On the Nature of Synaptic Receptors

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Abstract

This paper summarizes observations and speculations concerning the origin and chemical nature of the synaptic receptors, i.e., the transmitter- and drug-sensitive sites present in the postsynaptic membranes. Available evidence suggests that receptors are synthesized in the cytoplasm of the postsynaptic cell. However their appearance in a functional ("receptive") condition in the surface of this cell is under the direct control of the presynaptic nerve endings.

It is likely that such regulatory influence is exerted by a receptor-controlling, inhibitory compound which is released by the nerve endings, perhaps together with the transmitter. However, unlike the transmitter, the inhibitory compound would be taken up by the postsynaptic cell. The manner in which this inhibitory factor acts is unknown. It may block the synthesis of receptor molecules. Alternatively, it may exert a masking action on receptors already present in the surface of the cell, which would remain in a dormant or silent condition.

The changes which follow chronic denervation of skeletal muscle (a marked increase in the transmitter-sensitive areas of the membrane) can be explained by assuming that the neural inhibitory compound slowly disappears from the muscle cell after being disconnected from its motor nerve. As a consequence, silent receptors become active or new receptors are synthesized. The receptor-controlling compound would also leak from the muscle fiber at points where the muscle membrane has been damaged.

Since almost the only active receptors present in normal muscle are located at the subsynaptic area of the membrane, where the receptor-controlling factor should be expected to reach highest concentrations, the existence of a second factor which blocks or prevents receptor inhibition is postulated. This factor may be the transmitter itself.

Speculations on the chemical nature of receptors have favored the idea that they may be protein molecules bound to, or embodied into, the membrane structure. Attempts to isolated chemically the receptor substance have so far been inconclusive mainly because a receptor molecule, or molecular complex, separated from the cell ceases to behave as a receptor though it may still combine with the transmitter.

The idea that receptors may be protein molecules has received considerable support from recent work on the mechanism of the Schultz-Dale reaction in denervated, immunologically sensitized diaphragmatic muscle from the guinea-pig. With the aid of a microtap which allows the rapid application of protein to discrete areas of the surface of muscle fibers, it has been shown that antigens do exert a depolarizing action on sensitized muscle cells by decreasing the electrical resistance of their surface membrane.

It can be concluded from these experiments that antibody molecules bound to the surface of denervated muscle fibers behave as receptors for the specific antigens. The physical changes which take place in the muscle membrane, following their interaction with antigens, are similar to those elicited by acetylcholine on the cholinergic receptors of the same cells.

Artificial models of transmitter-receptor systems have been built by using artificial lipid films treated with antibodies and enzymes. The transverse impedance of such films, presumably coated with protein molecules, decreases when the specific antigens or substrates are introduced in the system.

The results support the idea that synaptic receptors are protein molecules which, synthesized by the postsynaptic cytoplasm, locate themselves in the membrane, the permeability of which they regulate, possibly by virtue of configurational changes. Such changes are in turn controlled by the combination of the active site of the receptor, i.e., the receptor surface, with specific molecules of transmitters and drugs.

Introduction

CLASSICAL HISTOLOGY, based on observations made with the light microscope, described few cellular membranes apart from the plasmatic or surface membrane and that surrounding the nucleus. In recent years, however, the electron microscope has revealed an increasingly large number of membranous structures in the cell (e.g., invaginations of the surface membrane, microvilli, endoplasmic reticulum, myelin sheaths, Golgi complex, mitochondria; membranes surrounding secretory granules, lysosomes, synaptic vesicles, etc.). Indeed, it has been estimated that between 60 and 80% of the dry weight of the cell is represented by membrane material.

Cell membranes are exceedingly tenuous structures, consisting basically of highly organized arrays of lipid molecules which, in association with proteins, form a relatively ion-proof fabric. The superb ability for insulation displayed by lipids is demonstrated by the fact that bilayers of phospholipid molecules formed on small holes drilled in a plastic septum offer a resistance to the flow of electric current as high as $10^9\ \Omega\ cm^2$ (23).

Because of the intrinsic impermeability of their lipid components, biological membranes are extremely well suited to separate and insulate cellular compartments, with an enormous economy of volume and weight, and to regulate the flow of materials between such compartments. Moreover, membranes serve as a framework which supports a variety of molecular mechanisms, often arranged in catalytic production lines.

The cell surface membrane controls the traffic of ions between the cytoplasm and the outside medium by offering a variety of specific pathways through which ions can flow. Some of these pathways appear to be aqueous channels permanently available to cer-

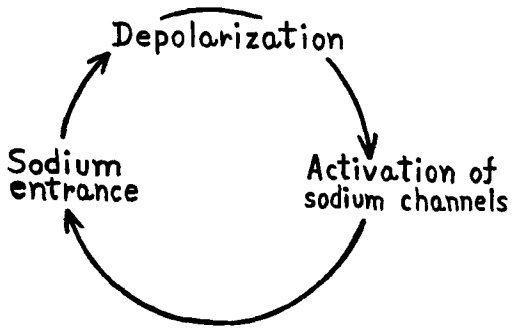

FIG. 1. Diagram representing the relationship between membrane depolarization and the activation of the sodium mechanism leading to the action potential. Owing to its influence on the sodium channels, causing the Na^+ ions to flow into the cell, depolarization is a regenerative, self-reinforcing process.

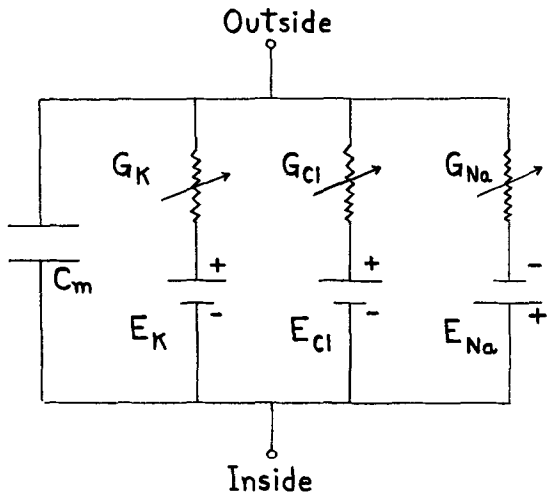

FIG. 2. Equivalent electrical circuit of the nerve and muscle membranes. C_m is the membrane capacitance in parallel with three e.m.f.'s, or ionic batteries, which are represented in series with their respective internal (variable) conductances. E_K, E_{Cl}, and E_{Na} are respectively the potassium, chloride, and sodium batteries, and G_K, G_{Cl}, and G_{Na} the membrane conductances to these ions. At rest, the potential difference between the inside and outside of the cell, or resting potential (inside negative), is determined mainly by E_K and E_{Cl} since their conductances are high whereas G_{Na} is exceedingly low. The action potential is caused by a sudden increment of G_{Na} that causes a reversal in the polarity across the membrane (i.e., inside positive).

tain ions and can be visualized as pores or holes through which the ions move under the influence of chemical and electrical gradients. At other sites in the membrane there are molecular mechanisms of enzymatic nature which push or pump ions against electrical and concentration gradients, a task requiring the expenditure of metabolic energy. Finally, channels are found which behave as ionic valving or gating devices. These are closed or "plugged" when the cell is at rest, but they open under the influence of different signals like a) physical and chemical stimuli from the outside, b) changes in the electrical gradient across the membrane itself, and c) chemical signals or messages sent from other cells.

Such ionic valving devices are responsible for the so-called excitation phenomena. These are rapid responses exhibited by a number of cells, mainly those of nerve and muscle, which involve sudden changes in the permeability and electrical potential across their surface membrane. Indeed, mechanisms a), b), and c) form the basis respectively of sensory reception, electrical excitation, and synaptic transmission.

