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Symposium

on

FRONTIERS OF MOLECULAR NEUROBIOLOGY

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SYMPOSIUM ON FRONTIERS IN MOLECULAR NEUROBIOLOGY

Chairman, FRANCIS O. SCHMITT

Presented before the Academy on April 22, 1968

By Invitation of the Committee on Arrangements for the 105th Annual Meeting

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INTRODUCTION

BY FRANCIS O. SCHMITT

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Mathematicians, physicists, and chemists, as well as neurological and behavioral scientists, have recently shown much interest in the problem of how the brain functions in subserving behavioral processes such as learning and the storage and recall of long-term memory. Neurophysiologists have traditionally studied these properties at the level of neuronal circuits, while behavioral scientists have been concerned with the performance of the whole organism. However, for effective investigation of such complex systems, basic studies are necessary at the level of brain cells and their molecular effectors. The purpose of this symposium is to portray recent advances in neurobiology considered primarily at or near the molecular level.

The biological unit of the nervous system is the *neuron*, which in various—sometimes bizarre—forms is closely associated with another kind of cell, the glia. The neuronal-glial interaction is vital for metabolic, neurophysiological, and possibly psychological processes. Indeed, a name might appropriately be given to the neuron-glial combination, since it is probably the real unit of the nervous system. This symposium will deal primarily with neurons.

The aspects of molecular neurobiology to be discussed in this symposium are characterized schematically in Figure 1. The illustration at the top is meant to

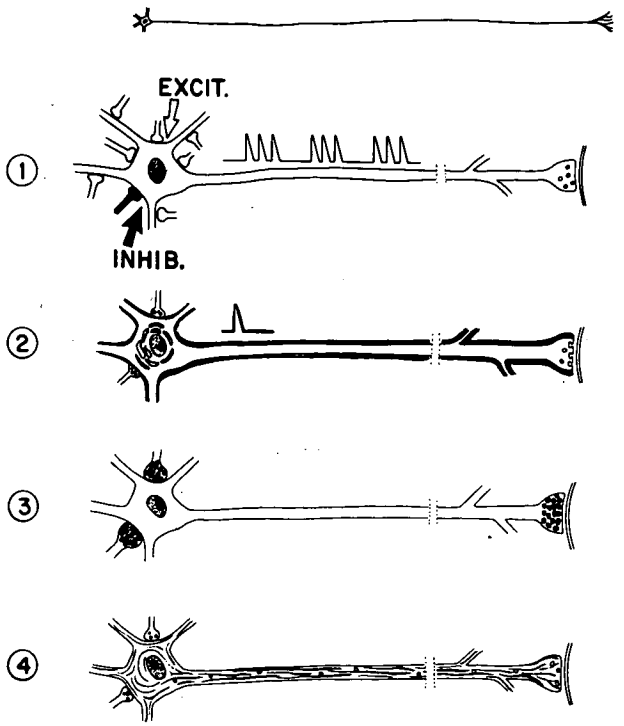

FIG. 1.

point out that the impulse-conducting axon, as a rapid-communications device, may be very long compared with the diameter of the cell; the ratio may actually be as high as $10^4:1$ or higher. The maintenance of this long cell process, the axon, so remote from the metabolic center of the neuron, requires explanation.

Neurons, in addition to their vegetative, maintenance, and energy-producing functions shared with other cells, have structures and properties that are unique to excitable tissues and that are concerned with communications and information processing.

Essential background for the biophysical and biochemical characterization of neuronal organelles is an understanding of the manner in which information is represented and processed within the microcosm of the neuron and in relation to neuronal inputs and outputs. Dr. T. H. Bullock will indicate levels at which

neural processing of information occurs within and between neurons. If a biological process is to be suggested by which the specificity in any type of macromolecule is to explain memory and learning, that process will probably be in the context of cellular mechanisms such as those described by Dr. Bullock. The neuron computes output signals appropriate to many inputs—there are as many as 10^4 to 10^5 synaptic endings on the dendrites of certain kinds of neurons—both excitatory and inhibitory in nature (see neuron *1* in Fig. 1). With knowledge of the mechanisms discussed by Dr. Bullock, the molecular neurobiologist may avoid vague generalities about molecular encoding of psychological information and may focus more effectively on specific processes likely to advance the field.

The limiting membrane, probably varying somewhat in its structure and composition in various parts of the neuron, is the site of excitation and its rapid transfer to fiber endings. An electric potential exists across surface membranes of all living cells. However, nerve membranes (and the excitable surface membrane of muscle cells), illustrated in neuron *2* in Figure 1, have the additional property of fast (*ca.* 10^{-4} sec) change in permeability which leads to the propagation of the self-regenerative action wave. Discovery of the molecular mechanism of this process is a major endeavor of molecular neurobiology. New ideas about this process and of the biophysics and biochemistry of cell membranes—a subject now widely discussed—will be presented by Dr. A. L. Lehninger.

Excitability is transferred across most synaptic junctions between neurons by chemical rather than electric means. Transmitter molecules are liberated in the synaptic cleft, presumably from vesicular containers or reservoirs (see neuron *3*, Fig. 1). The biochemistry and physiology of the biogenically active, excitatory, and inhibitory transmitter substances constitutes one of the most productive areas of present-day neurochemistry. Dr. V. P. Whittaker, who has developed methods of fractionating the transmitter-containing vesicles and synaptic endings as partial systems from brain homogenates, will portray current views on mechanisms of synaptic transmission.

Finally, I shall describe a system of fibrous proteins which have been under investigation in our laboratory for several decades and which, though highly differentiated in neurons, probably represent organelles vital to functioning of cells generally. Recent discoveries of the role of fibrous proteins in intracellular transport and of the transport of substances down the axon suggest a possible function for the ubiquitous fibrous proteins in neurons (see neuron *4*, Fig. 1).

Many other aspects might have been chosen for discussion in this symposium. However, reports from these four frontiers are illustrative of the vitality and relevance of molecular biological methods applied to neurobiology. Work of this kind helps build bridges between the three areas: molecular neurobiology, brain science, and behavioral sciences.[1] Progress is thereby accelerated both in the attack on mental disease and in the study of the most complex system known to man, the human brain. To the extent that success is achieved, science, the product of human thinking, may well be revised, as may other cultural institutions of modern man.

[1] Schmitt, F. O., *Arch. Neurol.*, **17**, 561 (1967).

REPRESENTATION OF INFORMATION IN NEURONS AND SITES FOR MOLECULAR PARTICIPATION

By Theodore H. Bullock

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The human brain is estimated to have 10^{10} nerve cells. These are not like so many grains of sand or even miniature switching relays but more like personalities—highly diverse, labile, and inclined to be active spontaneously. Each one has several parts: (1) receptive loci specialized for transducing in several ways the dozens of inputs that impinge on it, (2) pacemaker loci that inject spontaneous rhythms, (3) mixing and integrating loci, (4) threshold loci for initiating all-or-none nerve impulses in bursts and trains from 1 to 1000 per second, and (5) transmitter loci at each of the far ends of the nerve cell, where it influences up to several dozen others. The figure of 10^{10} gives no idea of the information flow, say in nerve impulses per second. Right now the 30,000 nerve fibers in your auditory nerve may be handling your auditory input with an average of a million or so nerve impulses per second; your visual input probably employs ten times as many; and each cascades into ten or more times as many between the incoming nerve and the cerebral cortex.

How is information about the world represented in the activity of single cells of the brain and are there strategic sites where chemical participation is most relevant to the communication functions, as distinct from the housekeeping functions and the growth or blueprint functions? While these functions may be related, they should be considered individually.

It will be my thesis here that we can now defend this proposition: There is not one code of nerve impulses, but several; and for each cell there is not one strategic site, but several.

First let me distinguish between the problems of the meaning or information content of neuronal messages (which I will not develop today) and those of the forms of representation of information. The nervous system must depend heavily upon labeled lines, that is, upon foreknowledge that any signals in the optic nerve are visual and in the acoustic nerve are auditory. Labels are of hierarchically more complex and biologically meaningful information content as we ascend toward higher centers. When certain units of intermediate complexity in the frog optic lobe discharge a few impulses, it is tantamount to the message, "An object of small size and sharp edges, darker than the background, has moved within a certain 3° cone in the visual field, in the virtual absence of movement elsewhere in the field." Insofar as these "meaning" labels are built in by heredity and normal development, they are not part of my concern today. Whereas practically any discharge of this frog unit has the meaning I just gave and called its label, the different degrees of discharge encode something quantitative about the movement, actually an ambiguous product of rate and extent and direction of movement, size and contrast of object, and amount of motion in the background. The next neuron or neuron of higher order, receiving converging input from many of these units and perhaps others with different labels, will sort out, not among the ambiguities I have thought of, but what the frog needs, e.g., "Very

interesting morsel of priority 2, at the latitude and longitude by which this line is labeled, has become active after a period of quiet." The problem I want to raise is the language of these messages, what parameters represent the different states that the cell is signaling from moment to moment. This is the general problem in a communications sense. It may lead us to recognition of the relevant cellular operations and locations of most interest for analysis of molecular componency. My concern today is the unit neuron and I will not deal with the representation of information by ensembles of neurons.

Sequence of Translations in Neurons.—Let us look at the sequence of translations in a single neuron, using as models of nerve cells two sorts of sensory cells because the relevant input is simple and controllable. There is ample reason to believe that all we shall say of these cells is equally true of cells in the middle of the brain.

When a normal physiological stimulus impinges on the receptive branches of a neuron, called the dendrites (Fig. 1), an unknown chain of events acts as a transducer on this stimulus to change the local properties of the cell in such a way as to bring about an alteration in the ability of specific cations or anions to cross the cell membrane. This in turn results in a flow of current across the membrane, focally in the stimulated region, either inward or outward according to the species of ions for which the membrane conductance has increased. This current is the

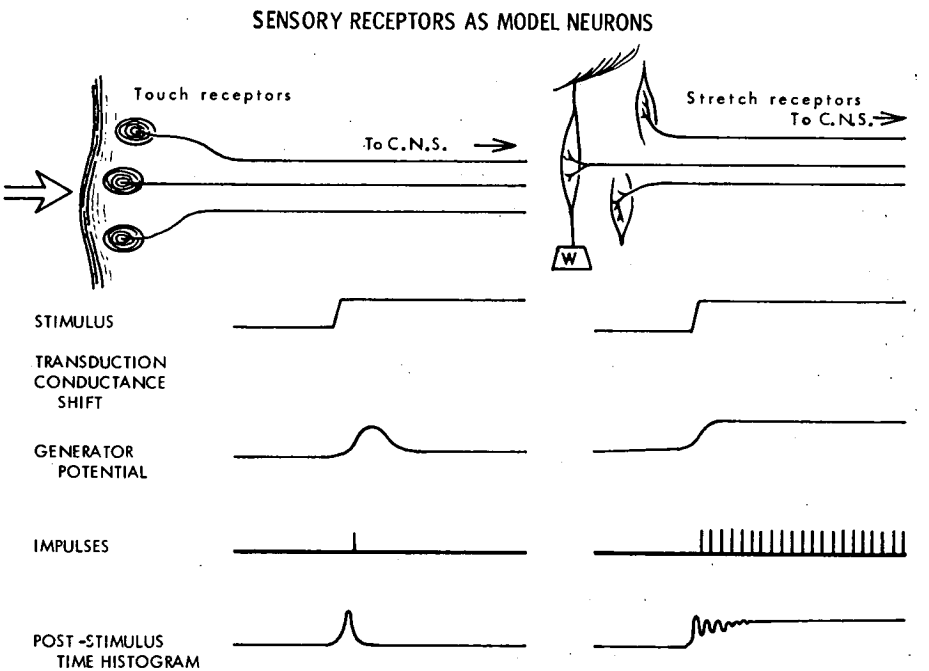

FIG. 1.—Two types of receptors, showing some of the events leading to a representation of some aspect of the stimulus in nerve impulses to the central nervous system (*C.N.S.*). "Transduction" and "conductance shift" are listed to indicate the series of intermediate steps not drawn.

measure of the so-called generator potential. This is a graded function of the intensity of the stimulus; it can be linear by certain measures and over a limited portion of the dynamic range, but it may also be nonlinear. Note that there seem to be several sequential steps in the chain of events between stimulus and generator potential. I want to focus today on the consequences of this kind of multiple-step encoding.

The generator current, though local in focus, spreads passively according to the cable properties of the nerve fiber for a millimeter or two, decrementing in amplitude and delaying its peak time due to the nerve cell membrane capacitance. At some point before it has decremented too far, it reaches a region of the nerve fiber membrane with a low threshold for nerve impulse firing, and suddenly a regenerative chain reaction occurs initiating the all-or-none impulse, or "spike," which is a transient reversal of the potential difference between inside and outside of the cell and its surface membrane due to a sequential change in its conductance for specific ions. This impulse now propagates actively along the nerve fiber like a lighted fuse, the local currents from the active region "igniting" the adjacent region. In the example of touch-sensitive neurons that we have chosen, the generator potential is not sustained; hence, there can be only one impulse per stimulus. The code is therefore simple and the information contained in that single spike is to the effect that at a certain moment a stimulus event occurred of the kind for which that fiber is labeled "touch." Notice that stored information about the state of excitability of the local mechanisms, and therefore about the various factors that influence this excitability such as the recent and the remote history, is embodied in the threshold for spike initiation and also separately in the amplitude of the generator potential for a given stimulus intensity.

The moment of occurrence of the spike may exhibit some "play" upon repetition of the experiment, and this scatter of latency or reaction time can be exhibited in the form of a so-called post-stimulus-time histogram which plots the probability of occurrence of a spike at each moment after the stimulus. This scatter does not necessarily indicate unreliability, since we can only control certain aspects of the total situation, whereas the internal states that are equally influential in determining excitability may be changing in a meaningful rather than a noisy manner. Moreover, the possibility that the fluctuation represents noise for the system cannot be excluded. The point of this remark is that evidence of essentially this kind has been used to draw the conclusion that the nervous system is basically probabilistic in operation since its units exhibit apparent play.

In another example, the physiological stimulus of stretch results in a sustained generator potential and, hence, in a sustained train of nerve impulses whose frequency or mean interval is a function of the degree of stretch. Here, then, is a new form of representation of the stimulus, the average-frequency. Frequency of spikes in a train so generally varies systematically with stimulus intensity and so obviously meets the requirements that it has long been treated as *the* form of representation of information, *the* language of the nerves. Recent evidence, however, reveals several other languages and compels a reconsideration of the familiar examples. For the present, and until we can show that the relevant

receiving cell really reads the proposed parameter, we must call the available forms of representation "candidate codes." Here, then, I give the candidate codes as I see them, in trains of impulses in single neuronal channels, and put aside for the present the representation of information in ensembles of nerve cells and the nonimpulse forms of communication between nerve cells.

