them. From this we learn that discontinuous and discrete elements can give us the illusion of continuity, but that the mere aspect of continuity alone is no test of inner coherence. Let me pursue this further.

Figure 2 is a picture of the spiral galaxy, Andromeda. Now, if you ask what Andromeda consists of, a census taker would reply, "So-and-so many stars"; a chemist might come forth with spectroscopically discernible distinctions. But would either of these answers add up to a definition of a spiral pattern? Certainly not. Nor would a scientist be happy if the additional feature were taken care of by invoking the magic act of "a spiralizer."

Closer to earth, Figure 3 shows photographs taken by the first weather satellite, Tyros. Note the cyclonic cloud pattern. But what are clouds in analytic view? Droplets of water. Now, could knowing all there is to be known about H_2O ever add up to a picture of this configuration? Of course not: the winds that have shaped it remain invisible. In Christina Rossetti's words,

> "Who has seen the wind? Neither you nor I.
> But when the trees bow down their heads,
> the wind is passing by."

So, here we meet the first caveat against willful isolation of an object from its natural context.

More spirals next, in Figure 4: the neurofibrils in the large motor cells innervating the electric organ of the torpedo fish. To say that these fibrils are made of protein will neither describe nor explain their spiral course. Indeed, from what we now know about their formation, the picture is just a photographic still, a momentary sample of a continuously unwinding record of motion in that cell. These spirals are but the residual traces of a moving stream of substance, the pattern of which must be sought in regularities of the underlying dynamics.

Here lies a general and basic lesson. What we perceive as static form is but the product, transitory or lasting, of formative *processes*. The features of the product—for instance, its geometry—can provide us with clues for the dynamics that underlie those processes. For instance, the counter-

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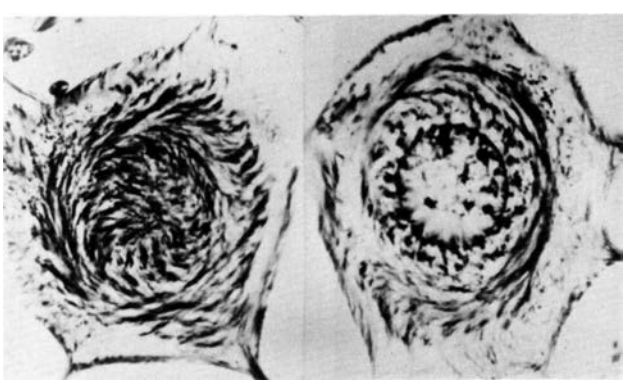

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clockwise spiral spin of water running off a bathtub drain is but an indicator of an asymmetry of forces resulting from the earth's rotation. The order we perceive in structured form thus is not primary, but the expression of the dynamic patterns that have engendered it. Yet this is only a beginning. Through their results, dynamics modify the setting for subsequent dynamics. Dynamically created forms, if somehow consolidated, become molds for the course of further activity. Free-flowing water grooves its bed until the bed begins to channel further flow, as in this instance (Figure 5) of a spiral spout carved by the twisted course of water that drained from a glacier bottom in the glacial age. Once formed, the spiral structure becomes self-perpetuating, gaining in polish and perfection by more erosion.

In passing, let me point out that by this dual action, a whorl can serve as a general model of how dynamic patterns tend not only to preserve, but in further consequence

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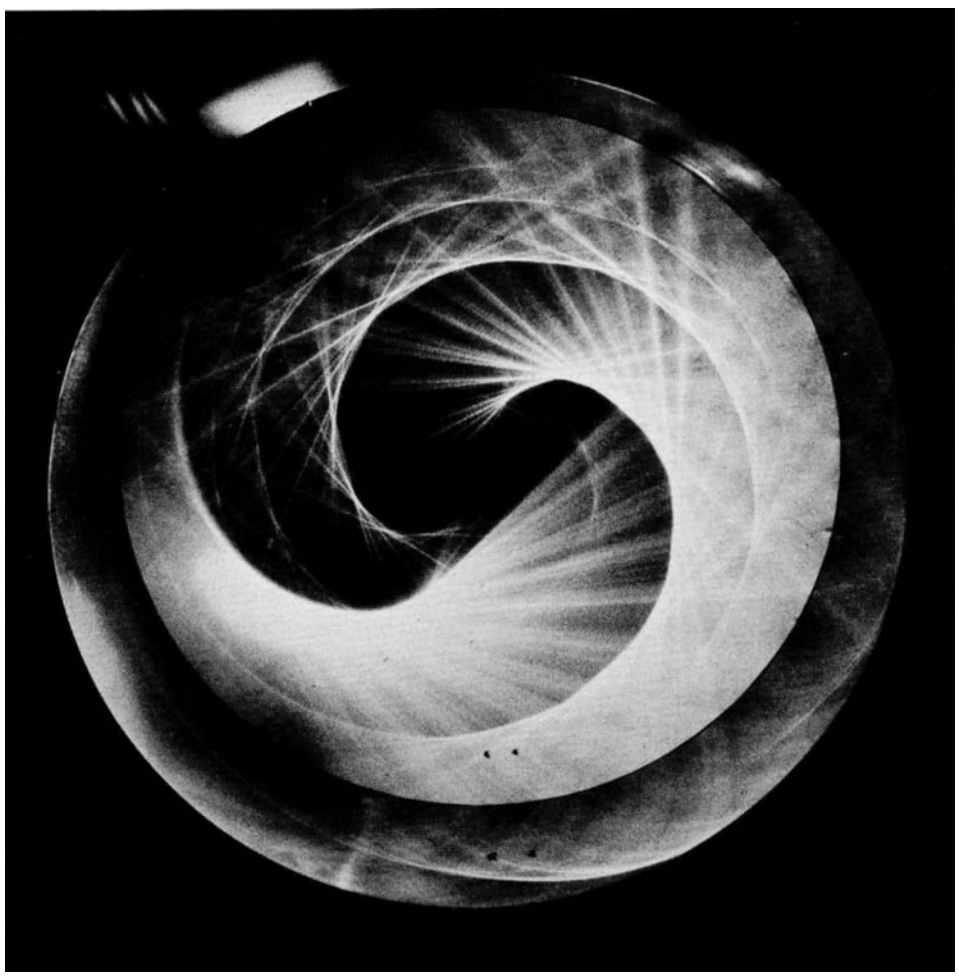
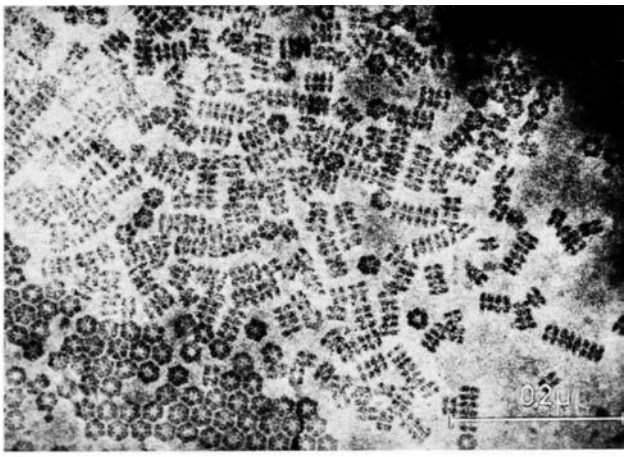


to accentuate, their self-engendered structures. I wish to stress this because of the obvious bearing the general two-step principle has on our understanding of brain mechanisms: of grooving, habit formation, facilitation, and learning theory. If taken seriously and followed up, it might dispose of the necessity of placing rigid fixation and plasticity of neural functions—instincts versus memory—into sharply divided categories, each run on a different principle.

Returning to our main line, we have recognized the spiral wall of the glacial mill as the dead effigy of the unitary dynamic sweep pattern that has created it. The spiral composition of an artist (Figure 6) is, similarly, the projection on dead canvas of some dynamic process, obeying mathematical terms for spirality, that has been going on in the artist's creative brain. Attempts to resolve this act to mere terms of numerical plurality, whether of neurons or of intraneuronal molecules, would seem to me to be as futile as to derive the spirality of a spiral nebula from our knowledge of single isolated stars.

By now I have exposed three propositions. First, that collectives tend to display novel features not discernible in their component units, hence justly called "emergent"; second, that such features are indicative of the existence of significant *relationships* among the members of the collective, such relationships being severed by physical or mental separation of the members from each other; and, third, that whenever one is faced with static geometric regularities of patterns, he ought to look beyond them—or, rather, behind them—for the rules in the play of forces that have shaped them. In thus raising the sights from statics to dynamics, static *interrelations* become dynamic *interactions*, and in the case of self-sustaining systems with the conservative features of wholeness, simple *interactions* become *interdependencies*. *States* then appear as but cross-sections through trains of *behavior* along the time-line, *scalar* values must be supplemented by *vectorial* interconnections, and vector systems of specifiable integral properties become realities. Let us then keep in mind that this progression from *elements* to *groups* objectively reflects the ascending scale of supplemental statements we need for adequate description of corresponding objects of our experience. I shall then present samples of such phenomena in that order. By choosing them from various points along that scale, I intend to blur the artificial dichotomy between modes of thought centered either on *elements* or on *continua*, each to the exclusion or invalidation of the other.

I shall use the example of *form* as master indicator of order. Its simplest examples are plain aggregates of identical units stacking up flank-to-flank or end-to-end, according to steric fitting, like key to lock, and chemical conformances. The macromolecular units of the blood pigment of a marine worm (Figure 7), each consisting of six subunits around a hole, stack up in contact: molecules as "modules." The only novelty by which the group differs from a mere sum of units is its predominantly planar array—a significant, yet low, degree of order. The stacking of virus particles (Figure 8), in its near-crystalline configuration, falls in the same class, although the forces interlinking the units so regularly are not equally obvious.



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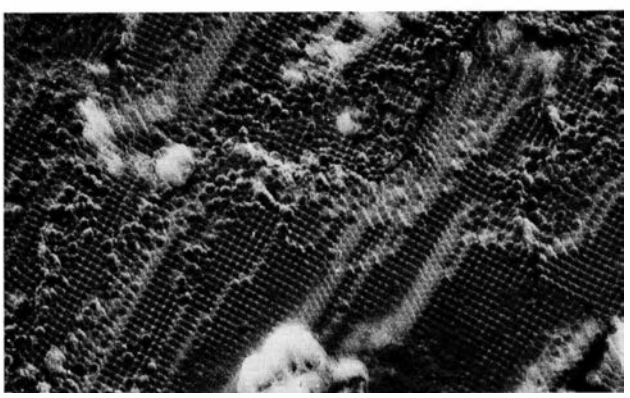
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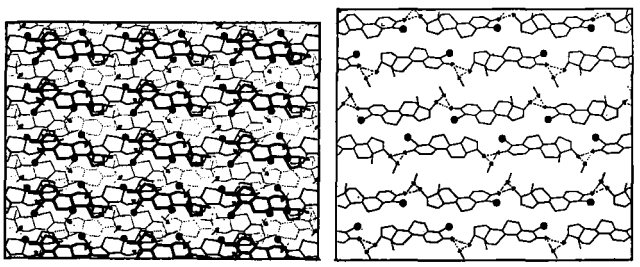
A polymer, as I shall show later, is a linear chain of identical links with couplings, end-to-end, like a railroad train. The linkage represents a first step of order in the assembly. The straightening of the chain from random coils to rectilinearity requires an additional step of ordering. For short lengths, intermolecular forces may serve as an explanation, but ruler-straight arrays over great distances undoubtedly must be referred to straightening effects from the environment; for instance, stretch. Common direction may be imposed by a further polarizing interaction with the environment. And so, in order to describe the formation of, for instance, a connective tissue fiber, we must construct steps of ever more specifications.



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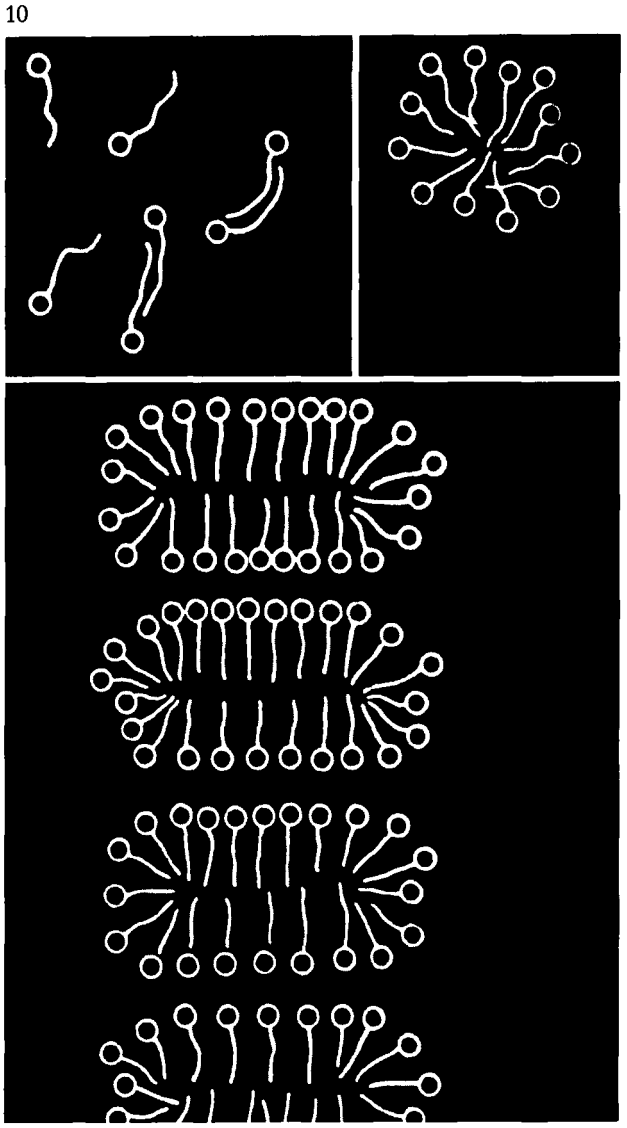

The stacking of lipid molecules into lamellar systems of the so-called smectic state (Figure 9) extends the same principle to *two* dimensions. Here, too, the environment enters as an ordering factor in that it offers to the molecules, as scaffolding for their own planar self-array, a planar interface between two immiscible media. Lacking such guidance, the molecules cluster into so-called micelles (Figure 10), yet by no means as random conglomerations, but in orderly structural patterns determined by their own collective interactions. For each of them the others are part of its environment—a forward reference to our conclusion that the notion of independent "elements" is, in itself, an abstraction, for in reality elements are part and parcel of a single, undivided continuum that embraces units and environment as one integral entity.

Yet, clearly, structural group order in these past examples can still be satisfactorily explained in essence by the microprecise automatic assembly of individual units and subunits, united like an erector set by their steric, chemical, and electrical properties. Such rigid compounding processes can hardly serve as star witnesses in our suit for a divorce from the one-plus-one-equals-two precept of thought. Therefore, let me proceed to a series of further samples, representative of higher-order systems. These are collectives of the following description: their features, "on the whole," show well-defined *regularities* of pattern, recurring consistently from specimen to specimen in each given class; but as one looks at smaller and smaller samples, similitude and regularity decline until, having descended to the elements, one can no longer find any hint of what the structure of the total complex might be like. This is because the *details* of pattern are in each case *unique*, no two microsamples being ever alike, even though the *composite pattern of the whole*, case after case, is of the same standard form. Contrary to the preceding examples, the order of the whole can here no longer be predicted from a simple upward projection of the elemental properties of single units stacked up in module fashion. The following illustrations will make this more specific.