This review deals with the last of these phenomena, in particular, with the experimental evidence which may cast some light on the nature of the chemical sensing devices, transducers, or receptors which are present in postsynaptic membranes. But as these receptors are only one of the links in the chain of events involved in the transmission of information between cells, a summary, perhaps lengthy but unavoidable, of the current ideas on the process of electrical excitation and synaptic physiology must be given in the guise of an introduction. For further information, the interested reader should be referred to the monographs of Katz (28) and Hodgkin (25).

Action Potential and Its Transmission

Excitation and Impulses

The Resting Potential. Nerve and muscle fibers share the property of generating and conducting action potentials. These are brief but intense changes in the ionic permeability and electrical potential difference across the cell surface membrane (also called the excitable membrane), which travel along the fibers with speeds of up to 250 mph.

At rest, both nerve and muscle membranes are selectively permeable to K^+ ions. Since the intracellular concentration of these ions is between 10 to 100 times higher than in the external solution, potassium tends strongly to leave the cytoplasm. However this tendency is balanced by an electrical potential difference, a Donnan equilibrium potential, which develops across the membrane, making the cell interior almost one-tenth of a volt negative with respect to the outside. Since the thickness of the excitable membrane is less than 100 Å, such potential difference, usually called the resting potential, amounts to a voltage gradient of about 100,000 V/cm, a value close to the breakdown point of the membrane dielectric.

The Sodium Mechanism. Besides the pores available to potassium, the excitable membrane has another system of channels permeable only to sodium ions. These differ from the K^+-channels by being provided with a valving mechanism which is under the control of the electrical potential difference across the membrane. In the resting cell these channels are closed; therefore, the permeability of the membrane to sodium is exceedingly low. But when the resting potential is reduced by any means, the molecular "plugs" which block these channels become free to move and the extracellular Na^+ ions rush into the cytoplasm, driven by concentration and electrical gradients.

The opening of sodium channels is a regenerative or self-sustaining process. A depolarization just sufficient to activate a fraction of the channels will lead through a positive feed-back process to the total activation of the Na-mechanism at that site of the cell (Fig. 1).

Another important feature of the Na-valving mechanism is its cyclic, self-quenching character, that is, the Na-channels do not remain open as long as the depolarization lasts but turn themselves off one millisecond or so after being activated and have to be reset by the resting potential before they can be opened again.

The Action Potential. The full activation of the Na-mechanism places across the membrane, parallel with the K-battery, a new e.m.f., a Na-battery which makes the cell interior positive with respect to the external solution. Since the internal resistance of this new battery is much lower than that of the K-battery, the potential difference across the membrane will be determined, at this instant, by the ratio of the

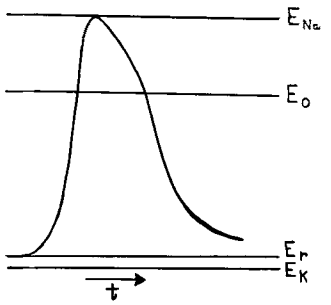

FIG. 3. A conducted action potential as recorded with an intracellular microelectrode. The horizontal lines indicate the sodium equilibrium potential (E_{Na}), the zero baseline (E_o), the resting potential (E_r), and the potassium equilibrium potential (E_K). During the action potential the potential difference across the membrane shifts from the resting level, close to E_K, to E_{Na}, returning to E_r at a slower rate.

concentrations of Na^+ ions on both sides of the membrane. (Cole and Curtis (9) have shown that, at the peak of the action potential, the membrane resistance of the squid giant axon fell from a resting value of 1,000 Ω cm^2 to an average of 25 Ω cm^2.) The potential variation from the resting potential to the sodium equilibrium potential, which is regenerative and self-conducting, is known as action potential or impulse.

An equivalent electrical circuit of the excitable membrane is given in Fig. 2 and Fig. 3 shows an action potential in which the potential difference recorded across the membrane shifts from the resting potential (E_r), close to the potassium equilibrium potential (E_K), to the sodium equilibrium potential (E_{Na}). It must be emphasized however that not all of this potential change is a consequence of the aperture of Na-channels at one point of the membrane. In fact, the foot and early part of the action potential is always caused by a passive depolarization, i.e., a reduction of the resting potential produced by an e.m.f. located outside the site from which the recording is done, such as a neighboring region of the same cell, where the Na-mechanism is already active, or in the battery or electronic pulse generator used by the experimenter.

The Conduction of Impulses. Nerve and muscle fibers resemble miniaturized versions of submarine cables. They possess a conductive core, the cytoplasm; an insulating sheath, the excitable membrane and they are immersed in a conductive medium, the extracellular solution, which plays the role of sea water. On account of the relative resistances of these materials, living cables are extremely inefficient passive conductors of signals; therefore, a potential difference imposed at one point between the core and the outside medium is propagated a few millimeters, at most, before it becomes completely attenuated owing to a current leak through the imperfect insulation. Yet such rudimentary cable-like properties are of prime importance in the conduction of action potentials.

The flow of Na^+ ions into the cell cannot take place without the simultaneous transport of an equal number of positive charges, K^+ ions, from the cytoplasm to the external solution; consequently an electric current flows in a local circuit whenever sodium enters the cytoplasm, and, owing precisely to the cable-like properties of the cell, such current spreads longitudinally along the fiber axis. For this reason, the outward potassium current flows through resting areas of the membrane, which are thereby depolarized. This determines the local activation of the Na-mechanism

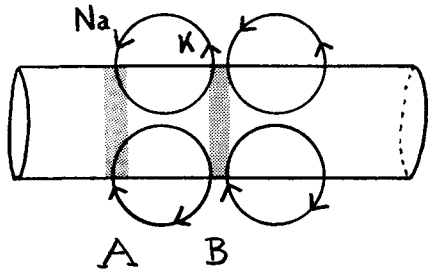

FIG. 4. Diagram illustrating the mechanism of conduction of the action potential along an excitable fiber. When the action potential is at point A, the sodium mechanism at this region is activated and Na^+ ions enter the cytoplasm. This inward flow of cations is accompanied by the simultaneous movement of K^+ ions from the fiber. Thus electric current flows in a closed local circuit. The outward flow of current at point B depolarizes the membrane at this level and, in turn, determines the activation of the sodium mechanism. The action potential has travelled therefore from point A to point B (see text).

and the firing of a new action potential. To the experimenter however it looks as if the action potential at point A (Fig. 4) has jumped, or has been conducted, to point B.

Thus, in virtue of a continuous circulation of current in local circuits, the action potential travels along the fiber. Its progression resembles, somehow, the movement of a wave on the surface of the ocean. In the sea each molecule of water moves in a circular path as the wave advances, though the net movement of water in the direction of the wave is zero. Likewise, as the action potential is conducted along the fiber, positive ions circulate in a continuously shifting wave. Their net movement along the axis is zero, though mixing between the intra- and extracellular cations, i.e., Na and K, takes place with the passage of each impulse.

The analogy between the action potential and the ocean wave must not be pushed too far; the latter represents flow or transmission of energy, but, in the nerve or muscle fiber, energy does not flow with the action potential. On the contrary, the energy stored in the ionic batteries of the cell is released locally, at each point of the fiber, by the interchange between intra- and extracellular cations (Na and K). The decreased concentration gradients are restored during rest by ionic pumps which use up metabolic energy.

Intercellular Transmission of Excitation

Synaptic Contacts. In nervous and neuromuscular systems, made up of large numbers of cells linked into complex networks, the action potentials are the standard unit signals utilized for the conduction of

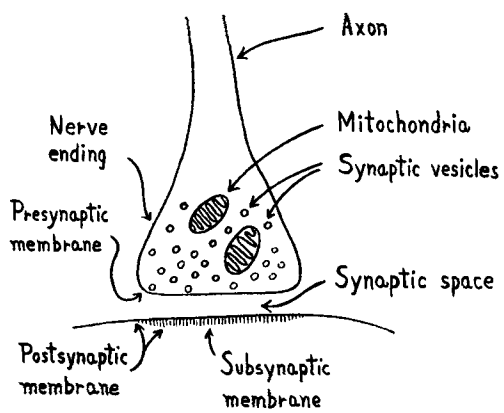

FIG. 5. Schematic drawing of a chemical synapse.