The usage of the word "code," thoroughly entrenched in the literature of neurophysiology, is of course different from a precise 1-for-1, alphabet-type code. It stands for an analogue or representation, interpretable by the normally relevant receiver. In this broad sense, the term is useful and introduces no ambiguity.

Candidate Codes in Trains of Nerve Impulses.—We have just seen the first type of code in the example of the touch receptor. The *instant of occurrence* of an impulse is all that is available. This is common in escape control systems and probably in others.

The second type, the classical average frequency code, is actually a class of distinct codes including (1) the instantaneous value, that is, the *most recent interval* between impulses; (2) a smoothed or a *weighted average* taken over some moving integration period; (3) the *frequency increment* expressed as additional impulses per unit time; and (4) the rate of change of frequency or, in other words, *frequency modulation.* Obviously these are not equivalent or interchangeable, and the parameter used will make a real difference to the system. The frontier in this field consists at present in recognizing the theoretical possibilities, looking for animals and situations within them that permit the isolation of one parameter from others, and building up a case for the candidacy based on the input-output function's having a reasonable sensitivity, a reasonable discrimination, a reasonable dynamic range, and a reasonable readability by the next neuron in sequence.

A third code of which we are quite sure is a spike *number* or *duration* of burst code. This can be thought of as a generalization of the first type. A class of cases is known, perhaps a very widespread one, in which there is repetitive firing of a fixed and noninformative frequency that carries information like a doorbell, represented by the instant of onset and the duration of the train (Fig. 2). Examples are the neurons controlling sound production in cicadas, electric organ discharge in the electric ray *Torpedo,* and electroreceptor input in knife fishes like *Hypopomus.*

Another possibility can be called *temporal microstructure.* Theoretically, a train of events at the same frequency might signal different messages by systematic manipulation of the succession of intervals, for example, in a simple case by alternating long and short intervals at a ratio of 5 to 1. This ratio could signal a different aspect of the input—for example, intensity—while the average frequency could signal another—for example, pitch of sound in the ear. We do *not* think that the ear works in this way and we do not know any instance in which this code seems to be used, but experiments on species that permit the necessary controls have shown that this code is readable by nerve cells in crayfish, lobsters, and mollusks. The dynamic range and discrimination are probably poor. Temporal microstructure grades into macrostructure when each cluster of im-

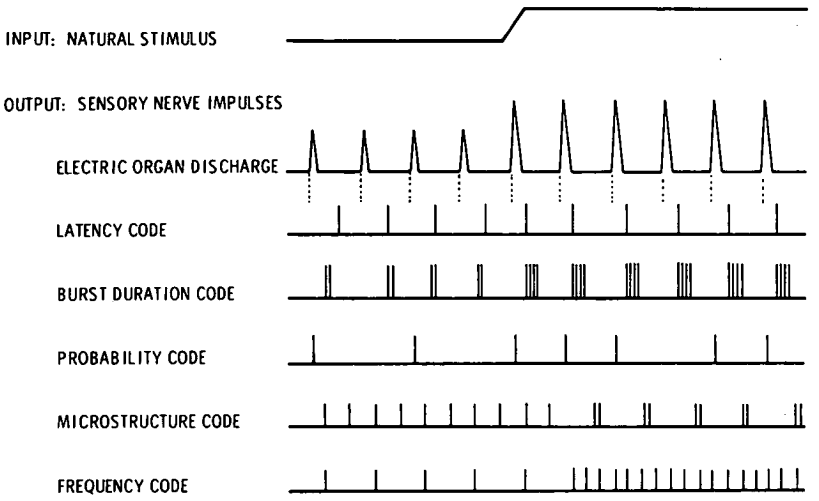

FIG. 2.—Summary diagram of several events plotted against time (in the range of 0.1–1.0 sec for the whole sweep), showing main types of nerve impulse codes, all of which probably exist in one place or another. Since several are best seen in electroreceptor nerve fibers of weakly electric fish, the chart includes a diagrammatic record of the fish's own electric organ discharge as seen in an electroreceptor on the skin, after the stimulus (approach of an object of different conductivity than the water) has modified its amplitude locally.

pulses itself represents a message, as in signaling vibration on the skin, or the beats of a hummingbird's wings.

Another available parameter is *interval variation*. This is not a generalization of the micropatterning just treated, since specific sequencing is not prescribed. Many nerve cells in different parts of the brain exhibit a characteristic degree and form of variation of intervals. Some have an asymmetrical distribution, with the modal interval shifted away from the mean, generally in the direction of a longer interval. There may be bimodal and multimodal distributions. It is clear that at least some of these differences, besides being available, are easily read by receiving neurons of ordinary properties, but we know of only a few neurons in which a systematic change in the form of the interval distribution takes place for a given neuron with different states or inputs. Certain cells in the retina change from a quite rhythmic to a very "bursty" distribution of impulses at about the same average frequency under illumination. Certain pyramidal cells in the cat motor cortex change from a more to a less regular pattern of firing at about the same average frequency when the cat goes from the waking to the sleeping state. Generally the variability of the intervals changes *pari passu* with changes of the mean frequency, and it is difficult to test the possibility that this systematic alteration of variability is itself useful to the system. There are great differences between types of nerve cells in the amount of variability. In electric fish, for example, there can be a play of less than 0.2 per cent in samples of many hundreds and even thousands of intervals and even at very low frequencies such as 10 per second, where the variation is usually largest. In a rattlesnake infrared receptor at 10 per second the range is several hundred per

cent, declining to about 10 per cent at 70 per second. Interval variation is perhaps usefully conveying information in some special cases, but, in general, experiments have not been done to test its possible contribution; it is usually regarded as noise.

Another type of code is illustrated by a class of units among the electroreceptors in peculiarly favorable electric fish that fire 1 for 1 in a strict phase relation with the discharge of the electric organ. There is no coding by number of impulses, frequency, variability, or microstructure, but as the normal physiological stimulus is increased and decreased by moving a dielectric or conducting object back and forth in the water beside the fish there is a systematic *shift in the latency* or phase of the nerve impulse relative to the electric organ discharge. It happens that there are other fibers that do not shift in latency and therefore give a point of reference for the measurement of the shifting units, so that this code is quite readable.

The last type I will mention may be called a *probability of missing* code, or sputtering. In the electric fish, for example, some units fire an impulse three out of ten cycles or four out of ten cycles or five out of ten cycles or six out of ten cycles, according to the intensity of the normal stimulus, an object in the water nearer or farther from the fish. This can be treated as a frequency code; we do not know how the receiving cells in the brain read it, but it is operationally distinct since the intervals change in a quantal manner.

Summarizing this section, we feel that there is reason to doubt that frequency is the only code employed, as has been the classical and textbook assumption. Five other properties of impulses in single axons are reasonable candidates: number, latency, variation, micropattern, and sputtering. At the same time, it is clear that there is no crucial experiment which proves that a candidate code is in fact used by the system, even when it is found to be available and readable. The criterion of relevance for the interest of the succeeding nerve cells is not met by a simple demonstration but rests on an accumulated understanding of the meaning and handling of information by the receiving units as in the reciprocal problem in communication science, of defining noise.

Sites of Lability: The Distinguishable Excitabilities.—How is the information in streams of impulses read? In particular, where are the sites that make decisions determining what weight is to be placed on different converging inputs, that mix and integrate these inputs according to preset biases, or that transfer functions to achieve filtering, abstraction, or recognition? These preset proclivities for weighting inputs represent the stored information from inheritance and past experience. Such questions compel a closer look at the nonimpulse forms of communication, beginning with the intracellular events leading up to the firing of impulses. Figure 3 repeats the main features of Figure 1 as an introduction to the steps where it is now known that lability can reside. I show a slightly more complex case here in having several receptive dendrites and a generator potential that exhibits a rapid and a slow phase of adaptation.

What happens to the resulting stream of nerve impulses? Figure 3 shows that a very important transition occurs in the terminals of the axon that carries the stream of impulses. At least sometimes, and perhaps generally, the impulses

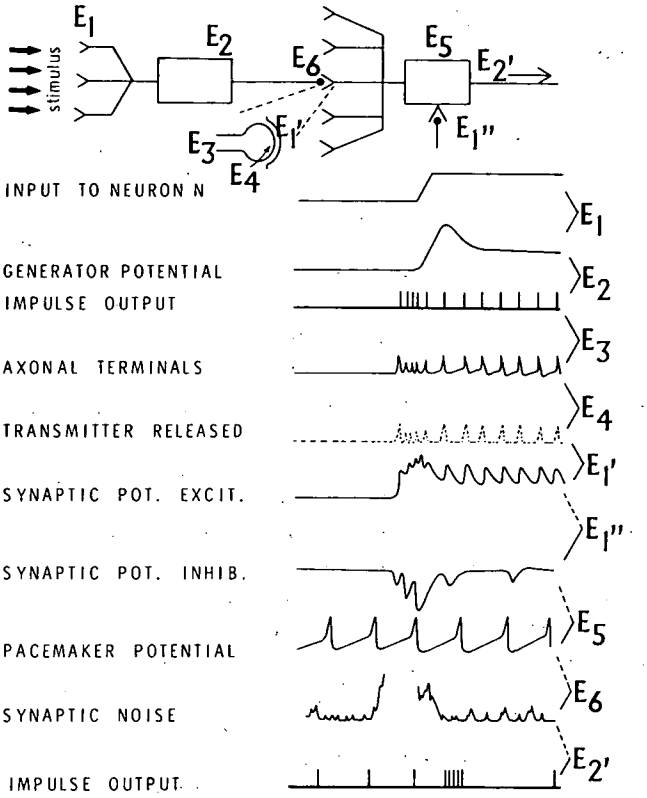

FIG. 3.—Summary diagram of events plotted against time (*ca.* 0.01–0.1 sec), showing several successive stages in the transfer of information from one nerve cell to another. The E's are the independent excitabilities or transfer functions and are the sites for possible molecular participation. Boxes above are two nerve cells of order N and $N + 1$; the synaptic contact between them (schematic) is enlarged to show the E's involved. A dashed line indicates transmitter released, to show that this record is hypothetical. Dashes on some of the E guidelines signify that the immediately preceding event is not the input for that E. See text for further explanation.

become decremental and slower-conducting; then follows a longer refractory period. The activity approaching the terminals is hence graded in amplitude, perhaps filtered against short intervals, and in many cases especially susceptible to alteration in amplitude in consequence of events outside the axon terminals that exert an enhancing or depressing effect.

Clearly we have already dealt with at least three distinct excitabilities. Contrary to the textbook statement, the neuron does not have *an* excitability measured by the threshold for the spike, but a series of separate excitabilities or transfer functions. The first one that we referred to was the function relating generator potential amplitude to impinging stimulus strength. This graded, local, nonlinear, actively amplifying response can be either excitatory or inhibitory in its consequences for subsequent steps. The second excitability and the only one

with a sharp threshold is that for spike initiation; it too is local since, once initiated, the spike is self-propagating with an excess amplitude such that the threshold is unimportant until conduction reaches the axonal terminals. The response in the terminals as a result of all the internal and external factors we have just mentioned is a third localized transform. Hereafter I will refer to these independent and localized excitabilities as E's.

In many if not most axonal terminals there is, then, a specific chemical transmitter released in an amount controlled by the arriving action potential and a set of events that are still poorly known (including the diffusion of this transmitter substance, its binding to specific chemical receptor molecules on the next neuron, called a postsynaptic cell, its decay by the action of a specific enzyme, and possibly feedback upon the terminal from which it was released). Because of our ignorance and not because there is only one, I refer to E_4 and subsume the overall transmitter effectiveness as a function of the presynaptic events.

It is a finding of sweeping importance that there are several E_4's, not just one. The diversity of submicroscopic forms and physiological properties of synapses makes it likely that they differ in this function even from place to place on the same postsynaptic cell and even when they employ the same chemical transmitter substance. In addition, there are at least two, and possibly more, different chemical transmitters in a given species of animal; these have separate release, diffusion, binding, decay, and possible feedback properties. In view of their parallel position I do not give them sequential numbers but refer to E_4', E_4'', E_4''' and so on, though this runs the risk of submerging their independence.

The postsynaptic membrane responds with a graded, local, specific membrane conductance change leading to a potential change called the postsynaptic potential. Note that this is therefore equivalent to the generator potential, and so I will again refer to it as E_1. Different transmitters have different effects in terms of the specific ions to which conductance is altered; hence, the magnitude and even the direction of the deflection of potential are also altered. Certain potentials tend to push the membrane toward the spike firing threshold and are therefore called excitatory postsynaptic potentials. Others tend to throttle this excitation, both by short-circuiting the excitatory postsynaptic potential and by countering it with a potential change of the opposite polarity, called the inhibitory postsynaptic potential. These have different properties and represent separate excitabilities, E_1', E_1''. Indeed, we should recognize that different excitatory synapses on the same neuron have different excitabilities, particularly in reference to their alterations over time as stimulation is repeated. Some show a facilitation with repetition, others the opposite, and both these types occur with faster and slower time courses in different cases, even on the same cell. Each of the processes and events that I am listing is a specific and localizable mechanism, presumably with a molecular basis and with an input-output function that is subject to various influences, as we shall see below.

There may be from one to thousands of synaptic endings on a given postsynaptic neuron. Each adds its contribution in synaptic potential—the fast ones and the slow ones, the facilitating and the antifacilitating, the excitatory and the

inhibitory, distal and proximal—each in proportion to preset transfer functions and the prevailing milieu.

Now we must bring in another domain of processes. It is of the utmost importance to realize the dramatic change in neurophysiological theory between the days when the system was principally thought of as a stimulus-response mechanism waiting like a complex telephone exchange to route messages when input is received, and the days when the ongoing background activity within the system became recognized. We are not sure that we know all the bases for this continual background activity, but we do know that in addition to whatever contribution may come from continual circulation of activity due to impulses in circuits and synaptic potentials like those just dealt with, there is also a class of events which is truly spontaneous in the strict sense of the term. That is to say, under permissive conditions in the medium, the nerve cell ticks like a clock, determining its moment of firing by its own intrinsic mechanisms. It is not independent of the environment for energy and bias, but independent of environmental pacemakers or triggers.

Figure 3 shows one form of such spontaneous pacemaker activity intrinsic to a nerve cell, in this case a relaxation oscillation form. This activity has two characteristics of importance for our argument. One is a locus from which it spreads its influence decrementally, to be mixed and added with all other forms of activity. The other is an excitability, E_5. This may be one of the most important forms of excitability of all, namely, the degree of alteration of the ongoing firing rate as a result of a given change in the conditions such as depolarization of the membrane due to current flow or ion accumulation. We know of several distinct types of slope to this relation and, indeed, different shapes of the curve with linear and nonlinear portions.

