THE ROLE OF CALCIUM IONS IN NEURAL PROCESSES

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INTRODUCTION

There is a certain artificiality in organizing a scientific review under the above title because one is thereby taking a very narrow view of the mechanism of nervous action. Perhaps this separate effort can be justified on the basis that calcium ions were barely mentioned in two excellent reviews, "The Ionic Basis of Electrical Activity in Nerve and Muscle" written by Hodgkin and "The Properties of the Nerve Membrane and Its Relation to Conduction of Impulses" written by Katz. There is a very real reason for this seeming neglect which will be made apparent by comparing the available evidence for the role of calcium ions in neural processes with that for the role of sodium and of potassium ions. However, this review will serve to re-emphasize that there are at least three important cationic constituents in ordinary Ringer's solution. Each of these has been assigned at least one special role in neural processes. Sodium and potassium ions are the principal current carriers in the membrane and their exchange accounts for the action current. In contrast, calcium ions seem to affect primarily the constraints imposed upon these ionic movements. In other words, calcium ions react with and become part of the surface structure of the cell. This circumstance makes the analysis of calcium action difficult in that it depends upon a knowledge of the surface structure. However, calcium ions seem to be in equilibrium with structures binding them. A molecular explanation of the role of calcium may, therefore, be simpler than the complete explanation for the role of sodium and potassium. For the properties of the latter stem from the initial spatial distribution of these two kinds of ions between the inside and outside of the cell, a separation far from equilibrium and dependent upon oxidative metabolism.

The review is organized on a simple plan that is repeated for each new topic. Each subject is introduced by reviewing prominent contemporary descriptions of a neural mechanism, with appropriate general references. The role of calcium ions is then exhibited in terms of its effect upon these cellular properties. This seemed to be a way to introduce some degree of organization into the array of information tabulated from the literature. To facilitate the qualitative analysis

of relations among measured properties and derived concepts two charts have been constructed. The first depicts proposed causal relations among properties of nerve cells and the other the effects of calcium ions upon these properties and upon the relations among the properties. Such a qualitative analysis is a useful preliminary to the formulation of experimental studies of the molecular mechanism of calcium action upon neural processes. The latter stage of the analysis lies before us. On the one hand, many actions of calcium upon nerve properties have been described and, on the other, the action of calcium on several of the more elementary cellular processes and molecular constituents have been reported. However, definitive experiments that convincingly relate the items in these two lists of phenomena have not appeared. For this reason the contemporary theories of the role of calcium in neural processes are purely formal or highly speculative rather than firmly based upon elementary physical processes demonstrable in nerve cells.

I. EXCITABILITY

a. Excitation and Response of Nerve Cells and Axons. The initiation and conduction of nerve impulses are manifestations of electrochemical processes localized in the surface structure of nerve cells and their axons (92). The existence of a threshold intensity of an adequate stimulus is evidence for a critical degree of change in the surface organization that permits the action current to develop. The change is a regenerative or explosive one, as revealed by the sub-maximal degree of disturbance required to elicit the full response (80, 83). The process is self-limiting and cyclic, being completed by cellular processes that restore the original excitable state. The molecular arrangements in the resting cell that are capable of such a transition from one state to another may be referred to as a metastable structure. In an experimental sense this structure is defined in terms of its responsiveness to imposed stimuli and in terms of the ionic fluxes controlled by it. The latter give rise to the potential changes recorded as the response to a stimulus, and are the physical means for propagating the impulse. After a response the metastable structure of the axon surface returns or is restored to its initial state by unknown processes. Associated with this restoration process are changes in ion flux which are terminated as the potential difference across the membrane returns to its initial value. Within the framework of these ideas, there exists a metastable structure imposing constraints upon the movement of ions across the axon surface, and there are forces which move these ions whenever these constraints are relaxed. Thus, the metastable structure and the sequence of ion fluxes it controls are conceptually distinguishable. Evidence for the physical reality of this distinction is that an environmental variable such as the concentration of calcium ions can produce large changes in the relative stability of the metastable structure without much change in the electrical response, measured as the action current of the conducted impulse.

The least intensity of an electrical current required to initiate a propagated nerve impulse is a measure of the stability of the excitable structure in the cell membrane. If the imposed stimulus is of subthreshold intensity there is a rapidly

developing decrease in stability followed by a gradual return to the initial value. The progress of this process of restoration can be estimated by measuring the threshold intensity for stimulation by a brief electrical shock as a function of time after an initial, or conditioning, stimulus (8, 46). In the same manner the sequence of changes in excitability following after an impulse can be traced. Regardless of the degree of disturbance to the excitable structure, subthreshold or maximal, some unknown processes in the nerve cell restore its stability to the initial value.