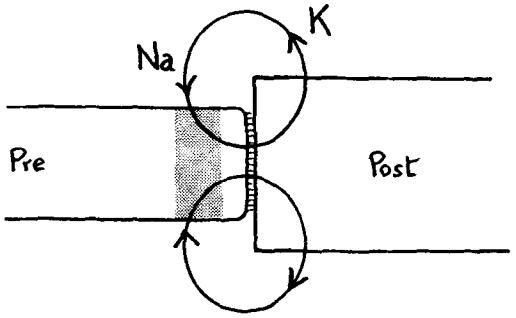

FIG. 6. Diagram illustrating the mechanism of operation of an electrical, or electrotonic, synapse. The shaded area in the presynaptic nerve fiber (Pre) represents the action potential; Na^+ ions flow into the presynaptic cytoplasm at this level. The ensuing ionic current flows into the postsynaptic cell (Post) across a specialized, low resistance, area of contact of both membranes. The outward membrane current carried by the K^+ ions leaving the postsynaptic cell causes its depolarization and excitation.

information. Messages are transmitted from cell to cell across specialized regions known as synaptic contacts or junctions. A synapse is that area where two nerve cells or a nerve cell and muscle cell enter into anatomical contact and functional nexus. The arrival of an action potential to the so-called presynaptic nerve-ending (Fig. 5) causes a change in the postsynaptic membrane. Often, and this is the only case to be considered, the incoming presynaptic action potential gives rise to a similar action potential in the postsynaptic cell. This process can take place by two different mechanisms.

Electrotonic Synapses. In one instance (Fig. 6) the depolarization of the postsynaptic cell, a condition *sine qua non* for the generation of an action potential, is caused by the flow of electric current from the presynaptic cell; i.e., the ionic current generated by the presynaptic action potential flows into the postsynaptic cytoplasm through the area of cellular contact and, upon leaving the postsynaptic cell, depolarizes its membrane.

For this mechanism to operate successfully two conditions must be met. First, low-resistance pathways between the pre- and postsynaptic cytoplasms are necessary; otherwise the current leaving the pre-

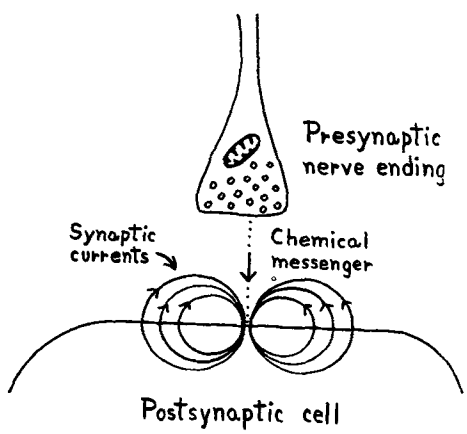

FIG. 7. Diagram representing the operation of a chemical synapse. The presynaptic nerve-ending does not supply energy to the postsynaptic cell but sends only an order or signal. This information is carried by a chemical messenger, or transmitter, which diffuses across the synaptic space, the width of which is grossly exaggerated. The interaction of this transmitter with the postsynaptic membrane causes the aperture of ion-permeable channels and the local flow of electric current in the circuit shown. The outward current depolarizes the membrane, causing the excitation of the postsynaptic cell.

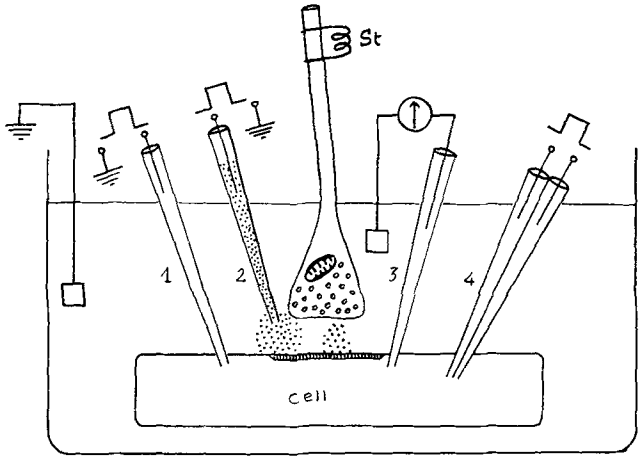

FIG. 8. Diagram illustrating various electrophysiological applications of glass micro-pipettes. Four pipettes, one of them a double-barrelled one, are shown dipped in a saline bath containing one cell with a presynaptic nerve ending. The bath solution is maintained at ground potential with the help of the electrode represented by the square at the left. Microelectrode No. 1 is used to inject current into the cell; a square voltage pulse is applied between a wire, in contact with the solution filling the pipette, and ground. Pipette No. 2 is filled with a solution of a drug or transmitter; by flowing a current pulse through its tip, a small amount of drug molecules can be applied to the receptors of the synaptic region of the cell. Pipette No. 3, inserted into the cell, is used to record the transmembrane potential, i.e., the potential difference between the cytoplasm and the bath solution. Finally, Pipette No. 4 is a twin-barrelled one. If the barrels are filled with solutions of different salts and a current is made to flow between both sides, it can be used to change the composition of the cytoplasm of small cells. Also, it can be used extracellularly to deliver two different drugs at the same site of the cell surface. *St* are the stimulating electrodes used to set up an action potential in the presynaptic nerve. Transmitter is liberated when this action potential reaches the nerve terminal.

synaptic cell would be lost into the extracellular space. Second, a quantitative matching should exist between the current generated by the presynaptic cell and that needed to depolarize, to threshold level, the membrane of the postsynaptic cell.

These two requirements are known to be fulfilled in a number of cases. The existence of low-resistance pathways between the two cytoplasms is associated with morphological specializations of the contacting cell membranes described by the electron microscopists as desmosomes, tight-junctions, etc. Furthermore, cells linked by electrical synapses are usually of similar sizes.

Chemical Synapses. The second type of synaptic mechanism is the so-called chemical transmission. In the synapses built according to this design, there is no flow of electric current between the two cytoplasms. The postsynaptic depolarization is not caused by an intercellular transfer of energy. Instead the presynaptic cell sends a mere signal or message of chemical nature, which causes a permeability change in the postsynaptic membrane and, as a result, depolarization and excitation (Fig. 7).

The chemical signals involved in this process are compounds, the synaptic transmitters, which, secreted by the presynaptic cell under the influence of the incoming action potential, diffuse across the gap between the adjacent cells and interact with the postsynaptic membrane. Such transmitters are molecules of relatively small size which belong to the group of the so-called biogenic amines (such as acetylcholine, epinephrine and norepinephrine, 5-hydroxytryptamine, dopamine, etc.).

The Synaptic Receptor

Microtechniques for Study of Synaptic Processes

Most of the available information on synaptic physiology derives from experiments performed with techniques built around the glass micropipette or ultramicropipette, as it has also been called to emphasize its exceedingly small dimensions (35). These are hollow needles made of Pyrex glass tubing, with an external tip diameter of less than 5,000 Å. When filled with concentrated electrolite solutions, they become conductive probes which can be driven into the cytoplasm of a cell without causing appreciable damage to its surface membrane (35). The membrane seems to have a semifluid structure, sealing around the glass when the cell is impaled and repairing itself when the pipette is withdrawn. Because of these unique properties, micropipettes can be used for a variety of purposes in electrophysiology, some of which are represented in Fig. 8.

Changes During Transmission

The application of these microtechniques to a number of synapses, particularly those with large postsynaptic cells, has provided a detailed picture of the physical properties of the postsynaptic membrane and the changes they undergo during the process of impulse transmission. These can be summarized as follows.

The ionic permeability of the postsynaptic membrane increases under the influence of the transmitter, as shown by a marked reduction in its electrical resistance. For example, in the neuromuscular junction of the frog, the transmitter (acetylcholine), released from the motor terminals by a nerve impulse, causes the appearance of an electric shunt across the endplate membrane (12,13,19). Indeed, the resistance between the inside of the muscle cell and the external solution (called the input or effective resistance of the fiber), which has an average value of about 200,000 Ω, is suddenly reduced to about 20,000 Ω by the released acetylcholine (Fig. 9).