Another form of spontaneous activity is illustrated by so-called synaptic noise. In the best-studied examples, quantal, miniature deflections of potential of the synaptic region occur in a random sequence. Their probability of occurrence is altered by presynaptic conditions, which leads to the view that this is a transsynaptic manifestation of spontaneous unit events. Here then is another E on our list.

The integration of all these graded local subthreshold processes depends on the decremental spread of each according to the geometry and conductance and capacitance of the local dendrites; their confluences and algebraic summations, which are not necessarily linear; and their smearing and dispersion in time due to the appreciable distances and capacitances, hence selective filtering of some high-frequency input arriving farther out on the dendrites. Here and there, active subthreshold local responses occur as the summed depolarization builds up close to threshold. Once more the transfer functions are subject to influence by alterations of the specific conductances due to agencies from within or without the cell.

General Conclusions about Candidate Sites for Molecular Participation.—So much for an enumeration of the distinguishable processes, steps, and events that are indicated by our present knowledge of mechanisms at the cellular level. What then can we say about the possible sites for molecular participation in the

nervous or communication aspects of cell function as distinct from the vegetative or maintenance aspects?

In the first place, the "polytheistic" conclusion is strongly underlined—that there are *many separate steps and loci* where lability can occur—as opposed to the classical "monotheistic" view that there is but one site of lability, the synapse. We have at least seven *E*'s, some of which actually include several separate ones in parallel. Each of these is a transduction, a translation or transfer function that presumably rests upon properties of molecular systems subject to initial determination by genetic mechanisms and subsequent modification by suitable conditions. Clearly, then, if we are tempted to refer to "lowering the resistance of a pathway" in explaining an example of learning, for instance, we have a wide choice of sites.

In the second place, the sequential nature of these excitabilities means that there is a kind of *equivalence* among them in the sense that an alteration in the over-all input-output function cannot in general be caused uniquely by an alteration in any one of the *E*'s, but the effect of a certain alteration in one of the *E*'s has a number of equivalents in certain alterations in others. This in turn means not only that there is an inherent ambiguity in an alteration in any given step, but also that such an alteration will only be effective if all the other *E*'s remain stable.

Our third general point, then, is the profound implication that so many separate processes must remain *essentially stable.* With all the opportunities for drift in the many sequential excitabilities, it is impressive that long-term constancy of calibration is in fact common. Examples are the discharges of high-frequency electric fish, the sound of neurogenic cicadas, the accuracy of many ballistic movements, our own body temperature, weight and pitch and color judgments, and so on. A probabilistic basis for this is apparently not available in the best-studied examples.

Fourth, all the opportunities for molecular participation are of the nature of *displacing continuous input-output curves* this way or that, or warping them. To be sure, creating discontinuities, gates, or quantal saltations is certainly possible as a special case. For some incipient models of the way in which molecules, with their wealth of specificity, might participate in neural coding, it may be important that in all these communication functions there has yet to be found a role for specificity of alterations in molecules. Are there roles not simply equivalent to a quantitative shift in the proportions of sodium and potassium conductances, but comparable to the role which specificity plays in establishing connections during growth and development? That molecular specificity can make a quantal or qualitative change in neuronal circuit function is really an article of faith. Models of how this could be done are quite imaginable but cannot be taken for granted until they are explicitly proposed in testable form in the context of the known excitabilities. The burden of asserting a proposition for a way in which macromolecular specificity could be brought to bear on learning and memory, in this context, is on him who invokes it. Such formulated propositions are virtually lacking.

Summary.—Four general aspects of molecular participation may be noted: the many loci, the equivalence of many changes, the stability of many excitabili-

ties, and the need for explicitly stated roles for specificity. Obviously, in a subject of interest to students of memory, macromolecules, chemotherapy, and psychology, these observations will not satisfy, but provoke progress. If the conclusions are corrected thereby, I will be delighted to change. The speakers who follow are among those most likely to compel me to alter these views.

My final hope is that the time has come when, instead of making vague references to molecular species suspected of playing "some" role in altering nerve cell properties, we can graduate to proposing one or another of the known steps or excitabilities. Indeed, with the rapid advances in neurochemistry we can expect the gradual reliability of criteria aimed at distinguishing between chemical findings directly relevant to the communication and coding aspects and those only indirectly so. Among these criteria would be a direct tie to one of the events, stages, or sites that I have listed.

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THE NEURONAL MEMBRANE

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In my contribution to this symposium, I propose to discuss the structural organization of the neuronal membrane in relation to one of its most characteristic and specialized functions, the propagation of the nerve impulse along the axon. Although considerable information is available on the electrical behavior of the neuronal membrane, very little is yet known of its molecular composition and structure because of the severe difficulties in obtaining for biochemical study sufficient material uncontaminated by the membranes of other types of brain cells, particularly the glia. However, in the last two or three years, some extremely promising experimental and conceptual advances have been made in the study of other types of membranes,[1–7] particularly those of mitochondria and erythrocytes, as well as of synthetic phospholipid bilayer systems. These developments permit formulation of some molecular models and hypotheses for the structure and behavior of the neuronal membrane.

Association of Neurons and Glial Cells—the Greater Membrane Concept.—In most neurons, the axon is in near contact with or is surrounded by glial cells whose plasma membranes may form a sheath around the axon. In peripheral nerves, the myelin sheath consists of many concentric windings of the plasma membrane of the Schwann cell. There is now increasing evidence that glial cells provide specific metabolic or biochemical support to the axon although glial cells are not components of neuronal circuitry. Because of this imputed metabolic and functional relationship, the molecular interface between the neuronal and glial plasma membranes becomes of special significance. This interface is comprised of the outer cell coats of each cell type and the intercellular space. Although the cell coat or wall is often thought of as merely supportive or protective, there is now much evidence that it contains highly specific and sensitive receptors for chemical or hormonal signals from other cells (cf. ref. 1). Recent biochemical studies also suggest that the cell coat merges with and is probably part of the supramolecular organization of the plasma membrane. Similarly, the intercellular space between the neuron and the glial cell, across which some form of metabolic interplay occurs, is not merely a dilute aqueous solution of amorphous character, but very likely a structured matrix endowed with a number of interesting molecular properties. The greater neuronal membrane thus consists of three zones—the plasma membrane, the cell coat, and the immediately surrounding intercellular space (Fig. 1).

The Plasma Membrane.—The plasma membrane constitutes the real permeability barrier of the cell and gives the membrane its very high electrical resistance and capacitance. The plasma membrane along the axon is not noticeably different under the electron microscope from that of other types of cells, and shows the two electron-dense lines characteristic of most plasma membranes after $KMnO_4$ fixation. The most widely generalized molecular model of the structure of plasma membranes is that first proposed by Danielli and Davson[2] and

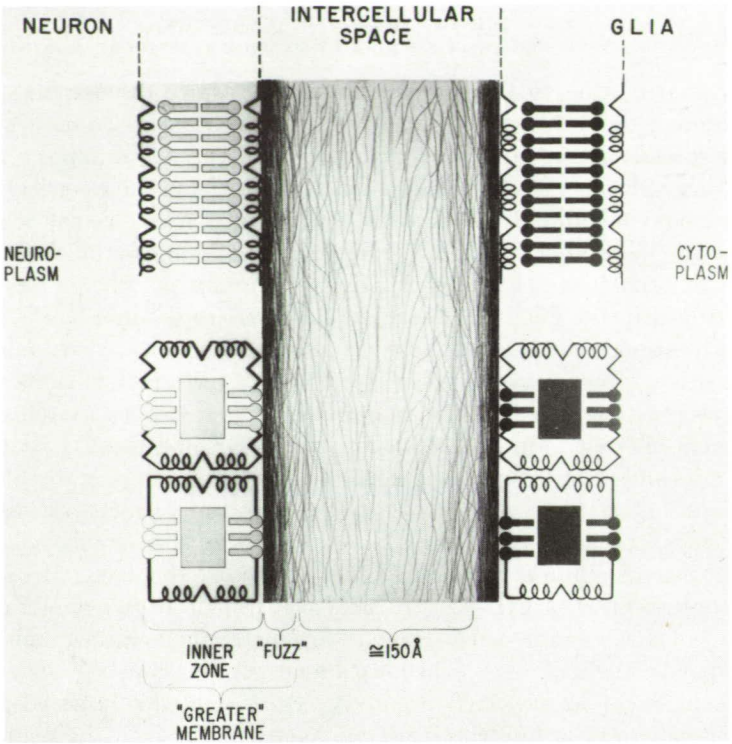

FIG. 1.—Diagrammatic representation of the "greater" membrane and intercellular space (courtesy F. O. Schmitt and the *Neurosciences Research Program Bulletin*).

later refined, particularly by Robertson,[3] as the unit-membrane hypothesis. It proposes that the lipids of the membrane, which comprise some 40–50 per cent of its mass, are arranged in a bilayer, with the hydrocarbon chains of the two lipid layers apposed to form a continuous, nonpolar hydrocarbon phase. On either side are monolayers of protein, which comprise some 50–60 per cent of the membrane mass. The dimensions of the unit membrane model are consistent with the results of X-ray diffraction studies of the myelin sheath and with the characteristic 35-Å clear space between the two 20-Å electron-dense lines visualized by electron microscopy after permanganate fixation. The clear zone is assumed to correspond to the hydrocarbon phase of the bilayer. Although the unit membrane hypothesis was originally postulated to account for the basic structure of all types of membranes, it is now certain that different membranes may vary substantially in molecular composition (particularly of the lipids), enzymatic activity, transport functions, thickness, and in the type of image yielded by high-resolution electron-microscopic techniques, such as negative contrast and freeze-etching.[4–7] Furthermore, the plasma membrane of any given cell type is not necessarily uniform over its whole surface and may be locally differentiated at desmosomes, tight junctions, and synapses, and may indeed possess

a microscopic nonuniformity as in a two-dimensional molecular mosaic. In addition, membranes may also change locally in structure as a function of activity, as will be discussed below. There is, however, one feature of the unit membrane structure, namely the lipid bilayer, which still accounts best for the characteristic permeability and electrical properties of plasma membranes, and it serves as a convenient point of departure for consideration of other membrane models.

Each type of membrane contains a characteristic set of complex lipids in a specific molar ratio, which appears to be genetically determined. The complete lipid composition of the axonal membrane is not yet known, but it differs significantly from that of the glia and of the myelin sheath.[8] Each of the polar or amphipathic phosphoglycerides of membranes contains a polar "head" and two nonpolar hydrocarbon "tails" contributed by C_{16}-C_{20} fatty acids or aldehydes, which are about 20 Å long when fully extended. The polar heads of the different membrane lipids differ quite significantly in size, conformation, and electrical charge. Just as the side chains or R groups of the 20 different amino acids of proteins are now known to be determinants of the three-dimensional structure of proteins, the characteristic polar heads of the 15 or 20 different types of polar lipids found in most membranes may similarly be determinants of membrane properties. Presumably each type of lipid may contribute some specific characteristic or property to the membrane. Most plasma membranes also contain considerable cholesterol, which forms hydrophobically stabilized complexes with those phosphoglycerides having unsaturated fatty acids. In such complexes the fatty acid tails of the phosphoglyceride become immobilized and rigid. Plasma membranes, because of their relatively high cholesterol content, are more rigidly structured and "tighter" than other types of membranes (cf. ref. 9).

One of the strongest pieces of evidence in support of the lipid bilayer model is the fact that, in the complete absence of protein, phospholipids in aqueous systems spontaneously form bilayers in the form of flat micelles, as large closed vesicles or bubbles, or as laminar, stacked structures called myelin figures. Most pertinent are the studies by Mueller and Rudin[10] and by Thompson and co-workers,[11] who have shown that phospholipid bilayers may be formed in apertures separating two aqueous phases. Such bilayers have low permeability to polar solutes, high permeability to water, and, significantly, extremely high electrical resistance and capacitance, comparable to that of the plasma membrane. Such lipid bilayers can also be made electrically excitable in the presence of an ion gradient,[12] and they have therefore become extremely important models of natural membranes.

Membrane Structure Proteins.—Little is known of the molecular structure of uncontaminated neuronal membrane protein, although the proteins of myelin have been examined in some detail. However, research on the structure protein of other membranes[13, 14] suggests that the monomeric form may have a molecular weight in the range 20,000–50,000 and that the monomers readily associate with each other to form insoluble aggregates stabilized by hydrophobic interactions. Optical rotatory dispersion and circular dichroism spectra of the proteins of different types of plasma membranes are very similar, which suggests that the

peptide chains of membrane proteins are folded in a characteristic manner and that they may contain some α-helical characteristics.[15, 16] Recent work also suggests that there are several, and possibly many, different molecular species of structure proteins in a given membrane. Membrane structure proteins are undoubtedly genetically coded, and they may in turn code or specify the specific content and ratio of the various lipids of the membrane. One hypothesis (cf. ref. 17) suggests that each species of membrane protein may be able to bind selectively a single type of membrane phospholipid. Thus a two-dimensional array of different species of membrane structure proteins might code a two-dimensional mosaic of specifically bound lipids.

Nuclear magnetic resonance measurements have revealed that the proteins are relatively fixed in the membrane structure, but that the lipids have considerable freedom of movement.[9, 18] The polar heads of the lipids behave as though they are in a dilute aqueous environment and they are at least partially susceptible to enzymatic attack by phospholipases.[19] The hydrocarbon tails are relatively free to move, although, as noted above, they are much more rigid and fixed in those membranes having a high cholesterol content.

Models of Membrane Structure.—Membrane models may be classified into bilayer and globular systems. Figure 2 shows some bilayer models, which differ largely in the conformation of the peptide chain and in the degree of penetration of the hydrocarbon zone by the peptide chain. The protein has been variously suggested to be in the extended or β-configuration, in partially α-helical form, and also in globular arrangements. Benson[20] has suggested a model in which the protein is wholly within the lipid bilayer, whereas Lenard and Singer[15] suggest that the peptide chain may form cross-links across the membrane. That some membranes may have a globular subunit construction has been suggested by high-resolution electron-microscopic observations (see refs. 4, 5, 7, 21–23). Membrane models containing globular lipid micelles, globular proteins, or combinations of both have been proposed (Fig. 3). Globular models as usually schematized do not appear to account as satisfactorily for the high electrical resistance of resting plasma membranes as do bilayer models, since they contain "soft" spots which could allow electrolytic conductance. It appears possible

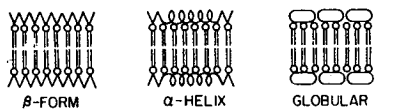

FIG. 2.—Some membrane models based on a lipid bilayer. Very recent infrared data appear to exclude β-conformations of the protein, at least in the erythrocyte membrane.[18]

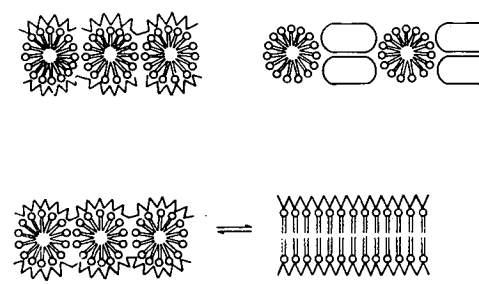

FIG. 3.—Some membrane models based on globular arrangements.

that neuronal membranes may undergo reversible transitions between two or more bilayer forms, or between bilayer and globular forms, during the action potential; if so, these forms must have similar electrical capacitance.[24] Such transitions during an action potential might be detectable optically, for example, by means of circular dichroism measurements.

