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PHASE TRANSITION IN MEMBRANE WITH REFERENCE TO NERVE EXCITATION

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Elucidation of the processes of nerve excitation on a physicochemical basis is essential for an understanding of the functioning of the nervous system. Because there are many undefinable quantities in a system consisting of an excitable membrane and its natural environment, however, there is frequently serious ambiguity in interpreting the results of physical measurements carried out on nervous tissues. Due to this ambiguity and to the ease with which high time resolution measurements of the electrical concomitants of the excitation process can be made, prevalent theories (*1*) of excitability are concerned mainly with a mathematical description of membrane currents and potentials. Such theories do not rely upon a well-defined physicochemical model of intramembrane mechanisms and are, therefore, not expected to yield any definitive information about the molecular processes involved in excitation.

The fluid media surrounding the axon membrane are, under ordinary experimental conditions, very complex. The external medium

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(blood, Ringer or sea water) contains a large number of uni- and divalent cations and anions. The internal medium (protoplasm) contains organic polyelectrolytes in addition to inorganic salts. The ensuing difficulties were eliminated several years ago when the perfusion technique was introduced into physiological studies of squid giant axons (*2*, *3*). It became possible to remove the protoplasm almost completely without affecting the excitability of the axon. The excitation of the axon could now be analyzed with simple, well-defined solutions on both sides of the membrane, and the necessary and sufficient conditions for the maintenance of excitability could be defined. One of the important conclusions derived from these studies is that the process of excitation is accompanied by a drastic change in the macromolecular conformation of the membrane, triggered by cooperative cation exchange at fixed negative sites in the membrane.

Recently, another powerful technique has been developed which promises to further increase our understanding of the mechanism of excitation. Changes were detected, with specifically designed optical and electrical equipment, in the optical properties of various invertebrate nerves during the nerve impulse (*4*, *5*); changes in turbidity, birefringence, and extrinsic fluorescence were studied also. Changes in thermal properties (*6*) have also been detected during nerve excitation. These optical and thermal studies offer additional evidence that the conformation of the nerve membrane macromolecules changes during excitation.

I. EXPERIMENTAL MANIPULATION OF THE MILIEU SURROUNDING THE SQUID AXON MEMBRANE

The natural milieu of squid axons contains about 450 mM NaCl, about 60 mM of the salts of divalent cations, mainly Ca and Mg ions, and a relatively small amount of other ion species. Although the external milieu of the axon membrane could be readily altered, a physicochemical approach to nerve excitation was not possible until the composition of the internal milieu of the axon could be controlled. It may be said that the physicochemical investigations on the excitation mechanism began with the introduction in 1961 of the technique of intracellular perfusion (*2*).

It is well known that complete removal of divalent cations from the fluid external to an axon causes loss of excitability. However, complete

elimination of univalent cations externally does not always lead to a complete loss of excitability. The first record in Fig. 1 illustrates the action potentials of an unperfused squid giant axon immersed in sea water. When the external solution was switched to a solution containing 100 mM $CaCl_2$ as the sole electrolyte and the axon was internally perfused with a 30 mequiv./l Na-phosphate solution (pH 7.3), the action potential shown in the middle of the figure was observed. Although these fluid media are not " normal " for the axon, the axon membrane did not change irreversibly. When the external medium was switched back to natural sea water and the internal medium to a 400 mequiv./l K-phosphate, the axon again produced action potentials with normal size and duration as shown in Fig. 1 (right).

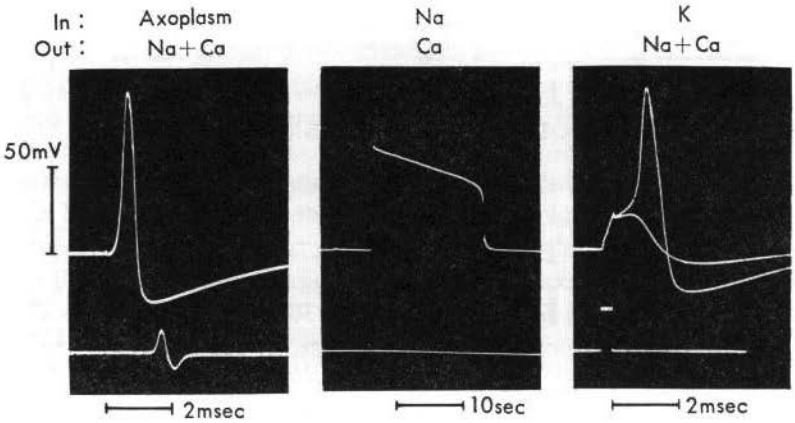


Fig. 1. Oscillograph records of all-or-none action potentials in an axon under different conditions. *Left:* Record taken before initiation of internal perfusion; the external medium contained 300 mM NaCl and 100 mM $CaCl_2$. *Middle:* Record taken approximately 12 min after the onset of internal perfusion with 10 mM sodium phosphate; the external medium was 100 mM $CaCl_2$. *Right:* Record obtained from the same axon after switching the internal perfusion fluid to 400 mM K-phosphate and the external medium to the solution used in the left record; sub- and suprathreshold responses are superposed. The time markers in the right and left records are 1 msec apart. The time marker in the middle record represents 10 sec. The stimulus duration was 0.1 msec for the left record, 100 msec for the middle record, and about 0.3 msec for the right record. Axon diameter: Approximately 400 μ, 21°C (Watanabe, Tasaki and Lerman, *Proc. Natl. Acad. Sci. U.S.*, **58**, 2246 (1967)).

In squid giant axons immersed in a 100 mM $CaCl_2$ solution, the ability to produce action potentials has been demonstrated with the salt of one of the following univalent cations internally: Na, Li, Cs, choline, tetramethylammonium, *etc.* (As we shall see later, either the phosphate or fluoride salts of these cations were most suitable for this type of experiment; sucrose or glycerol was added to the media to maintain the normal osmolarity.) Various salts of Sr or Ba can be used instead of $CaCl_2$ in the external medium (*7*). The action potentials obtained under these conditions are essentially similar to those shown in Fig. 1 (middle).

The analysis of the results obtained from a membrane system containing only two different species of cations is far easier and less amenable to misinterpretation than that from a system with three or more cation species. Therefore, a great emphasis will be placed in this article on the analysis of electrochemical properties observed under these " bi-ionic " conditions.

II. ASYMMETRY OF THE AXON MEMBRANE

When discussing the electrochemical properties of axon membranes, it is important to point out that the inner and outer surfaces of the axon membrane have very different properties. Surrounded with sea water externally, squid axons internally perfused with isotonic KCl solution, maintained excitability for only about 30 min. However, such axons perfused with K-glutamate, K-phosphate, or KF internally maintained excitability for many hours. Different survival times are obtained with different anion species perfused internally (at the same K-ion concentration). The survival time gave the following sequence of anion species (*8*):

$$F > \text{phosphate} > \text{glutamate, aspartate} > SO_4 > Cl > NO_3 > Br > I > SCN.$$

The fluoride ion is most favorable and the SCN ion least favorable for maintaining excitability. The same anion sequence was found with the salts of other cations. This sequence is identical to that of anions arranged according to their lyotropic numbers (*9*) and is consistent with the fact that polypeptides are the major components of the excitable membrane. Similarly, the order of favorability of internally perfused univalent cations for axons was determined by the same method as for anions, and was as follows:

$$Cs > Rb > K > NH_4 > Na > Li\,.$$

This type of sequence is the same as that found by Bungenberg de Jong for phosphate colloids (*10*).