It can be concluded that, under the influence of acetylcholine, new ion-permeable channels open in the postsynaptic membrane. There are reasons to believe that these new channels are not only permeable to sodium and potassium but, in experimental conditions, to other small diameter cations such as NH_4^+ and $(CH_3)_4N^+$ (46). Their aperture provides a pathway for the discharge of the resting potential, i.e, the depolarization and excitation of the postsynaptic cell.

The electrophoretic application of acetylcholine to skeletal muscle fibers has allowed the accurate mapping of the transmitter-sensitive regions of the membrane, which were found to be restricted to the areas immediately subjacent to the nerve endings, extending only a few μm beyond them (38).

Furthermore, it has been established that the muscle membrane is asymmetrical from the viewpoint of its sensitivity to the transmitter. Acetylcholine depolarizes only if applied to the external or neural surface. Contrariwise, the administration of this compound to the cytoplasmatic surface of the membrane, even in the subsynaptic region, has no effect on its properties (12,13).

Pharmacology and Biophysics

In spite of the progress summarized above, a most important aspect of synaptic physiology is still poorly understood, namely, the chemical nature of the transmitter-sensitive sites in the postsynaptic membranes and the mechanisms responsible for the increased ionic permeability brought about by the transmitter. So far, little more can be done than pose problems and formulate questions, borrowing a concept which has been essential to the pharmacologists for almost half a century, that of chemo-receptor or receptor.

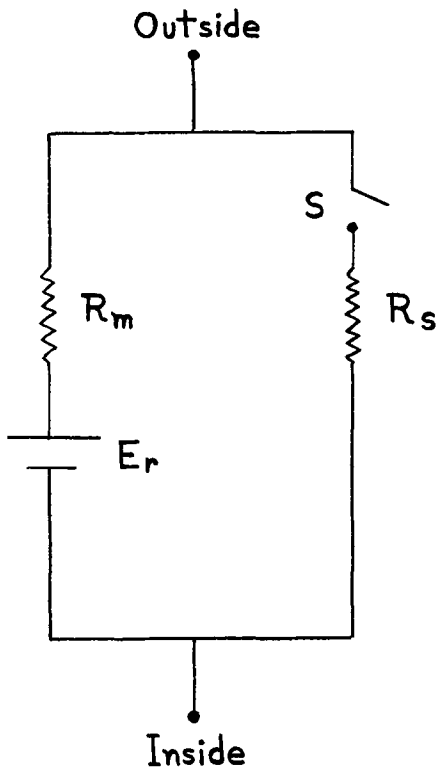

FIG. 9. Equivalent electrical circuit of a postsynaptic membrane in a chemical synapse. The resting potential of the cell is represented as a battery (E_r) in series with its internal resistance, i.e., the resistance of the cell membrane (R_m). The synaptic receptors are represented by a switch (S) in series with the resistance of the activated synaptic channels (R_s). At rest, i.e., in the absence of synaptic transmitter, S is open; therefore the potential recorded across the membrane (V) is equal to E_r. The released transmitter acts by closing the switch S, i.e., by opening the ion-permeable channels at the postsynaptic membrane. The membrane resistance is then shunted by R_s.

Historically, Ehrlich and Langley are credited with the introduction of the idea of receptor. Ehrlich developed it during his studies on chemotherapy. Drugs, he reasoned, act by combining with chemical groups, side chains, or receptor sites at the cell surface (18). Langley (33,34) used similar arguments to interpret the results of his experiments on the effect of different drugs on different types of muscle. If a drug acts on a given type of muscle, he argued, it is because a receptive substance, i.e., one which has special affinity for the drug, is present in those cells.

Since Ehrlich and Langley, the concept of receptor has become one of the basic assumptions in pharmacology. Based on it, Clark laid the quantitative foundations of this discipline. His "occupation theory" (8) asserted that the effect of a drug is directly proportional to the fraction of the receptors occupied, at a given time, by the drug molecules. Accordingly the relationships between dose and effect could be described with the help of the Michaelis-Menton equation or the Langmuir adsorption isotherm.

In spite of the immense amount of experimental work generated by this hypothesis, much of it has been

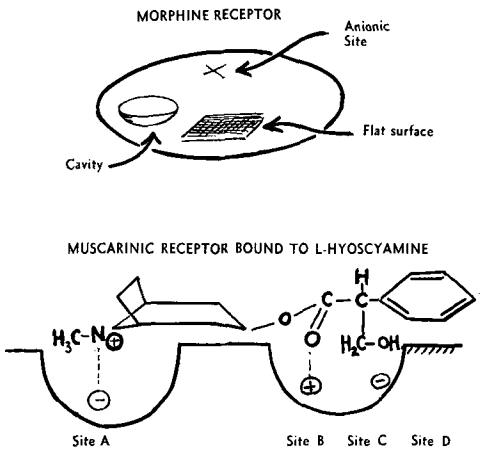

FIG. 10. Two receptor surfaces.

done in the absence of precise information on the physical or physiological nature of the receptors involved and their location within cells and tissues. Indeed, receptors have been treated as sites of unspecified nature and position linking, by equally unspecified mechanisms, the drug molecules with physiological processes, the change or variation of which is generally known as the pharmacological effect of the drug.

The sites where drugs abut on cellular structures, i.e., the receptors, have often been visualized as an interface or surface, an area which, regardless of its nature and location, can be mapped by comparing the pharmacological effects of series of homologous compounds in which the shape of a pharmacologically active molecule is distorted, step by step. In this manner the topography of the receptor surface can be described in terms of chemical functions and electric charges, the nature, relative positions, and absolute distances of which can be determined by reference to complementary groups in the drug molecules. Two receptor surfaces are illustrated in Fig. 10.

The localization and identification of receptor surfaces within the cell has usually depended upon the advances achieved by disciplines other than pharmacology. For example, the discovery of the various roles played by enzymes in cell physiology led to the finding that enzymes are the site of action of some drugs, i.e., the receptor surfaces have been identified with the active site of catalytic proteins.

Similarly, the electrophysiological analysis of the changes which take place in the postsynaptic cell during the process of transmission, and its pharmacological modification, demonstrated that both physiological transmitters and a number of important drugs act by combining with specific regions or sites of the postsynaptic membrane. It was concluded that the combination of those compounds with a specific receptive substance located in the membrane was responsible for the occurrence of the observed resistance changes.

The Membrane Receptors

During the last two decades a detailed picture of the receptors present in postsynaptic membranes has emerged as the result of a re-statement of pharmacological concepts in the light of biophysical experimentation. Such picture can be summarized as follows.

The receptor, or the area or region of the receptor which combines with the molecules of the transmitter or drug, is located at the interface which separates the cell surface from the extracellular space.

The receptors however do not move freely in this interface but are imbedded or rooted into the membrane. The receptor surface is often visualized as protruding above the cell surface, like the exposed part of an iceberg, while the bulk of the receptor would be ensconced within the membrane material.

No receptor surface comparable with that jutting above the outer surface of the cell would be found in the inner surface of the plasma membrane since transmitter or drug molecules applied from the cytoplasm seem to be inactive.

Because of the characteristic chemical topography of their exposed surface, receptors have a high degree of chemical specificity with regard to their reactions or interactions with compounds dissolved in the adjacent aqueous phase. Receptors would combine only with molecules possessing a chemical configuration and charge distribution which somehow complements that of their exposed surface.

The interaction between drug and receptor surface results in the formation of a reversible drug-receptor complex, in which the complementary groups, functions, or electrical charges in both moieties are joined by relatively weak bonds.

As a result of the formation of such a complex, a steric change of some sort occurs in the receptor and/or surrounding structures, causing an increase in the ionic permeability of the membrane. This is followed by the flow of ionic currents and a change in the electrical potential difference across the membrane.