The Outer Coat of the Neuronal Membrane.—The outer coat of the neuronal membrane, when normally fixed for electron microscopy, manifests low electron density. Its presence may be inferred from the fact that the dense outer lines of the plasma membranes of adjacent neurons and glia ($KMnO_4$ fixation) are not observed to touch or fuse, at least along the axon. The minimum spacing between the outer lines of two adjacent cells (150–200 Å) suggests that each coat must be at least 75 Å thick. The outer coat can be partially penetrated, however, by certain electron-dense materials such as ruthenium red or lanthanum hydroxide (cf. ref. 1).

The outer coat of neurons appears to contain little or no collagen and no chondroitin or dermatan acid mucopolysaccharides such as are present in the coats of other mammalian cells. There are, however, two classes of carbohydrate-rich structures present in the cell coat, namely the glycolipids and glycoproteins. Like the phospholipids, the glycolipids possess polar heads and two long hydrocarbon chains, which are probably present in the outer layer of the lipid bilayer structure. Simple glycolipids contain monosaccharides or short oligosaccharides as their polar heads; they have no electrical charges. Among these are the cerebrosides, which are not found in neurons, but are present in glial membranes and the myelin sheath. Of far greater interest are the gangliosides, which are characteristic of neurons and not found in glia.[26] Although gangliosides are relatively minor components of the neuronal lipids (<5% of the total), they are probably the most specific lipids of neurons. The gangliosides, like other lipids, have two hydrocarbon tails and long branched polar head groups composed of oligosaccharides containing one or more residues of sialic acid, a generic term referring to N-acetyl or N-glycolyl derivatives of the nine-carbon sugar derivative neuraminic acid, which is negatively charged at pH 7.0. There are several classes of gangliosides, containing one, two, three, or more sialic acid residues. Monosialogangliosides predominate along the axon, whereas polysialogangliosides are more profuse at nerve endings.[27]

Gangliosides very likely play a dynamic role in neurofunction and are not merely structural lipids. Brain tissue incubated *in vitro* in the absence of oxygen loses its electrical excitability and simultaneously loses sialic acid residues from its gangliosides. Addition of gangliosides to such inactivated brain tissue restores its electrical excitability.[28] Moreover, it has been found that injected C^{14}-labeled glucosamine, a precursor of sialic acid, is quickly incorporated into the glycoproteins and/or gangliosides of nerve endings.[29] Gangliosides bind univalent cations such as Na^+ and K^+, divalent cations such as Ca^{++} (cf. ref. 26), and polycations such as protamine very strongly, presumably at the negatively charged carboxyl groups of the sialic acid residues. In fact, the electrical excitability of neurons can be blocked by adding protamine and can then be restored by the addition of gangliosides.

The glycoproteins of cell coats share an important common denominator with the gangliosides; they also contain sialic acid residues. One class of glycoproteins contains 40–50 per cent carbohydrate, in the form of a small number of long, branched oligosaccharide side chains containing sialic acid termini; these side chains are linked covalently to the amide group of asparagine residues in the peptide chain backbone. The other type of glycoprotein contains a much larger number of short disaccharide side chains covalently linked to the hydroxyl groups of serine, threonine, and possibly hydroxylysine residues. Most of the side chains contain a terminal residue of sialic acid. Cell surfaces possess a net negative charge which in a number of cases has been shown to be contributed largely by sialic acid residues (cf. ref. 30).

Figure 4 shows a hypothetical model of the cell coat and plasma membrane.

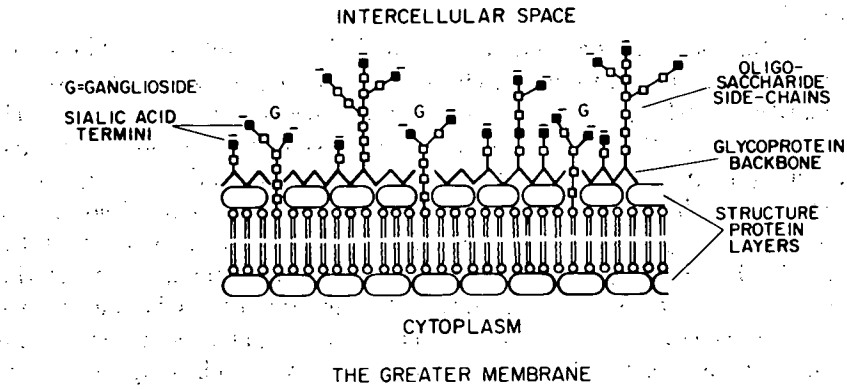

FIG. 4.—A model of the plasma membrane and cell coat.

It suggests that the negatively charged heads of the gangliosides may extend well outside the cell coat and into the intracellular space, like antennae. Their sialic acid groups are thus exposed to extracellular Na^+ and Ca^{++} ions. Changes in concentrations of these ions in the neighborhood of these antennae could cause conformational charges which might be transmitted mechanically to the hydrocarbon bilayer and induce local changes in its packing arrangement. It is suggested that the glycoproteins are superimposed on the monolayer of structure protein.

The plasma membrane–cell coat complex is shown as having molecular asymmetry, or sidedness, in keeping with the fact that plasma membranes bring about vectorial enzymatic activities such as energy-linked uptake of K^+ and discharge of Na^+. Such anisotropy allows the coupling of vectorial transmembrane processes to scalar enzymatic activities in the cell, in accordance with the Curie principle. Another noteworthy feature of this model is its composite or layered structure, which has a number of theoretical advantages for vectorial transport of ionic solutes.[31]

The Adjacent Intercellular Space.—In addition to glucose, amino acids, inorganic electrolytes, and minor amounts of protein, the intercellular aqueous phase

of the brain, which is about 15 per cent of its total volume, contains hyaluronic acid. This linear polysaccharide has a molecular weight in the millions; every other monosaccharide unit bears a negative charge at pH 7.0. Because of the like electrical charges along its length, hyaluronic acid does not assume a compact, highly folded conformation, but rather an extended rigid form which tends to fill the entire volume of a dilute solution. Hyaluronate thus occupies a large domain, whose volume may exceed the volume of the hyaluronate molecule itself by several thousandfold. This highly charged and highly hydrated molecular "jungle" impedes the flow of water and tends to exclude other macromolecules from its domain.[32] The many negative charges on hyaluronate also have the capacity to immobilize Na^+, Ca^{++}, and other cations; conversely, other cations cause partial collapse of the rigid, extended structure of hyaluronate with profound modification of its viscosity and space-filling properties. During passage of the action potential along an axon, the rapid movements of Na^+ and Ca^{++} ions between the axonal membrane and the surrounding intercellular space can be expected to cause transitory changes in the conformation of the acidic polysaccharides in the intercellular space which can in turn modulate their own tendency to immobilize water and simple cations, possibly in a feedback manner.

That the intercellular space may play a dynamic and important role in neurofunction has been suggested by the interesting work of Adey[33] and his colleagues. They have shown that learning in animals is accompanied by characteristic changes in the electrical impedance of certain areas of the brain. They suggest that these impedance changes occur in the intercellular space and reflect changes in the cation-binding properties of hyaluronic acid and other negatively charged macromolecules. They suggest further that the learning engram may be extracellular, in effect the result of the smoothing of a pathway through the molecular jungle of the intercellular space.

Membrane Structure, Divalent Cations, and the Action Potential.—For many years it has been thought that movements of Na^+ and K^+ are specific elements in the excitation of the neuron and the propagation of the action potential, particularly since the classical work of Hodgkin[34] and Huxley showed that the sum of the electrical currents carried by the entry of Na^+ and the exit of K^+ can be quantitatively related to the transmembrane potential and the transmembrane conductance at all points in the action potential. Specifically, it has been assumed that the first result of stimulation is the entry of Na^+. However, more recent experiments on the squid giant axon, particularly by Tasaki and his colleagues,[35] are opening a new chapter in our understanding of the role of cations in the excitation process.

After removal of the internal cytoplasm from the squid giant axon by mechanical extrusion, the empty axon can be continuously perfused internally with a solution of known electrolyte concentration. Simultaneously, the electrolyte composition of the external bathing solution can also be varied. The electrical excitability of such axon preparations (which, however, still retain a myelin sheath) can be preserved for long periods, provided an appropriate ion gradient is maintained across the membrane by perfusion. The most surprising finding with

this approach is that neither Na^+ or K^+ is required on the inside or outside of the membrane to support excitability. Axons perfused internally with the phosphate salts of the univalent cations Li^+, Rb^+, or Cs^+, or substituted ammonium ions, can maintain excitability and show an action potential if they are externally bathed with a divalent cation such as Ca^{++}, Ba^{++}, or Sr^{++}, despite the completely "unphysiological" nature of the internal and external ions. Na^+ or K^+ are thus not specific requirements for exciting the membrane. In fact, the axon membrane is still excitable when internal K^+ is replaced with Na^+, so that the normal outward → inward Na^+ gradient across the membrane is reversed.[35]

Tasaki and his colleagues have concluded that their data are not in accord with any hypothesis for the excitation process that requires as the first event a specific increase in Na^+ permeability and influx of Na^+ into the axon.[35] They propose that in the resting state, the outer region of the axon membrane contains bound divalent cations, probably Ca^{++} derived from the external medium. On stimulation by an outward-directed current, some of the bound Ca^{++} is replaced by univalent cations derived from the internal medium (K^+ in normal nerve). This ion-exchange process is suggested to trigger a change in the conformation of membrane subunits to a second state, in which membrane conductance is increased, which permits Na^+ to diffuse in and K^+ to diffuse out. At the end of the action potential the membrane-bound K^+ is displaced by Ca^{++} again, returning it to its original resting state of conformation. Such an exchange of divalent and univalent cations at specific anionic sites on the membrane with accompanying change in membrane properties also appears to occur in the membrane of isolated mitochondria.[36] It is possible that the Ca^{++}-binding sites, which are small in number, may be provided by specific sialic acid residues of the membrane gangliosides. Actually, only a very small fraction of the membrane area need undergo loss of Ca^{++} to cause its all-or-none excitation.[35]

Another important opening is the discovery that the action potential can be blocked by extremely minute concentrations of tetrodotoxin, the highly lethal toxin of the ovaries of the fugu, or Japanese puffer fish. This toxin, as well as a similar one from a California salamander, blocks the exchange of univalent cations for divalent cations at anionic binding sites, preceding the influx of Na^+ and efflux of K^+ once excitation has taken place.[37] The site of action of tetrodotoxin is on the outer region of the axonal membrane; tetrodotoxin perfused inside the axon has little effect.[38, 39] In titration experiments, Moore and Narahashi[38] have concluded that there are only about 13 such tetrodotoxin-sensitive cation binding sites per square micron of membrane surface in the lobster axon, confirming Tasaki's view that cation exchange at only a few molecular units in the membrane suffices to convert a very much greater number of units into a conformational state allowing passage of Na^+ and K^+.

Cooperative Interactions in Membrane Functions.—Two properties of the neuronal membrane suggest that it undergoes cooperative transitions. One is the finding that interaction of only a few molecular sites in the membrane is required to trigger a change of the whole membrane. The other is the all-or-none nature of the response of the neuron to increasing stimulus intensity (Fig. 5). These properties are suggestive of the behavior of certain solid-state systems studied by

the physicist[40] and of the behavior of allosteric or regulatory proteins and enzymes studied by the biochemist.[41] At least three investigators (Nachmansohn,[42] Tasaki,[35] and Changeux[43]) have postulated that electrical or chemical perturbation of a few specific membrane subunits causes a change in their conformation and that this change in conformation is then physically transmitted to neighboring subunits in a cooperative manner. The membrane subunits are considered to exist in two states: the resting and the excited states. The free energy difference between these two states cannot be large, since only a very small energy input suffices to trigger the change. Presumably there is a phase transition, or a transition between two metastable states. In principle, the membrane can be visualized in the same terms as the hemoglobin molecule when it undergoes oxygenation, with its classical sigmoid dependence on oxygen partial pressure.

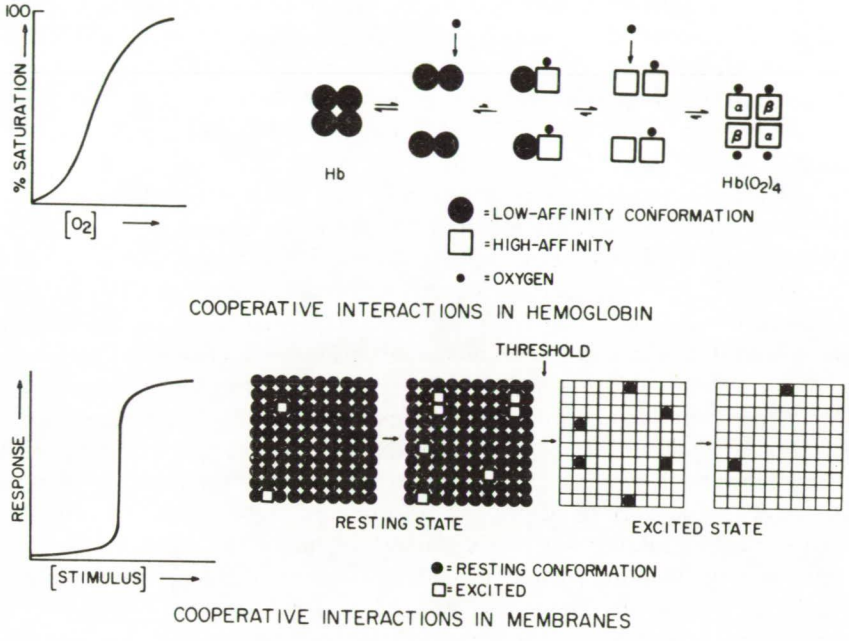

FIG. 5.—Cooperative interactions in hemoglobin and in membrane structure.