In contrast with the difference among anions in the intracellular perfusion fluid, no significant differences in the effect on excitability were observed among different external anion species. It is well known that there is a marked difference in behavior among external cations. If one calls an external cation " favorable " when it tends to increase the action potential amplitude, the following cations have the sequence:

$$Li,\ Na > NH_4 > Cs > Rb > K\,.$$

(Note that the criterion for determining this cation sequence is different from that for internal cations and anions.)

As pointed out above, divalent cations are indispensable for maintaining the excitability of axon membranes. However, intracellular perfusion with a solution containing divalent cations leads to immediate and permanent loss of excitability. The effects of various univalent cations upon the action potential amplitude are different on the two sides of the axon membrane. All these experimental facts show that there is a marked difference between the physicochemical properties of the inner and outer layers of the axon membrane.

III. ELECTROCHEMICAL PROPERTIES OF AXON MEMBRANES AT REST

Under continuous perfusion with an isotonic solution containing KF and sucrose, a squid giant axon maintains its excitability for many hours in an isotonic medium containing $CaCl_2$ and NaCl. When no electric current passes through the membrane, the transmembrane fluxes of all ion species in the system satisfy the following conditions:

$$J_{Na}+J_K+2J_{Ca}-J_{Cl}-J_F=0$$

where each flux J is expressed in mole$\cdot$cm$^{-2}\cdot$sec^{-1}.

Replacement of the chloride ion in the external medium with other anions, *e.g.*, Br, SO_4, ethylsulphate, *etc.*, does not change the functions of the axon. This fact implies that the axon membrane has a high fixed density of fixed negative charges. When the membrane has such a high density of fixed negative charges, the fluxes of anions should be small.

Furthermore, it has been found experimentally that the flux of the divalent cation, J_{Ca}, is far smaller than the fluxes of univalent cations (*11*). Consequently, the above equality is simplified approximately to

$$J_{Na}+J_K \cong 0.$$

Radio-tracer studies carried out under these experimental conditions reveal that the relation given above is approximately correct in the resting state of axon membranes. Namely, the resting efflux of the K-ion is roughly equal to the resting influx of the Na-ion, irrespective of the concentrations of K- and Na-ions. Under the conditions that the fluxes of anions and divalent cations are negligibly small, integration of the Nernst-Planck flux equations leads to (*12*):

$$J_{Na}=J_K=\frac{RTXu_Ku_{Na}A}{(u_K-u_{Na})\delta}\ln(u_K/u_{Na}), \qquad (1)$$

where X is the fixed charge density of the membrane, δ and A are the effective thickness and area, of the membrane, and u_K and u_{Na} stand for the average mobilities of the K- and Na- ions in the membrane. (Note that A/δ is related to the compactness of the membrane.) In Eq. 1, R and T are the gas constant and the absolute temperature, respectively.

Under the same conditions, the electric resistance across the membrane is represented by the equation:

$$r=\frac{(u_K-u_{Na})\delta}{F^2Xu_Ku_{Na}A}[\ln(u_K/n_{Na})]^{-1}, \qquad (2)$$

where F is the Faraday constant. A comparison between Eqs. 1 and 2 leads to the following equation:

$$rJ_i=RT/F^2 \qquad (\text{for } i=K,\ Na) \qquad (3)$$

This equality is valid under the assumption that the membrane is highly permselective, *i.e.*, that the anion fluxes are negligibly small. The experimental data for the squid giant axon in the resting state show that Eq. 3 is valid, taking into account the possibility of experimental errors (*11*). This finding is consistent with the view that the membrane of the squid axon in the resting state is a cation exchanger membrane.

Now, let us consider a uniform cation exchanger membrane that sep-

arates two electrolyte solutions containing both univalent and divalent cations. The membrane is assumed to be permselective. The activities of uni- and divalent cations in the outer solution are a_1' and a_2', and those in the inner solution are a_1'' and a_2'', respectively. The integration of the Nernst-Planck equation yields the following equation for the membrane potential, $\Delta\varphi$ (*13*):

$$-\Delta\varphi = \frac{RT}{F}\left\{\ln\frac{a_1''}{a_1'} + \frac{u_2}{2u_2-u_1}\ln\frac{\gamma''+1}{\gamma'+1} + \frac{u_2-u_1}{2u_2-u_1}\ln\frac{u_2\gamma''-u_2+u_1}{u_2\gamma'-u_2+u_1}\right\}, \quad (4)$$

where γ' and γ'' denote the values of $(1+8KX\,a_2/a_1^2)^{1/2}$ at the membrane outer and inner surfaces, K is the equilibrium selectivity coefficient defined by

$$\bar{C}_2/\bar{C}_1^2 = Ka_2/a_1^2. \quad (5)$$

The other notations are the same as in the previous equations, except that the subscripts 1 and 2 refer to the univalent and divalent cations species, respectively. In Eq. 5, $\bar{C}_2$ and $\bar{C}_1$ are the average concentrations of divalent and univalent cations in the membrane phase which equilibrates with a solution containing divalent and univalent cations at the respective activities a_2 and a_1. In the derivation of Eq. 4, K, X and u_2/u_1 are assumed to be constant throughout the membrane. When the chemical composition of the solution on one side is held constant and the concentrations of uni- and divalent cations on the other side are varied, Eq. 4 is simplified to

$$\Delta\varphi = (RT/F)\left[\ln a_1' + \frac{u_2}{2u_2-u_1}\ln(\gamma'+1) + \frac{u_2-u_1}{2u_2-u_1}\ln(u_2\gamma'-u_2+u_1)\right] + \text{const}. \quad (6)$$

The constant in Eq. 6 is related only to the composition of the solution of the side denoted by $''$. It is easy to show that when either K or u_2/u_1 approaches infinity, Eq. 6 becomes

$$\Delta\varphi = (RT/2F)\ln a_2' + \text{const}. \quad (7)$$

In either case, the membrane potential is determined by the activity of the divalent cation species in the external solution only. Conversely, when K or u_2/u_1 approaches zero, Eq. 6 becomes

$$\Delta\varphi = (RT/F)\ln a_1' + \text{const}. \quad (8)$$

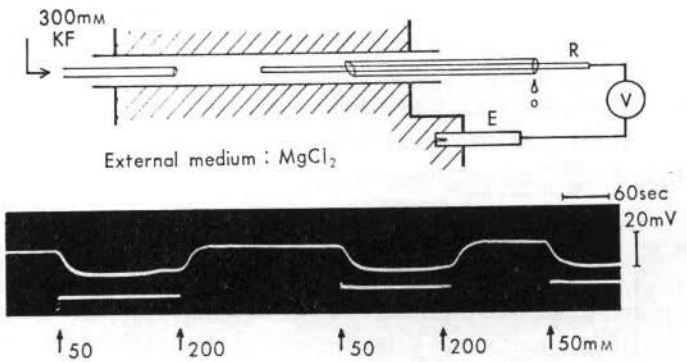


Fig. 2. *Top:* Schematic diagram showing the experimental setup used to determine the effect of the reduction of the external divalent cation concentration on the resting membrane potential; E represents a calomel electrode and R a glass capillary electrode filled with 600 mM KCl. *Bottom:* Oscillograph records taken from an axon internally perfused with 300 mM KF (glycerol) solution (phosphate buffer). Downward deflections represent increased negativity within the axon. External media are prepared by mixing a 400 mM $MgCl_2$ (Tris-buffer) with 12% (v/v) glycerol (Tasaki, Watanabe and Lerman, *Am. J. Physiol.*, **213**, 1465 (1967)).