The metastable structure has been given some physical properties by a variety of experiments. It is well-known that a change in potential difference across the cell surface changes the threshold for excitation. Thus, the distribution of electric charges across the cell membrane is one physical variable related to the stability of the excitable mechanism. Furthermore, the threshold degree of change produced by an electrical stimulus has been correlated with a critical degree of depolarization of the membrane (152, 153, 154, 155, 81, 166). The change in structure is such that the conductivity of the membrane changes (31). In particular, there is, in the giant axon of Squid, a marked change in rate of transfer of sodium ions across the membrane, permitting a rapid influx of sodium ions (80, 93) and a transient reversal of the membrane potential difference (36, 82). The gradual return of the excitability to its original value is related to a gradual return of the ease of producing the required changes in sodium permeability (80, 81). The relation of threshold intensity of current to membrane potential difference, measured between an inside and an outside electrode, has been examined more thoroughly in muscle than in nerve (48, 89, 130). This evidence indicates that a critical value of membrane potential difference characterizes the liminal state of excitation. Thus, the relation of stability to initial membrane potential difference is explained for muscle. It seems very probable that the same rule holds for nerve cells since much of the ancillary phenomena are observed therein (cf. 18).

The metastable structure thus appears to exist in various physical states determined in part by the membrane potential difference and characterized by the capacity to undergo transient changes of structure revealed by changes in impedance. These physical aspects of the process of excitation and response thus clarify the notion of a metastable structure by giving it specific properties regulating ion traffic in the membrane and a stability determined by the charge distribution in and across the membrane. In addition, the stability will be determined by the specific molecular organization of this structure and will be subject to variation by every environmental variable that can react directly with the structural molecules involved.

In Figure 1 some of the general relations derived from a study of the literature on excitation and response of nerve cells are reproduced as an aid to the exposition of the action of calcium upon processes in nerve cells and axons. The previous paragraphs will clarify the significance of the arrows in the diagram. The items numbered 11, 12, 13, 14, are experimentally observed properties defining excitability and here interpreted as evidence for a metastable molecular struc-

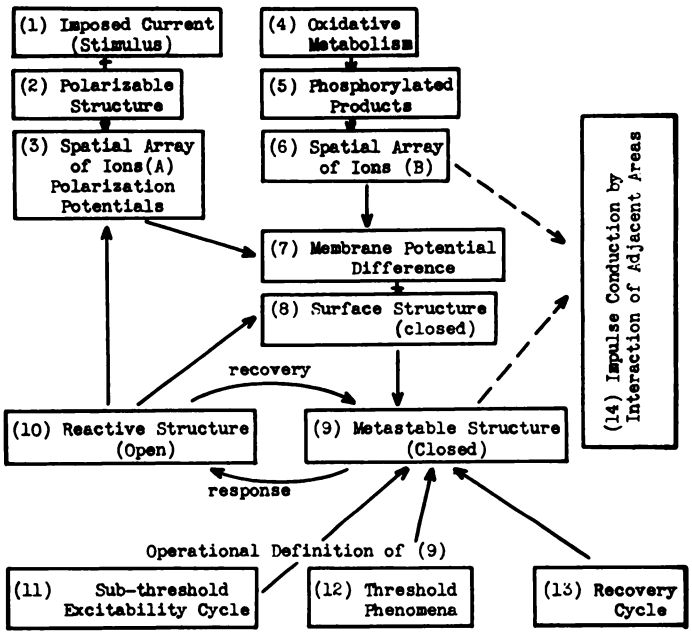

FIG. 1. *Diagram of some relations among measurable properties of axons.*

ture 9 within the cell surface. Item 7 is an experimentally observable property from which 3 and 6 are inferred by means of electrostatic laws. The existence of 2 and 8 are inferred from a wide range of experimental evidence, including impedance measurements. Obviously, the molecular significance of the structures 2, 8, 9 and 10 is unknown. However, this diagram provides a rough guide to a systematic study of the literature on excitation and conduction. It suggests alternative pathways for recovery of excitability 10 → 8 → 9 or 10 → 9. In particular, it provides a framework within which the action of calcium ions on neuronal properties can be discussed despite the fragmentary nature of the available information. [The general references (14, 80, 92, 111, 131, 149, 165, 172) will supply the experimental basis for these statements and further references to the voluminous original literature. The above brief formulation of the results is this author's point of view.]