In the neuronal membrane the sigmoidicity is of a much higher order, so that it shows all-or-none and amplification characteristics (Fig. 5). Changeux has treated the response of neurons to acetylcholine by a thermodynamic formalism similar to that developed for allosteric enzymes.[44] Recent work in Nachmansohn's laboratory suggests that the Ca^{++}-binding subunit of the neuronal membrane is the acetylcholine receptor molecule. Binding of acetylcholine to this receptor is proposed to cause a conformational change of the latter, leading to release of bound Ca^{++}, which sets off a membrane change of the kind suggested by Tasaki. Nachmansohn has recently reviewed the properties of acetylcholine esterase and acetylcholine receptor proteins.[45]

Permeability-Inducing Antibiotics.—Recent work on respiration-linked ion movements in isolated mitochondria has led to another advance in the understanding of molecular aspects of the excitation of the neuron. Work of Lardy,[46] Chappell, Pressman, and others has shown that there is a large class of toxic antibiotics that can induce the movement of K^+ or Na^+ through mitochondrial membranes. Most of these antibiotics are circular molecules. One of the best studied is valinomycin, a circular peptide of 12 amino acid residues; it permits K^+ but not Na^+ to penetrate the membrane.[47] Another is nonactin, a macrocyclic tetrolide which forms a complex with K^+, the configuration of which has been analyzed by X-ray methods;[48] the K^+ is bound by anionic groups inside the "doughnut hole." These antibiotics are believed to bind in the membrane to create ion-specific tunnels or channels. It is of the greatest significance that these antibiotics, as well as other uncoupling agents of oxidative phosphorylation, such as 2,4-dinitrophenol, induce specific ion permeability not only in the intact mitochondrial membrane, but also in protein-free synthetic phospholipid bilayers. The mitochondrial uncoupling agent 2,4-dinitrophenol, for example, can make artificial membranes specifically permeable to H^+ ions, so that they behave quantitatively like a proton-sensitive glass electrode;[49] valinomycin makes such membranes behave like a K^+ electrode.

One of the most significant recent papers in membrane science is the report by Mueller and Rudin[12] that the macrocyclic antibiotic alamethicin, which is a charged circular molecule, can impart electrical excitability to a synthetic phospholipid bilayer in an aperture separating two aqueous phases having different concentrations of K^+. Such an artificial membrane shows an action potential on electrical stimulation and an increased cation conductance. This effect of alamethicin was found to be highly cooperative; the electrical response of the synthetic membrane was found to be proportional to the sixth power of the electrical stimulus or to the sixth power of the alamethicin concentration. Such synthetic bilayers containing alamethicin were found to simulate the electrical behavior of a wide variety of natural membranes. Mueller and Rudin have postulated that a group of five or six alamethicin molecules, each binding a K^+ ion, may aggregate into a stack which may extend through the membrane. Normally, in the resting state, this stack does not allow free passage of K^+. However, when the membrane is perturbed by the electrical stimulus, one of the charged alamethicin subunits is assumed to undergo conformational change as a result of the electrical stress. This change is cooperatively transmitted to the entire stack of alamethicin molecules and results in its directional opening and vectorial discharge of the K^+ out of one side of the membrane. This rationalization of the action of alamethicin suggests that a similar principle underlies the operation of the normal Na^+ and K^+ gates of the neuronal membrane. These gates may be comprised of analogous stacks of circular Na^+-binding and K^+-binding molecules which are normally closed and do not allow Na^+ and K^+ to pass through when the membrane is in the resting state. When stimulated, they change conformation in a cooperative manner and allow unidirectional passage of Na^+ and K^+ down their gradients, and thus produce an action potential. Another important consequence of the work of Mueller and Rudin in reconstituting

electrical excitability of synthetic bilayer membranes is that it provides striking and compelling support for the lipid bilayer as the central low-dielectric core of natural excitable membranes.

Summary.—Recent advances in the study of natural membranes suggest some models for the supramolecular structure of the neuronal membrane that may account for at least some of its characteristic properties. The negatively charged groups in the outer surface or coat of the greater neuronal membrane, as well as the occurrence of negatively charged acid mucopolysaccharides in the intercellular space, may be fundamental to the exchanges of Na^+ and Ca^{++} ions occurring during excitation and recovery, and possibly in learning and memory. The hypothesis that the neuronal membrane is a two-state system capable of undergoing changes of state by cooperative conformational transitions of its subunits, analogous to the behavior of allosteric proteins, appears to account for the all-or-none nature of neuronal excitation and for the fact that alterations in only a few specific receptor sites, possibly involving acetylcholine and Ca^{++}, can produce profound changes in Na^+ and K^+ movements. These developments, as well as the capacity of certain antibiotics to confer electrical excitability on synthetic phospholipid bilayers, promise to open the door to a molecular biology of neuronal transmission.

After this manuscript had been completed, Keynes and his colleagues reported (Cohen, L. B., R. D. Keynes, and B. Hille, *Nature*, **218**, 438 (1968)) that the light-scattering properties and birefringence of the squid giant axon undergo significant change as the action potential passes, an observation that strongly supports the occurrence of conformational or phase transitions.

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SYNAPTIC TRANSMISSION

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Nerve cells communicate with each other through specialized regions of contact called synapses. There are estimated to be about 4×10^{11} such contacts per gram of guinea-pig cerebral cortex;[1] this suggests that the human brain may contain as many as 10^{14} contacts, a level of connectivity truly remarkable in both its complexity and its degree of miniaturization. Contacts morphologically similar to synapses occur between nerve and muscle or secretory cells, and since much of our knowledge of synaptic transmission has been obtained with the latter types of contact, it is convenient to consider all of them together.

Types of Synapse.—Chemical synapses: The contact which a motor nerve makes with striated muscle, the neuromuscular junction (Fig. 1), is perhaps the synapse—in this broader sense—about which most is known. The axon of the motor neuron undergoes an expansion at its ending to form a flattened bag, the under surface of which is closely applied to, but does not touch, the postjunctional membrane. The cleft between the pre- and postjunctional membranes is enlarged by the existence of folds in the latter; its function seems to be to prevent the excitation of the postjunctional element by the electrotonic spread of current from the presynaptic nerve when impulses traveling down the axon reach the terminal. Transmission of excitation across the gap is achieved instead by the release of a specific chemical transmitter substance, the identity of which is known in this case: it is acetylcholine. If acetylcholine is applied in minute amounts to a junction through a micropipette, it generates a graded postjunctional potential similar to that seen during synaptic transmission; the synaptic and evoked potentials respond similarly to the presence of drugs.[2]

Some years ago, the important observation was made[3] that in the resting junction there were small, transient postjunctional potentials occurring randomly in time, similar to the full-scale potentials that signalize transmission, but too small to generate a contraction. The known electrogenic properties of acetylcholine showed that these miniature potentials could not be caused by the resting diffusion of acetylcholine out of the presynaptic nerve terminal molecule by molecule, but rather that "packets" or quanta of acetylcholine variously estimated to contain between 1,000 and 10,000 molecules were being released in random fashion. The full-scale potential recorded during transmission was shown to be due to the synchronized release of a large number of these quanta over a very short time interval. Similar "minipotentials" have been detected at central synapses,[4] and it is thought that transmitter release is quantized there too.

About the time that minipotentials were discovered, the first high-resolution electron micrographs of synapses were being made.[5–8] These showed that a highly characteristic feature of the presynaptic terminal cytoplasm was the presence of large numbers of small vesicles about 500 Å in diameter (Fig. 1*B*). It was natural to speculate that these might be the morphological counterparts

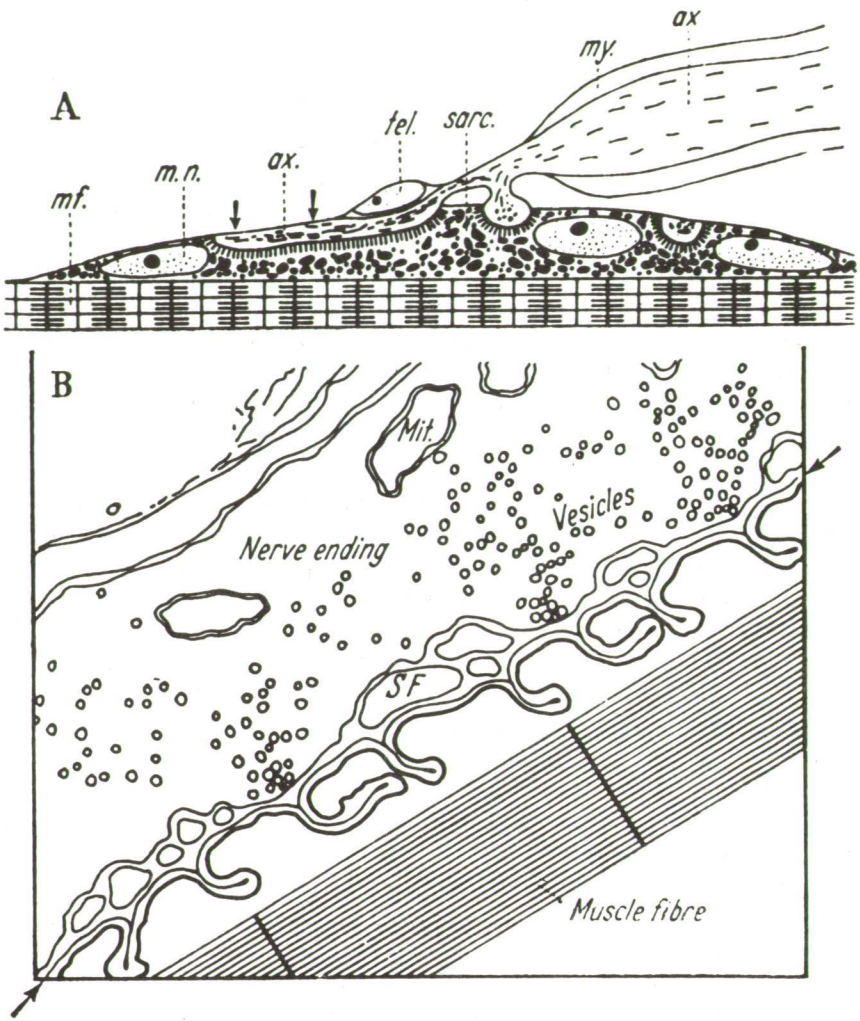

FIG. 1.—(*A*) Diagram of a motor endplate showing axoplasm (*ax.*) and myelin (*my.*) of motor nerve, and saclike terminals (*arrows*) lying in gutterlike depressions of the mitochondrion-rich muscle sacroplasm (*sarc.*). The terminals are protected by teloglia (*tel*), and muscle nuclei (*m.n.*) and myofibrils (*mf.*) are also diagrammed.

(*B*) Tracing of an electron micrograph of a portion of the nerve terminal similar to that between the arrows in (*A*). Note the highly folded postsynaptic membrane extending into the muscle sacroplasm, the fingerlike projections of teloglia (*SF*), and the numerous vesicles and mitochondria (*Mit.*) in the terminal cytoplasm.

of the quanta. Structures of this size could contain the requisite amounts of acetylcholine. It has since proved possible to isolate very pure preparations of synaptic vesicles and to demonstrate directly that they contain acetylcholine in roughly the right amount.[9]

In the central nervous system (Fig. 2) the terminal axons and their presynaptic swellings are much smaller than at the neuromuscular junction, and considerable

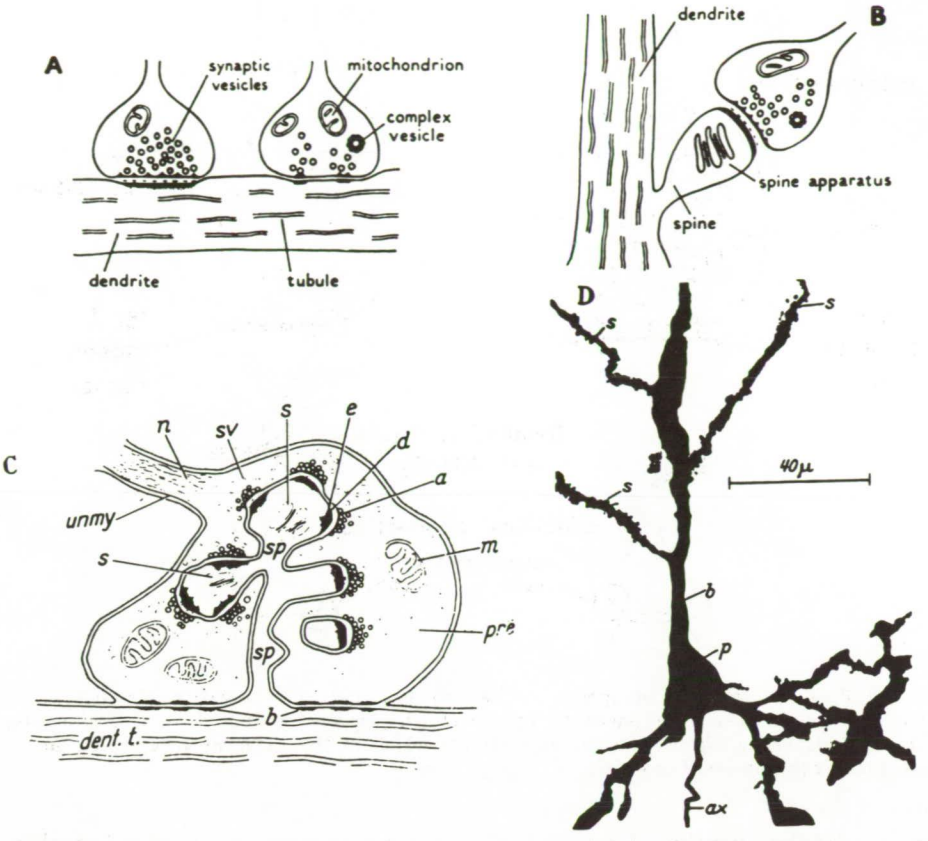

FIG. 2.—Types of central synapse. (*A*) Type 1 and type 2 synapses on a dendrite. (*B, C*) The attachment is with a dendrite spine (*sp*) which, in hippocampal pyramidal cells (*C*) may invaginate the presynaptic ending (*pre*). Synaptic vesicles (*sv*) often form clusters (*a*) opposite thickenings (*d, e*) on the spine, which is seen to contain an array of flattened vesicles and membranes (*s*). The unmyelinated preterminal axon (*unmy*) contains neurofilaments (*n*); the dendrite, anchored to the presynaptic nerve terminal by symmetrical attachment plaques (*b*), around which there is no special concentration of vesicles, is filled with neurotubules (*dent. t.*). (*D*) shows a Golgi preparation of a cortical neuron in which the perikaryon (*p*), dendrites (*b*) with spines (*s*), and axon (*ax*) are visible.

morphological variations occur. However, in most vertebrate synapses the clearly defined gap between pre- and postsynaptic membranes and the presence of numerous 500-Å vesicles in the presynaptic terminal cytoplasm are constant features which lead us to believe that such synapses utilize chemical transmitters also, though not necessarily acetylcholine.