In this case, the activity of the univalent cation species in the external bulk solution determines the potential difference across the membrane. Therefore, observations of the dependence of the membrane potential on the concentration of cation species are expected to reveal whether or not the membrane has a very large selectivity coefficient K for divalent cation over univalent cation species.

The effect of the dilution of the external medium was examined by using internally perfused squid giant axons (*14*). An example of the results is shown in Fig. 2. A thoroughly cleaned axon was internally perfused with a solution containing 300 mM KF and 30 mM K-phosphate at pH=7.3. The external medium was a rapidly flowing solution containing 200 mM $MgCl_2$. The external medium was then switched to a rapidly flowing 50 mM $MgCl_2$ solution. As can be seen in Fig. 2, there was an immediate fall in the intracellular potential referred to the potential of the external medium. When the original 200 mM $MgCl_2$ solution was reintroduced, there was a prompt rise in the intracellular potential. In most cases, the effect of dilution was completely reversible. The magnitude of the potential change observed was 14 to 18 mV for a four-

fold change in the concentration. This observed value agrees very well with the value expected from Eq. 7. Similar results were obtained when $CaCl_2$ was used instead of $MgCl_2$ in the external solution. In the concentration range between 100 and 400 mM, the replacement of the Mg-ion with the Ca-ion produced no change in the membrane potential.

On the other hand, a change in concentration of the normally predominant Na-ion in the external medium at a fixed concentration of divalent cations hardly affects the intracellular potential in the resting state. For example, the addition of univalent cations, *e.g.*, Na-ions, to the external medium produces no significant change in the potential. A tenfold variation of the Na-ion concentration, *i.e.*, from 10 to 100 mM in the presence of 200 mM $MgCl_2$, brings about only a few mV changes in the intracellular potential instead of the 58 mV expected from Eq. 8. A similar result is observed for K-ions, provided that the K-ion concentration does not exceed a certain critical concentration, as will be discussed in detail in the subsequent section.

The experimental results mentioned above imply that the axon membrane in a resting state has a high selectivity for divalent cations over univalent cations. (Note that the membrane potential is insensitive to the external univalent cation concentration when $K \gg 1$.) In other words, the resting state of the axon membrane may be regarded as a divalent cation-rich state. At lower concentrations of divalent cations, however, the membrane potential frequently becomes unstable; the fluctuation of the membrane potential under these conditions is probably related to the process of action potential production, as will be discussed below.

IV. ABRUPT DEPOLARIZATION

In squid giant axons internally perfused with Cs- (or Na-) phosphate and immersed in an external solution containing $CaCl_2$ as the sole electrolyte, the membrane potential can be abruptly changed when the K-ion added to the external medium exceeds a certain concentration. Figure 3 shows an example of the experiments of this type (*15*). The axon is electrically excitable under these conditions as noted previously; but no electrical current was delivered to the axon during the following process.

The axon was kept in a steady state by rapid circulation of both

internal and external solutions. At the onset, no univalent cation was present in the external medium. Then, portions of a nonelectrolyte (glycerol or sucrose) in the external medium were replaced with KCl and the external K-ion concentration was raised in small steps. No significant change in the membrane potential was observed when the external KCl concentration was raised successively from 0 to 5 mM. However, when the concentration of KCl reached a certain value, 10 mM in the case shown in Fig. 3, the intracellularly recorded potential increased

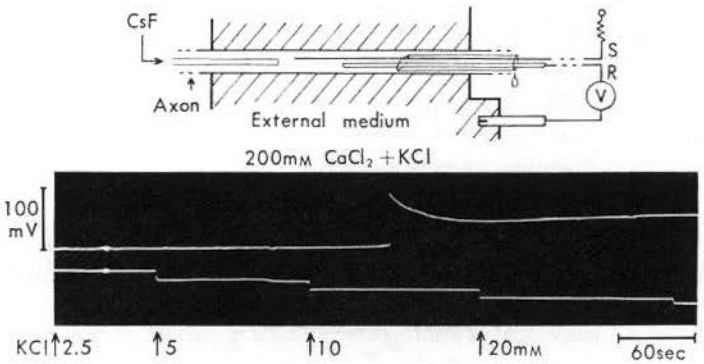


Fig. 3. Abrupt depolarization induced by external application of KCl. The KCl concentration in the rapidly flowing external fluid medium was raised by a factor of 2 at times marked by arrows. A silver wire electrode (S) was used to test the excitability of the axon before the experiment. R represents a glass pipette recording electrode. No electric stimuli were delivered while continuous recording of the membrane potential (V) was made. The potential jump produced by 10 mM KCl was 83 mV, and this was followed by a gradual potential fall of approximately 40 mV. Temperature, 16°C (Tasaki, Takenaka and Yamagishi, *Am. J. Physiol.*, **215**, 152 (1968)).

abruptly by more than 80 mV. This was followed by a gradual potential fall of about 40 mV. Once the potential jump had been observed, a further increase of the external KCl concentration caused no more sudden changes in the membrane potential. Instead, the potential changed smoothly with the KCl concentration and followed approximately Eq. 8: a tenfold variation of KCl concentration produced about a 58 mV change in the membrane potential.

In the field of electrophysiology, a rise in the internal potential level

is called " depolarization." Following an " abrupt depolarization " caused by KCl, the axon cannot respond with an action potential to a stimulating (outward-directed) current through the membrane. When the axon membrane is depolarized, the lowering of the external KCl concentration produces " abrupt repolarization." The K-ion concentration required for repolarization is lower than that for depolarization.

When the external KCl concentration is only slightly above the critical concentration that caused an abrupt depolarization, an increase in the external Ca-ion concentration without changing the KCl concentration brings about repolarization.

As argued above, the observations indicate that the selectivity coefficient K of Eq. 5 suddenly changes from a very high value to a low value when the critical level of external KCl concentration is reached. In other words, the resting membrane is considered to be in a Ca-rich (or divalent cation-rich) state; and the depolarized membrane is regarded as being in a K-rich (or univalent cation-rich) state.