b. Calcium Ions and the Excitability of Axons. A familiar phenomenon in neurology, both experimental and clinical, is the change in excitability of peripheral nerve that is correlated with changes in the concentration of ionized calcium in the bathing solution. The available data for A-fibers in the sciatic nerves of the frog show that the stability, measured by the threshold intensity of direct current, decreases markedly when the concentration of ionized calcium is below about 0.8 mM while a negligible change occurs in the range from 1 to 5 mM (121, 17). The stability of the excitable mechanism in the isolated giant axon of the Squid decreases in a similar manner with decrease in the concentration of ionized calcium in the bathing solution (17). However, the concentration

range within which the changes occur is much higher for nerves from Squid than for those from frog (10–70 mM in artificial sea water). The generality of this phenomenon is further emphasized by the correlation between the measured excitability of the ulnar nerve and the concentration of calcium in the blood of humans with hypoparathyroid disease (162, 97, 178). It is perhaps worth emphasizing that these changes in sensitivity can be exhibited by electrical, mechanical and chemical stimuli.

The least intensity of electrical current required to initiate a nerve impulse depends upon the duration of current flow. The rheobasic current decreases with decrease in the external calcium concentration, as described above. The changes in excitability are also detected by brief (50 microsec.) electrical stimuli (123, 125). The relation of threshold intensity to duration of direct current for durations of a millisecond or less (in frog A-fibers) is characterized by an empirical time constant k (77). This time constant is insensitive to changes in calcium ion concentrations (78, 77) but does change if the concentration is high enough. Thus, if frog nerves are bathed in solutions containing higher than normal concentrations of calcium ion (14 $\times$) the rheobase is increased and so is the minimum quantity of charge required for brief threshold shocks (9). In these experiments the chronaxie and, therefore k, was decreased. However, it is important to note that no significant changes were observed for variations in calcium concentration around Ringer's proportion. It is precisely in this range (0–5 mM for frog nerve) that marked changes in excitability occur.

The threshold intensity of an electrical current is greater the slower the rate of rise of this current to its final value (77, 162). Thus, the threshold intensity of an exponentially rising current increases with the time constant determining its rate of rise. This phenomenon, called accommodation, is a measure of the rates of processes that oppose the excitatory action of the stimulating current. It has been measured in isolated single fibers of frog (151, 166), as well as in nerve bundles. The rate of the process of accommodation depends markedly upon the concentration of ionized calcium in the solution bathing a nerve (162). In solutions with low calcium concentration the nerve processes are less effective in opposing the stimulatory action of a current. These actions of calcium appear also in experiments upon ulnar nerve in humans (97, 162, 178).

In the neighborhood of Ringer's proportion of calcium ions in the bathing solution the rheobase is relatively insensitive to changes of this ion. In the lower concentration range the nerve becomes more excitable and below 0.3 mM, on the average, alpha fibers in the sciatic nerve of frogs may become spontaneously active (17) (see later). At high hydrogen ion concentrations (13) or in the presence of 2 per cent carbon dioxide (111) this spontaneous activity is minimized even after prolonged bathing in calcium-free solution. Under these conditions the change in rheobase can be measured from near zero concentration up to 5 mM calcium chloride (13, 17). The relative lack of effect of changes above one millimolar concentration suggests that the ion-binding structure with which calcium ions react has become saturated. Below one millimolar there is a gradual loss of bound calcium from these structures, and the sensitivity to stimuli in-

creases. If the concentration of calcium chloride is increased to 10–15 mM then the threshold for excitation again rises (9, 121, 111). The significance of the action in this range of concentration is obscured by irreversibility of the effects (121, 111) accompanied by histologically revealed changes in the myelin sheath (111). The initial actions of such high calcium may be upon the nodal regions, accounting for the threshold rise, while the irreversible effects are in the myelin layer, lowering the resistance therein (167).