Newer Approaches

A synaptic receptor could be said to be fully understood not only if one could account for the chemical specificity of its reactive surface, and the manner in which it interacts with transmitter and drug molecules, but also if one knew the mechanisms by which such interaction is reflected in a change of the effectiveness of the postsynaptic barrier as an ionic barrier.

To be able to answer all these questions one should have a picture of the receptor as a structural or molecular entity. Are the receptors single molecules or aggregates of monomeric units? Or do they consist of a local contamination of the membrane by compounds of low molecular weight?

These problems have generated a considerable amount of speculation. On the whole, most informed guesses have favored the idea that synaptic receptors are protein molecules attached to, or imbedded into, the fabric of the cell membrane. Indeed, protein molecules possess the necessary features to account for the known properties of the receptors. The high chemical specificity of the latter is, after all, similar to that exhibited by antibodies against antigens or enzymes towards substrates. It is conceivable also that proteins could be incorporated into the structure of membranes in such a fashion that small conformational changes in their molecules would cause large changes in the diameter, or number, of channels available for ionic flow.

Furthermore, it is known that the formation of both antigen-antibody and enzyme-substrate complexes causes a change in the configuration of the proteins. So it is reasonable to believe that the formation of the drug- (or transmitter-) receptor complex could result in a change in the tertiary structure of the receptor protein, which would be responsible for the so-called receptor activation.

Thus Nachmansohn (44) has explained the activation of cholinergic receptors by acetylcholine as caused

by a small local folding of a section of a protein which removes a positively charged amino group from a strategically located point, thereby permitting the flow of cations through a pre-existing channel or site of increased permeability of the membrane.

The fact that many enzymes are known to be located at cell interfaces where they control the traffic of ions across the underlying membranes, even against concentration or electrical gradients, strongly supports the above ideas. Nevertheless speculation alone, no matter how sound it may be, is clearly insufficient to settle a problem which is beyond the reach of purely pharmacological and biophysical experimentation.

Chemical Isolation

For anyone biochemically inclined, the most obvious method of inquiring into the chemical nature of receptors would be to homogenize the cells or tissues and isolate the receptor substance. This has been, after all, the basic approach in enzymology; indeed, the reactions catalyzed by an enzyme can only be ascertained after the catalytic protein has been purified.

Yet this approach has not been entirely successful when applied to synaptic receptors since their function cannot be dissociated from the surrounding cell environment. Chemical isolation of receptor molecules is feasible in principle, but as soon as the cellular structures and compartments are destroyed, there is no way of finding out whether or not the receptor substance is still preserved in the glassware. The fact that a drug or transmitter combines with a fraction of a biochemical preparation does not prove that one is dealing with receptor molecules. After all, drugs and transmitters are known to combine with extrajunctional sites in postsynaptic cells (51).

Attempts to isolate acetylcholine receptors from the electric organ of fish have been made by Chagas and his collaborators (7,24) and by Ehrenpreis (16,17).

Destruction by Chemical Agents

Woolley and Gommi (52) reported that the serotonin receptors of the rat uterus and stomach can be selectively destroyed by briefly incubating the tissue with small amounts of the enzyme neuraminidase in the presence of EDTA. After these agents were washed off, their preparations were found to be insensitive to the stimulating action of serotonin. However the receptors, destroyed or rendered dormant by this method, could then be reactivated by treating the preparation with crude lipids extracted from the same tissues with chloroform-methanol.

These findings were in disagreement with the "protein hypothesis"; in fact, they supported the idea that serotonin receptors are of lipid nature, a conclusion already drawn by these authors as a result of previous work. According to them, serotonin would bring about an active transport of calcium ions through the cell surface membrane by means of a sequence of specific, reversible, and cyclic reactions with the receptor lipid.

Attempts to modify receptors have also been made by exposing visceral muscle preparations to the influence of protein-denaturizing agents, such as urea or high temperatures (20). The fact that, after such treatments, the contractions elicited by a given drug were reduced or abolished has been regarded as an indication that the specific receptors were inactivated and that the receptor substance was a protein. Yet the amplitude of the contractile responses of a tissue to a given drug depends on many factors, including not only the availability of appropriate receptors in a reactive condition but also the presence of an adequate membrane potential as well as sufficient calcium concentration in intracellular stores and the integrity of the contractile proteins. All these factors should be analyzed separately before conclusions on the condition of the receptor substance could be drawn.

Control of Chemical Sensitivity

A good deal of information on the behavior and possible origin of postsynaptic receptors has been provided by experiments dealing with the influence of the motor-nerve endings on the chemical sensitivity of skeletal muscle fibers.

So far, in this paper, the presynaptic cell has been regarded merely as a source of synaptic transmitter. It is known however that a number of metabolic and organizational patterns of the postsynaptic cell are under the control of the presynaptic nerve-endings. These influences are particularly easy to demonstrate in skeletal muscle by cutting the motor nerve and allowing its peripheral end to degenerate.

One of the most important changes observed in muscles deprived from their motor innervation is the enlargement or spread of the acetylcholine-sensitive areas in each fiber. The surface of normal, innervated muscle fibers is only sensitive to the depolarizing action of the transmitter in the subsynaptic region though small acetylcholine-sensitive areas can also be found at the ends of the fiber, at the muscle-tendon junction (30,31).

As first demonstrated by Ginetzinsky and Shamarina (22), the whole surface of the denervated muscle fiber becomes sensitive to acetylcholine, a phenomenon which was further studied with electrical techniques by Axelsson and Thesleff (5) and by Miledi (36–40). Following denervation, the cholinergic receptors appear to proliferate by spreading out from the synaptic region. The opposite process takes place if the motor nerve is allowed to regenerate. Indeed, the receptors regress and revert to their initial subsynaptic distribution.

The work of Diamond and Miledi (15) has shown that such changes cannot be dismissed as pathological processes but are in harmony with the normal patterns of motor development. The entire surface of fetal muscles in the rat is depolarized by acetylcholine. But when the motor innervation is established, the receptors recede and adopt the adult pattern of distribution.

These observations suggest that the cholinergic receptors are synthesized by the muscle fibers rather than originated at the motor-nerve endings, a conclusion which received further support from experiments by Katz and Miledi (29,31). These authors severed the pelvic end of the frog sartorius muscle, a region normally deprived of nerve endings, and left it in situ within the animal. After about one week this part of the muscle, switched off from nervous control, became sensitive to acetylcholine.

Since, as also shown by Miledi (36), the immersion of a denervated muscle for 24 hours in a solution of acetylcholine does not cause a regression in the acetylcholine-sensitive areas, one is led to believe that the absence of the transmitter is not the immediate cause of all the changes which take place in denervated muscle. It can be surmised that, in addition to the transmitter, the motor nerve terminals liberate a second compound. This substance would inhibit the synthesis of acetylcholine receptors or, alternatively, block their activity.

Thesleff (49) has shown that a muscle chronically paralyzed by botulinum toxic develops all the changes typical of denervation. Since this toxin is known to produce a complete block of acetylcholine liberation (both its spontaneous release and that induced by the nerve impulse), though the integrity of the nerve endings is maintained, it seems reasonable to assume that the hypothetical "receptor-controlling" inhibitory compound may be released together with the transmitter, in other words, that each packet of acetylcholine molecules released from the nerve endings is accompanied by an amount of the inhibitory staff.

This idea is in disagreement however with the observations of Miledi (38) on the properties of regenerating neuromuscular synapses in frog muscle. Indeed, the acetylcholine sensitivity of some muscle fibers was found to have regressed to the normal pattern of distribution before the synapsis was able to transmit impulses and even before the spontaneous liberation of acetylcholine quanta (i.e., the so-called miniature end-plate potentials) had regained the characteristics typical of normal fibers.