Measurements of the membrane impedance indicate that there is

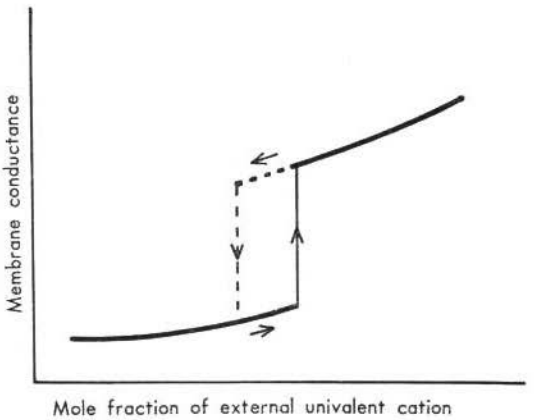


Fig. 4. Simplified diagram showing abrupt changes in the membrane conductance produced by continuous changes in the mole fraction of the external univalent cation. This diagram is based on the results from experiments with squid giant axons internally perfused with CsF or Na-phosphate. Note the hysteresis loop in the diagram (Tasaki, Barry and Carnay, in " Physical Principles of Biological Membranes," ed. by F. Snell, J. Wolken, G. Iverson and J. Lam, Gordon and Breach, New York, (1970)).

a sudden and large fall in membrane resistance when the axon membrane is abruptly depolarized by the addition of K-ion to the external medium. This fall of the membrane resistance is followed by a gradual, partial restoration of the resistance with time, as will be shown later (see Fig. 7B). As noted in Sec. III, the electric resistance of a membrane is determined by the mobilities and concentrations of the mobile ions within the membrane. Hence the sudden fall in the membrane resistance associated with abrupt depolarization is a reflection of a sudden increase in the concentration of univalent cations in the membrane and/or a sudden decrease in the compactness of the membrane. The fact that a continuous rise in the external K-ion concentration produces an abrupt change in the membrane structure is a sign of the cooperativity of the process involved. In fact, a plot of the membrane resistance as a function of the mole fraction of the external univalent cations also shows an abrupt change, as shown in Fig. 4.

There are, in addition to the K-ion, other univalent cations which cause abrupt depolarization. The sequence of univalent cations that depolarize the membrane is

$$K > Rb > Cs > NH_4 > Na > Li\,.$$

The K-ion has the strongest depolarization power, *i.e.*, the lowest critical concentration for abrupt depolarization. A difference in the chemical species of anions in the external medium does not appreciably alter the observed depolarization power of univalent cations. It is interesting that the above ion sequence is the reverse of that for the favorability of external cation species, and that the sequence is similar to that determined for the so-called sodium-pump mechanism (*16*). The reason for this similarity will be explained in the later section.

V. AXON MEMBRANE IN THE EXCITED STATE

The excitability of a squid giant axon can be maintained for many hours with an external medium containing 200 to 500 mM NaCl and 50 to 100 mM $CaCl_2$ and an internal perfusion fluid consisting of 400 mM KF. Then the amplitudes of the action potential vary with the external Na-ion concentration in accordance with Eq. 8. Note, however, that such an amplitude-augmenting effect is not limited to the Na-ion. It is known

that the excitability of the axon can be maintained when the Na-ions in the external medium are replaced with polyatomic cations, such as hydrazinium, guanidinium, NH_4, or other appropriate univalent cations. The amplitude of the action potential varies logarithmically with the concentration of these polyatomic cations.

Changing anions in the external solution does not affect the excitability or the amplitudes of the action potential. In other words, the axon membrane behaves like a reversible, univalent cation-sensitive electrode in the excited state, irrespective of the external cation species. In this sense, the excited state of the axon membrane is considered to be equivalent to the depolarized state of the membrane discussed in the previous section.

Isotope measurements have been carried out on axons internally perfused with a K-salt solution and immersed in a solution containing both NaCl and $CaCl_2$. Such measurements indicate that the fluxes of anions, J_{Cl}, and of divalent cations, *e.g.*, J_{Ca}, during the excited state are almost negligible compared with those of interdiffusing univalent cations, J_K and J_{Na}. Both J_K and J_{Na} increase by a factor of about 200 at the peak of an action potential compared with the levels in the resting state. The influx of Na-ions, J_{Na}, is roughly equal and opposite to the outflux of K-ions, J_K.

Impedance measurements as well as determination of the current-voltage relationship of the axon in the resting and excited states show that the electric resistance of the axon decreases by a factor of about 200 at the peak of action potential. This implies that the electric resistance-flux product, *i.e.*, $r \times J_i$ (i=K or Na), remains roughly unaltered during excitation. It then follows from Eq. 3 that the axon membrane behaves as a permselective cation exchanger membrane in the excited state, as well as in the resting state. Thus, we may conclude that anions in the external medium are nearly perfectly excluded from the membrane phase both in the resting and excited states.

As noted previously, a stimulating current is directed outward through the axon membrane. Such a current tends to transport internal univalent cations, K-ions, into the axon membrane and to increase the mole fraction of the K-ions in the membrane. This increase in the mole fraction of K-ions is similar to that obtained with the addition of K-ions to the external medium. The favorable internal cations (K, Rb, Cs) have a stronger tendency to depolarize the membrane than the

usual external univalent cations (Na, Li, *etc.*). Consequently, the transport of the internal, more depolarizing cations into the critical layer of the axon membrane by a stimulating current pulse is very effective in bringing the membrane to the depolarized state. Thus, it is considered that the initiation of an action potential by a stimulating current is an abrupt depolarization triggered by electrophoresis of the internal univalent cation into the membrane.

VI. STABILITY OF RESTING AND ACTIVE STATES OF AXON MEMBRANE

Both the resting and the excited states of an axon membrane are stable. This stability is examined by applying a small perturbation from an electric field across the membrane. Figure 5 illustrates the procedure

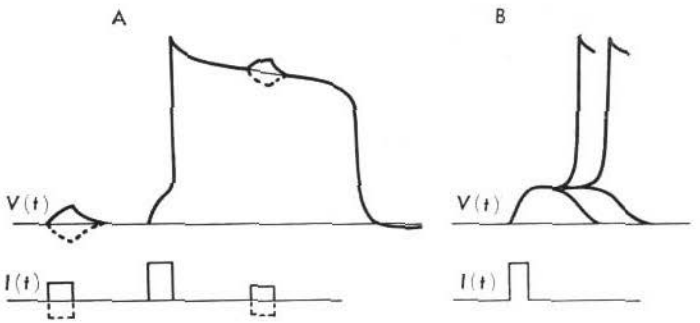


Fig. 5. A: Diagram showing the " stability " of the squid axon membrane in the resting state and during the plateau of a prolonged action potential. The lower trace, marked $I(t)$, shows the time courses of the rectangular current pulses applied to the axon. The upper trace, marked $V(t)$, shows the variation of the membrane potential produced by the current pulses. B: Diagram showing the variability in the potential-time curve for stimuli at threshold intensity; the four traces represent the membrane potentials observed at the same stimulus intensity (Tasaki, in " Nerve Excitation," Charles C. Thomas, Springfield, Ill., p. 106 (1968)).

for such an examination. The lower trace in the figure represents the time course of the rectangular current pulses applied to the axon membrane. The upper trace represents the resulting variations of the intracellularly recorded membrane potential. When the intensity of a

perturbing current pulse is low, the transmembrane potential of the axon in the resting state varies exponentially with time. The potential variation brought about by an inward-directed current is approximately equal in magnitude (and the opposite in sign) to the variation caused by an outward-directed current pulse of the same intensity. The termination of the applied current leads to the return of the potential to the initial level. A similar potential change can be demonstrated during the plateau phase of a prolonged action potential (*17*).

When the current pulse applied to the axon membrane at rest raises the intracellular potential level by about 20 mV, an action potential is produced. After a current pulse of threshold intensity, the transmembrane potential is constant for a variable period of time before it approaches either the upper or the lower value of the membrane potential. In Fig. 5B, the four oscillograph traces show the time course of the membrane potentials with the same stimulus intensity. These observations indicate that both the upper (excited) and lower (resting) levels in the membrane potential are stable. Furthermore, the critical potential may be considered to be an intermediate, unstable level between the two stable levels. It is known that a pulse of inward-directed current delivered during the plateau of a prolonged action potential can produce a transition from the upper potential level to the lower. In order to produce this transition, the membrane potential has to be displaced beyond the unstable level between the upper and lower stable potential levels.