Take, for instance, the bed of blood capillaries in a tissue (Figure 11). They branch and re-anastomose almost at random, yet the resultant network offers an aspect of great over-all regularity. Descriptively, the regularity is reflected in the near-constancy of distances between the branches; dynamically, it reflects a growth pattern elaborated by interactions of the component branches, both among one another and with the cellulated matrix they pervade. In oversimplified terms, the interactions involved are a type of *competition*. This, then, is interaction no longer in contiguity, but at a distance. Each branch may be viewed as surrounded by a shell of influences of graded strengths—*domains*, which keep each other at a re-

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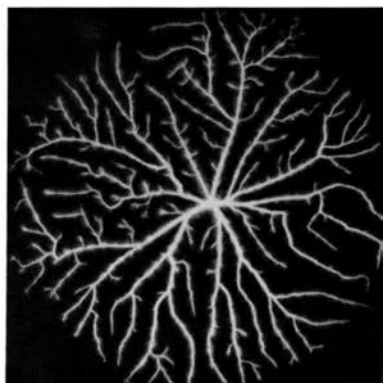

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spectful standard distance. The term "domain" is used in forward reference to subsequent comments on field and gradient principles.

A botanical counterpart to the capillary bed is the venation of a leaf (Figure 12). But let me at once dispel the notion that growth patterns of this type are a preserve of organisms. The next picture (Figure 13), for instance, shows the lightninglike pattern of an electrostatic discharge from a point source. Ideally, it should, of course, be radial but in reality, the unpredictable variations of conductance and resistance, resulting from the random inhomogeneities of the medium through which it has to travel, establish spearheads for separate and competing ionization tracks. Despite this capriciousness in detail, the total picture still emerges as one of systemic order. Growth patterns of snowflakes (Figure 14) also show infinite variation of detail within a high degree of constancy of the over-all form of the growing crystal.

The growth pattern of a nerve cell from the cerebellum (Figure 15) reveals the same rule: the same degree of unpredictability of the details of ramification, yet at the ends great uniformity of distribution among the terminal branchlets. While it is their environment—the matrix of the brain—that offers to the advancing branches a warp and woof of easy pathways, the decision of which of them will be utilized, and in what force and microdistribution, is indeterminate, left to be decided at each branching point by the actual local competition for the limited supply of substance arriving from the common cell body. So, if the common source may have had a "program" for the attainment of the highly regular end result, the precise way of how to get there could certainly not have been spelled out in it in great detail. For those ways are different and unique for each of the millions of cells. The double meaning of the word "design" comes to our mind: design as purposeful planning at the start, ending in stereotyped design as accomplishment, but countless ways of execution leading from one to the other.

Extending our examples upward, Figure 16 pictures a lace coral—a limestone housing development of both

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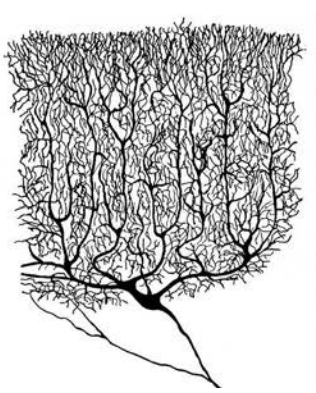

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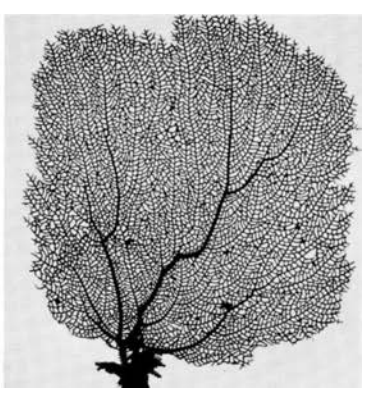

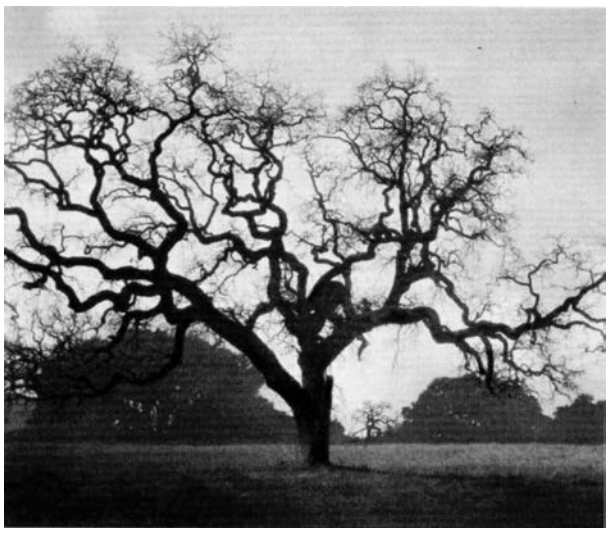

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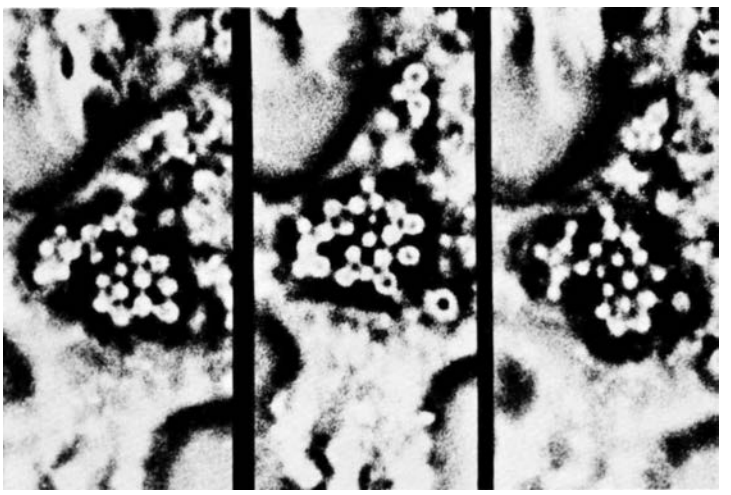
great over-all regularity and individual uniqueness, built by thousands of separate little animals in the colony in a concerted pattern of behavior. You may sense already my own design. It is to reorient thinking from static *form* to formative *behavior* across all orders of magnitude. The range extends beyond the coral colony to human society and to what I take to be its design for living, and indeed, survival: namely, to recognize that individual freedom in the *small* is compatible with the existence of collective order in the *gross*, which reconciles self-determination of the individual with the much stricter frame of rules descriptive of his group.

Were it not for this principle of nature, were the development of every part or branch allowed to pursue its own capricious course without constraints, without a frame of integral interdependencies, we could not have

trees (Figure 17) that we could categorize distinctly by their shapes as oaks or pines or poplars even though each specimen is individually unique. Such stereotyped end form defies any logical attempt to regard the product as just the blind outcome of a bunch, or call it a sum, of microprecisely programed cause–effect sequences of linear chain reactions in the sense of a naive mechanical machine concept.

The conclusion that countless constellations of convergent microevents may yield macroproducts of essentially the same standard pattern makes it, by the same token, gratuitous to assume that similar terminal patterns must have had similar mechanisms and histories in common. The treelike pattern of the Colorado River delta (Figure 18) will prove the point. Let us go on then and reverse the outgrowth pattern of the tree and we obtain the picture of a river forming by junctions of tributaries from many sources; but also, similarly, the inverse "arborization" of cracks in crystals advancing from edge to interior in stepwise confluence (Figure 19) from top to bottom.

Let us now move up to the next step of complexity. Our past examples have been relatively simple. They dealt with interactions among parts of systems which, after all, were still connected and continuous in substance—blood vessels, leaf veins, trees, rivers, etc. Order emerging in complexes of disconnected, discrete units taxes our explanatory faculties far more severely. As introduction to the subject, I chose Figure 20—three frames from a motion picture film of one and the same cell in tissue culture at brief intervals. They show a group of granules, each about one micron in size. These granules are separate bodies, freely mobile, bouncing around in the soft cell plasma. But as they change positions, they assume preferentially characteristic geometric group configurations—mostly hexagonal, but intermittently an occasional square. Now, since they are separated by appreciable distances, we must infer that in their random buffeting by Brownian motion, they are transitionally stabilized—trapped, as it were—at equidistant equilibrium points in a field of



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forces established by their mutual interactions, like partners in a square dance or quadrille. If this sounds vague, consider that we can at least describe the various fleeting configurations with relative precision, even though we do not know the actual dynamics defining the grid.

There are other cases, however, in which the operation of group dynamics has lent itself to more concrete definition. Since they demonstrate most cogently that going beyond the one-plus-one-equals-two rule does not mean giving up scientific discipline for the outer space of supernaturality, I shall dwell on them somewhat more extensively.

Let us take two bodies (Figure 21), as centers of emanations and force fields extending radially into the environment, and let them move toward each other from a great distance. Beyond a certain range, interactions between the two are as negligible as the effect of gravitational attraction by the moon is on our stance. Yet as we bring them closer (Figure 22) and as the overlap of their domains increases, their joint effects depart increasingly from the result one would expect from a sheer superposition and algebraic summation of their single contributions. They mutually distort each other's sovereign patterns of action.

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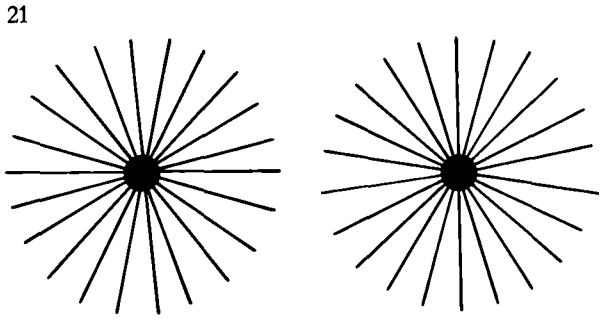

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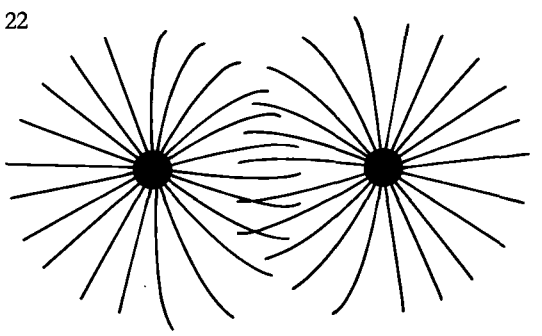

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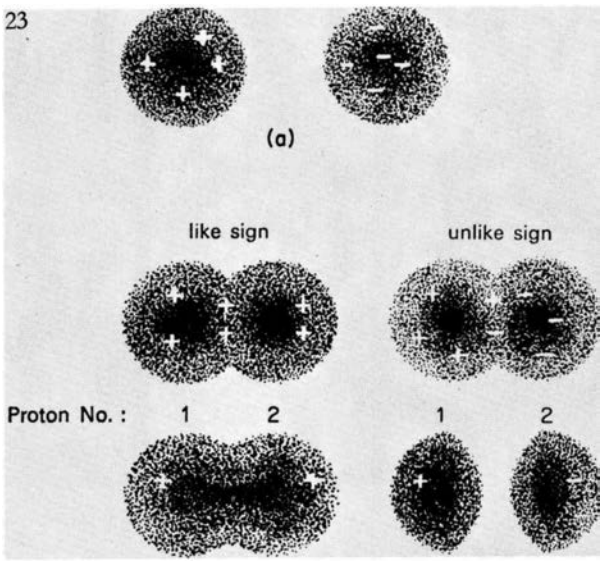

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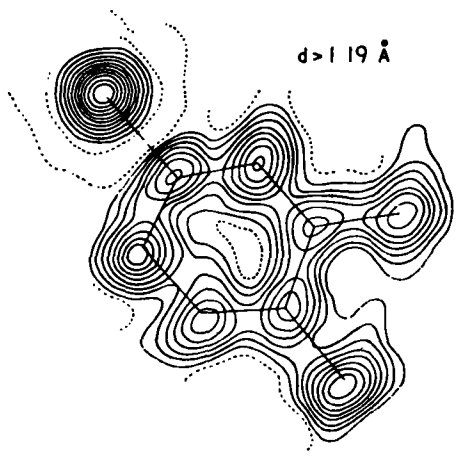

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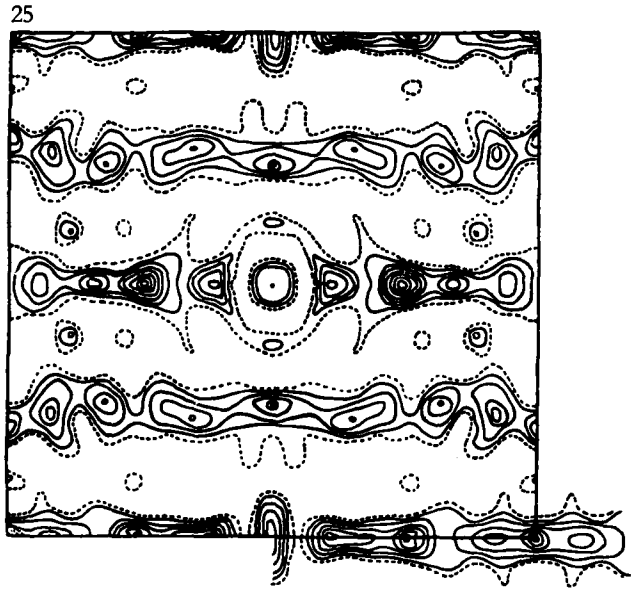
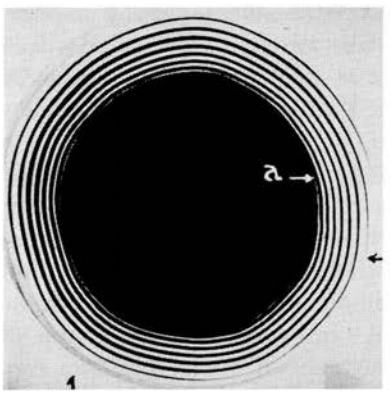


In atomic dimensions, for instance, this yields the redistribution of electrons between atoms (ligand fields), deforming the erstwhile spherical electron clouds, as shown in Figure 23 (bottom line). The dumbbell-shaped interaction pattern seen in the figure at the left is typical of many cases. The next picture (Figure 24) shows the mapping of electron distribution in a small organic molecule (specifically a diaminochloropyramidine). Such a continuous field pattern emerges from the group interaction of the constituent atoms and atomic groups, which formerly were envisaged and represented as discrete, neatly bounded entities. More complex molecules—for instance, the protein, myoglobin, shown in Figure 25 in the so-called Patterson projection of its subunit fields—yield maps of still more sophisticated collective fingerprints. One is reminded of the contour maps of mountain ranges. Domains of particles are no more truly isolated than are mountain peaks.