The fact that, in normal, innervated muscle, the sensitivity to acetylcholine is highest at the subsynaptic region, an area where the concentration of the inhibitory compound should be expected to be maximal, poses a new problem. To explain this paradox one can postulate that, besides the receptor-inhibitory compound which acts on the whole muscle fiber, the nerve endings release another compound which, acting only at short range, neutralizes or antagonizes the receptor-controlling factor in the subsynaptic region of the membrane.

Since one feels reluctant to multiply the number of hypothetical compounds released by the nerve endings, the possibility should be considered that the compound which protects the receptors against the inhibitory influence of the presynaptic nerve, keeping them in an active condition, is the transmitter itself.

In addition to the changes in the sensitivity of the muscle fibers to acetylcholine, chronic denervation gives rise, at least in certain species, to the appearance of receptors to compounds which lack pharmacological effects on normal, innervated muscle. Thus denervated diaphragmatic muscle of the guinea-pig not only becomes sensitive to acetylcholine but also shows a singular and unexpected sensitivity to histamine and bradykinin, compounds which do not stimulate normal mammalian muscle. In addition, if the denervated muscle is taken from immunized guinea-pigs, it is also extremely sensitive to immunological reactions, that is, it contracts in the presence of small concentrations of the homologous antigen (4).

Apart from its effects on the chemical sensitivity of the muscle membrane, denervation exerts an interesting influence on still another aspect of muscle behavior, namely, the formation of new neuromuscular junctions with growing motor-nerve endings. If the central regenerating end of a cut motor-nerve is surgically implanted in a normal muscle, no new synapses are formed; however, if the same experiment is performed using a denervated muscle, new neuromuscular junctions are readily established. The same occurs (39,40) if a regenerating motor-nerve is brought into contact with the pelvic end of a frog sartorius muscle surgically severed from the innervated areas.

Morphological Integrity of Muscle Surface

When the pelvic end of a frog sartorius is severed from the rest of the muscle and cut off from the receptor-controlling influence of the motor-nerve endings, it becomes sensitive to acetylcholine. One should add now that new receptors appear also in the still innervated segment of the muscle, close to the point of injury (31). It seems that the neural inhibitory factor leaks out of the muscle or becomes somehow inactivated in the neighborhood of the injury.

Moreover, Miledi (41) has shown that local injury of the muscle renders its whole surface ready to form new synapses with regenerating motor-nerve endings, an observation which indicates that a change localized in a relatively small area of a muscle fiber is able to remove the inhibitory influences exerted by the nerve endings on the entire cell. Whether or not this effect is related to those described in the previous paragraph remains to be seen.

Finally mention should be made of the observations of Vrbová (50), showing that the implantation of the peripheral regenerating end of a mammalian motor-nerve on the surface of a normal, innervated muscle, at a region devoid of neuromuscular synapses, induces the development of chemical sensitivity even though no synaptic contacts are formed.

Immunological Induction of Chemoreceptors

The anaphylactic contraction of immunized visceral muscle occurs not only when antigens are injected into the whole animal but also takes place in vitro. Thus, if a piece of ileum or uterus taken from a sensitized animal is suspended in warm, oxygenated, saline solution of appropriate physiological composition and a small amount of the antigen is added to the bath, a shortening of the muscle follows. This phenomenon has been known as the Schultz-Dale reaction since it was independently described by these two authors more than 50 years ago (10,47).

It is generally admitted that the appearance of immunological responsiveness is caused by the incorporation of antibody molecules into the tissue. Indeed, one can immunize smooth muscle not only actively, i.e., by injecting the antigenic protein into the animal, but also passively, that is, in vitro by incubating the tissue with a solution of antibody molecules. The sensitization developed in this fashion seems to depend upon the adsorption of antibody γ-globulin (42). Both antibody and non-antibody γ-globulins compete for specific cellular attachments, or receptors, and according to Mongar and Schild less than 1% of such receptors have to be occupied by the antibody γ-globulins to achieve full sensitization.

As emphasized by Sir Henry Dale (11) in his Croonian Lecture on the biological significance of anaphylaxis, one can regard the antibodies as specific receptive substances for the antigens. Indeed, immunological reactions offer to the biologist an unique opportunity to experiment with well-defined and easily available molecules which, when incorporated into certain tissues, induce in them the appearance of specific chemoreceptors to equally well-defined antigenic molecules. However it is only recently that the contractile responses of muscle elicited by immunological reactions have been studied with electrical techniques from the viewpoint of receptor physiology.

During the last decades the anaphylactic reaction of sensitized muscle has been explained as caused by the liberation of pharmacologically active compounds (biogenic amines) within the tissue as a consequence of the antigen-antibody interaction. Such compounds are believed to be stored in nonmuscular elements of the tissue, mainly in the mast cells, from which they

would be released under the influence of the antigen. Implicit in this view is the notion that mast cells do possess chemoreceptors for the antigens. However, because of their small size, such cells and their receptors have not been amenable to direct electrophysiological experimentation.

Nevertheless, if one critically reviews the extensive literature on anaphylactic phenomena, one finds that many observations cannot be fitted easily into the above hypothesis (1). On the whole, these observations reveal the existence of not uncommon disagreements between the pharmacological properties of different tissues (i.e., their responses to biogenic amines) and the information on the chemical mediators released in the same preparations by the antigen-antibody interaction.

Such discrepancies could be accounted for, separately, by *ad-hoc* hypothesis, involving the liberation of new, still unknown, chemical mediators. Yet a more simple and general explanation is to assume that, besides releasing chemical mediators from the mast cells, antigenic proteins also exert a direct action on the ionic permeability of the muscle membrane, i.e., that this membrane is provided with specific chemoreceptors for the antigen.

This conjecture could, in principle, be tested with the same microtechniques described in Fig. 8, i.e., by the rapid application of minute doses of antigens to sensitized smooth muscle cells while recording their electrical activity. Nevertheless initial attempts to perform such experiments were unsuccessful a) because of the small dimensions of those cells and the syncytial character of the visceral tissue, and b) because of the impossibility of delivering detectable amounts of protein by electrophoresis from micropipettes (tip O.D., 1 μm or less). It became clear that such tests would require both a new and effective technique for the micro-application of protein and a search for a muscle preparation which, while showing Schultz-Dale reactions, would have a cell size significantly larger than that of visceral muscle.

Both conditions were eventually fulfilled. An electrically controlled microtap or microvalve (6) was designed which allows the application of proteins from micropipette tips with an O.D. of 5 to 10 μm. Moreover the need for larger muscle cells was met by the observation that chronically denervated diaphragmatic muscle of immunologically sensitized guinea-pigs does contract in the presence of small concentrations of antigen (4).

Experiments in which antigenic proteins were applied with a microtap to the surface of single denervated muscle fibers demonstrated that the antigen does exert a powerful depolarizing effect on the sensitized muscle membrane. Thus, if the tip of a closed microtap is placed near the cell surface and a short energizing current pulse is applied to the instrument, causing the tap to open, a transient membrane depolarization is observed (3). Though slower, these "antigen-potentials" (Fig. 11) are similar in shape to the acetylcholine-potentials elicited electrophoretically on the end-plates of normal muscle.

Control experiments in both innervated diaphragms of sensitized guinea-pigs and denervated diaphragms of nonimmunized animals gave negative results, demonstrating the immunological nature of the processes underlying the antigen-potentials. Moreover the repeated occurrence of such potentials at the same site of the cell surface is accompanied by a progressive and irreversible desensitization.