We shall now discuss the stability of the axon membrane that has been depolarized by application of KCl externally. When weak perturbing current pulses are applied to such a membrane, it is found that the depolarized state of the membrane is stable. Also, a strong pulse of outward-directed current does not evoke an action potential. But, a strong pulse of inward-directed current shifts the membrane potential from the depolarized level to the repolarized. Electric responses (*i.e.*, large, abrupt changes in the membrane potential) observed in depolarized axons are reversed in sign (*18*).

VII. EFFECTS OF TEMPERATURE CHANGES ON EXCITABILITY

It has long been known that an action potential can be evoked by sudden cooling in various excitable tissues. This implies that the process in

the axon membrane underlying depolarization (excitation) is exothermic, and that repolarization is endothermic. Since an endothermic reaction is encouraged by heating, a rise in temperature should lead to repolarization of the excited membrane. This was tested for a prolonged action potential by applying a brief heat pulse (*19*). It was found that the action potential could be terminated by a heat pulse in an all-or-none manner. This observation supports the view that the termination of the action potential (and, hence, repolarization) is a cooperative and endothermic reaction. Recent studies on temperature change during action potential also support this view (*6*).

VIII. STRUCTURE AND MODEL FOR AXON MEMBRANE

Since the adherent Schwann cell and connective tissue cannot be removed from the external surface of the 100 Å thick axonal membrane, it is difficult to chemically analyze the squid axon membrane. However, considering the experimental results described in the preceding sections, we may visualize a physical picture of the excitable membrane (*20*).

The excitable membrane is represented by a network of macromolecules held together, at least partly, by intra- and intermolecular salt-linkages between adjacent charged groups. The membrane may be subdivided into two distinct layers: The outer layer is compact with many fixed negatively charged groups on its component macromolecules; these negatively charged groups are probably carboxylates and confer cation exchanger properties on the outer layer. The inner layer of the membrane is less compact and contains a small number of fixed anionic sites, probably phosphates. The major diffusion barrier is considered to be the outer layer of the membrane.

In the outer layer, the complex formation between the fixed charged groups and the divalent cations from the external medium, as well as hydrogen and hydrophobic bonds, keeps the structure compact in the resting state. However, this structure is labile and can readily be disrupted by univalent cations, especially by the invasion into the outer layer of the univalent cations that have a strong depolarizing power, like the K-ion. The disruption leads to a decrease in compactness and to an increase in hydrophilicity of the outer membrane layer. The change in the membrane during excitation (or depolarization) may be attributed to this disruption. The decrease in compactness of the membrane in the

depolarized state partly stems from the strong repulsive forces between the charged groups of the membrane skeleton released by disruption of complexes. The rise in hydrophilicity may be due to the rearrangement of water molecules around the negatively charged sites exposed by the removal of divalent cations.

It is reasonable to assume that the structure of the membrane is not perfectly uniform along the axon surface. The distribution of the depolarized and repolarized portion in the membrane is considered to fluctuate with time due to the thermal motion of ions and membrane macromolecules. When the axon (as a whole) is in the resting state, the major portion of the membrane surface is compact and rich in divalent cations. When the axon is excited, the major portion of the membrane surface is in a loose, univalent cation-rich state.

The potential across a membrane depends on both the salt compositions of the external and internal media and on the structure of the membrane. Therefore, the membrane potential in the excited conformational state of the membrane differs from that in the resting state even when the chemical composition in the milieu stays constant. Then, if the membrane is composed of a mixture of patches in the excited state and of patches in the resting state, the local membrane potential will vary from one position to another along the membrane surface.

A depolarized (excited) patch has a smaller negative membrane potential than a resting patch has. This non-uniform distribution of patches with different emfs along the membrane surface creates local or eddy currents even when there is no net electric current across the membrane. The local currents are inwardly directed in excited patches, and outwardly directed across resting patches. The inward current through the excited patches of the membrane tends to induce repolarization by bringing Ca-ions into the membrane from the outer solution, while the outward current through the resting portion tends to bring about depolarization by carrying K-ions into the membrane from the axon interior. As a consequence of this long range interaction between resting and excited portions of the membrane, an excitable axon which is as a whole in its resting state, is considered to possess active (excited) spots which appear and disappear spontaneously.

When the major cation species in the external medium are Na- and Ca-ions, the axon membrane can be in the resting state even at a relatively high Na-ion concentration because of the weak depolarizing

power of the Na-ions. If K-ions invade the external surface of the membrane by the application of a stimulating current or by an increase of the KCl concentration in the outer solution, the membrane fraction in the depolarized state rapidly increases because of the strong depolarizing power of K-ions. When the fraction of the membrane in the excited state reaches a certain critical value, the membrane becomes predominantly active. A simple calculation based on the difference in membrane potential and conductance between the excited and resting states of the axon membrane indicates that the critical active fraction is less than 1% of the total membrane for the case of the squid axon membrane. This indicates that excitation of only 1% of the entire surface of the membrane is sufficient to throw the remaining 99% of the membrane into the excited state.

In the above discussion, it is assumed that every surface element (subunit) of the membrane is either in the excited (active) or the resting state (*20–22*). We now consider possible physicochemical bases for this property of the membrane macromolecules.

Barrer and Falconer (*23*) have shown that the X-ray diffraction pattern of Na-rich zeolite is different from that of K-rich zeolite. As the mole fraction of K-ion in a solution bathing Na-rich zeolite is continuously increased, the X-ray pattern changes. In the reverse direction where the K-rich form is converted to the Na-rich form, the transition occurs at a different mole fraction. A similar discontinuous process is encountered in the exchange between divalent and univalent cations in certain synthetic zeolites (*24*). These discontinuous ion-exchange processes can be explained in terms of statistical mechanics. When the occupancy of the neighboring sites by cations of the same kind is energetically more favorable than the occupancy of the adjacent sites by different cation species, a cooperative ion-exchange transition is expected. Making use of the Bragg-Williams approximation on the two dimensional lattice, we can derive an ion-exchange isotherm for a two cation species system (*23*).