My reason for showing these diagrams is that they express symbolically that patterned processes in space and time form *continua*. To single out and fence in mentally, in such continua, peaks, centers, foci, or what not, corporeally isolated from their context, has long been a legitimate abstraction of immense tactical and practical utility in science. Yet, he who forgets that it is basically an abstraction could as well end up trying to extract the center of gravity from a body. One recognizes a kinship between this trend and some of the old notions, still not totally extinct, about brain centers as the "seats" of specific functions.

Familiar and accepted as the preceding propositions are for the molecular realm, their equal validity for higher levels, through the cellular to the social, has rarely been pointed up, let alone studied and conclusively proven.

Let me again start from an inorganic model, the so-called rings of Liesegang (Figure 26). A drop of silver salt dropped on a gelatin plate that had been soaked in a chromate solution lays down, as it slowly diffuses, periodic concentric rings of insoluble silver chromate. The rhythmic character stems from some sort of threshold phenomenon, formally comparable to the rhythmic response of nerve tissue to a constant stimulus. If we place *two* such diffusion centers sufficiently far apart on a common plate (Figure 27), the total pattern still adds up, in the main, to one-plus-one. Yet, if we narrow the original distance between them, their mutual interference becomes conspicuous (Figure 28): the twin set of circles becomes distorted, the more peripheral ones merge to single dumbbell-shaped contour lines, and the outermost and farthest advanced form a smooth enclosure of both. The two domains have fused. Carrying on the tests, I then placed seven drops on a plate in the configuration of the Great Dipper. Figure 29 shows the outcome of an actual experiment.



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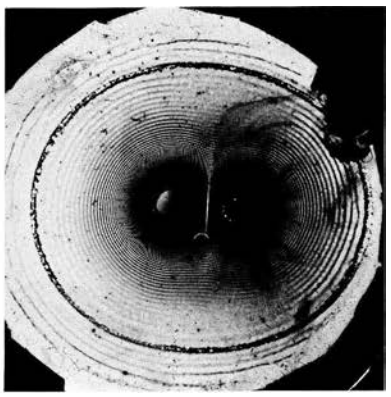

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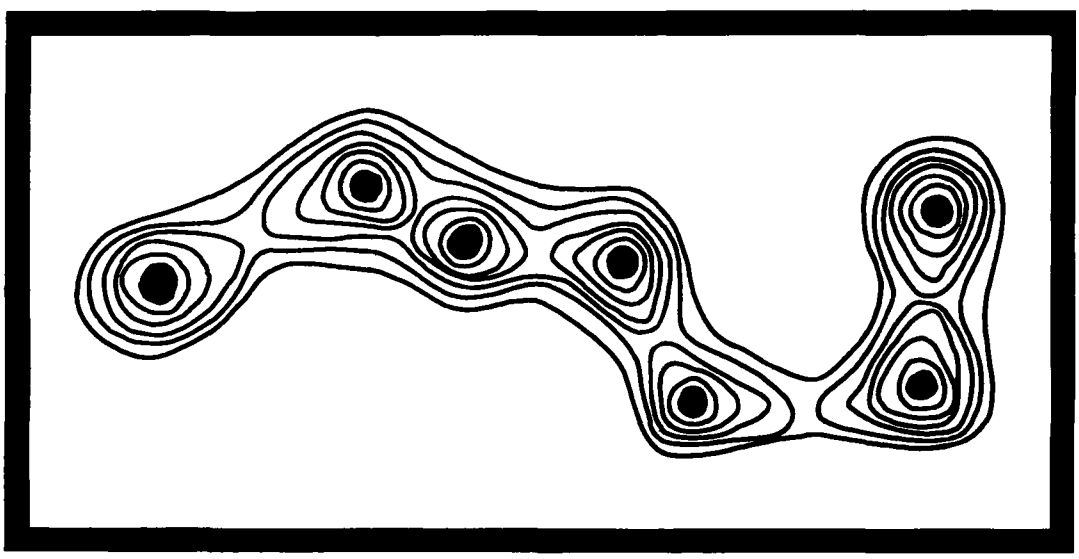
As one notes, the resulting pattern of silver lines coincides with the pattern of point connections that led man to give the stellar group its name. Does not the unequivocality of this correspondence intimate that a similar dynamic interaction pattern in man's brain had guided his interpretation? Stars do register on retina and brain as single points, but may not the several neural processes thereby actuated engender, on a higher brain level, dynamic interactions that integrate an erstwhile mosaic of local dots into a unitary spatial pattern?

At times, proposals for such physiological underpinnings of Gestalt phenomena have been set forth, couched mostly in symbolic terms of field concepts and related models. Experimental verification, however, has remained scanty. Because cell types other than neurons have furnished far more factual examples of how pattern-determining field effects can arise, I shall turn to those. I shall present two major types of patterning interactions among dispersed cells, first, in a liquid (thermally agitated) environment and second, in a firm cohesive matrix.

The earliest morphogenetic step in the egg of the seaweed *Fucus* is the sprouting of a rootlet on one side. In isolated eggs this sprouting takes place at quite a random spot. When several eggs are combined in groups, how-



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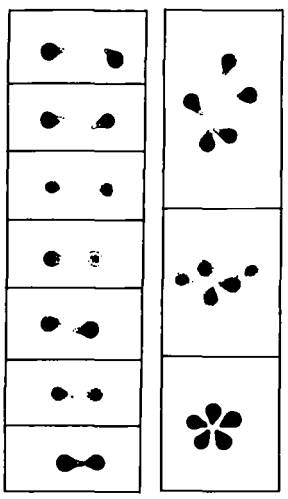

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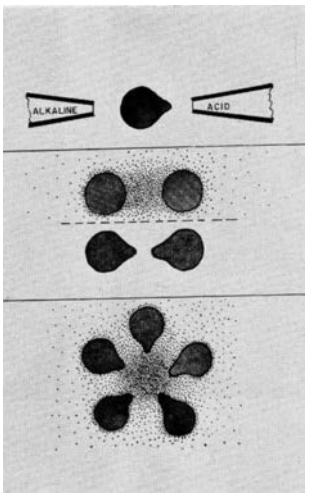

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ever, sprouting is patterned. As Whitaker has shown, pairs of eggs in close proximity sprout at the sides facing each other (Figure 30). However, huddles of more than two sprout toward the geometric center of the group (Figure 30). Thus "mutual attraction," which might still have been conceivable within pairs, is clearly ruled out as explanation for groups of more than two. How then can one account for the phenomenon? Quite simply. When Whitaker placed undivided eggs into a *p*H gradient (Figure 31), the rootlets sprouted at the more acid side, perhaps because the egg membrane was weakened on that side and sprang a leak through which the rootlet could hatch. Now, eggs may be assumed to secrete their own acid as a metabolic product. This acid cannot diffuse from the confined space within a cluster as rapidly as it can from the outer shore. As a result, an inner-to-outer concentration gradient of acid will develop and polarize the members of the group toward their common center. The additional step that the experimenter had to take to localize root formation the eggs perform among themselves in concert, thus adding what makes two different from one-plus-one, and even three different from the new two-plus-one.

In clusters of explanted embryonic nerve cells (Figure 32), Stefanelli observed a similar convergent growth of dendrites toward the common center, but only if they were not near the outer edge of the drop of culture medium; for near that border, competition between the inner medium and its outer environment apparently reversed the gradient, and the dendrites consequently grew outward. The actual agents involved here have not been determined. For other tissue cells, however, we could prove (Figure 33) that bipolar cells in culture, acidified at one end, withdraw that process, thus becoming unipolar. This fairly reproduces what two cells exuding acid will do to each other as soon as they come close enough. Of course, exudates other than acids could have the same polarizing group effect.

Group patterns among cells in semisolid media arise differently. Most tissue cells, as well as nerve fibers, need the support of solid structures—fibrin or collagen fibers, for instance, along which they move and grow, like plants along a trellis. The diagram in Figure 34 summarizes the gist of four decades of experiments on this principle of "contact guidance." An untreated protein coagulum, e.g., fibrin in a blood clot, is a random tangle of fibers (top of figure). As illustrated in the lower part, stretch orients the mesh in the direction of the lines of stress. Depending for guidance, like blind men, on the fibrous tracks, the cells then trace the underlying structure. Cell group patterns thus have their precursors in the fibrous matrix in which they are enmeshed. Therefore, if cells could do to the ma-

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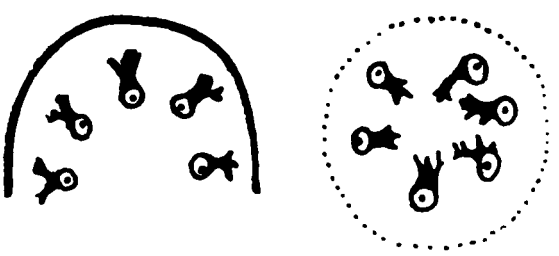

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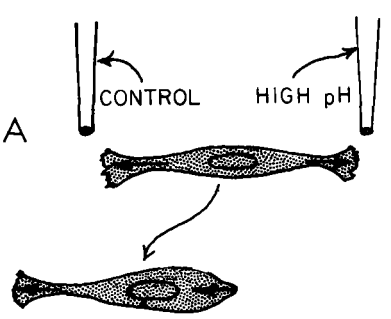

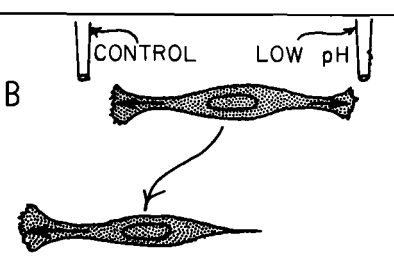

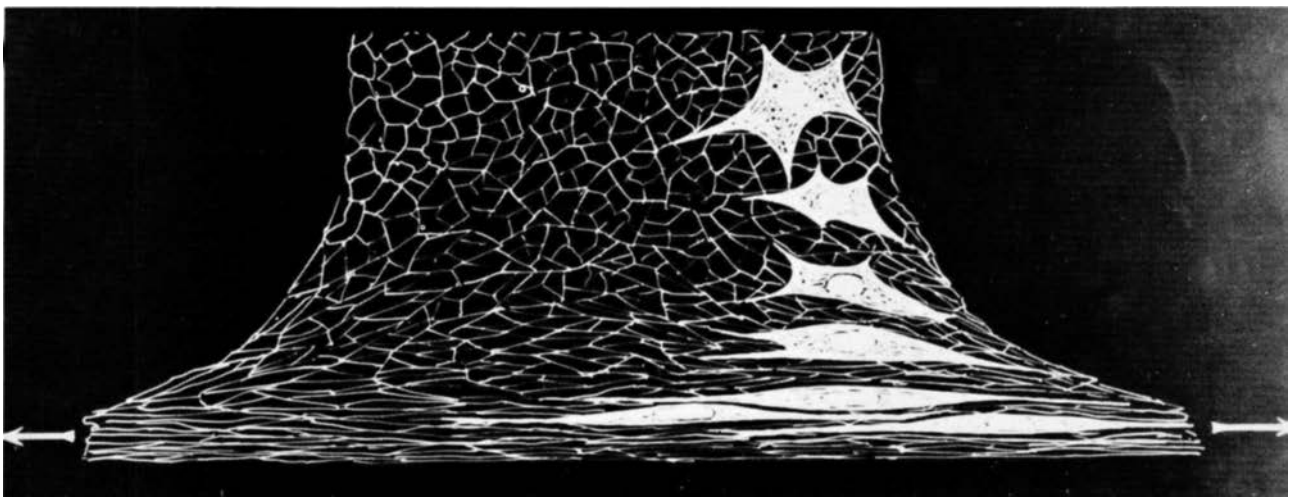

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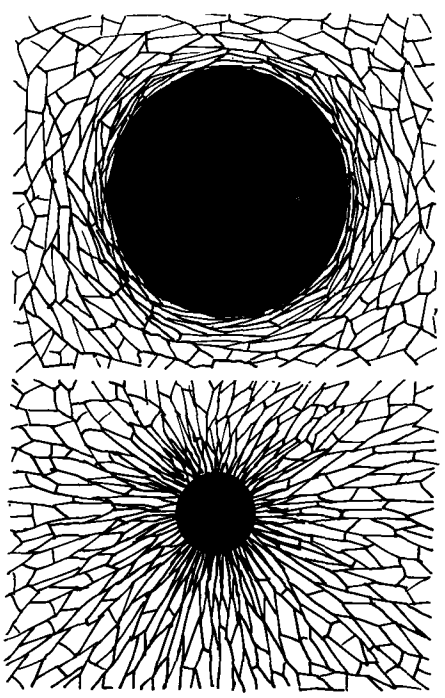
trix what the experimenter does in applying stretch, they could evidently manage to set up their own physical interconnections and group patterns. And indeed, they can do this. Here is how.

A cohesive fibrous network is under internal tension. Any local disruption of the net makes the surrounding meshes retract to form a ring around the hole, as any lady knows from holes in her stockings. Some spiders (Figure 35) use this as a trick to build a strong-walled nest. Now, cells can achieve the same result, where needed, by local liquefaction through proteolysis of their matrix, or just by local expansion (Figure 36). Many fibrous and muscular coats around hollow organs owe their circumferential orientation to this effect. More pertinent for us here is the bottom diagram, which illustrates local shrinkage. The meshes are gathered purse string fashion, assuming a radial orientation with focus on the shrinking center. Now, some cells, especially proliferating ones, make their surrounding matrix shrink in just this fashion by the release of chemicals that make the meshes squeeze out water, like setting jelly. If cells subsequently emigrate from such a center, they naturally follow their self-created radial routes.

Now, if there are two such cell clusters in a common matrix, will the resulting growth pattern turn out two overlapping stars? Of course not. Let me recall the diagram with which I introduced the two-center theorem earlier in the article (Figures 21 and 22). Two local centers



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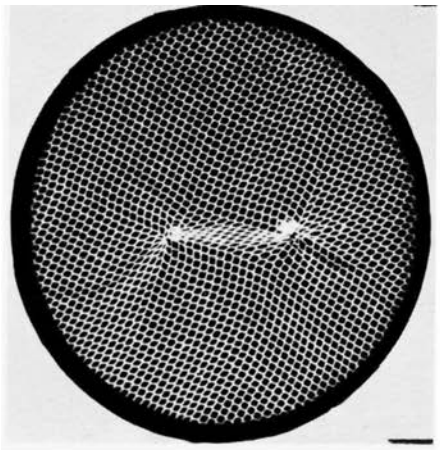

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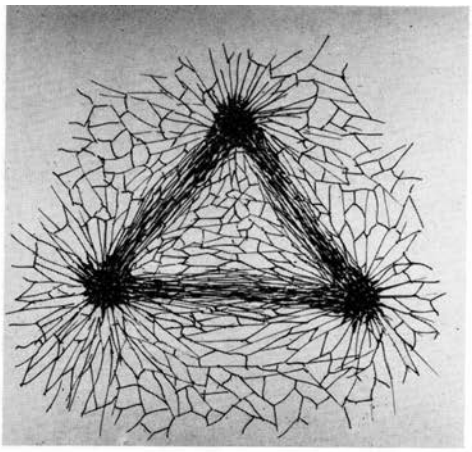

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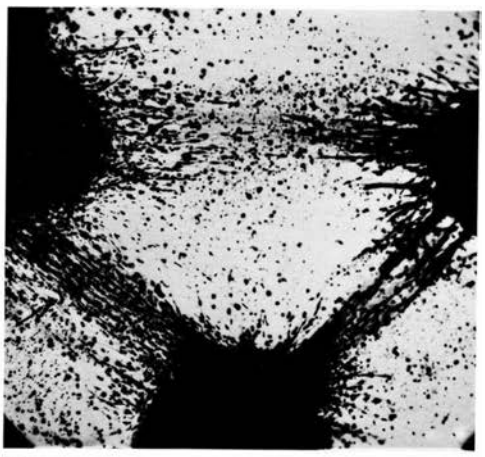

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of contraction in the net decidedly do not add up in their effects, as can be demonstrated readily by gathering a taut piece of mesh fabric at two points (Figure 37): the meshes between the two centers are distorted lengthwise into a straight course along the connecting line. In the same way, two clumps of growing cells force fibers in their common colloidal medium to assume a straightline orientation along the shortest distance between them. Three centers generate a structural triangle in their matrix (Figure 38), which then serves as roadway for emigrating cells. Thus, three scalar and erstwhile unrelated local chemical activities become upgraded through vectorial interaction into a well-defined space pattern emerging *de novo*. Figure 39 shows an actual case of such an automatically established triangular interconnection among three embryonic spinal ganglia in vitro.