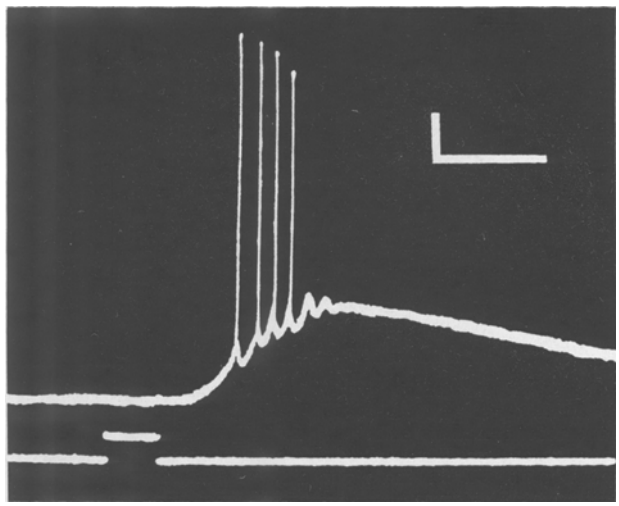

FIG. 11. "Antigen-potential," elicited by the application of ovalbumin, with an electrically operated microtap, to the surface of a muscle fiber of a denervated hemidiaphragm preparation taken from a guinea-pig immunologically sensitized to this protein. The upper trace is the transmembrane potential recorded with an intracellular microelectrode. The lower trace monitors the current which opens the microtap. The tip of this instrument was placed close to the surface of the muscle cell. The aperture of the tap, allowing the diffusion of protein, caused a depolarization of "antigen-potential" and the firing of four spikes. Calibrations: 10 mV and 1 sec. (2).

The amplitude and time-course of the antigen-potentials depend critically upon the distance between the tap and the surface of the cell. As this distance is reduced, both the peak amplitude of the potential and the rate of its rising phase increase. This indicates that the potentials follow the changes of the protein concentration at the surface of the cell rather than being the effect of the liberation of chemical mediators from tissular stores under the influence of the released protein. Indeed, these experiments suggest that the immunological receptors for the antigen are not restricted to the mast cells but also exist in the surface of the sensitized muscle cells. Their interaction with the antigen molecules would be responsible for the depolarization.

Furthermore, it has been shown that the antigen-potentials are associated with a large reduction in the electrical resistance of the muscle membrane. For this reason, it can be surmised that the depolarization induced by the antigen is caused by a sudden increase in the ionic permeability of the muscle membrane when the immunological receptors are activated.

Since Mongar and Schild (42) have shown that the antibody molecules involved in the anaphylactic reactions are attached to the surface of the cells rather than being trapped in intra- or extracellular spaces (21), it seems reasonable to identify those molecules with the receptors for the homologous antigen, the activation of which is responsible for the changes in membrane potential.

The link between the immunological reaction and the increased membrane conductance may be found in the molecular changes known to be associated with such reactions. The combination of the antigen with the antibody which used to be regarded as the smooth coupling of two complementary molecular configurations, fitting into each other like adjacent pieces of a jig-saw puzzle (hence the key and lock models), is now visualized by the immunochemists as the forced matching of two quasi-complementary structures.

As a result of the lack of complete correspondence between the two molecules, their reaction is accom-

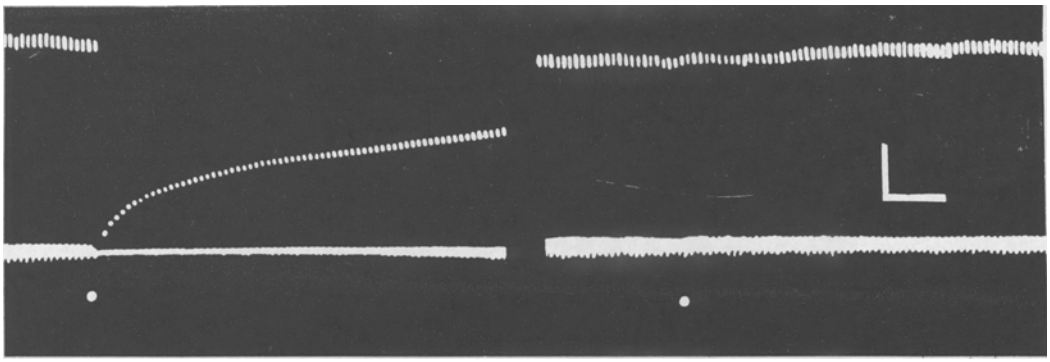

FIG. 12. Effect of ovalbumin on the transverse electrical resistance of an artificial lipid film treated with anti-ovalbumin serum. The addition of this serum to the aqueous phase in contact with the lipid, in a concentration of 1/40 v/v, before the beginning of the record, had no effect on the film resistance. However the subsequent addition of the homologous antigen (10 μg) to the same phase caused a marked drop in the amplitude of the voltage pulses produced by a constant current, flowing through the film. The record on the right side of the figure, taken after 10 min, shows full recovery of the film resistance. A second and equal dose of ovalbumin failed to influence the film resistance, indicating the immunological desensitization of the system. Horizontal calibration: 10 sec. Amplitude of vertical calibration is equivalent to a film resistance of only 1,600 Ω cm^2.

panied by large stresses which cause a deep distortion in the shape of the reacting proteins. Thus it has been demonstrated that the volume, the optical activity, and the number of exposed sulfhydryl groups in the antibody molecule change markedly upon its reaction with the antigen (45). It would not be surprising that, if antibody molecules firmly attached to the cell membrane undergo a partial denaturation upon reacting with the antigen, a derangement may occur in the structure of the membrane, large enough to decrease its effectiveness as a barrier to ionic movements. In fact, this hypothesis is based on Ishizaka and Campbell (26,27), who suggested that the anaphylactic reaction is attributable to the change in the configuration of antibody molecules combined with tissue constituents.

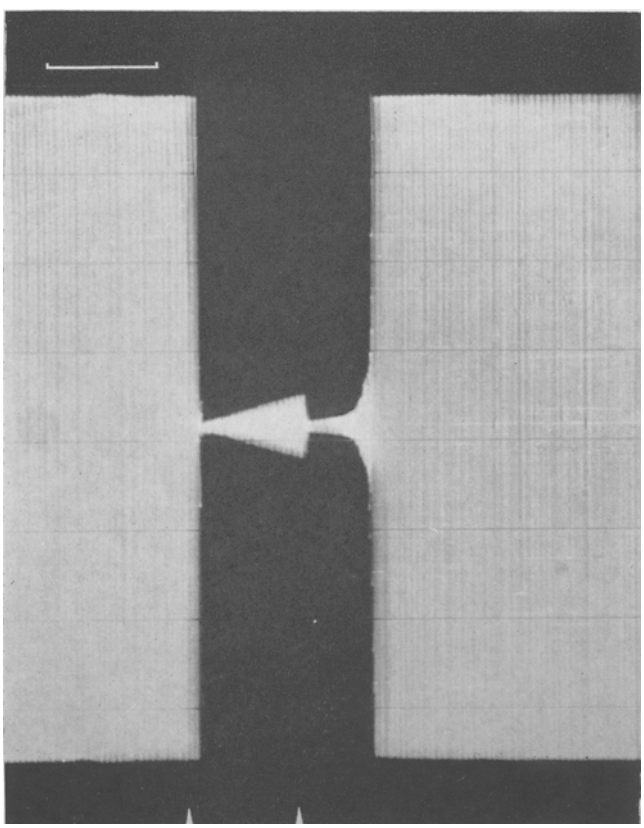

FIG. 13. Sudden reductions in the transverse electrical impedance of an artificial lipid film exposed to chemotrypsin (30 μg/ml) when a substrate for this enzyme, denatured ovalbumin, reaches the lipid-water interface. Each arrow marks the effect of adding a droplet of substrate solution, containing 10 μg of protein, to the same aqueous phase in contact with the lipid in which the enzyme was dissolved. Horizontal line, 0.5 sec.

Experiments with Model Systems

Although the successful reproduction of some of the outward characteristics of biological phenomena does not vouch for the views and assumptions which led to the chosen models, these may be useful insofar as they may suggest new ideas and fresh experimental approaches. In this regard, the recent development of techniques to form lipid films with a thickness and other physical properties resembling those of cell membranes offers an interesting challenge, that of incorporating in such structures mechanisms similar to those operating in natural membranes, for example, chemoreceptors.