An example of a theoretical ion-exchange isotherm for divalent-univalent cations on a two-dimensional lattice is illustrated in Fig. 6 (*20*). The abscissa represents the equivalent fraction of univalent cations in the solution phase, and the ordinate represents the equivalent fraction of univalent cations within the membrane phase. On the line marked ABC, the fraction of divalent cations in the membrane phase is much

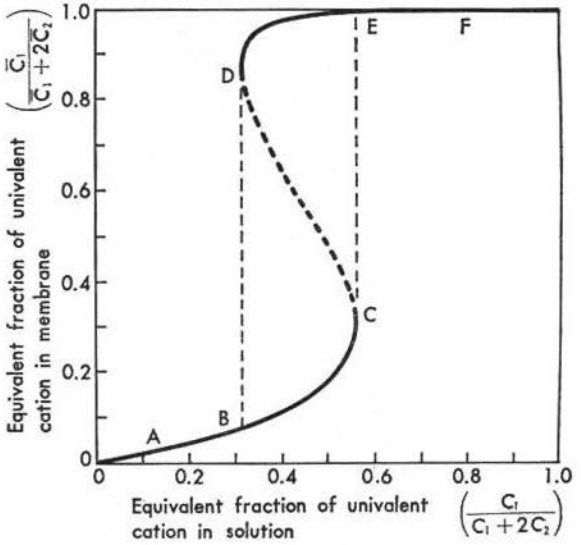


Fig. 6. Theoretical ion exchange isotherm calculated for a cation exchanger membrane immersed in a salt solution containing univalent and divalent cations. In the calculation, it was assumed that occupancy of two neighboring charged sites in the membrane by two cations of different valences is energetically unfavorable. C_1 and C_2 represent the concentrations in the solution of univalent and divalent cations, respectively; $\bar{C}_1$ and $\bar{C}_2$ represent the concentrations within the membrane. When the equivalent fraction of the univalent cation in the solution is increased continuously from 0 to 1, the corresponding fraction in the membrane increases along the course 0, A, B, C, E, and F. When the equivalent fraction in the solution is decreased from 1 to 0, the corresponding fraction in the membrane changes along F, E, D, B, A, and 0 (Lerman, Watanabe and Tasaki, in " Neurosciences Research," ed. by Ehrenpreis and Solnitzky, Academic Press, New York, Vol. 2, p. 71 (1969)).

larger than that in the bathing solution phase, that is, the selectivity coefficient K defined by Eq. 5 is much larger than unity, $K \gg 1$. When the external univalent cation fraction reaches C in Fig. 6, there is a discontinuous increase in the fraction of the univalent cations in the membrane phase. A further increase in the fraction of the external univalent cation leads to a continuous increase in the intramembrane univalent cation concentration. At this stage, the selectivity constant K is much smaller than unity, $K \ll 1$.

The isotherm shown in Fig. 6 implies that a hysteresis loop exists

in this type of cation-exchange process. When the external univalent concentration is lowered continuously from the point marked F, a sudden fall in the intramembrane univalent fraction is expected to occur along the line D-B. The external univalent fraction at point B is much lower than that at point C. A hysteresis is also observed in the abrupt depolarization experiments on the squid giant axon membranes as discussed above (see Fig. 4).

IX. TWO STABLE STATES OF AXON MEMBRANES

The resting state of an axon membrane corresponds to the segment ABC in Fig. 6, where the negative sites of the axon membrane are predominantly occupied by divalent cations derived from the external solution. The depolarized or excited state of the membrane corresponds to the segment DEF. Here, the equivalent fraction of univalent cations in the membrane is greater than that of divalent cations. The discontinuous relationship between the external univalent cation concentration and the membrane potential in abrupt depolarization experiments, as well as in production of action potentials under bi-ionic situations, can thus be interpreted as representing a phase transition within the membrane associated with a cooperative ion-exchange process involving univalent and divalent cations at anionic membrane sites.

As mentioned in the previous section, the value of the selectivity coefficient K defined by Eq. 5 varies discontinuously from a very large value on the segment ABC to a small value on the segment DEF at point C. A sudden decrease in K, and thus a sudden increase in the univalent cation fraction at the outer membrane surface, causes a sudden rise in the intracellular potential, as seen in Eq. 4. Since the intramembrane mobility of univalent cations is greater than that of divalent cations, the sudden rise in the intramembrane univalent cation fraction is accompanied by an abrupt fall in membrane resistance.

When membrane resistance suddenly falls following transition, there is a corresponding rise in the cation fluxes as described in Eq. 3. This rise in the cation fluxes gradually decreases the concentration difference (across the membrane) of the cations contributing to the membrane potential, resulting in a gradual shift of the potential.

The results of impedance measurements on axons under bi-ionic conditions (shown in Fig. 7) indicate that membrane resistance progres-

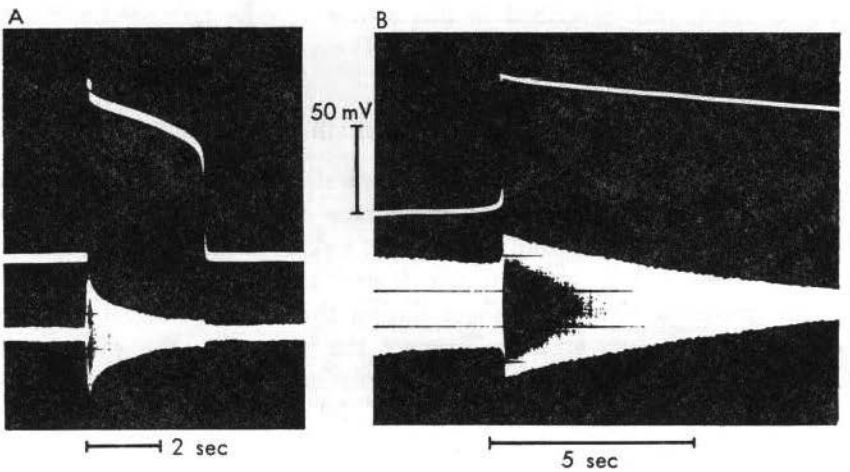


Fig. 7. A: Changes in the membrane impedance associated with the electrically induced action potential of an axon internally perfused with 25 mM CsF solution and immersed in 200 mM $CaCl_2$ solution. The impedance bridge was balanced with the membrane impedance in the resting state. The room temperature was 18°C. B: Changes in the membrane impedance produced by an alteration of the composition of the external medium from a mixture of 200 mM $CaCl_2$ and 25 mM CsCl to a mixture of 100 mM $CaCl_2$ and 450 mM CsCl (abrupt depolarization). The temperature was 19°C. Note that the sudden rises in the membrane potential were associated with simultaneous changes in the membrane impedance (Tasaki, Takenaka and Yamagishi, *Am. J. Physiol.*, **215**, 152 (1968)).

sively increases during action potential. Thus the interdiffusion fluxes of cations through the membrane would gradually fall while the axon is in the excited state. This fall of cation fluxes in turn raises the divalent cation fraction in the outer membrane surface. Eventually, the membrane undergoes a transition from the univalent cation-rich state to the divalent cation-rich state (D-B transition in Fig. 6), bringing about an abrupt repolarization. Thus the phenomena of abrupt depolarization and repolarization and, hence, the production of action potential are adequately explained on the basis of cooperative ion-exchange processes.

As was shown in Sec. VI, both the excited and resting states are stable in the sense that small perturbations do not trigger large changes in the properties of the membrane and that the membrane returns to the original state when the perturbations are removed. For this reason, the

logical framework described in this article may be termed the "two stable states theory."

X. OPTICAL PROPERTIES OF AXON MEMBRANES

Until quite recently, rapid physicochemical changes occurring during excitation in and near the axon membrane could be detected only by electrophysiological techniques. Now it is known that light scattering, birefringence (*4*, *5*), and extrinsic fluorescence (*5*, *25*) of axon membranes undergo transient changes during the action potential. These optical observations strongly support the view that the process of excitation is accompanied by a conformational change of the macromolecules within the axon membrane. In this article we are mainly concerned with the changes in extrinsic fluorescence, because such changes are readily interpreted on a molecular basis.