Besides the 500-Å vesicles, four other types—larger vesicles about 1000 Å in diameter, elongated vesicles,[10] dense-core vesicles, and "coated" vesicles—occur in certain types of terminal and in terminals fixed in certain ways. These differences in vesicle morphology may reflect differences in the type of transmitter utilized; thus there is good evidence that dense cores are associated with the monoamine transmitters noradrenaline and 5-hydroxytryptamine,[11] and some

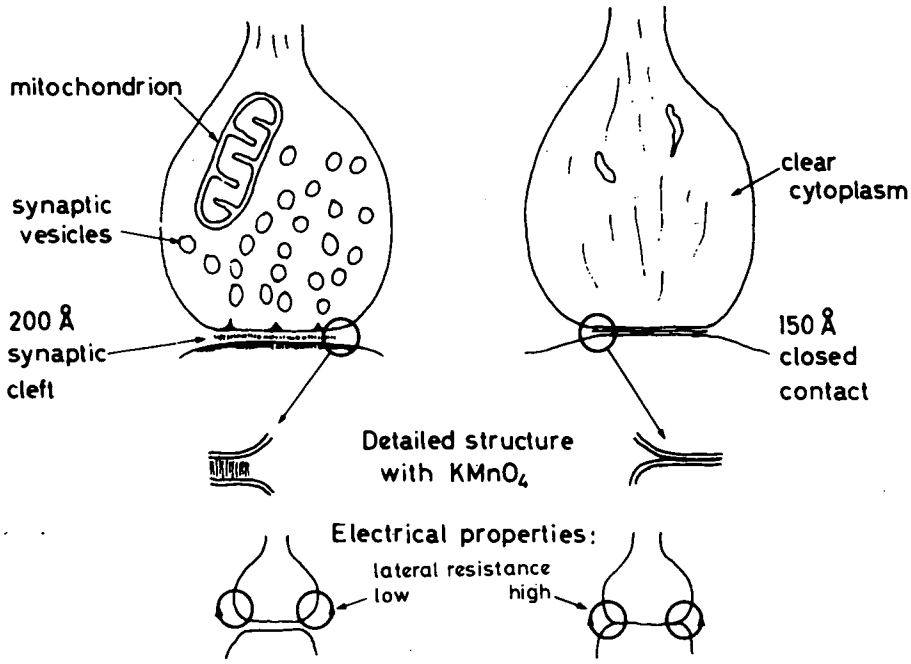

FIG. 3.—Characteristics of open, chemical synapse (*left*) and closed, electrical synapse (*right*). Note in the latter synapse the absence of synaptic vesicles and cleft and the invasion of the postsynaptic elements by action currents which in the chemical synapse are short-circuited by the low-resistance cleft.

reason for thinking that elongated vesicles (observed most clearly after formaldehyde fixation) may be characteristic of synapses exerting an inhibitory effect on the postsynaptic cell.[12] The presumed chemical transmitter at such synapses brings about a hyperpolarization, that is, an increase in the membrane potential of the postsynaptic cell (probably by increasing the permeability of the membrane to chloride ions), thus making it more difficult to depolarize by excitatory transmitters.[13]

Electrical synapses: The identification of synapses as chemical on the basis of their morphology has been strengthened by the discovery[14] of true electrical synapses, that is, synapses in which transmission occurs as the result of the electrotonic spread of current from the pre- to the postsynaptic cell. Such synapses are characterized morphologically[15] (Fig. 3) by presynaptic swellings filled with a watery cytoplasm from which vesicles are absent and by close contact, without any intervening 200-Å cleft, between the pre- and postsynaptic membranes. Mixed types of synapse are also known: significantly, the vesicles in such synapses occur in the neighborhood of the 200-Å cleft; the cytoplasm adjacent to regions of close contact is free of vesicles.

The Identity of Central Transmitters.—Acetylcholine: Besides being the transmitter at the neuromuscular junction, acetylcholine is also the transmitter at autonomic ganglia, at certain postganglionic autonomic endings, and at very

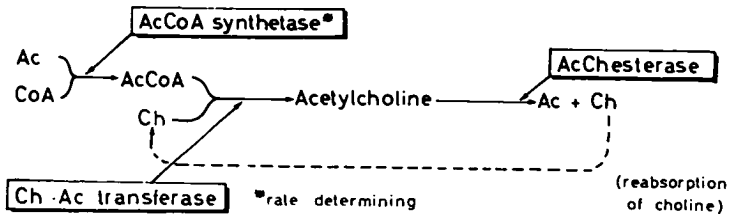

FIG. 4.—Synthesis and destruction of acetylcholine by nervous tissue. (*Ac*, acetate or acetyl; *Ch*, choline.)

many central synapses. It is synthesized in nervous tissue (Fig. 4) by the enzyme choline acetyltransferase from acetyl-coenzyme A and choline, and rapidly hydrolyzed after release to acetate and choline by the enzyme acetylcholinesterase. Since the products have less than one ten thousandth of the transmitter activity of acetylcholine, this hydrolysis very effectively terminates the transmitter action. Degeneration studies show that all three components—choline acetyltransferase, acetylcholine, and acetylcholinesterase—are constituents of cholinergic neurons and, although concentrated in the nerve endings, are distributed in smaller amounts throughout the length of the neuron. However, there is evidence that acetylcholinesterase is functionally on the outside of the neuron and only comes into contact with acetylcholine after the latter has been released. Acetylcholinesterase hydrolyzes acetylthiocholine as well as acetylcholine, and in the presence of copper the thiocholine released is precipitated. This may be used as the basis of a histochemical method for cholinesterase, in which the copper thiocholine is converted to a black deposit of copper sulfide.

With the use of this method, it has been shown that many of the cholinergic neurons of the central nervous system form a great ascending system of neurons originating from cell bodies in the reticular formation and radiating to all parts of the forebrain: to the hypothalamus, thalamus, visual pathway, striatum, hippocampus, and neocortex.[16] This system appears to be identical with the ascending reticular activating system, which is responsible for electrocortical arousal and which may well be involved in consciousness and the wakened state.

The monoamines: Noradrenaline, dopamine, and 5-hydroxytryptamine make up a group of related monoamines to which a central transmitter role has been ascribed. Unlike acetylcholine, these amines can be directly visualized histochemically by taking advantage of the green or yellow fluorescent compounds that they form with dry formaldehyde gas. This method has been brilliantly developed and used by a large group of Swedish workers to trace peripheral and central monoaminergic neurons. Once again, the transmitter is found to be highly concentrated in the nerve terminals, but by special methods cell bodies and axons can also be rendered fluorescent. Such studies show that central neurons containing these transmitters are organized into fairly well defined tracts.[17] The ascending tracts are less extensive than, and distinct from, the cholinergic tracts, but like the latter, they start from the midbrain and innervate many of the same forebrain regions.

Amino acids: There remain many central synapses not accounted for by cholinergic and monoaminergic neurons. There is increasing evidence that certain amino acids, particularly glutamate, γ-aminobutyrate, and glycine may have a transmitter role. Many central synapses are concerned not with excitation but with inhibition. Recent work, using iontophoretic application and intracellular recording from single units by means of micropipettes, shows that γ-aminobutyric acid in the cerebral cortex[18] and glycine in the spinal cord[19] have all the requisite properties for inhibitory transmitters in those areas. Another amino acid, glutamate, has the property of firing most nerve cells, but the changes in the electrical properties of the cell membranes that it produces do not exactly parallel those produced by synaptic stimulation. Both glutamate and γ-aminobutyrate are effective in extremely small amounts; about 10^{-15} of a mole will cause a detectable effect on a single cell. These amino acids, with certain others, exist in nervous tissue in uniquely high concentration; and γ-aminobutyric acid and the enzyme glutamate decarboxylase, which forms it from glutamate, do not occur in other mammalian tissues.

Many drugs that affect the central nervous system are believed to do so by interfering in one way or another with chemical transmission, and some of the most powerful are structural chemical analogues of putative transmitters. In Figure 5 the chemical relationship between certain transmitters and some well-known hallucinogens and tranquilizers is indicated. Significantly, the hallucinogenic agents are chemical analogues of either acetylcholine or the monoamines, substances which, as we have seen, are transmitters in ascending systems possibly concerned with consciousness and perception.

We can only surmise why the evolving nervous system turned to chemical transmission in order to achieve communication between nerve cells. Storage of transmitters in presynaptic nerve terminals may be a device to ensure unidirectionality of transmission: a property of the synapse but not of the axon, and probably essential for the orderly processing of information in the central nervous system. Antidromic impulses traveling up axons into cell bodies are unable to travel further, because the postsynaptic cell does not contain the transmitter whose release is essential for communication across the gap.

Another reason for chemical transmission may be to reduce unwanted "cross-talk" between intertwined but functionally distinct systems. Iontophoretic application of putative transmitters to single neurons of the nervous system shows how chemically specific nerve cells are. A single unit in the cat cortex may be excited by low doses of glutamate, but not at all by acetylcholine. If the tip of the pipette is moved only a few microns deeper, contact may be made with a cell that is responsive to both compounds.

The Isolation of Presynaptic Nerve Terminals and Their Component Organelles.—Synaptosomes: When brain tissue is homogenized in iso-osmotic media (e.g., 0.32 *M* sucrose) under conditions of relatively mild shear, most of the acetylcholine of the original brain tissue remains bound to particulate material, in which state it is both immune to the action of cholinesterase and pharmacological inactive. It may readily be released by procedures that disrupt lipoprotein membranes. The particles bearing the bound acetylcholine may be separated

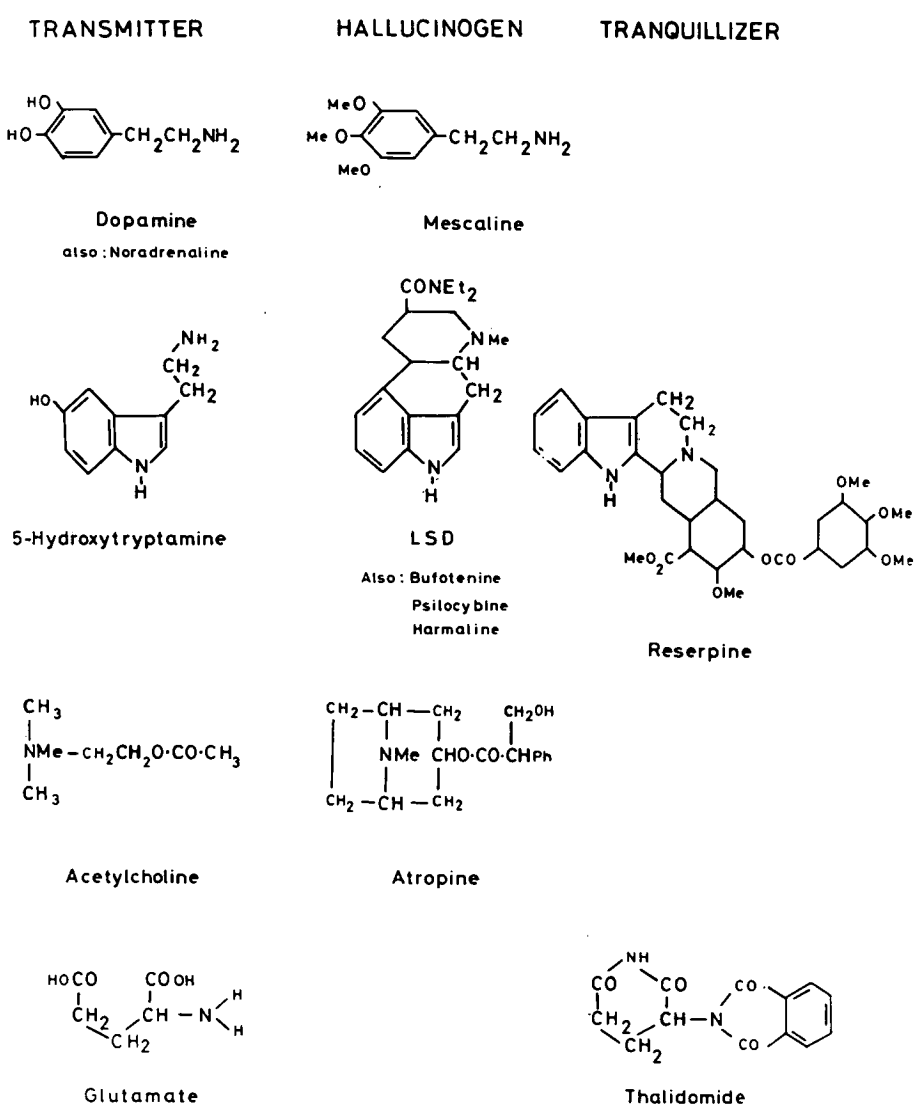

FIG. 5.—Structural relationship between central transmitters and psychoactive drugs.

from nuclei, glial and neuronal fragments, mitochondria, microsomes, and other products of homogenization by differential and density-gradient centrifuging.[20] The fraction so obtained is also rich in other putative transmitters such as noradrenaline and 5-hydroxytryptamine.[21] The significance of these findings became clearer when it was discovered that the fraction consists of an enriched preparation of detached presynaptic nerve terminals[22, 23] to which the name "synaptosomes" has been given.[24] Apparently the physical properties of the presynaptic terminals are such that they survive the general cellular disruption that accompanies homogenization, and "pinch off" to form detached, sealed

structures that retain both the morphology and the transmitter content of the original endings (Fig. 6).

The successful isolation of presynaptic nerve terminals has provided a new type of *in vitro* preparation with which to study the molecular processes involved in the synthesis, storage, release, and ultimate inactivation of transmitter substances,[25] and the effects of drugs on these processes. Other problems that may be studied with synaptosome preparations include the dynamics of axonal flow[26] and the intraterminal synthesis of protein,[27] which may be of considerable importance in relation to the problem of the plasticity of synaptic connections and the mechanisms of memory and learning. The preparation should also serve as a source of new transmitters, provided suitable means can be devised for their detection.[28]

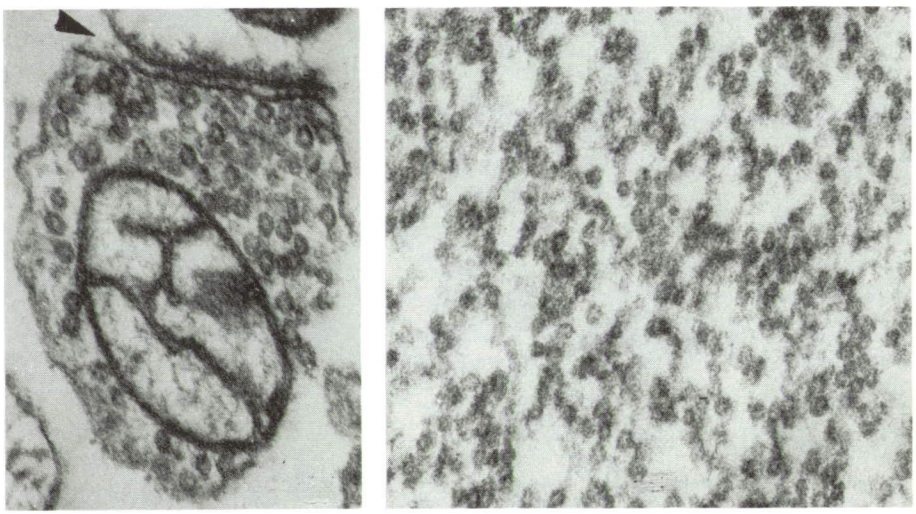

FIG. 6.—Electron micrographs of (*left*) synaptosome (×85,000) showing external membrane, cytoplasm containing synaptic vesicles and a small mitochondrion, and (*arrow*) adherent length of postsynaptic membrane; and (*right*) isolated synaptic vesicles (×70,000).