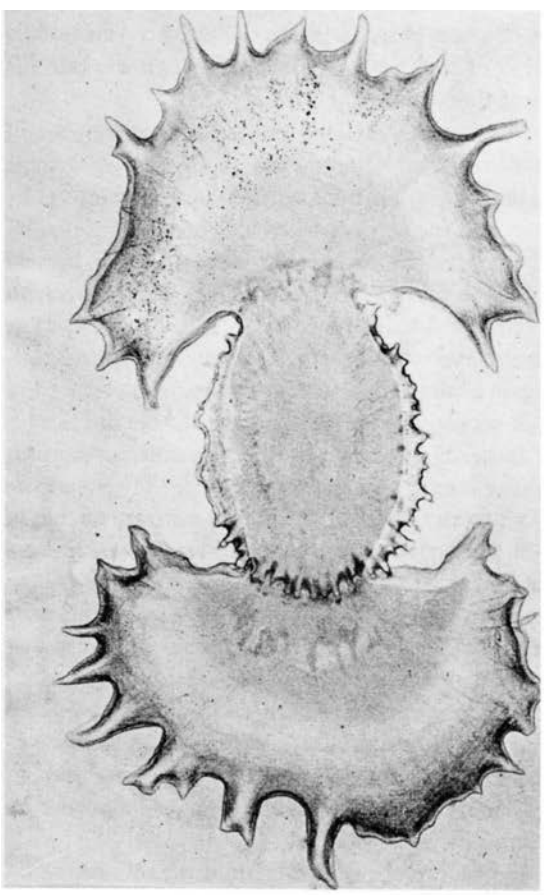
The "beeline" taken by outgrowing cells toward the distant colonies is simply a result of contact guidance. It is definitely not to be credited to any hypothetical "alluring" substances that might have emanated from the distant sources acting as beacons; but there is recent evidence that straight fiber bundles can expedite chemical traffic along their surfaces. So, secondarily, the cell population along the fibrous bridges does gain ulterior benefits from its earlier highway construction. Quite generally in development, structural order, once it has been established, creates conditions for its self-promotion and further elaboration.

The architectural effects of a two-center interaction are noticeable in populations at all levels from the molecular to the human. The earliest chromatograms, made by the chemist Runge more than a century ago by letting mixtures of substances diffuse on filter paper, are an example (Figure 40). The confluence of the edges of diffusion from two separate centers immediately sets up a communicating channel, which then drains further substance seepage into its bed. The resulting pattern, reproduced here from one of Runge's original experiments, closely resembles the pattern of mitotic spindles in cell division. Coincidence? Perhaps, but worthy of attention anyhow. And curiously, a recent architectural proposal by Catalano (Figure 41) for the most efficient structure of a modern growing bicentric city embodies a remarkably similar symmetric pattern of settlement along pressure and flow lines of communication. Population and market distribution between two actual towns, mapped to scale by Isard (Figure 42) bears further witness to the operation of the two-center effect in the dynamics of human ecology.

This mention of ecology brings me to my last set of examples. Ecology is group behavior in free interaction with other groups and with environment. It epitomizes the lifting of subject matter from sheer catalogues of items to paramount concern with their typical differential distribution and, digging deeper, with the patterns of the underlying dynamics. Behavior patterns are ecology's instruments. To explain life, static cell anatomy must become molecular ecology, organisms be comprehended through cell ecology, and societies through the dynamics of human ecology. They all provide us with examples of rigorous scientific propositions that hold for groups but dissolve when efforts are made to reduce them to elemental properties. Here resolution becomes sheer dissolution.

Let us then be emphatic: True, scientific history has grooved our habit of explaining group behavior in terms of the interactive behavior of quasi-independent unit actors, whether molecules or men. Yet this pragmatic, conceptual artifact has serious limitations, and once we reach the limits of its applicability we must relax our historical commitment to exclusive legal recognition of conclusions arrived at analytically and must concede equal explanatory

status to collective statements of fact in their own right. Modern physics has done it implicitly in adopting thermodynamics, quantum theory, relativity, and statistical mechanics, and the time has come when the life sciences had better follow suit for their own good. They must learn to accept recognized orderliness of the behavior of systems on its own terms. If they can reduce it to analytical terms, well and good. If not, the reality of nature still must be allowed to prevail and override pet micromechanistic preconceptions based on predilections. Therefore, regardless of whether ordered behavior patterns of systems will yield to obstinate analytical efforts at piecing them together through sheer assembly of component pieces—whether of molecules joining to form specific macromolecular systems, of cells to compose organs, or of neuronal circuits to yield adaptive functions—the integral formulations will retain their claim to reality and primacy. Both conceptually and historically, reductionist description is a secondary and limited tactical convenience. Its limits are to be determined empirically and not by prejudice.



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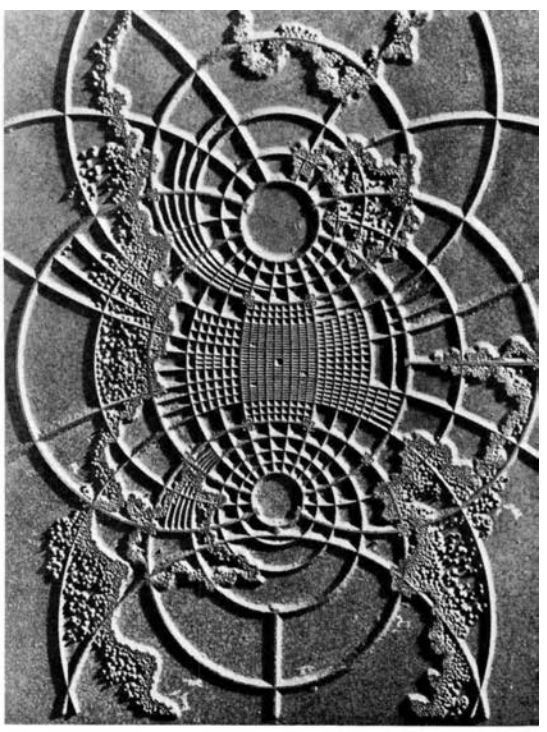

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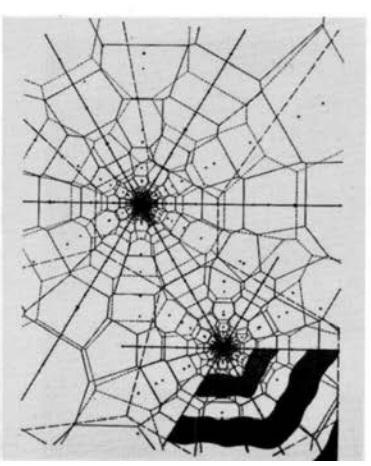

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Let me briefly pursue this matter on the example of linear arrays, already mentioned briefly earlier. Collagen fibers (Figure 43) are bundles of polymeric chains of protein molecules, linked head to tail and flank to flank. Under certain conditions, homologous subgroups of neighboring chains line up in register, resulting in conspicuous cross-banding. Each molecule is characterized by a specific sequence of amino acids along its backbone. The sequential order being commonly referred to as a code, it justly can be compared to the sequence of letters in a word. Register, then, signifies the sliding of identical letters into alignment (Figure 44), and there is good reason to expect that the molecular mechanism of zipping collagen fibril to fibril by site-matching will be revealed before long. This instance of higher group order then resolves itself simply into a case of assemblage by the orderly stacking of contiguous elements, not unlike our initial examples. But what if similar ordered arrays arise in collectives of linear units without the benefit of mutual contact? How to explain, for instance, the corresponding group pattern of freely mobile, well-separated, linear units in Figure 45, which shows an assembly of trout evenly spaced in parallel and register on the bottom of a stream? Clearly, current flow lines and interindividual signals combine in yielding this behavior pattern in grid form, but just how is wholly obscure. How far, then, can we carry the notion of synthesis of such group patterns through the free interaction of their unit elements?

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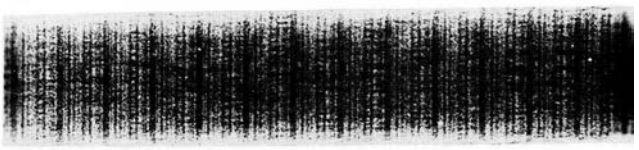

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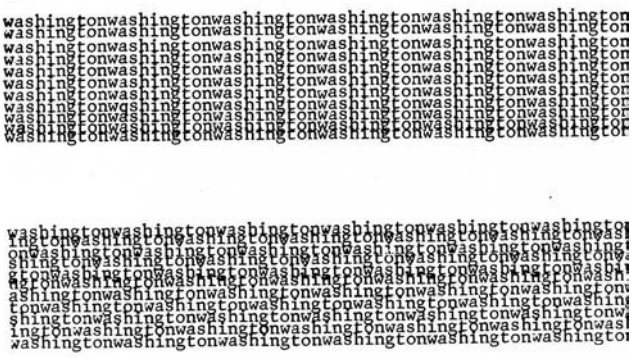

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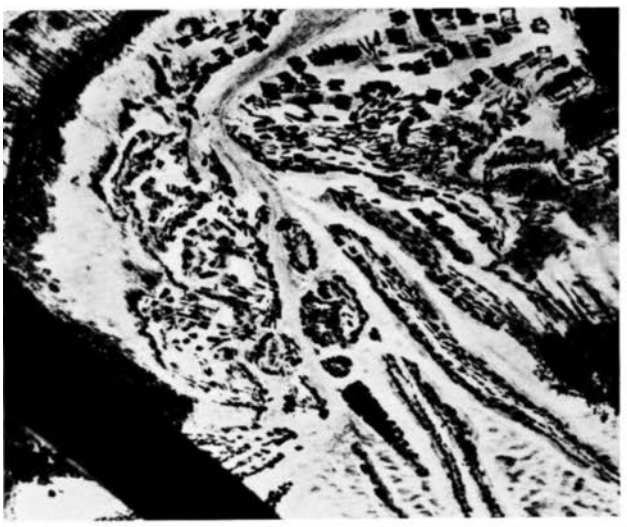
Figure 46 presents an aerial photograph of logging in the Columbia River. The logs are all aligned by current flow and shore lines. Their deposit in register is man-made. In other words, the collective order is strictly imposed by outside forces, which makes it irrelevant to our present context. Not so the next example. Figure 47 is an electron micrograph of rod-shaped tobacco mosaic virus. The units are clustered, and within each cluster the component rodlets are again both in alignment and in register. They look like match sticks; and indeed a group of matches can by analogy serve as a model of the physical mechanism through which such a simple step of order in self-assembling groups can come about. If one scatters matches at random on the surface of water in a dish and then agitates the surface by continuous tapping on the container, the floating matches get into motion and as they collide, they turn into positions of mutual alignment and register—positions evidently satisfying an equilibrium (minimum surface energy) requirement for that particular three-phase (water-wood-air) system. Three stills from a motion picture film of such a model experiment are reproduced in Figure 48, to show the progress in the increase of order by "self-ordering."

Being an instance of ordered group behavior emerging visibly from elementary interactions, the case surely is heartening to reductionist faith. Unfortunately, the argument is open-ended, as can be readily observed in motion pictures of a remarkable rod-shaped microbe, *Bacillus circulans*. Loosely scattered bacilli start out by assembling in physical arrays just like those matches (Figure 49), but once the group has enlarged to a certain critical size, the whole mass adopts a totally different course of behavior: it begins to circle around its own geometric center as fulcrum and keeps on rotating for indefinite periods of time (Figure 50), like a revolving disk, regardless of whether



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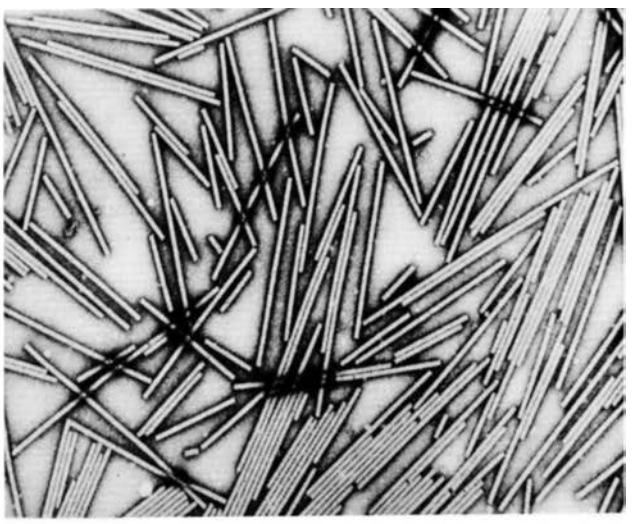

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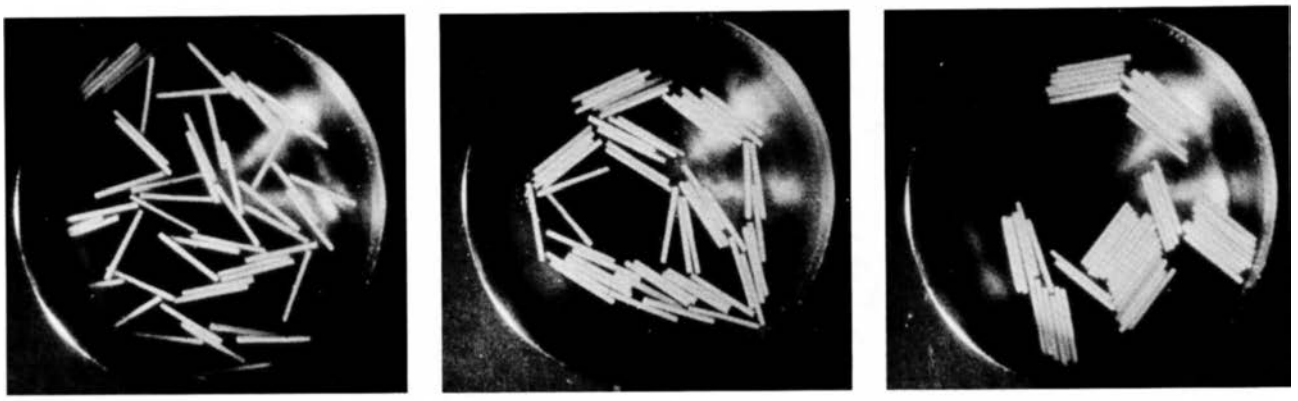

