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

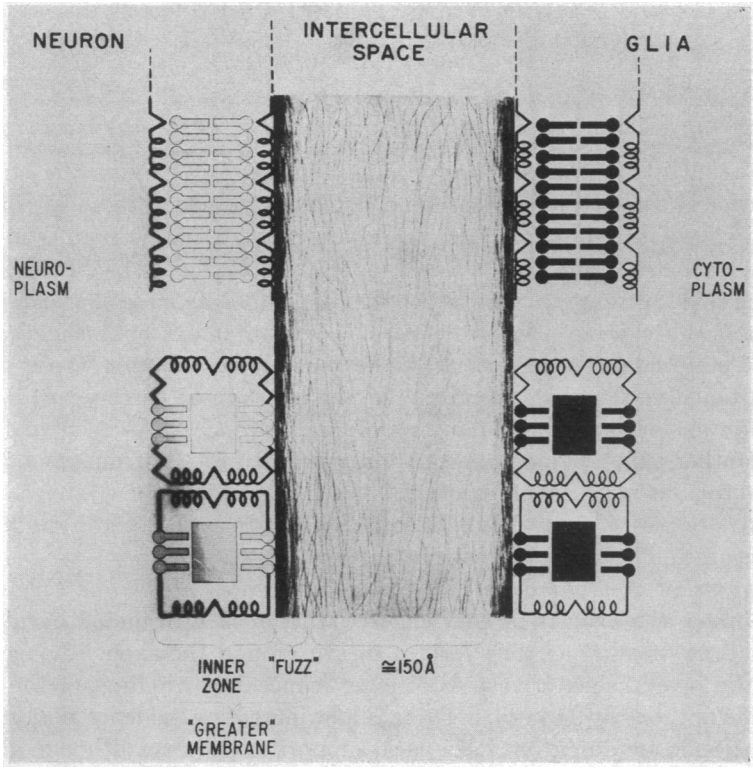

FIG. 1.—Diagrammatic representation of the "greater" membrane and intercellular space (courtesy F. O. Schmitt and the *Neurosciences Research Program Bulletin*).

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

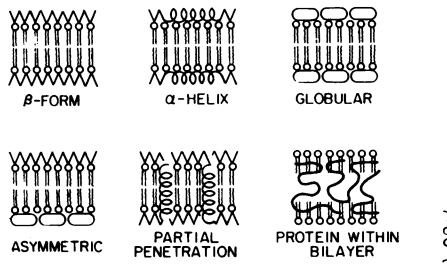

FIG. 2.—Some membrane models based on a lipid bilayer. Very recent infrared data appear to exclude β-conformations of the protein, at least in the erythrocyte membrane.[18]

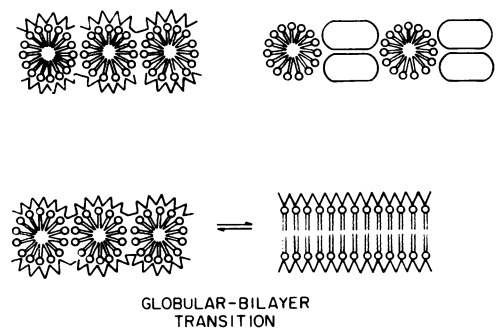

FIG. 3.—Some membrane models based on globular arrangements.

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.

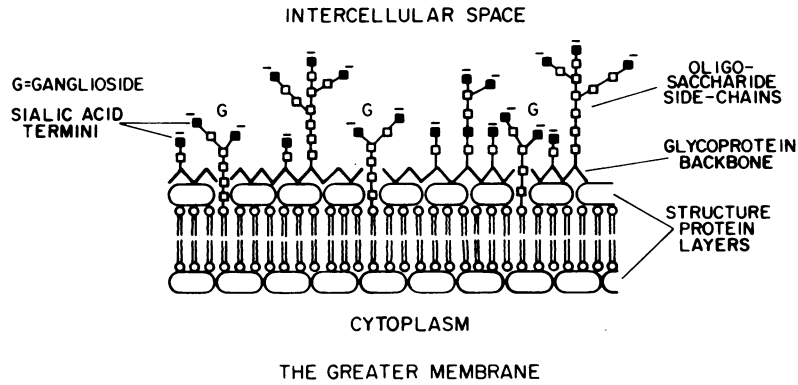

FIG. 4.—A model of the plasma membrane and cell coat.

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.

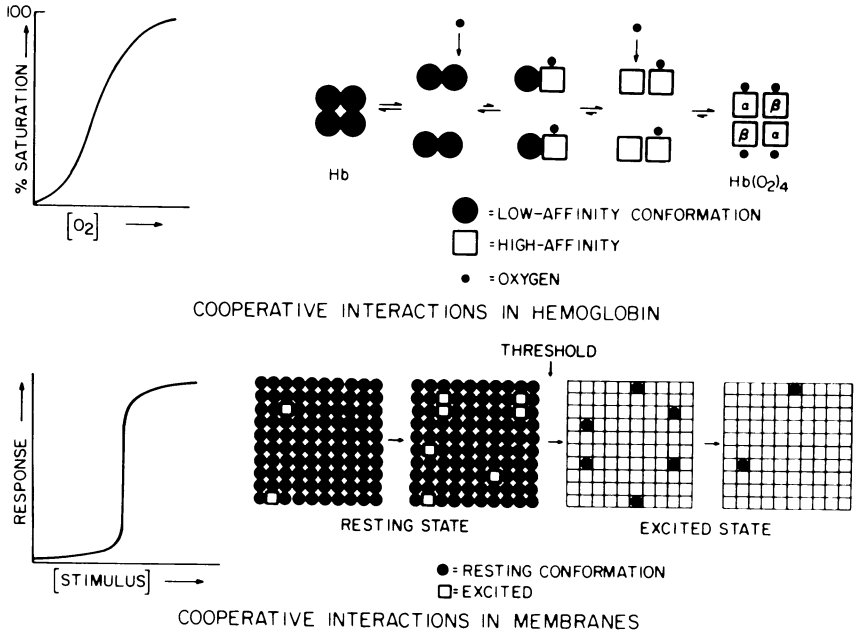

FIG. 5.—Cooperative interactions in hemoglobin and in membrane structure.

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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