Recent work with gel filtration through Sephadex[29] in the author's laboratory has confirmed earlier impressions that synaptosomes are sealed structures and contain constituents of soluble cytoplasm, including glycolytic enzymes. Though rather labile, they respire well if incubated with substrates (especially an equimolar mixture of glucose and succinate) and cofactors at 25–30°C, and this respiration is coupled to the synthesis of high-energy phosphate compounds. Actively metabolizing synaptosomes take up choline by a Na^+-dependent, hemicholinium-sensitive mechanism similar to that found in brain slices.[30] The permeability of the external membrane to ions is similar to that of mammalian C-fibers. Thus, in many of their properties, synaptosomes behave as miniature cells and can be used as model neurons.

Synaptic vesicles and external synaptosome membranes: Synaptosomes are osmotically sensitive, and on suspension in water swell up and burst, releasing a

proportion of their cytoplasm, synaptic vesicles (Fig. 6), and intraterminal mitochondria. Density gradient centrifuging permits the separation of these components from each other and from the external membranes of the synaptosomes.[24]

Such preparations enable the chemical organization of the presynaptic terminal to be studied.[31] Acetylcholine exists within the synaptosome in two fractions, a cytoplasmic and a vesicular; it has been estimated that there are about 2000 molecules of acetylcholine per cholinergic vesicle, which is within the lower range of estimates of the size of a quantum of acetylcholine.[9] The two fractions are quite distinct, as has been shown in labeling experiments,[32] the vesicular fraction being negligibly labeled under conditions that result in considerable cytoplasmic labeling. Even in whole brain, the vesicular fraction is more slowly labeled than the cytoplasmic when tritiated choline is the precursor.[33]

By contrast, at the ionic strength prevalent in intact synaptosomes, the enzyme synthesizing acetylcholine, choline acetyltransferase, is all in the soluble cytoplasmic compartment of the synaptosome.[34] The mechanism whereby vesicular acetylcholine is synthesized remains obscure.

The enzyme and lipid composition of vesicles and external membranes has been compared and found to differ in several ways.[35] External membranes contain Na^+, K^+-activated adenosine triphosphatase, cholinesterase, and ganglioside in considerable concentrations; these components are not present in vesicles. External membranes, like myelin and the plasma membranes of other cells, such as liver cells, have a cholesterol:phospholipid molar ratio close to unity; synaptic vesicles have a ratio of about 0.4, similar to microsomes, which are mainly derived from internal membranes.

These differences in composition are relevant to the still unsolved problem of how the transmitter is released from the terminal. On the assumption that release involves the vesicular fraction as the quantal hypothesis requires, it seems unlikely that the vesicles simply fuse with the external wall, but rather likely that they form a membrane system distinct from, but communicating through, the external presynaptic membrane. There might well be an intercommunicating system of fine tubules—hard to see by normal histological methods—composed of protein or another macromolecule that could open or close in response to ionic changes induced by action potentials. In negative staining, fine interconnections between vesicles, as well as detached and exceedingly fine fibrillar material,[35] are sometimes seen.

Postsynaptic membranes: Synaptosomes are not infrequently seen with portions of postsynaptic membranes adhering to them[23, 9] (Fig. 6). The synaptosome preparation is thus a useful potential source of postsynaptic membranes and of the material responsible for the adhesion of the pre- and postsynaptic membranes, a knowledge of which may well be important for our understanding of how specific contacts are made in the developing nervous system.

Summary.—Electron-microscopic, histochemical, and iontophoretic techniques support the concept that transmission at most synapses is brought about by the release of small amounts of chemical transmitter substances. There is now much circumstantial evidence that acetylcholine and noradrenaline, long established as

transmitters in the peripheral nervous system, are central transmitters also. Other central synapses, excitatory and inhibitory, may utilize certain amino acids; among the most potent pharmacologically when applied to single cells are glutamate, aspartate, γ-aminobutyrate, and glycine.

There is also evidence that certain major systems of neurons utilize particular transmitters. Thus acetylcholine and the monoamines appear to be involved in ascending systems concerned in some way with the conscious state; by contrast, γ-aminobutyrate and glycine are the putative transmitters of inhibitory cells in the cortex and spinal cord, respectively.

Chemical transmission may thus be thought of as a kind of chemical coding and may have developed as a device for elimination of unwanted "cross-talk" between functionally distinct systems of neurons. The localization of the transmitter in the presynaptic nerve terminal is also a guarantee of unidirectionality of synaptic transmission—a prerequisite for the orderly relaying of information in the central nervous system.

Many drugs that act on the central nervous system are structural analogues of chemical transmitters, and a useful hypothesis is that they act by interfering at one point or another with the molecular mechanisms involved in the synthesis, storage, release, and ultimate destruction of central transmitters. Certain hallucinogens, e.g., mescaline, LSD, and atropine, are structural analogues of the transmitters (noradrenaline, 5-hydroxytryptamine, acetylcholine) believed to be utilized in the neuron systems involved in consciousness and affective states. The possibility is also being actively discussed that certain psychoses may result from the impaired or abnormal metabolism of some of this group of transmitters.

In recent years, a new approach to the problems of the mechanisms of transmitter metabolism, storage, and release has been made possible by the discovery that presynaptic nerve terminals can be isolated from nervous tissue. If the tissue is dispersed under appropriate conditions in isotonic sucrose, the nerve terminals are pinched off and torn away from their attachments to form sealed structures termed synaptosomes. Centrifugal isolation techniques permit the synaptosomes to be separated from other tissue elements.

Synaptosomes retain both the fine structure and the transmitter content of the original terminals. From them may be prepared samples of terminal cell sap, external membranes, intraterminal mitochondria, and synaptic vesicles. These last are the characteristic organelles (about 500 Å in diameter) of the presynaptic nerve terminals. A study of these organelles is permitting conclusions to be drawn regarding the molecular basis for the storage and release of transmitter substances and the mode of action of drugs and toxins on these processes.

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*FIBROUS PROTEINS—NEURONAL ORGANELLES**

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At the turn of the century, thin fibrous structures, having thickness close to the resolution of the light microscope, were seen in silver-stained preparations of many kinds of nerve cells. Called "neurofibrils," these fibrous proteins were thought to be vital organelles of neurons and at first were believed to conduct the nerve impulse. After the surface membrane was found to be the site of bioelectric properties, the role of the fibrous protein had to be reassessed. Physiologists tended to regard neurofibrils as artifacts of fixation. Polarization optical evidence supported the existence in fresh axons of a fibrous protein having thickness small with respect to the wavelength of light.[1] At midcentury the fibrous protein was observed by electron microscopy in unfixed preparations[2, 3] and subsequently, by several investigators, in thin sections of fixed preparations. The protein became known as the *neurofilament;* the term *microfilament* is a generic term for similar structures found in many types of cells.

For many years the fibrous proteins of axoplasm have been studied in our laboratories and attempts made to characterize them physicochemically and to determine their function. Progress was slow because only small amounts could be obtained from the giant fibers of the squid *Loligo pealii* from Cape Cod waters during the summer months. Brain as a source of protein was not practical at that time because of the lack of an identifying marker constituent such as a particular amino acid (as collagen is characterized by hydroxyproline, for example). The problem of year-round supply of adequate amounts of fresh axoplasm was solved by procuring it from the large squid *Dosidicus gigas*, available during most of the year at the Marine Biological Station of the University of Chile near Valparaiso.

Microtubules, recently shown to be present generally in plant and animal cells, represent a portion of the fibrous proteins of neurons and probably play a role qualitatively similar to that played in other cells, i.e., to subserve processes of motility and transport of solutes and particulates directionally within cells. Microtubules are also thought to provide mechanical strength and rigidity which could be of importance in neurons, with their enormously extended axonal and dendritic processes. A more detailed presentation of the evidence concerning the probable function of microtubules and neurofilaments will be given in a report of a Neurosciences Research Program work session by Schmitt and Samson.[4]

Electron-Microscopic Characterization of Neurofilaments and Microtubules.—With the electron microscope two fibrous forms are observed in neurons: neurofilaments and microtubules. The two types resemble each other in fundamental ultrastructure and in chemical composition. Both types are shown in the electron micrograph of Figure 1. Neurofilaments are about 100 Å in diameter; microtubules are 240 Å in diameter, with a wall thickness of 50 Å. In longitudinal sections both structures are straight, apparently rigid, and unbranched, extending

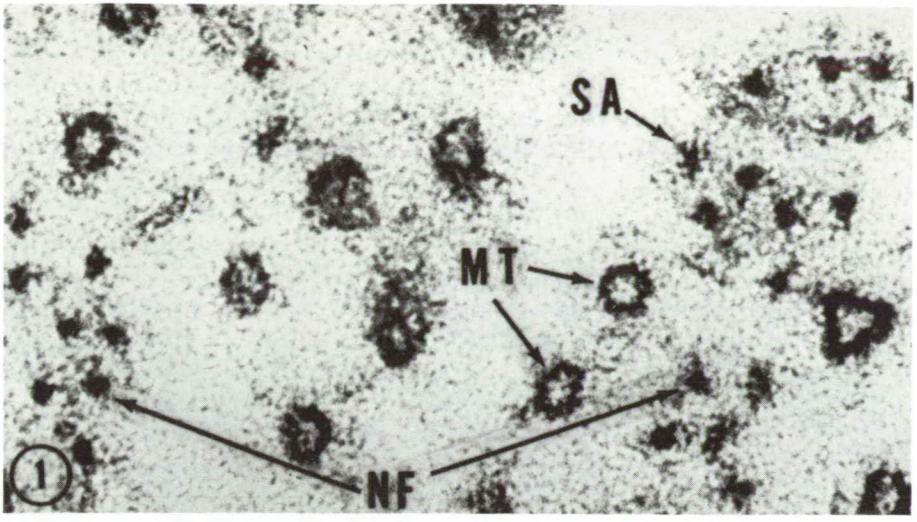


FIG. 1.—Electron micrograph of anterior horn cell of the rat, showing microtubules (*MT*) and fascicles of neurofilaments (*NF*) in a dendrite. Neurofilaments are similar to those in cell bodies and axons. Notice subunits in neurofilament walls, also radiating side arms (*SA*). Magnification, 280,000. Original electron micrograph courtesy S. L. Palay and comparable with those shown by Wuerker and Palay.[5]

continuously in the axon or dendrite. Cross-bridges between microtubules have been observed by Kohno,[6] who found them particularly evident in the region of the axon hillock. Radially directed outgrowths like spokes, which may represent cross-bridges, are frequently seen on the neurofilaments (Fig. 1). As in the case of muscle, cross-bridges may be important functionally.

Overproduction of fibrous protein is characteristic of brain cells in the presenile dementia called Alzheimer's syndrome and in certain abnormalities such as neurolathyrism, produced experimentally in animals by feeding compounds like iminodipropionitrils or by treating the brain with aluminum salts.[7] Characteristically, twisted microtubules or bifilar neurofilaments pack the cells.[8, 9] This overproduction of fibrous protein may be so great as to lead to the exclusion of other vital organelles, such as mitochondria, and to subsequent cell death.

Because some ten million people in this country alone suffer from senile manifestations of various kinds, the problem is one of much clinical concern. It is also of importance in aging processes. Investigation of factors controlling the production of fibrous proteins is therefore a critical one of great human concern as well as scientific interest.

Biophysical and Biochemical Characteristics of Neurofilaments and Microtubules.—During the last decade improved methods have been developed for the purification of the fibrous proteins from axoplasm extruded from the giant nerve fibers of *Dosidicus gigas*. In the presence of 0.1 *M* mercaptoethanol the unrefrigerated material is stable for shipment from Chile to our laboratories in Cambridge. Neurofilaments such as those shown in the electron micrograph in

Figure 2 may be obtained by zone sedimentation in a sucrose gradient or by freezing, which causes the filaments to separate upon thawing.

Dried cylindrical extrudates of axoplasm have high positive birefringence.[12] However, wide-angle X-ray diffraction patterns obtained from such preparations show no evidence of orientation of the fibrous proteins; only two rings characteristic of unoriented backbone and side-chain spacings are recorded. These findings indicate that neuronal fibrous protein is not of the α or β type.

P. F. Davison and E. W. Taylor,[13] of our laboratory, suggested in 1960 that neurofilaments are built of globular subunit protein molecules, somewhat along the lines of tobacco mosaic virus (TMV). Their model is illustrated in Figure 3. Globular subunits of 30–40 Å in diameter are depicted as forming strands which are wrapped about each other to form a filament 80–100 Å in diameter. Unlike TMV protein, the neurofilament has neither RNA nor nucleotide component. A central aqueous channel *ca.* 30 Å in diameter, though unnecessary for the theory, was pictured.[14] Several investigators have confirmed the general features of the model by high-resolution electron micrographs obtained from transverse sections of axons.

Because the protein subunits, as in TMV and other viruses, have a strong affinity for each other, isolation of monomer subunits in the native state is extremely difficult. This pronounced affinity may be due primarily to bonding of hydrophobic residues within the subunit molecules. Increase of ionic strength or of pH leads to partial depolymerization of neurofilament strands. Reversible aggregation of these subunits into the native helical structure has not yet been achieved in such preparations.

In our laboratory, Davison and F. C. Huneeus[15] have studied the protein subunits constituting the neurofilaments. Dissociation of the filamentous structure may be accomplished by treatment with detergents, by dissolution in concentrated 6 *M* guanidine hydrochloride, or by succinylating the protein and blocking the thiol groups. Chromatography of such dissociated preparations on agarose columns[16] has resolved two components, the smaller of which has an amino acid composition similar to that of microtubules and probably makes up the subunit skeleton of the neurofilament. The second component, also an acidic protein, has a high content of serine (*ca.* 12%) and may be an asymmetric protein (giving hypersharp peaks in the ultracentrifuge pattern). This protein may conceivably represent the fuzz, spikes, or cross-bridges intimately associated with the neurofilament protein as seen in the electron micrographs of thin sections of neurons of various kinds.[5, 6, 17] The function of this material remains unknown.