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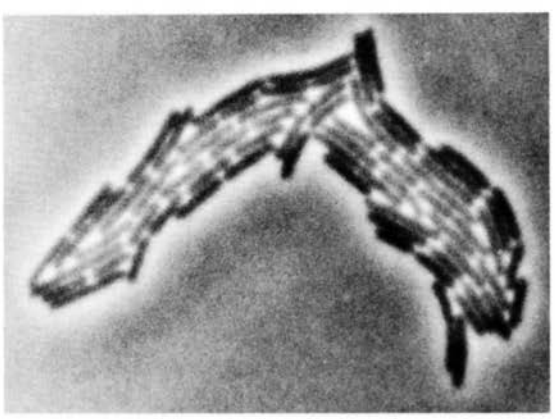

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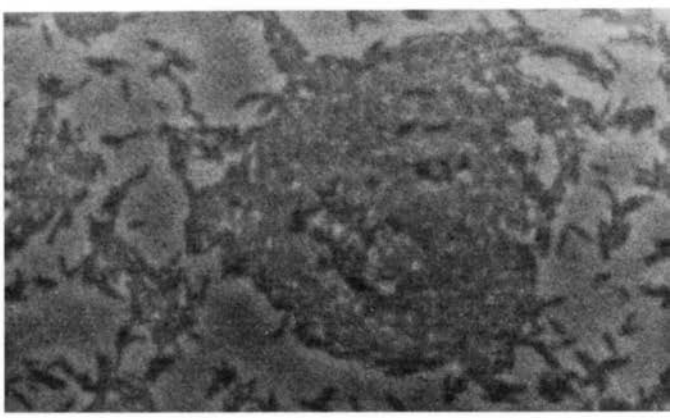

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the number of individuals are counted by the hundreds or hundred thousands. There is no sign in the behavior of individuals before their assembly that would have intimated the future rotatory performance of the collective. In fact, although each spinning mass tends to keep its sense of rotation once it has been started, whether clockwise or counterclockwise, fragments that split off may change to spinning in the reverse direction. So evidently the individual units are totally uninstructed as to the direction in which they will be made to spin by their assembled com-

munity. Here we are faced with true emergent novelty

The circling motion of masses of army ants is equally instructive (Figure 51). They keep running round and round a central column, drawing in stragglers, leaving others behind, in an interminable mass circus movement. The infectious pattern of these insect dervish exercises is spectacular. Unfortunately, this running in circles finds a counterpart in human affairs. In fact, in general, there is not too much difference between the laws of human ecology and the ecology of cells and molecules. Dependence on environment, self-sorting by segregation, compounding in groups, recombining for symbiotic reciprocity—in short, self-patterning of groups—occurs among molecules and men alike.

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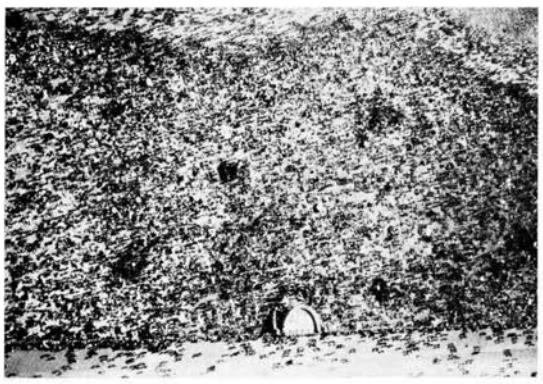

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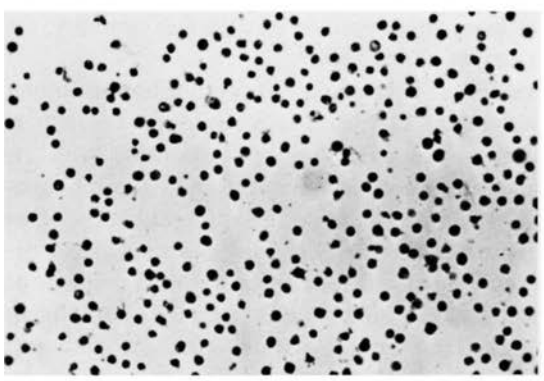

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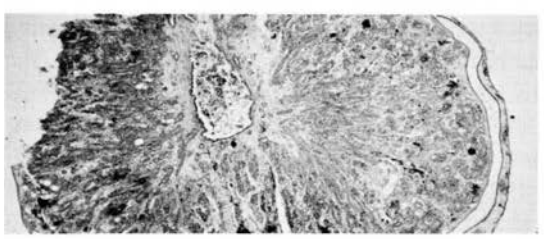

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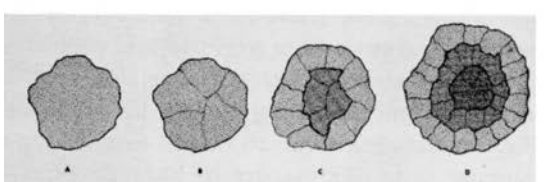

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Take, for instance, this aerial view of Coney Island on a Sunday (Figure 52). Consider the people as molecules. The heavier border on top is the condensed belt of hydrophobic bodies adsorbed to the water-beach interface. The dark clusters inside the mass clearly mark domains of attractive forces, presumably emanating from sources of nutrient and stimulant attractants. Their equidistant spacing indicates mutual repulsion through forces of competition; and so forth. The analogy is not at all facetious. It cuts deep into the heart of our topic, for it exemplifies basic features of self-organizing systems. I could have gone on, for instance, to relate how a random mixture of isolated single cells (Figure 53), obtained by dissociating an already functioning embryonic kidney, then scrambled, lumped and properly nourished, can reconstitute itself into a remarkably well-organized miniature kidney (Figure 54); or how similarly scrambled cells of embryonic chick skin in tissue culture can grow into normal feathers (Figure 55); all of this entirely by "do-it-yourself" methods.

Examples of "self-organization" of this kind are numerous. To label them is easy, although gratuitous. To understand them is a long way off. Exclusively reductionist tactics will never get us there, if they persist in "going it alone"; nor, on the other hand, will sheer verbal soporifics. What the task calls for is, first of all, a job of thorough conceptual overhauling and renovation. It requires that we drop self-imposed blinders and admit to view the higher perspective of the whole—not just its bogus literary versions, but its hard scientific core, expressed in such phenomena of emergent collective order as I have illustrated. The venal preoccupation with bits of the materials of nature as such—with "what there is"—must give way to a broader concern with the manner of their operation and use—with "how it all works." And, in this shift of emphasis, one discovers that all the bits hang together; that they are all intermeshed in webs of subtle interactions forming domains or subsystems within the over-all continuum of the universe.

To emphasize the "systems" character of the dynamics of living entities I have, ever since 1923, couched their description in terms of the concept of "fields." Lest this symbolic term again arouse alarm, let me decontaminate it instantly by the following simple example (Figure 56). Let us take a circumscribed body, depending for its maintenance on active exchange with its environment; for instance, an egg in the ocean, a cell in a tissue, a human individual in society. Then let the unit multiply into a few more units; they all continue to have a share in the common interface of exchange and communication with the medium. But let the number of units keep on increasing, whether by subdivision or accretion, and all of a sudden a critical stage arises at which some of the units find themselves abruptly

crowded inward, cut off completely from direct contact with their former vital environment by an outer layer of their fellows. The latter thereby acquire positions not only geometrically intermediary, but functionally mediatory, between the ambient medium and the now inner units. From then on, "inner" and "outer" units are no longer alike. A monotonic group of equals has become dichotomized into unequal sets. With the emergence of the distinction between innerness and outerness, the $1 + 1 = 2$ rule becomes inapplicable.

The train of events to follow such a "differentiation" of a radially symmetrical core-crust dichotomy is easy to envisage. Interactions between the "outer" members and their newly established "inner" neighbors would expose to another set of new conditions any fresh units arising subsequently in the intermediate zone between them, and hence call forth in them a third type of reaction. Moreover, polarized influences from the environment (e.g., gradients such as illustrated above for cell orientation) would impose an axiate pattern upon the group. Thus would ensue a train of sequelae of ever-mounting, self-ordering complexity. In all these steps, the fate of a given unit would be determined by its response to the specific conditions prevailing at the site in which it has come to lie, those conditions varying locally as functions of the total configuration of the system—its "field pattern," for short. This principle—long recognized empirically as a basic criterion of systems but not always fully appreciated in its implications—is commonly referred to as "position effect."

The main point to bear in mind is that none of the component members of the group, all erstwhile alike, can know their future courses and eventual fates in advance; can know whether they would become "inner" or "outer" or "intermediate." Nor does it matter for the resulting pattern of the complex as a whole, as is best illustrated by the process of twinning. By cutting in two the cluster of cells that constitutes an early embryo or an organ rudiment, one can obtain two fully formed embryos or two fully formed organs, the way the sorcerer's apprentice, in trying to kill the water-carrying broom by splitting it down the middle, got two busy whole brooms instead. What had been destined to form a single typical organism or organ has yielded two instead, each half assuming the organization of a well-proportioned whole. In principle, we can now understand why: because bisection through the middle has resulted in "innermost" cells coming to lie "outermost" again, whereupon the whole pattern of subsequent dynamic interactions has proceeded, reduced to half-scale in harmonious proportions. (Of course, the individual parts can respond to their new local cues appropriately only if their original positions in the undivided framework have not already single-tracked them into courses unresponsive to the new demands.)

An inorganic model of this process is, for instance, a sitting drop of mercury. Its convex, lens-shaped form results from equilibrium between opposing sets of forces—gravitation and adhesion, tending to spread the mass, and cohesion and surface tension, tending to hold it together. Disturb the equilibrium by cutting the liquid drop in two, and each half immediately restores its own equilibrium by assuming a convex lens-shape. But freeze the original lens-shaped drop solid before cutting, and then bisect, and each half will retain its former shape of half an oblate; the dynamics that do the remolding in the liquid drop are still at work, but deprived of their free mobility, the elements can no longer yield.

The example of twinning is just one illustration among many for the thesis that strict determinacy (or invariance) of a collective end state is fully reconcilable with indeterminacy (or variance) in detail of the component courses of events leading up to it—a thesis I have tried to contrast with the basic reductionist doctrine that a determinate end can only be reached as the blind outcome of a microprecisely determined tandem chain of component microevents. This latter doctrine, "microscopic" and micromechanistic in the old sense, just is not tenable in the light of facts unobscured by artificial blinders; and yet its popularity has grown steadily because of the indisputable proof that in the progress of science, as I said before, the artifact of reductionist abstraction has had a most signal pragmatic merit. But the time has come when we must check back with *real* nature to find what we have missed by adopting the short-sighted view of close-range analysis as the sole legitimate approach to insight into nature. My early introduction of the "field" concept into biology has aimed at no more than at offering a semantic therapeutic against the spread of this epidemic of myopia and constriction of the visual field, which leaves so many burning problems in the life sciences unattended. The "field" is a symbolic term for the unitary dynamics underlying ordered behavior of a collective, denoting properties lost in the process of its physical or purely intellectual dismemberment. Being descriptive of a property of natural systems, it must not be perverted into a supernatural principle; the study of those properties is, of course, an empirical task and not a literary pastime.

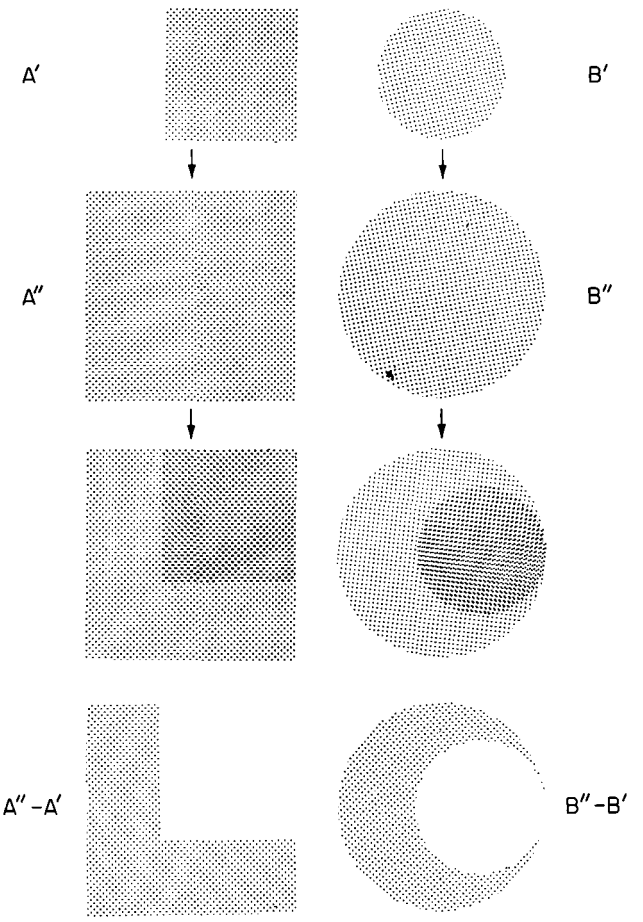
If the young generation were only to realize the origin of the microdissectionists' claim for a monopoly of insight into nature, more of them might turn to problems now kept out of their purview. So let me close with a brief anamnesis of the prevailing conceptual deficiency disease. To me, the crux seems to lie not so much in *a priori* reasoning as in our practice of phrasing experi-

mental results in some sort of shorthand language. I shall explain this on the schematic model of an experiment (Figure 57), right.

An experiment is motivated by our curiosity about the relationship between two phenomena of nature, A and B. We study them by changing A from A′ to A″ and observe a correlated change of B′ to B″. We then proceed to correlate the difference (A″—A′) with the difference (B″—B′), these differences being represented by the cross-hatched areas in the diagram. And basically this is all we can extract from the experiment. But this is not the point at which we commonly stop. We usually go on to endow the *differentials* with an existence of their own, dissected from the context from which they were abstracted in the first place (bottom line in the diagram), and before we realize it, we have personified them as "actors." Genes for the difference between a white and a pink pea became simply genes for white and pink, respectively, throwing the peaness into discard; the differences between integrated brain functions before and after local lesions became transliterated to domiciles for specialist subfunctionaries, as if the rest of the brain were uninvolved; and so on.

In trying to restore the loss of information suffered by thus lifting isolated fragments out of context, we have assigned the job of reintegration to a corps of anthropomorphic gremlins. As a result, we are now plagued—or blessed, depending on one's party view—with countless demigods, like those in antiquity, doing the jobs we do not understand: the organizers, operators, inductors, repressors, promoters, regulators, etc.,—all prosthetic devices to make up for the amputations which we have allowed to be perpetrated on the organic wholeness, or to put it more innocuously, the "systems" character, of nature and of our thinking about nature.