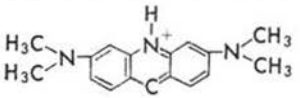
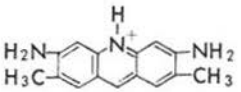
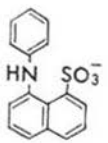
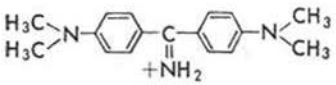
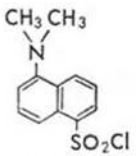
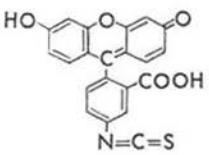
Under natural conditions, nerves from squid or spider crabs, do not show any detectable fluorescence in the visible range of the spectrum. When nerves are vitally stained with the appropriate fluorescent dyes and are irradiated with quasi-monochromatic light, the emission of fluorescent light by the nerve can be detected. When the stained nerves are stimulated electrically, a small transient change in the intensity of the fluorescent light occurs when the nerve impulse arrives at the irradiated portion of the nerve.

The fluorescent compounds listed in Table I give optical signals (*25*). Some of these dyes give rise to negative signals, representing a transient decrease in fluorescence during the excitation (negative sign in Table I). The names and chemical structures of dye molecules and the wave lengths of the excited quasi-monochromatic light used are listed in the first and second columns of the table. The signs and the relative magnitudes of the observed optical signals, which are between 5×10^{-4} and 10^{-5} times the fluorescence of the unstimulated nerve preparation, are given in the table for three different nerve preparations. The dyes were applied intracellularly in the case of squid giant axons; with squid fin nerves and crabn erves, staining was performed by immersion.

Nerves stained externally with Pyronin B and Rhodamine B gave rise to a transient decrease in fluorescence intensity during action potentials. It was not expected that intracellularly applied Pyronin B

TABLE I

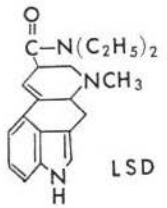
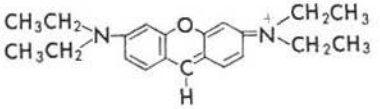
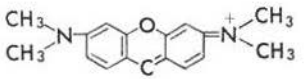
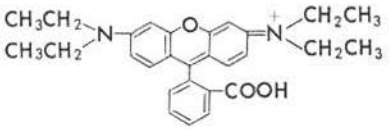
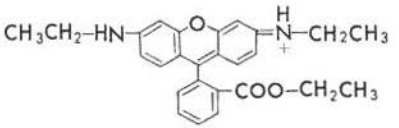
Compounds Used to Demonstrate Fluorescence Changes in Nerves

Compound	Excitation wavelength in nm	Giant axon	Fin nerve	Crab nerve
 Acridine orange	465 (20)	+++	+++	+++
 Acridine yellow	450 (20)	0	+	++
 ANS	365 (10)	—	++	+++
 Auramine O	450 (5)	0	0	++
 DNS	365 (10)	0	0	+
 FIT	500 (20)	+ (ext)	++	++

continued

TABLE I

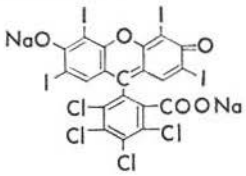
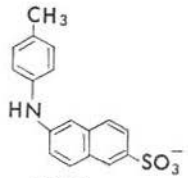
Compounds Used to Demonstrate Fluorescence Changes in Nerves (continued)

Compound	Excitation wavelength in nm	Giant axon	Fin nerve	Crab nerve
 LSD	365 (10)	0	+	++
 Pyronin B	550 (5)	++	—	—
 Pyronin Y	550 (5)	0	—	—
 Rhodamine B	550 (5)	—	—	—
 Rhodamine G	500 (20)	0		++

continued

TABLE I

Compounds Used to Demonstrate Fluorescence Changes in Nerves (continued)

Compound	Excitation wavelength in nm	Giant axon	Fin nerve	Crab nerve
 Rose bengal	550 (5)	0		++
 TNS	365 (20)	–		–

Symbols: ANS, 8-anilinonaphthalene-1-sulfonate; DNS, 1-dimethyl-aminonaphthalene-5-sulfonyl chloride; FIT, fluorescein isothiocyanate; LSD, lysergic acid diethylamide; TNS, 2-*p*-toluidinylnaphthalene-sulfonate. The half band widths of filters used are given in parentheses. Strong, medium, and weak optical signals are shown by the difference in the number of + (increase) and – (decrease) symbols; the absence of a measurable signal is shown by 0 (adopted from Tasaki, Carnay and Watanabe, *Proc. Natl. Acad. Sci.*, **64**, 1362 (1969)).

would give rise predominantly to an increase in fluorescent light during excitation. It was also of great interest that the sign of the signal from squid giant axons perfused with ANS was opposite to that of the ANS signals obtained from squid fin and crab nerves.

The measurement of "*fluorescence polarization*" offers direct information about the viscosity of the medium surrounding fluorescent molecules. Figure 8 shows the principle of the technique used for measuring the fluorescence polarization. The white light from a strong incandescent lamp was converted into quasi-monochromatic light by an interference filter F_1. The monochromatic light was polarized by inserting a polarizer P between F_1 and the nerve, so that the electric vector

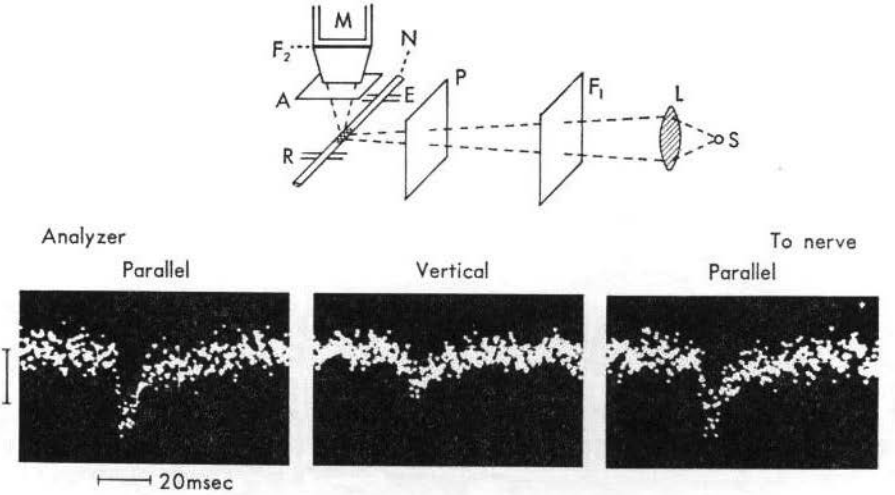


Fig. 8. *Top;* Schematic diagram showing the experimental setup used to measure changes in fluorescence polarization during excitation in a crab nerve stained with Pyronin B. The letter S represents the light source; L, lens; F_1, primary filter; F_2, secondary filter; M, photomultiplier tube; P, polarizer; A, analyzer; N, nerve; E, stimulating electrodes; R, recording electrodes. *Bottom;* Optical signals obtained with the principal axis of the analyzer (E-vector of fluorescent light) parallel to the long axis of the nerve (right and left records) and perpendicular to the nerve (middle record). The quasi-monochromatic light used for excitation of dye molecules was polarized with its E-vector in the direction parallel to the nerve. A CAT computer was used for recording. For right and left records, the vertical bar represents 2×10^{-4} times the background intensity. The middle record was taken under the same experimental conditions except that the analyzer was rotated by 90°. The temperature was 19°C (adopted from Tasaki, Carnay and Watanabe, *Proc. Natl. Acad. Sci. U. S.*, **64**, 1362 (1969)).

of the incident light wave was parallel to the longitudinal axis of the nerve. The light was focused on the stained nerve by a lens L. The fluorescent light from the nerve was detected at 90° to the incident beam with a photomultiplier tube; a secondary filter F_2 and an analyzer A were placed between the nerve and the photomultiplier. The analyzer could be rotated 90° so that fluorescent light, polarized either parallel or perpendicular, to the nerve could be detected. To improve the signal-to-noise ratio in recording, a digital computer was used for averaging multiple signals. The nerve was stimulated with brief pulses of electric

current applied to the nerve near one end, and the action potentials were recorded externally at the other end.