Microtubule protein has not yet been isolated from squid neuroplasm, though its colchicine binding is high.[18] Colchicine binds strongly to microtubule protein (as originally demonstrated for the microtubules of the mitotic apparatus) and also to neuroplasmic protein, presumably microtubule protein, according to Borisy and Taylor.[18] Weisenberg and Taylor[19] used this property to isolate microtubule protein from mammalian brain homogenates. According to Taylor,[4] the protein thus isolated, having a molecular weight of about 60,000, has amino acid composition similar to that of microtubules generally, to squid neurofilaments, and to actin. Microtubules in certain tissues are unstable at

low temperatures and high pressures, but are stabilized by D_2O, suggesting that subunit interbonding is primarily by hydrophobic forces.

Recently, Kirkpatrick[20] has fractionated microtubules, having characteristic appearance in electron micrographs, from homogenates of mammalian brain.

For comparison with the model of neurofilament structure, the model for microtubules is shown in Figure 4 (see also Tilney and Porter[21]).

Functioning of Microtubules in Cells Generally.—Microtubules are thought to give rigidity to long slender cell processes such as the axopods of Heliozoa;[21] to provide motility in the case of cilia and sperm tails, perhaps by some kind of sliding-filament mechanism;[22] and to mediate transport of cell particulates, e.g., in the movement of melanin granules into and out of the elongate arms of melanocytes[23] and of secretory vacuoles from the Golgi apparatus to the periphery of the rhabdite in *Planaria.*[24]

One of the most graphic examples of microtubule-implemented intracellular transport has been demonstrated by Rudzinska[25, 26] in the feeding process of the protozoan *Tokophrya.* The feeding apparatus in this sessile suctorian consists of tentacles—fine, thin, stiff tubes which broaden considerably at the tip. At the periphery of the knoblike tip are complex missilelike particulates containing enzymes. When a swimming ciliate such as *Tetrahymena* touches the knob of the tentacle, the ciliate becomes attached and immobilized. Apparently the protruding ends of the particulates are sticky and release a toxic substance into the prey upon contact. At the same time a stream of additional particulates starts to move rapidly outward through the tentacle from the cytoplasm of *Tokophrya.* Shortly thereafter, connection is made between the tentacle and the prey cell; constituents of the cytoplasm of the prey pass through the tentacle into the body of the predator. The two streams move in opposite directions, though not simultaneously: the outward stream of the particulates precedes the inward flow of the prey's cytoplasm. Forty-nine microtubules, arranged in seven clusters each consisting of seven microtubules, divide the thin cylindrical tentacle roughly into an outer tube, in which the missile-shaped particulates travel centrifugally, and an inner tube, in which cytoplasmic constituents pass as food from the prey centripetally into the predator cell.

It seems improbable that such directional movement is due to flow resulting from gradients of hydrostatic pressure; rather the microtubules might provide a structural basis for the active transport of food from the prey to *Tokophrya.*

Neuroplasmic Transport.—During the last 20 years, P. Weiss and his collaborators[27–29] have shown that neuroplasm constantly flows unidirectionally from the cell body down the axon. It is estimated that neuroplasm equivalent to one to three times the volume of the nerve cell is synthesized per day. Weiss has interpreted the phenomenon as one of maintaining metabolism of parts of the axon remote from the cell center. This discovery, which has given a new dynamic dimension to neurobiology, calls for explanations at the molecular level. The phenomenon has now been confirmed by radiolabeling experiments in many laboratories.[30] In addition to the slow, 1–6 mm/day velocity,[29–32] much faster rates (hundreds of millimeters/day[33–35] and even up to 2800 mm/day[36]) have been observed. This fast movement, which appears to be specific with respect

to the substance transported, may be saltatory, i.e., discontinuous translatory movements, quite different from Brownian movement.[37] Neuroplasmic transport, which is bidirectional in thin channels containing a colloidal system of high structural viscosity, is not fully understood at the present time. However, it is improbable that particulates are transported by gradients of hydrostatic pressure; rather, a local topochemical reaction on the surface of the microtubule seems responsible. One of the most striking cases in support of this conclusion is the rapid (100–200 mm/day) movement of catecholamine storage granules down the axons of sympathetic C-fibers which are only 0.5–1.0 μ in diameter.[33] Hydrostatic pressure gradients can probably be ruled out; they would have to overcome great shear forces to move particles so rapidly in such thin tubes. Microtubules appear to be the chief or only fibrous structures seen with the electron microscope in such thin nerve fibers. It is reasonable, therefore, to suggest that a local interaction on the surface of the microtubules is responsible for such rapid, probably saltatory movements. The hypothalamic neurons, in which secretion granules move at high velocities (e.g., 2800 mm/day or 32 μ/sec[36]), have recently been shown to contain many microtubules;[38] this finding supports the view that the transport is caused by the interaction between microtubules and secretion granules.

Mechanochemical Coupling in Transport.—How can rapid, saltatory transport of particulates such as vesicles, secretion granules, melanine granules, and even whole nuclei be explained at the molecular level? Clearly a transduction of chemical to mechanical energy is involved. The detailed mechanism by which mechanochemical coupling occurs, though unknown, is of great biological interest.

Perhaps by reference to muscle—the tissue differentiated for mechanochemical coupling—we may gain some insight into the coupling process. According to the sliding-filament theory,[39–42] the fibrous proteins myosin and actin do not themselves contract when muscle shortens. Rather the filaments slide by each other due to the thrust exerted on the actin by the heavy meromyosin cross-bridges. At the end of the cross-bridge is the enzyme adenosine triphosphatase (ATPase). Bound to the actin is ATP, the source of energy for the contraction. At the binding site, actin combines with myosin, forming actomyosin. The ATPase, activated by Ca^{++}, presumably at another site on the actin, splits off

FIG. 2.—Electron micrograph of neurofilaments isolated from axoplasm of squid giant axon, as described by Maxfield.[10, 11]

FIG. 3.—Model of subunit organization of neurofilament, after Davison and Taylor[13] and Schmitt and Davison.[14] (*A*) Monomer subunit; (*B*) subunit strand; (*C*) neurofilament, longitudinal view; (*D*) transverse section.

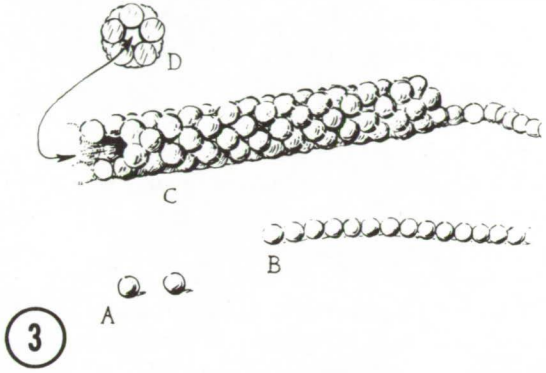
FIG. 4.—Model of subunit organization of microtubules.

FIG. 5.—Postulated mechanism of active transport of vesicles by interaction with microtubules; model based on the sliding-filament hypothesis of mechanochemical coupling in muscle. On the left is shown a subunit strand of a microtubule; on the right are subunits in the protein wall of a vesicle. Significance of sites for binding the two proteins and for activating the ATPase (or GTPase) is described in the text.

FIG. 6.—Diagrammatic illustration of the roles of the axonal surface membrane in propagating the action potential and of the axoplasm in transporting certain vesicle-enclosed transmitters to synaptic endings by means of fibrous proteins.


2


MICRO-
TUBULE
240 Å
4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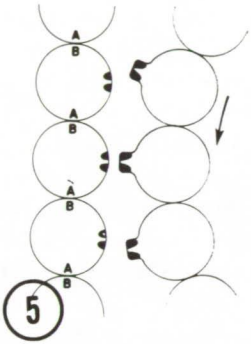
A
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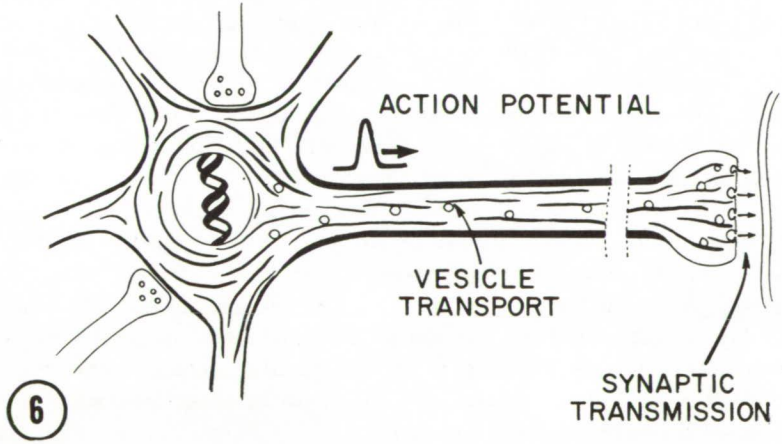
ACTION POTENTIAL
VESICLE
TRANSPORT
SYNAPTIC
TRANSMISSION
6

ATP; and energy is transferred, causing conformational change of the myosin cross-bridge of a kind that delivers a thrust to the actin, which then moves with respect to the myosin. In this mechanism, precise dimensional and steric conformance is required between actin and myosin subunits.[42]

It is not obvious how a sliding-filament system could cause transport of substances in neurons; nor is there evidence that the microtubules and neurofilaments interact through a sliding movement of one with respect to the other (though cross-bridges have been observed between microtubules[6]). However, on the basis of the circumstantial evidence of the presence of microtubules and their conjectured ability to transport particulates in other tissue, a speculative model (Fig. 5) may be suggested as a tentative working hypothesis.

Substances to be transported, such as certain transmitter molecules and enzymes or secretions, are contained in protein vesicles. It is suggested that the walls of the vesicle contain protein monomers, the diameters of which are congruent with those of the monomer subunits of the microtubule which presumably contain the source of energy, ATP or guanosine triphosphate (GTP). On the vesicle monomers, it is postulated, is a binding site for the microtubule subunit and another site where the ATPase (or GTPase) is activated. When the ATP is hydrolyzed, energy is coupled, and conformational changes occur which provide a thrust, moving the vesicle, so that a different vesicle subunit is in apposition with a different subunit of the microtubule, and the thrusting process is repeated. The vesicle thus moves or jerks along the microtubule or neurofilament in a direction dependent on the sense of the helix of the microtubule or neurofilament.

A similar mechanism may be responsible for the saltatory and directional movement of the particulates within cells generally. Holmes and Choppin[43] find that infection of hamster kidney cells with parainfluenza virus SV5 produces multinucleate syncytia in which nuclei, arranged in long parallel rows, migrate straight to the center of the syncytium. The cytoplasmic channels between the nuclear rows are birefringent, probably due to bundles of microtubules, shown by electron microscopy to be present in the channels and intimately associated with the nuclei. Colchicine treatment blocks nuclear movement. Holmes and Choppin suggest that the microtubules may determine the direction of nuclear migration and may provide the motive force for their movement. Such a transport of vesicle-enclosed, biochemically potent compounds directionally to particular intracellular locations may be evolutionarily older than the sliding-filament mechanism of muscle, which was presumably differentiated to develop large contractile force rapidly and on a macroscale.

Role of Fibrous Proteins in Molecular Neurobiology.—The slow, 1–6 mm/day, unidirectional, nonspecific flow may be effected by a mechanism different from the fast, specific, potentially bidirectional transport. Weiss[29] attributes the former to hydrostatic forces exerted upon the axoplasmic column as a whole, presumably by peristaltic contraction of a sheath structure; alternatively the slow movement may be actuated by neurofilaments. The fast transport, however, is most reasonably attributable to the microtubule system, which is associated with saltatory transport in cells generally.[37] Whether there is any coupling

between the propagated action potential wave and the transport of particulates, a process which may seem reasonable teleologically, is unknown; according to Kerkut *et al.*,[34] fast transport of glutamate occurs only in stimulated nerves.

As is illustrated diagrammatically in Figure 6, transport of transmitters down the axon to synaptic terminals is as vital to interneuronal transmission as is the action potential, at least in nerve-fiber types where the transmitter is not synthesized in terminals and must make the journey down the axon. Vesicles are in this category, and it is interesting that Weiss[44] has observed accumulation of vesicles in axons, the peripheral flow of which had been dammed by compression. The situation is clear in the sympathetic neurons in which catecholamine storage granules carry the transmitter norepinephrine, which is thought to be synthesized in the cell body and perhaps also within the storage vesicle that is transported down the axon to the ending. Lysosomes containing enzymes, possibly involved in protein metabolism at terminals, are known to be transported down the axon.[45]

Dendrites are rich in microtubules. Experiments have not yet been devised to test whether transport occurs in dendrites, but it seems probable that such may be the case.

If the synthesis of specific proteins is involved in memory consolidation, their transport to synaptic endings may play a vital physiological role in the plastic regulation of cell-to-cell interaction, as in learning, for example. In connection with this transport, Barondes[46] recently found that protein synthesized from labeled amino acid precursor passed down the axon to endings where, he suggests, the protein combines with mucoid constituents at the synapse; these proteins, he thinks, may be concerned with plastic alterations on which learning depends. Bogoch[47] has also speculated along these lines.

By means of the fibrous proteins of microtubules or neurofilaments, materials biosynthesized in the cell center may be directionally transported to various sites on or within the neuronal membrane; thus the neuron's ongoing bioelectric activity may be modulated not only by synaptic inputs, but also by the neuron's genetic and biosynthetic mechanisms. Substances impinging extracellularly on the neuronal membrane or generated near it may be rapidly transported to the cell center, there influencing gene expression. Such processes may be especially important in the development of the brain, possibly through the action of specific nerve growth factors.

Further study of the fibrous proteins may lead to a better understanding of neuronal processes at the molecular level and provide a basis for a more effective investigation of the biological, neurophysiological, and psychological phenomena.

Summary.—Neurofilaments, *ca.* 100 Å in diameter, and microtubules, *ca.* 240 Å in diameter, form the basis of the fibrous structures in neurons described in classical neurohistology. Not unique to neurons, these two fibrous types are found in cells generally and function as important organelles.

Neurofilaments from squid axoplasm have been isolated, their probable ultrastructure has been deduced, and the composition and properties of the protein subunits have been determined.

It is suggested that neuronal microtubules are functionally concerned with the movement of particulates and organelles, specifically with the fast (10^2 to 10^3 mm/day) transport of specific neuroplasmic constituents down the axon and possibly also within the cell body and dendrites. A tentative hypothesis is proposed for the molecular mechanism of such transport. Neurofilaments may also be involved in neuroplasmic movement, possibly of the slower (1–6 mm/day) type of neuroplasmic flow.

Transport of certain transmitters, neurosecretions, and trophically active substances from the site of synthesis in the cell body to axon endings appears to be mediated by the fibrous proteins. Regulation of the synthesis of these proteins is therefore vital to normal neuronal function.

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