When it was found that the immunization of denervated diaphragmatic muscle of the guinea-pig induced the appearance of membrane receptors for the antigenic proteins, we were tempted to see whether a similar phenomenon could be elicited by using, instead of the denervated muscle membrane, the thin lipid films described by Mueller et al. (43). Preliminary experiments gave surprisingly successful results. The films were first exposed to antibodies dissolved into one of the two aqueous compartments in contact with the lipid phase. After a short interval, while the transverse impedance of the film was continuously monitored, antigen was added to the same aqueous phase containing the antibody. This was seen to be followed by a large but reversible reduction in such impedance, a phenomenon comparable with that observed in the denervated muscle membrane (14).

The responses of "immunized" lipid films exhibited a remarkable similarity to those shown by sensitized living tissues. First, they are highly specific, taking place only if the homologous antigen or a closely related protein is employed. Second, the responses show a typical and irreversible desensitization, that is, the membrane responses elicited by addition of the antigen could only be shown two or three times. Finally, the addition of antigen-antibody complex to the system, instead of adding separately the two proteins, had no effect on the impedance of the lipid films. No responses were observed when the antigen was added to one side of the lipid film and the antibody to the other.

Fig. 12 illustrates one of these experiments. The impedance of the film was monitored by recording the

voltage drop caused by the flow of square pulses of current across the membrane but, because of the slow speed of film, the individual pulses merged, giving the impression of continuous traces.

To test the hypothesis that the observed impedance changes could be attributable to configurational changes that take place in antibody molecules during their interaction with the antigen, experiments were conducted with enzyme-substrate systems. It has been proposed by Koshland (32) that the enzyme-substrate interaction is also accompanied by a change in the configuration of the catalytic protein, which would be necessary for the proper alignment of the groups that form the active site. Thus, it was expected that, if enzyme molecules attached to the film were still able to react with their substrates, the subsequent changes in their configuration might be reflected in the transverse resistance of the lipid.

Experiments using several catalytic proteins (both proteolytic and metabolic) confirmed these expectations and gave results resembling those obtained with antigen-antibody systems (Fig. 13). They differ from the latter however as far as the impedance changes elicited by substrates on enzyme-treated membranes could be brought about a number of times in succession without obvious desensitization.

The responses of enzyme-treated films were also highly specific and could be blocked by agents known to inhibit the activity of the enzymes. The results of these experiments suggested that the impedance changes reflect the formation of the enzyme-substrate complex. Unfortunately, the occurrence of such responses, working with both enzyme-substrate and antigen-antibody systems, depends critically upon the composition of the crude lipid mixture used to form the artificial films, composition that varies unpredictably from batch to batch.

Some factors are known to block the responses of the films. However the exact conditions which the lipid mixture must meet to produce responsive membranes are still unknown. For these reasons, further empirical use of these techniques should await a better knowledge of both the chemistry of the lipid extracts and the structure of the films.

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Discussion

DR. J. E. MULDREY (Tulane Medical School, New Orleans, La.): I would like to ask, have you considered fluorescent antigen as perhaps a way of defining a little more closely the actual location of the sites on the denervated muscle which pick up the antigen? What is the combined action of antigen and acetylcholine or antigen and histamine on this kind of system? Can you get from this kind of study on denervated sensitized muscle any index of the possibility that you might be dealing with two or more different types of sites? Finally, with respect to your apparatus for measuring the properties of lipid films, is there any possibility that this might be practical for the quantitative assay of serum levels of FSH and LH?

DR. DEL CASTILLO: To answer your first question, we have not used fluorescent antibodies. However, we have explored carefully the surface of the muscle with antigen-filled micro-taps. In our best preparations, taken from young animals, the entire exposed surface of the muscle fibers seemed to be uniformly sensitive to the antigen. In older animals the connective tissue is thicker, and we do know that this tissue represents a significant barrier to the diffusion of protein. Therefore, the responses to antigen are often very poor and vary considerably at different areas of the membrane, Your second question dealt with a problem we have not studied; that of the interaction between histamine and antigens. We have some observations, however, on histamine sensitivity of the denervated muscles and found that it is different topographically from that to acetylcholine. The electrophoretic application of the transmitter always elicits membrane depolarizations; however, histamine-potentials are only occasionally seen with the same technique.

As to your last question, I believe that this method will ultimately find practical applications. Before

this can be done, however, one has to know more about the chemistry of the lipid extracts and learn how to obtain them consistently. Only a small fraction of the extracts of brain lipids form responsive membranes. Most of them yield films which, while having similar appearance, do not react to immunological or enzymatic reactions. We have found some factors which prevent or block the responses of the films, such as oxidation derivatives of cholesterol. However, attempts to find systematic differences in the composition of active and inert extracts have failed so far.

DR. SMALL: Time for one last short question.

DR. EHRENSTEIN: I was going to make a suggestion about the reasons for the increase in resistance. Perhaps what happens is that in the first and second applications the antigen is preferentially being absorbed.

DR. DEL CASTILLO: This, however, was not antigen. We have never seen an increase in the resistance of the membrane working with antigen-antibody systems. We observed it only when adding physostigmine to cholinesterase-treated lipid films (see Del Castillo, J. A. Rodriguez and C. A. Romero, Ann. New York Acad. Sci. *144*, 803–818 (1967).

DR. EHRENSTEIN: I see. Perhaps the same argument would still apply: that the molecule you are adding would absorb preferentially to the receptive sites during the first and second applications, but if at that point the receptive sites were saturated, then a subsequent addition would no longer allow additional adsorption to these sites. If one assumes that the adsorption at the preferential sites is going to lower the resistance, then you would not get any more lowering, and perhaps the increase in resistance is just some non-specific effect of adding material to the membrane as a whole.

DR. DEL CASTILLO: Well, this is a possibility worth exploring. So far, however, we have not attempted to explain such an effect, though we have used this observation as an argument against the possibility of the resistance drop being an artifact. A decrease in the resistance of the lipid could be attributed to a mechanical breakdown of the membrane. However, it is difficult to see how an *increase* in the resistance could be produced in this way.

DR. EHRENSTEIN: One way of checking this is to put smaller and smaller doses of material in, so that it would take more doses to saturate the sites. In this way it may be possible to assay how much material is required for the effect to start, and thus to determine the number of available sites.

DR. BURTON: Let me paraphrase several questions which have been asked—one, can you see any evidence of adsorption of the protein to the bilayer membrane surface? Two, what is your technique of adding your reagents to the chamber media and, have you measured the rate of mixing, and perhaps, adsorption of the added protein or substrate?

DR. DEL CASTILLO: Evidence on the adsorption of protein to the lipid layer has been obtained by several workers. With regard to the time effects, we purposely have chosen concentrations of substrate which would produce fast transitory effects.

To obtain them we found that the best technique was to use very small droplets (10 μl) of a relatively concentrated substrate solution, adding them to the chamber at a point where the substrate has a high probability of coming into contact with the lipid membrane while still at a high concentration. Since the solution in the chamber is slowly stirred by means of a small magnetic bar, we added the droplets a few millimeters 'upstream.' We have checked the movements of the solution by injecting droplets of ink and could see streaks of dye reaching the membrane area. Therefore, we interpret the transient effects we have recorded as due to the build-up of high, but fleeting, concentrations of the substrate in contact with the lipid; the amount of substrate added with each droplet being too small to cause a significant concentration in the bulk of the solution. When a large, uniform concentration in the solution is reached we observe very long lasting effects, in fact, the membrane breaks often before the resistance comes back to the initial value.

Conclusion

DR. BURTON: I am personally very happy with the fine development of this three-day symposium from its beginning, discussing physical chemistry, up to today, where we have discussed a natural membrane and a model of this natural membrane. It is very apparent that the speakers have spent a great deal of time preparing their lectures for this symposium. For that, I thank them very much. In addition, I am impressed by the tactful moderating of the discussions that occurred during all of these sessions. It contributed a great deal to the success of the meeting. I think our discussion was quite good and to that end I think you, as the audience, deserve also to be complimented because your participation in this discussion is of the utmost importance. Many points came out in discussion that were not in the papers and that were very pertinent, so I thank all of you.