May my presentation have succeeded in documenting that party lines drawn between emphasis either on the whole or on the parts are based on the artifice of predilection, rather than on the realities of nature. And may this realization find its way into human ecology, particularly its political branch, to prove that society is not called upon to choose between two extremes: either a license for anarchic random excursions of its component individuals, or the enforced subordination of individual members to a rigid group order dictated from above, but that, as in all organic systems, order in the gross emerges, not only in spite of, but as a result of, the interaction of free elements with freedom in the small, restrained only by common purpose—or call it program—and respect for nature, which after all, to speak in pre-Galilean terms, abhors not only a vacuum, but disharmony.



57

Neuroscience and Human Understanding

STEPHEN TOULMIN

IN HUMAN ACTIVITIES of all kinds, the short-term demands of effective tactics can be distinguished from the longer-term demands of sound strategy and are liable to conflict with them. In the practical conduct of war or medicine, a particular victory, or cure, may be obtained using means that frustrate the overall aims of the whole military or medical campaign, while the most effective short-term political solutions often defeat the longer-term goals of the very men who formulated them.

Science is no different. And why should it be? Here too, as we work our way towards larger intellectual goals, each of us must commit himself for a month, for a year—even for a lifetime—to immediate problems of a detailed and localized sort; and must, if necessary, devote all his working time and energies to the tussle with them. The resulting day-to-day concern with the tactics of our own special areas may, understandably, distract us from the longer-term strategy of our sciences, with the resulting danger, once again, that we are sometimes tempted to choose the tactical good rather than the strategic better.

Still, among ourselves—uncommitted, as we can be only when on neutral ground—we may surely (as Bishop Butler put it) "sit down in a cool hour" and consider our more general and distant goals—what they are, what they can be, and what they should be. We can surely, that is, lift our eyes from immediate problems about the cerebellum, the ribosomes, and the neurons and look at such particular problems as these in a larger and longer perspective.

Such a task is never easy. The strategic considerations underlying science are apt to be elusive, for several reasons. Within any single generation, they are commonly left unstated; they form a body of agreed presuppositions, which, for the time being, there is no occasion or motive for calling into question. Again, the changes that take place at this deeper level often show up only in the transmission of science from one generation to the next; and frequently the result is not a meeting of the minds between the generations, but an exchange of uncomprehending insults—with an Einstein, for instance, criticizing the Copenhagen physicists as shallow positivists, and the pupils of Bohr reproaching him in return for letting "nostalgia for nineteenth-century causality" blind him to the true significance of the quantum mechanics he himself helped to found. (In a recent issue of the *New York Review*, the late Robert Oppenheimer even declared that the work of Einstein's old age was "not really physics at all.")

Nevertheless, these presuppositions *do* change. The slowly shifting patterns in the basic ideas and ideals of science may be perceptible only when we look back at the historical development of our concepts; but they are there all the same, and they can be mapped. And it is only when we do take this larger view that we can see how (to give only one example) simple particulate or atomistic theories have, in all centuries and in every science, evoked continuum or field concepts as a reaction, in order to explain the interactions of the theoretical atomic units. This is equally so, whether one considers the attractions and repulsions Newton introduced into his matter theory, so changing the traditional system of atoms and the void into a hybrid theory of atoms and central forces, or the buffering that modifies the action of genes, or the exchange forces between nucleons, or the developmental gradients of embryology, or the position-effects now becoming apparent even at the macromolecular level.

So let me begin this chapter by putting one thing back on the record. Seen in the perspective of science since the year 1600, our many and varied inquiries into perception, language, and behavior, and into the neural and cerebral mechanisms they involve, have been conducted within the framework of a broader intellectual policy; the overall, strategic aim of that policy has been to establish what the traditional rubric calls "the nature of human understanding." The ambition to understand ourselves—notably, to understand the nature of our own thoughts and perceptions—is one of those large aims to which scientists have been committed ever since the first speculations of the pre-Socratics in Ionia. Like cosmology, the origin of life, and the common structure of all material things, the problem of human understanding has a continuous history of 2,500 years; and perhaps it is the very choice of so compelling (not to say obsessive) a theme that explains the fascination of the Neurosciences Research Program as a focus for the reintegration of science.

Yet, by stating my topic in these terms, I clearly run the

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risk of alienating both scientists and philosophers. Surely, you may respond, "the nature of human understanding" is the traditional subject matter of "epistemology," and so philosophical, while the experimental sciences of psychology, neurology, and molecular biology are something quite else. What good does it do (the scientists may be wondering) confusing decent, honest, neurological issues by bringing in this . . . this metaphysics? And can we not (the philosophers may be asking) prevent our discussions about the intrinsic character of percepts, concepts, and beliefs from becoming entangled with contingent, factual questions about the causal machinery associated with them?

My first purpose here will be to sidetrack these objections. Considered as a phenomenon in the History of Ideas (I shall argue) this very separation—between a *philosophical* theory of knowledge and the *empirical* neurosciences—turns out to have been a by-product of seventeenth-century physics; and the seventeenth-century ideas about Matter and Mechanism that necessitated the separation are ones which natural scientists have by now abandoned. If this can be established, then, as a matter of intellectual strategy, the chief obstacle to a reconciliation between neuroscience and the theory of knowledge will have been removed; and before I finish I hope to indicate—at any rate, roughly—the distance we still have to go in order to reach this goal. But that will be only the third stage in my argument. Before I can usefully put this question, two preliminary problems must be tackled. For here, as elsewhere, the best way to see how the traditional schism might be overcome is to consider how it originated in the first place.

Matter, mind, and mechanism

The men who initially stated the intellectual aims of modern science, and who conceived a strategy designed to fulfill them, began with very clear ideas about what *matter* is, what a *machine* or *mechanism* is, and so what a *mechanical* or *mechanistic* explanation must be. True, in detailed content, the theories of such different men as Galileo and Descartes, Newton and Boyle, were very different and, in many respects, quite incompatible. But at the deeper level these men shared a common aim—to give a complete account of any natural phenomenon by demonstrating the mechanical processes it involved. And they also shared certain common assumptions; notably, a belief that all genuinely physical processes, arising out of the properties of matter alone, should ultimately prove explicable in "mechanistic" terms.

At a quick, casual reading, these positions look quite acceptable and very like our own. Taken at face value, indeed, their words could perfectly well be ours. Like them, we want to explain all natural phenomena in terms of the material constitutions of things; like them, we accept such explanations as complete only when those phenomena are related back to the laws governing the properties of matter; and, like them, we think of the processes by which these laws operate as being (in some sense) machine-like or mechanistic. It is only when we dig behind the common forms of words that we discover how far science has moved in the last 300 years, even at the deepest, strategical level. And, once we start cashing in the key words matter, machine, and mechanistic, for harder intellectual currency, this face-value agreement vanishes.

What, then, did the men of the seventeenth and eighteenth centuries mean by the terms matter and machine? One might quote any of a dozen definitions from the period but, as a representative example, let me cite Ferguson's *Lectures*, a standard eighteenth-century exposition of Newtonian physics:

> By the word *matter* is meant every thing that has length, breadth, thickness, and resists the touch. The inherent properties of matter are solidity, inactivity and divisibility.

As for machines:

> *Machines* [or] *mechanical powers* . . . help us mechanically to raise weights, move heavy bodies, and overcome resistances, which we could not effect without them.

The type-examples of a machine for seventeenth- and eighteenth-century physicists were the six basic or "simple" machines familiar since Archimedes:

> The *lever*, the *wheel* and *axle*, the *pulley*, the *inclined plane*, the *wedge*, and the *screw*.

On these definitions both matter and machines were simply *instruments* by which actions initiated from outside could be transmitted or modified. Ferguson underlines the point for us: "That matter can never put itself in motion is allowed by all men."

Whatever else they disagreed about, all the new seventeenth-century physicists shared one goal: to show that the Sun and planets and other such physical systems operated as "compound machines," in the sense explained. The properties of all physical systems were, ideally, to be accounted for by combining the actions of the six basic machines, and a seventeenth-century scientist who was satisfied with anything less was reproached by his colleagues for invoking "miracles" or "occult qualities." When Newton put forward his theory of gravitational attraction, for instance, both Leibniz and the followers of Descartes complained in those very terms: he had, they objected, left unspecified the *mechanical linkages* by which

gravitational force was supposedly transmitted.

At a tactical level Newton rejected this criticism: it was enough for the time being, he replied, to demonstrate how the planetary motions, and many other phenomena, matched against the inverse-square law mathematically. At a strategic level, however, he conceded the point. *Of course,* he replied through his spokesman, Samuel Clarke, we must make it our business to investigate the linking mechanisms by which gravitational attraction is propagated, just as soon as phenomena turned up throwing light on these chains of mechanical influence. For *of course* action at a distance was inconceivable—" 'Tis a contradiction to suppose a body to act where it is not"; so we could ultimately rest content only with linear chains of cause-and-effect, involving at every stage the transmission of forces by contact, impact, direct pressures, and collisions alone. And off Newton went into private speculations about possible "aether-corpuscles" filling the otherwise empty space between the heavenly bodies and perhaps interacting to produce gravitational forces as the result of their multiple contact-pressures.

Suppose one was limited to these conceptions of matter, mechanism, and mechanistic explanation—conceptions that recognized as authentic physical processes *only* transfers of momentum or energy by the contact or collision of solid, impenetrable particles (no independent fields-of-force, no action-at-a-distance, no patterns-of-organization more permanent than the pressure of "aether-corpuscles" could maintain). Given only such conceptions as these, an exhaustive physical explanation of the processes going on in living things faced inescapable difficulties. After all, the very idea of "living" or "self-propelled matter" ran counter to Ferguson's axiom, "allowed by all men"—viz. "that matter can never put itself into motion." As for the suggestion of "thinking matter" or a "thinking machine," in seventeenth-century terms these phrases involved flat contradictions. A machine was simply an instrument for transmitting an outside action; it was not a "prime mover." (Notice how that useful Aristotelian phrase has become a technical term in the transportation industry, for distinguishing self-propelled locomotives, which are prime movers, from merely passive coaches and box-cars, which are not.) So how could a machine, in seventeenth-century terms, be spoken of as self-powered, self-propelled, or self-regulating, still less as calculating, weighing alternatives, or making original discoveries? These possibilities were for the time being ruled out, not because seventeenth-century scientists had made any empirical discoveries that compelled them to take this position, but rather because of their own self-imposed definitions of the terms matter and machine.

The consequences for physiology of basing a mechanistic science on these definitions can be illustrated very nicely from Giovanni Borelli's strictly mechanical treatise, *On Animal Motion* (1680). In discussing the action of the muscles on the limbs Borelli says:

> Muscles are the organs and machines by which the motive faculty of the soul sets the joints and limbs of animals in motion. In itself a muscle is an inert and dead machine, which is . . . put into action solely by the access of the motive faculty.

Since the muscles were only passive material instruments, the true source of animal motion had to be sought for elsewhere, outside the realm of material things.

To put my first historical point in a nutshell, Descartes and his successors (Newton included) landed themselves with *animism* in physiology and with *dualism* in psychology as a direct result of their own restrictive definitions of matter and mechanism. The "motive faculty of the soul" could not itself be the property of a machine, since a machine was never self-moving: still less could the reasoning powers of man. The Bodily Machine might serve as a passive physical instrument through which the Active Mind might interact with the world of Matter, but the Mind itself had to be something of quite another, non-Material kind. In short, the philosophical problems about Life and Mind (with capital initial letters) which have bedeviled biology and psychology from the seventeenth century on are not perennial features of our scientific tradition. On the contrary, they played no more than a minor part in the older Aristotelian system, and they arose in their familiar form chiefly as *artefacts* or *by-products* of the definitions on which seventeenth-century physics was founded.

As such, the problems were inescapable only for so long as those definitions remained unquestioned, and on a general level they were challenged at once. As a matter of principle, argued Thomas Hobbes, the proper program for Descartes' mechanistic science should not be to exclude mental phenomena from its subject matter, but rather to aim at a mechanistic theory of *ideas.* Hobbes' reward for this suggestion was to be denounced from the pulpits of England as "an Epicurean atheist" and to be debarred from election to a Royal Society that could find room for literary figures like John Dryden and Samuel Pepys. More cogently, Julien de la Mettrie argued in his *L'Homme Machine* (1747) that the Cartesian definition of matter was at fault:

> There is nothing base about Matter, except to those crude eyes which fail to recognize Nature in her most brilliant Creations. . . . Thought is so little incompatible with *organised matter* as to be apparently one of its properties—along with *Electricity, Mobility, Impenetrability, Extension,* and so on.

But de la Mettrie, too, was execrated and ignored, and had no immediate influence on the development of

physiology. At the level of working science, it took a century and a half before anything very much changed. For the time being, the standard definitions of matter and mechanism seemed indispensable to the new physics. They had been adopted in the first place for convincing methodological reasons, as part of the revived alliance between physics and mathematics; and the payoff from this alliance, in terms of Newton's *Principia* alone, had been vastly impressive. So there were strong reasons for standing firmly by them—even though the price might be to exclude from science the study of Mind for certain, and possibly also that of Life.

Still, these limitations could not be accepted indefinitely. By the mid-nineteenth century the axioms and strategy of physical science were being seriously reconsidered, along with current assumptions about the nature and potentialities of machines and mechanistic processes. Once field concepts were securely established in gravitation theory and electromagnetism, the basic seventeenth-century demand for actual physical contact had to be modified. Meanwhile, the interconvertibility of different kinds of energy (thermal, electrical, chemical, and so on) made *self-powered machines* conceivable in theoretical terms, as well as the practical reality they had been for some time, while the invention of the steam governor provided a prototype for the kind of self-regulating "feed-back" mechanism that Claude Bernard soon used to explain heat regulation in warm-blooded animals.

By comparison with the scientific orthodoxy of the seventeenth century, nineteenth-century physics had greatly extended the range of activities that matter and machines could be conceived of as performing. The physics of the twentieth century has transformed the situation entirely. We live now with fundamental physical theories in which action by contact and collision have entirely lost their earlier significance. All the basic interactions of quantum physics represent species of field processes and (as Boscovitch foresaw in the eighteenth century) mechanical collision itself is now re-interpreted as the effect of force fields between the colliding bodies.

So, if we set out today to produce a mechanistic science, including a mechanistic physiology, we face a very different task from that which Descartes and Borelli faced. Throughout the last 200 years the terms machine and mechanism have been shifting their meanings slowly, but in the long run drastically, and the aims and strategies appropriate to a mechanistic science of physiology have shifted with them. As a result, the battle line in physiology between mechanists and the vitalists, or between holists and reductionists, has never been definitively drawn—and never could be. (All the key terms involved—"force," "cause," "nerves," even "molecule"—have been subject to the same conceptual evolution.) We can now recognize as machines and mechanical processes systems which the physicists of 1700 would unanimously have dismissed as "either miraculous or imaginary"; machines which not only violate Ferguson's definitions by being self-moving, but are also self-regulating, self-adjusting, and even in some cases self-programing and self-debugging. Faced with a mid-twentieth-century computer, Descartes, Newton, and even Leibniz would have had no option but to say, "That's not what we mean by a *machine* at all!"