Figure 8 (bottom) shows recordings from a crab nerve stained externally with Pyronin B. The dye molecules in the stained nerve were excited with a monochromatic polarized light 550 mμ in wave length.

Changes in the fluorescent light from the nerve were first measured with the analyzer parallel to the nerve axis (right and left records in Fig. 8). Thenthe analyzer was rotated 90° and the change in emitted light during the action potential was again recorded (middle record). It was found that the change in the fluorescent light with its electric vector parallel to the nerve is approximately twice as large as that polarized vertically to the nerve.

The degree of polarization of the fluorescent light from the resting nerve is defined by

$$P_r = \frac{I_r' - I_r''}{I_r' + I_r''}, \tag{9}$$

where I_r' and I_r'' are the intensities of the fluorescent light polarized longitudinally and vertically relative to the nerve axis. The degree of polarization during excitation, P_a, is defined similarly in terms of the intensities, I_a' and I_a'', observed during an action potential:

$$P_a = (I_a' - I_a'')/(I_a' + I_a'').$$

It is difficult to measure P_a directly, because the simultaneous determination of I_a' and I_a'' during an action potential is required. However, the variation in polarization associated with nerve excitation, *i.e.*,

$$(P_a - P_r),$$

can be calculated by the use of the following expression:

$$P_a - P_r = \frac{1}{2}\left\{\frac{I_a' - I_r'}{I_r'} - \frac{I_a'' - I_r''}{I_r''}\right\}(1 - P_r^2), \tag{10}$$

where subscripts a and r stand for the active (excited) and resting states of the nerve. The quantity $(I_a' - I_r')/I_r'$ in this equation represents the size of the optical signal observed with the principal axis of the analyzer parallel to the nerve; $(I_a'' - I_r'')/I_r''$ denotes the signal size obtained after the rotation of the analyzer through 90°. In the derivation of Eq. 10,

the fact that the variation of the fluorescent light intensity during excitation is far smaller than unity, *i.e.*,

$$(I_a - I_r)/I_r \ll 1 ,$$

has been used.

In crab nerves stained with Pyronin B, the degree of polarization in the resting state, P_r, was found to be approximately 0.14. During excitation the intensity of the emitted light decreases; I' decreases much more than does I''. This means that the degree of polarization decreases.

In the example shown in Fig. 8, $(I_a' - I_r')/I_r'$ is approximately 1×10^{-4} at the peak of the action potential. Introducing these values together with the value of P_r given above into Eq. 10 we obtain

$$P_a - P_r = -0.25 \times 10^{-4}$$

for a crab nerve stained with Pyronin B. The optical signals and, hence, the variations in polarization are very small. However, since only a small fraction of the cellular elements in the nerve is involved in nerve excitation, the observed magnitude of the optical signals does not necessarily indicate that the structural change is very small. The following calculation may serve to clarify this point.

The thickness of the major diffusion-barrier in the axonal membrane is less than 10 mμ. Individual axons are covered with layers of Schwann cells and connective tissue. There are some fibrous materials which are stained by the dye in the intercellular space. Therefore, the amount of dye molecules within the major diffusion-barrier is probably 10^{-3} to 10^{-4} times the total amount of dye molecules in the nerve. There is no doubt that the optical signals shown in Fig. 8 were derived from only a very small portion of dye molecules located within the membrane.

By using the equation derived by Weber (*26*), the degree of polarization for a nerve may be written in the following form:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right) \Big/ \left\{ \frac{f_1}{1 + 3\tau_1/\alpha_1} + \frac{f_2}{1 + 3\tau_2/\alpha_2} \right\} , \tag{11}$$

where P_0 is the limiting polarization, τ_1, the lifetime of the dye molecules in the axonal membrane, α_1, the relaxation time for rotational motion of the dye molecules in the membrane, and f_1 is the fraction of the dye molecules contributing to the optical signal. The symbols τ_2, α_2 and f_2

represent the corresponding quantities for the dye molecules outside the axon membrane; these quantities are assumed not to change during the process of action potential production. Since f_1 is considered to be 10^{-3} to 10^{-4} times f_2 (and $f_1+f_2=1$), it is found that the observed small decrease in P during nerve excitation derives from a large increase in τ_1/a_1 associated with production of an action potential. The optical signal observed with Pyronin B is negative; therefore, the lifetime, τ_1, must either decrease or remain unchanged during nerve excitation. Thus, it is inferred that there is during nerve excitation a marked decrease in the relaxation time, a_1, for rotational Brownian motion of the dye molecules in the membrane.

The order of magnitude of τ_1 is 10^{-9} sec (*26*, *28*), and P_0 is known to be very close to 0.5 for Pyronin B (*27*). The relaxation time for rotational diffusion of a fluorescent oscillator in the resting axonal membrane is considered to be on the same order of magnitude as that in a concentrated polymer solution; therefore, it may be reasonable to assume a_1 is of the order of magnitude of 10^{-6} sec (*29*). If it is assumed that the optical signal in nerves stained with Pyronin B is due to changes in static quenching (*27*), τ_1 would remain unaltered during the action potential; the observed decrease in P is attributed in this case solely to a marked decrease in a_1, namely, to a large increase in the rotational motion of the intramembrane dye molecules during the action potential. If τ_1 decreases during an action potential, a greater reduction in a_1 is expected.

It is well known (see Secs. IV and V) that the mobilities of ions in the axonal membrane drastically increase during an action potential. This increase is related, by virtue of Walden's rule (*30*), to a fall in the viscosity of the media surrounding individual ions. The fall in the degree of polarization of the fluorescent light discussed in this section may be regarded as an additional piece of evidence to indicate a fall in the intramembrane viscosity during the process of nerve excitation.

XI. CONCLUSION

The development of new techniques to study the electrical and optical properties of nerve membranes has helped to elucidate the molecular events occurring in the membrane during nerve excitation. The results obtained by the use of intracellular perfusion of squid giant axons have

led to a better understanding of the ionic environment that is required for the production of action potentials. Axons are found to maintain excitability in external media completely devoid of univalent cations. All-or-none action potentials can be elicited in axons with Na-phosphate internally and $CaCl_2$ as the sole external electrolyte. From these and other results, it is proposed that a conformational change in the nerve membrane macromolecules, caused by cooperative univalent and divalent cation-exchange at fixed negative sites within the membrane, is the primary event in nerve excitation.

Changes in turbidity and birefringence and changes in the intensity of fluorescence of stained nerves have been detected during excitation. These changes in optical properties may reflect a conformational change in nerve membrane macromolecules coincident with the nerve impulse.

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