The neurological context of epistemology

So much for the first of my two preliminary arguments. The Cartesian view of Mind as something quite distinct from Matter was a by-product of ideas about Matter and Mechanism that twentieth-century physics has abandoned; the axioms and definitions of seventeenth-century science made psycho-physical dualism—so foreign to Aristotle and his followers—almost inescapable. In my second argument, I shall focus more precisely on the mechanism of the nervous system. For in the seventeenth and eighteenth centuries the connection between neurology and the theory of knowledge was extremely close. In their discussions of perception, knowledge, and understanding, men such as John Locke and Isaac Newton took for granted a *physiological* picture of neural activity that largely determined the form of their *epistemological* theories, and this form lasted unchanged up to the time of Kant.

What was this neurological model? We may look at Descartes or Locke, at Borelli or Newton or Boerhaave, at any of those scientists who were committed to the new "mechanical philosophy"; in each case, we find a similar picture of nervous structure and action. (The odd-men-out were Leibniz and Harvey, both of whom retained strong affinities with Aristotle.) According to the general view, a sensory nerve was a material fiber or tube through which some intermediate causal agency was transmitted linearly to the depths of the brain, where all the mental processes of "perception" were actually initiated; while the corresponding function of a motor nerve was (as Borelli put it) to "communicate the commands of the appetites to the muscle, thereby stimulating its motion." The new scientists were uncertain what, exactly, these intermediate causal agencies were—whether the transmission of "the ideas of sense" inward and "the commands of appetite" outward involved "an incorporeal force, a vapour, wind, humour, motion, impulse or something else." They were uncertain, too, at what precise stage there took place the crucial transition from "material" causes in the Body to "non-material" causes in the Mind. But somewhere in

the deepest recesses of the brain, it was generally agreed, there must come into play that inner Theatre of the Mind known as the *sensorium commune;* and all effective operations with ideas and sense-impressions were conceived of as occurring in this purely mental region, causally associated with some point within the depths of the brain. (Descartes boldly guessed that this union of Mind and Body was located in the pineal gland, and he is often laughed at for doing so. But he went beyond other seventeenth-century scientists only in chancing some definite hypothesis, where the rest left this question in the air; all of them alike were committed to the idea of a sensorium acting *somewhere* within the brain.)

Once this neurological model is reconstructed, the development of epistemology during the seventeenth and eighteenth centuries is readily understood. Let me show you what happened, in terms of an analogy or parable. Imagine a man compelled to spend his whole life shut up in an interior room of a house. His entire conception of the world in which he lives has to be built up from the sounds and pictures on a television set that is located in this interior room and fed from cameras and microphones fixed onto the exterior of the house. Now, suppose our man asks himself the question: How far are the sounds and images available to me fair representations of actual objects and events in a world outside the house; and how far are they misleading artefacts of the intervening communication system, with no real counterparts in the world beyond?

If he does ask this question, he will have only a few options. He may (1) try to reason his way, on first principles, to a rough working distinction between certain aspects of the sounds and images reaching him which do, and certain others which do not, share some formal similarity (or isomorphism) with their external counterparts. Geometrical and kinematic relationships, he may decide, should be transmitted to his room with least change; so the relative shapes, sizes, and movements of the images on the screen will give a more reliable clue to the shapes, sizes, and movements of the corresponding objects than, say, their colors or textures will give to any counterparts in the outside world.

Alternatively, (2) he may decide that any attempt to draw a distinction between reliable and misleading properties of the images involves an unjustified extrapolation. There is no possibility of his comparing those images with the supposed "realities" to which they allegedly "correspond," in order to check the degree of resemblance between them: indeed, the very idea of making such a comparison seems to lack any "operational meaning." The very existence of any such independent "realities" may, in this way, come to appear a barren hypothesis—an empty speculation; and he may end by questioning whether *any* external reality can be claimed beyond that of the sounds and images themselves.

Again, (3) while conceding that this last radical doubt about the world outside the house is the only fully consistent position, he may yet draw back at the last moment. Practical necessities, he may argue, compel him to organize his sensory images and sounds according to certain rules and patterns, even though, from a strict theoretical point of view, the validity of the patterns cannot be established; and these rules and patterns form an important element in what he calls "human nature."

Or finally, (4) with growing sophistication, he may argue that these organizing patterns themselves are a function of the whole communication system; that a coherent set of sounds and images can reach him in his inner room only if (so to say) the inputs to his receiver are passed through synchronizing circuits that impose a particular spatial and temporal organization upon them.

If our Man-in-the-Inner-Room follows through these positions in turn, he will be re-enacting the development of epistemology from John Locke up to Immanuel Kant. For anyone who speculates about human understanding within the limits of seventeenth-century physics and neurology, the situation of the Man-in-the-Inner-Room becomes a plain description of human experience. According to the seventeenth-century neurological model, *we are that man.* Our own position *vis-à-vis* the world of material objects outside our own bodies is just that of my imagined Man *vis-à-vis* the world outside his house. The house is our body; the causal connections from the cameras and microphones to the television set are our sensory nerves; and the Inner Room, in which all the perceiving and thinking has to be done, is our *sensorium.* Finally, the four possible ways I have outlined of interpreting the sounds and images reaching the Inner Room are those introduced in the philosophical debate by John Locke, Bishop Berkeley, David Hume, and Immanuel Kant, respectively. (There is, however, another side to Kant, and we shall have to return to him later.)

So we face a paradox. Throughout the seventeenth and eighteenth centuries, anyone who tried to piece together a single, consistent set of scientific ideas embracing (at one extreme) force, motion, and gravitation, and (at the other) thought, perception, and knowledge, was bound to end up in difficulties. If he prided himself on being up-to-date and on operating with the latest definitions of Matter and Mechanism, he was then compelled to divorce mental processes from material ones and to explain intellectual operations in terms of the Man-in-the-Inner-Room model. The effect of such a view (and on this point I agree with Gilbert Ryle) was to replace a difficult but intelligible

problem by an outright and absolute mystery. The problem of how a complete human being directly recognizes, classifies, and describes the world immediately around him was by this step translated into the far more intractable question: how a Mind within a *sensorium* within a brain within a complete human being can do the same things indirectly and at a remove, given only the sensory inputs displayed to Mind on arrival at the screen of the *sensorium*.

On the other hand, if a seventeenth- or eighteenth-century scientist questioned the foundation of the Inner Room model, as Julien de la Mettrie did, he could do so *only* at the price of also questioning the conceptions of Matter and Mechanism seemingly fundamental to the whole "new science" of Galileo, Descartes, and Newton. Either way round, the accepted categories of matter, force, and motion failed to square with the accepted categories of mind, perception, and knowledge.

So if, looking back, we are tempted to scorn those philosophers whose account of human understanding took the Inner Room model for granted, we cannot justly criticize them for arguing too much *a priori*, or for ignoring the scientific knowledge of the times. If anything, the trouble was that they paid *too much* attention to contemporary science; their theories treated the latest scientific ideas about Matter and Mechanism with unquestioning respect at just the points where those ideas had been generalized too rashly and hastily. Because matter, according to the accepted scientific definitions, was inert and passive, the ultimate sources of all thought and activity were, by definition, banished from the "material" world; and the operations of human understanding, once lodged within the elusive *sensorium*, became "a mystery wrapped in a riddle wrapped in an enigma."

The activity and organization of material systems

So much for preliminary and destructive arguments. From this point on, let me try to make more positive and constructive suggestions. If the problems of traditional epistemology are (as I have claimed) artefacts and by-products of seventeenth-century physical science, one might expect philosophers today to be asking: How differently should a theory of human understanding be built up, given the physics and physiology of the mid-twentieth century, rather than that of Newton, Descartes, and Borelli?

That is the question I shall be concerned with, in one way or another, for the rest of this chapter. Yet it is one to which surprisingly little attention has been paid hitherto. For, in intellectual as in practical affairs, there is a certain inertia. Once securely established in men's minds, models, dichotomies, and distinctions can maintain their influence, even after their original justification has evaporated; and this is one way in which tactical preoccupations outlive the changes at the longer-term, strategic level that have made them irrelevant. So, at any rate, it seems to be with the ideas that concern us here. Not only in the writings of philosophers, but to some extent also in those of physiologists and psychologists, one can still detect the aftereffects of Cartesian dualism and the Inner Room model.

When the men of the seventeenth and eighteenth centuries speculated about "ideas" and "impressions" in terms of the *sensorium commune*, they were (as we saw) doing the best they could within the scientific framework of their time. Yet, despite all that has happened in natural science since 1800, many of our contemporaries still work within philosophical terms of reference that are effectively identical with those of David Hume. Philosophers of the "sense-datum" school, for instance, whose ideas derive from Ernst Mach and Bertrand Russell, frequently argue as though perceiving, thinking, and understanding were processes taking place entirely in some mental sphere located *at the far end* of the nervous system; Russell himself, for instance, put this view bluntly when he declared that "all we ever directly see is a part of our own brains"—a conclusion that revives the *sensorium* theory in its purest and crudest form. In return, there is a temptation for physiologists of perception to reply impatiently, "No, it is not our *minds* that perceive, discriminate, learn, calculate and respond. . . . It is our brains," and to argue that, given a sufficient understanding of the cerebral mechanisms, we could quietly drop the word mind, leaving the rest of our system unaltered.

But one cannot simply abolish one term of a distinction (such as Mind) while retaining the other, correlative term (Matter) with its meaning unaffected. If the Cartesian view of the Mind-Body relationship is unacceptable today, that is not because Descartes' notion of Mind has turned out to be merely superfluous. What is at stake here is the whole distinction between active Mind and passive, inert Matter, from which seventeenth-century physics began. In destroying Cartesian dualism, we do not show that living and thinking beings are, after all, compound machines (in the seventeenth-century sense) made up of essentially passive, material units; on the contrary, we show that Matter itself cannot, after all, be properly defined as essentially passive and inert. Recall de la Mettrie's words: "Thought is so little incompatible with organized matter as to be apparently one of its properties—along with Electricity, Mobility, Impenetrability, Extension, and so on." We have not banished the Ghost from the Machine, only to be left with an untenanted Machine; rather, we have discovered that, in many crucial respects, the Ghost and the

Machine are indissoluble—the activities we call "mental" being inextricable from the structure we call "material"—a very Aristotelian thought. This being so, to drop the word mind in favor of the word brain, leaving everything else unchanged, would do no more than to replace the Man in the Inner Room by an Automaton in the Inner Room, the Inner Room model itself being left intact.

So minor a change, in any case, does less than justice to the strategic changes that have taken place in physics since 1700—notably, to the physicists' new views about Matter. For, regarded as a general theory of matter, quantum mechanics enables us to go one step further. Right up to 1925, scientists regularly distinguished between the basic units or "bricks" out of which material things were supposedly made, and the patterns subsequently to be found in the behavior of these units. The strategy of exposition in physical science was always to *begin* by defining the structure of material systems and then *afterwards* to investigate and discuss the laws governing the interaction of atoms, molecules, cells, or other constituent units. In strict quantum-mechanical terms, however, this distinction can now no longer be drawn.

A physical system, or mechanism, is now specified by a wave-equation that characterizes in one step both the material constitution of the system and its mode of operation —both its structure and its activity. There is no procedure for specifying the one independently of the other, and to speak of either in isolation is a mere abstraction. The wave-equation of a water molecule specifies at once both its structure and its activities; the more complex wave-equation for a complete macro-molecule should likewise necessitate its capacity to replicate; and if, *per impossible,* we were ever able to set up an overall wave-equation to characterize a complete organism, then in the very act of specifying its structure we should have to entail its physiological and psychological capabilities as well. Therefore in principle, at any rate, the possibilities that Descartes and Newton ruled out have become legitimate options. The capacity for thought is clearly no more "incompatible with *organized matter*" than are the properties of electricity and mobility; to our eyes, indeed, an appropriate organization must *entail* that capacity.

For the time being, of course, the idea of a comprehensive wave-equation for a complete organism is merely visionary, and it certainly has no direct relevance either to our sensory mechanisms or to the problems of human understanding. Very likely, indeed, it is more than simply visionary. Like Laplace's description of the entire universe as a predictable Newtonian mechanism, the idea may turn out to be internally inconsistent. Indeed, even the idea of a macro-molecular wave-equation is scarcely practical politics, although there are rumors that a first, approximate quantum-mechanical treatment of replication has been worked out at Berkeley.

These possibilities are worth mentioning here simply for reasons of intellectual strategy. For the explanatory ideal that dominated physical science from the mid-seventeenth century until a few decades back—the model of solid, impenetrable, essentially inactive, unit bricks, exchanging energy and momentum by collision—has been displaced, even within physics, by a quite different explanatory ideal, which traces back to the Stoics in antiquity, rather than to Democritus and the Epicureans; and this requires us to consider the activities of a *whole system* in terms of the overall pattern of interactions by which it is defined and maintains itself—treating the system as the "product" of its component factors, rather than as the "sum."

A molecule has a wave-equation that is strictly related to the wave-equations of its component atoms; but it is a *new* wave-equation nonetheless, and no purpose is served by attempting to locate, say, the characteristic properties of the water-molecule within the equations for any of its component atoms. In the very nature of things, a new, higher-order system will display integrated patterns of activity that can never be exhaustively explained in terms of isolated causal interactions of its sub-parts. For the quantum physicist, any stable association of elementary units, from an atom up, is already an "organized" system —if the word had not already been pre-empted, one might almost have said "organism." Even mathematically, the properties of such an integrated system are properties of the *whole,* rather than being attributable to any of its component *parts.*

This is a worthwhile reminder for anyone concerned with the broader problems of human understanding, especially with the attempt to clear the aftereffects of seventeenth-century physics out of the theory of knowledge. You recall the old adage calling on us to adopt realistic attitudes and expectations in ethics: "A man is a man, and neither a monk nor a monkey." In the same way one might say, "A human being is a human being, and neither an observer trapped in his own *sensorium,* nor a brain with arms." Nothing in physical science imposes on us any longer the absolute seventeenth-century distinction between the physical properties of the body and the psychical activities of the "motive faculty" within it; and nothing in contemporary neuroscience does so, either. So we can now restate our questions about perceiving and understanding in terms of the inputs and outputs of complete human beings, instead of being driven willy-nilly up the nerves and into the thalamus—or the pineal gland. Certainly there are sub-systems in the brain and the eye that play especially delicate and critical parts, as "correlates" or

"substrates" of perception, consciousness, and the rest; but these sub-systems need to be thought of as elements in the larger systems by which an organism's activities are linked up with relevant parts of its environment, and the complete system is indispensable in every case for the genuine phenomenon. (Leibniz, incidentally, argued all along that the *âme*—that is, the totality of our mental capacities—acts, not through any single point in the body, but in a distributed way, through many different organs.)

So our subject matter need no longer be the "inner man" peering at the sense data arriving in his *sensorium* and wondering what he is entitled to make of them. (We see, not *through* our optical system, but *by its use*.) Rather, the theory of human understanding is concerned with the complete man, face to face with the world of objects around him and capable of interacting with it directly. And the trouble with speaking, even metaphorically, of the brain as "facing problems," of cells as "talking to each other," of the central nervous system as "taking decisions," or of molecules as displaying "memory" is that, by seeming to credit one or another of a man's parts or organs with a function belonging to the whole man, such shorthand expressions threaten to drive us back, conceptually, into the *sensorium* from which we have so recently escaped.

The cultural and historical dimension

What agenda, then, could we imagine for an adequate account of human understanding, if it escaped completely from the influence of out-of-date physics? To introduce this question, let me digress for a moment and refer to an essay, "Time in Biology," written some ten years ago by the late J. B. S. Haldane. In this essay Haldane emphasized the internal complexity of all biological phenomena, and the same theme has been taken up more recently by C. H. Waddington. Every biological event, they point out, can be considered against four different time scales. Certain aspects of the event are variables within systems whose significant changes take a few minutes, a few hours, or a few days; these are the variables of gross physiology. Within these changes we can detect more minute biochemical or microphysical processes whose time scales are commonly much shorter—a few fractions of a second at most—and in studying these sub-processes we can often treat the gross physiological variables as being, for practical purposes, *in*variant. Biochemical phenomena are thus abstractions from actual systems, which are subject to a very much slower physiological change.

Conversely, the phenomena of normal physiology are abstractions from, or cross-sections of, complete life histories that have time scales of the order of one to a hundred years; and once again, given the great difference between the effective time scales of, say, respiration and ageing, this abstraction is legitimate, at any rate for most practical purposes. Finally, the development of individual organisms displays features demanding an explanation in terms of the evolution of the species; and this means relating the changes taking place over a few decades to evolutionary and genetic processes of kinds which, characteristically, take hundreds of thousands of years. The single biological event, in all its historical particularity, is neither an evolutionary, nor a developmental, nor a physiological, nor a biochemical *event*; but, by abstracting from the complete historical fact variables whose significant changes take place over the appropriate timescale and "freezing" the rest, we can treat such an event as either a biochemical, or a physiological, or a developmental, or an evolutionary *phenomenon*. (Even the bare description of a phenomenon thus involves an element of abstraction; and this point is of importance if one is concerned with the relations between the *theoretical* and the *historical* aspects of science.)

If we approach the theory of perception and understanding on a similar strategic level, what should we expect? Such a theory must have all the variables—all the dimensions and degrees of freedom—characteristic of general biology, but *it must also have one more*. Suppose I am walking through a New Hampshire wood with Ernst Mayr and he stops to draw my attention to a rattlesnake plantain, or to a broad-tailed hawk seated on its nest in the fork of an old tree. Once again, we could find in the moment at which he recognized the bird, or orchid, for what it was, aspects calling for analysis on a micro-physiological or biochemical time scale; others involving processes at the level of normal physiology or psychology; others again requiring references to intellectual development and learning over a period of decades; and, finally, genetical and ethological aspects having much longer-term implications. But in this case there is a further, additional time scale to be considered. The capacity to recognize at a glance, and to name, a particular species of bird or plant exploits and presupposes a taxonomic system that is itself the product of an *historical* or *cultural* sequence some hundreds of years in length. Our concepts and categories being products of cultural history, the varieties of human perception are products not only of physiological and psychological constants but also, to a substantial degree, of cultural and historical variables.

This point was first made emphatically by Kant's idealist successors in Germany, but for justice's sake I must really take up the story again with Kant himself. Earlier on, I implied that Kant regarded the spatial and temporal aspects of our experience as reflecting the character of our sensory transmission system, but this was only a half-truth. Janus-like, Kant's theories faced both ways. He

tried his best to struggle free from the Man-in-the-Inner-Room model, but he never completely succeeded. The structure of experience could not be settled (he argued) by the physiological or psychological mechanisms of our sensory apparatus alone, as that would have meant treating the theorems of geometry (for instance) as contingent by-products of our bodily makeup. Instead, he claimed, this structure was determined by the pattern of categories, concepts, and forms we find indispensable for classifying and describing the world we perceive. To perceive something, in the full sense of the term, it was not enough simply to receive sensory inputs; we must *make* something of those inputs, in terms of categories, concepts, and forms that *we* bring to their interpretation. "Concepts without percepts are empty," he declared in a famous epigram, "but percepts without concepts are *blind*." In this strong sense of the term, we can *see* what is the case, only if we could in principle *say* what is the case; and from this it follows that an adequate account of perception and understanding must dovetail with our ideas about language and the use of concepts.

This was already a significant new twist; and, if Kant had not still been committed to a certain fundamental dualism (of mind and matter, reasons and causes, *noumena* and *phenomena*), he might have taken the next step also. But he had begun as the leading physical cosmologist of his generation, and he retained one foot in the Newtonian physics of his earlier career; so he was determined to protect reason and all essentially mental concepts from contamination by the contingent world of material causes and natural phenomena. The concepts and categories that preoccupied him (for instance, those of geometry) *must* be as they were, he argued, if we are to have any coherent experience at all; they *must* be characteristic of all rational experience whatever. Just as they were not by-products of physiology and psychology, so they must not be treated as by-products of history and sociology either. The world of "pure reason" was still, for Kant, the world of the mind; and this had to be considered aside from all causal questions, whether about the workings of the body or about the chances of history. So, when all was said and done, Kant's basic problem was still the same as Descartes'. The Man remained in the Inner Room; the Reason *inside* still had the same difficulty in coming to terms with the Thing-in-Itself *outside*.

For the first thorough-going philosophical renunciation of the Inner Room model, we must look to, of all people, Hegel. (Hegel's philosophy may be deservedly unfashionable, but it does not follow that all his insights were unsound.) A student reportedly asked Hegel whether he agreed with Berkeley's criticisms of Locke's theory of knowledge, and his reply was sarcastic: "What was previously said to be 'outside' is now said to be 'inside'—as though that changed anything!" In the first place, Hegel insisted, perception, recognition, and understanding could not usefully be considered as simple relationships between an inner mental world deep within the skull, and an external material world outside the skin. It was necessary, from the very outset, to consider how men employ concepts and categories to organize and interpret their sensory inputs, because without categories and concepts, nothing could ever be *said* on the basis of those inputs. (As the late J. L. Austin put it, "Sense-data are dumb.") Furthermore, the systems of interpretation and concepts that men currently employ in this way have not remained unchanged throughout history. On the contrary, the concepts we now use to shape our experience of the world are the current outcome of a long and continuing process of historical and cultural development. So the problem of perception itself, involving as it does a *conceptual* component, turned out to have an extra historico-cultural dimension, over and above the four dimensions characteristic of regular biology. (This insight has, of course, had a long history of subsequent development, through Karl Marx, Emile Durkheim, and Max Weber up to our own day.)

When we move from general biology to the theory of human understanding, then, we are compelled to extend Haldane's analysis and say, "In every perceptual or cognitive event there intersect processes, not of four, but of *five* different kinds—processes taking place on *five* different time scales." At each level up to that of the complete historical fact, the range of possibilities is delimited more narrowly. We are *capable* of perceiving or recognizing anything only when it gives physical indications to which our sensory equipment can respond; this depends on the chemical and physical sensitivities of our sensory systems; and these in turn have been selected out from all the conceivable genetical combinations during the time scale of organic evolution. But what we *in fact* end up by perceiving or understanding will be something very much more specific than this; it will be something we have found out how to discriminate, recognize, categorize, describe, and connect with other things by applying the criteria and classification systems that we have either picked up or been formally taught, within a particular phase of cultural and conceptual history.

An agenda for epistemics

We can now come round at last to our central question: "How far have we to go to achieve an adequate account of human understanding?" Supposing that epistemology, treated as an autonomous branch of philosophy, took its

traditional form as the result of an historical accident; and supposing that we are determined to bring our theory of knowledge back into relation with the neurosciences; what problems will the resulting account have to face?

Such a theory—what one might naturally call "epistemics," rather than epistemology—must satisfy a number of conditions. First and foremost, it must set aside all the absolute distinctions that were by-products of seventeenth-century theory (mind and matter, physical and psychical, etc.), since these represent false and needless antitheses. The basic systems of modern physical theory are quite unlike the corpuscularian mechanisms of the seventeenth century, and naive linear-contact action need not dominate a twentieth-century theory of perception and understanding either. This done, it will need to escape finally from the Cartesian Inner Room model. That means posing all problems having to do directly with intellectual and perceptual capacities in terms of the total relationship between our sensory systems, our brains, and the environment with which we have to deal—which includes the conceptual patterns we inherit or acquire. (This must be done, I believe, even in the case of those questions that involve the notion of "consciousness": it requires of us, not behaviorist slogans, but simply a behavioral methodology.) We may call the resulting explanation mechanistic if we please, but this no longer matters. Once the Cartesian antitheses have been cleared away, we achieve nothing by asserting that our systems are material, physical, or mechanical—for these adjectives no longer have any opposites, and their use in no way alters the fact that we have chosen to study mental activities in the first place.

With these preliminary remarks, one can state a number of general problems involving *epistemic* issues in the sense here explained, and in all these areas we have a long way to go. Some are problems for historians and philosophers: others necessarily involve the natural sciences also. For instance, those of us who work in analytical philosophy and the history of ideas have only recently begun to study systematically the ways in which the growth of our discriminatory capacities interlocks with the development of our linguistic equipment—what one might call the field of conceptual ontogeny. Nor have we yet analyzed in nearly enough detail the processes of conceptual phylogeny, by which intellectual and linguistic innovations are selectively transmitted from generation to generation within a culture. Still, if some of my professional colleagues in philosophy are now treating the history of ideas and the psychology of learning as areas of real significance, that is very largely because of their relevance to the development of our fundamental concepts, categories, and forms of understanding.

So much for history and philosophy: beyond this point, we shall need the cooperation of the natural scientists. For the conceptual element enters, by implication, not only into intellectual history, but into sensory physiology and psychology also. How does the growing child develop perceptual skills—the ability to recognize and name different kinds of objects and systems, or to make appropriate responses, both linguistic and of other kinds, to different situations? Is it, as Erikson and Lorenz have argued recently, by a kind of "ritualization" of learned habits? And, if so, how is this ritualization selectively registered in the electrical and biochemical correlates of the learning process? (At this level, concepts play an indispensable part in the organization of experience, and the study of learning leads on into the study of language acquisition and use.)

Again, how does conceptual training affect the actual responses of the human observer, as compared with the role played by, for instance, permanent structures in the sense organs? Presumably the "bug-detecting" structures that Lettvin and Maturana described in the frog's eye act in a way in which "cultural" content is next to nothing, and in which genetical component is predominant. Presumably, we must distinguish those physiological structures whose operations are independent of learning from those whose effectiveness is dependent on appropriate "drilling." Yet can a distinction between the permanent hardware of the brain and nervous system, and an instructional programing by which conceptual habits are imposed on it, be maintained consistently, and insisted on? Or does the learning process itself leave a permanent mark on neuro-physiological structure? Do mental exercises modify the body's neurological development in the way that physical exercises modify the development of its musculature? When we talk, say, of a painter as developing his own individual "eye," do we perhaps mean—literally—what we say?

Or suppose that a comparative ethologist studies the behavioral capacities of different groups of animals. Presumably these capacities—and the physiological endowments associated with them—have been evoked by evolutionary demands as surely as more straightforward genetical and morphological features; yet, at the moment, our understanding of the evolution of behavior is at the level of early nineteenth-century paleontology. Still, such an understanding could be very illuminating in a number of ways. The human capacity for rational deliberation—such as it is—and language, regarded as the instrument of this deliberation, certainly have a back history; and behavioral likenesses and continuities between different groups and species should throw light on this back history, in the same way that comparative anatomy and morphology—as re-interpreted after Darwin—threw light on the back history of our bodily structures and mechanisms.

(Von Frisch and Lindauer have shown that something, at any rate, can be done in this direction.)

Once we have begun to consider how far animals as well as humans can be said to conceptualize, a further possibility arises. For we shall now need to ask how far the working of our own intellectual equipment is limited by evolutionary and genetic factors. Do our capacities for rational thought and conduct, for instance, display patterns having an evolutionary origin? At a certain level the answer is obviously "Yes." But where are we to draw the line? Is there perhaps something in Chomsky's hint that the doctrine of innate ideas—long discredited among empiricist philosophers—may have to be revived, as a fact of epistemic physiology? Is it the case, as he argues, that the basic grammar of all human languages displays uniform features that reflect genetically determined aspects of our intellectual equipment? (In that event, one might have to take more seriously Wiener and McCulloch's suggestion that Kant's "synthetic *a priori*" might, after all, have a physiological, rather than—as Hegel claimed—an historical and cultural basis!) Finally, may not historical changes in the character of human and pre-human rationality themselves have served as a selective factor in the course of evolution? Any meeting of scientists such as this proves, at any rate, one thing: when the human species adopted an upright posture, its hands were freed, not merely to hunt and to make weapons and to kill, but also to figure and to construct and to devise instruments.

Those are some of the questions for whose answers a philosopher interested in the theory of knowledge in the 1960's must look to the empirical scientist. Many of them, no doubt, are questions for the future—items on the agenda of a possible science that we are not yet in a position to tackle. All the same, it can occasionally be worthwhile to frame the questions we would *like* to see answered, especially when we are concerned with the long-term strategy of science, rather than with its immediate tactics—and this can sometimes be legitimate even when we have scarcely the ghost of an idea what answers to expect.

One thing we can say. Suppose we knew the immediate biochemical and electrical substrates, both of sense perception and of intellectual deliberation. Suppose we understood the processes by which concepts and standards of judgment became internalized in learning. Suppose, too, that we could see clearly how social and intellectual considerations interweave in the conceptual history of different cultures. And suppose we could piece together the evolutionary sequence by which the human species developed its conceptual, linguistic, and intellectual capacities. If all these things were taken into account, and we had explored in detail all their inter-relationships, then—and only then—we should be approaching the point at which one might say, with some confidence and justification, "So *that* is what is involved in human understanding!"

GUIDE TO PAGE LOCATION

NOTES

COMPONENTS OF THE NERVOUS SYSTEM [pages 5–31]

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The Ribosome—A Biological Information Translator Alexander Rich

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Conformational Change and Modulation of Enzyme Activity Daniel E. Atkinson

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Dynamic Aspects of Information Transfer and Reaction Control in Biomolecular Systems M. Eigen

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Neuronal Specificity in Neurogenesis

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Some Recent Developments in the Biochemistry of Membranes EUGENE P. KENNEDY

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Membrane Ultrastructure in Nerve Cells

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Synaptic and Ephaptic Transmission

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Comparative Physiology of Dendrites

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The Problem of Sensing and the Neural Coding of Sensory Events VERNON B. MOUNTCASTLE

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BRAIN CORRELATES OF FUNCTIONAL BEHAVIORAL STATES [pages 499–633]

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Neurophysiological Aspects of Rhythms

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Neurophysiology of the States of Sleep M. Jouvet

The bibliography of sleep is extensive. Excellent books and symposiums concerned with sleep states have recently been published:

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Unit Activity in Sleep and Wakefulness

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Intrinsic Organization of Cerebral Tissue in Alerting, Orienting, and Discriminative Responses W. R. Adey

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BRAIN CORRELATES OF LEARNING [pages 637–775]

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Effects of Ablation K. L. CHOW

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Postnatal Growth and Differentiation of the Mammalian Brain, with Implications for a Morphological Theory of Memory

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The Enhancement of Learning by Drugs and the Transfer of Learning by Macromolecules

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Agents That Block Memory B. W. AGRANOFF

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