The description of the state of the excitable mechanism as modified by calcium ions is more detailed when outlined in terms of the temporal evolution of the excitation process following a brief conditioning stimulus of subthreshold intensity. After such a stimulus the excitability at the cathodal region rises abruptly to a maximum and then falls to a minimum and gradually approaches the initial level (8, 46). Monnier has emphasized that this excitability cycle is sensitive to changes in the concentration of calcium ions in the solution bathing the axon (123, 125, 126, 127). If the calcium ion concentration is decreased there may be several oscillations of excitability before the processes restore the original level characterizing the unconditioned axons. This is a property of Squid axons bathed in sea water (5, 157a). The cycle of changes resembles a damped oscillatory process and is characterized by a damping factor, G, measured as the ratio of the amplitude of the enhanced phase of excitability to that of the first phase of depressed excitability (123, 125, 127). As mentioned before, the extreme effect of removal of the diffusable calcium from a nerve is the development of spontaneous activity (5, 15, 16, 17, 52, 108). This is revealed by conduction along the axon of impulses that arise in the calcium-deficient areas. This state of the nerve cell presumably corresponds to tetany observed in intact organisms with low serum calcium (97, 98). Such rhythmic self-excitation arises in the experimental preparations from intrinsic oscillatory processes (5, 15, 17). The depletion of calcium, presumably by decreasing some damping factor, merely permits the amplitude of a rhythmic excitatory process to grow to threshold size. Such rhythmic excitation is facilitated by the concomitant lowering of threshold. The required source of energy is in the axon (Figure 1).

On the basis of these observations calcium ions in the environment seem to enter into a reversible association with molecules in the frog nerve at concentrations below 5 mM. Two states of the nerve have been identified in terms of the changes in sensitivity to electrical stimuli. At very low concentrations the state of the axon permits self-excitation. At higher concentrations (including the physiological range) the excitable mechanism is metastable, and a response occurs only as a result of a sufficiently intense stimulus. In addition, over this entire range of concentrations, the process of accommodation is less effective in opposing the stimulatory action of current flow the lower the concentration of calcium ions.

The physical nature of the accommodation process is not known. Recent studies on single frog fibers have re-emphasized that the stimulatory effectiveness of a gradually increasing current is related to the rate of increase as it passes the rheobasic intensity (53, 166, 151). If the rate of rise is too slow there is no regen-

erative membrane change and no propagated action current. In frog axons subjected to such an ineffective current the nodes become inexcitable as this current passes the rheobasic intensity (53). This suggests that a gradual rather than an explosive transition from the metastable to a stable, inexcitable state, has taken place (from state 9 to either 8 or 10 in Figure 1). Perhaps this process is analogous to the inactivation of the increased sodium permeability that occurs in the membrane of Squid giant axons (80, 81). No changes in membrane potential have been reported to occur during the latter process, which profoundly affects the excitability. Further study of these phenomena is needed because the action of calcium excess or deficit on excitability and accommodation may be related to changes in the rate of the inactivation of the enhanced sodium permeability. It is just here that two stages (8 and 10 of Figure 1) of recovery of excitability are suggested. The suggestion is that a higher than normal rate of this inactivation process, $10 \rightarrow 8$, may permit a transition from an excitable to an inexcitable state without the regenerative or explosive increase in sodium leakage associated with the action potential. The transition from state 8 to 9 follows after the reestablishment of the membrane potential difference. Accommodation to electric current flow occurs in Nitella (176). There it is associated with a gradual diminution of the increment of polarization potential developed by a given increment in membrane current. Thus the accommodated state of the membrane has a smaller variational resistance, presumably that paralleling the effective capacitance.

In résumé, the excitation processes can be modified by changes in external concentration of calcium ions. In accordance with the relations indicated in Figure 1 these processes are evidence for a metastable structure within the cell surface. Perhaps the actions of calcium ions upon excitation processes arise from a primary reaction with ion-binding molecules in the metastable structure that change its intrinsic stability. Other suggestions are that calcium ions react with organic ions required for transport of sodium ions across the cell membrane (83) or react with molecules controlling the permeability of the membrane for potassium ions (156, 157 a, b). In either mechanism the effects on the threshold for excitation by electrical current would arise indirectly from changes in the polarization potential developed by a given current. To test this possibility the critical membrane potential for threshold excitation must be measured as a function of the calcium ion concentration bathing a nerve cell. [In muscle this critical potential did not change when the excitability was changed by calcium depletion (89).] These and other possibilities for explaining the observed actions of calcium ion upon excitability of nerve cells are evident from the relations of Figure 1. Studies of excitation processes alone define the problems but are not adequate for making a choice among the physical explanations.

II. EXCITABILITY AND MEMBRANE POTENTIAL DIFFERENCE

a. Calcium Ions and Subthreshold Electrical Polarization. The polarization potentials developed by current flow across the cell membrane are of fundamental significance in the process of excitation. For this reason alone the action of cal-

cium ions upon the polarization properties of nerve cells are of great interest. An additional consideration is that such studies can be carried out with subthreshold intensities of current. Thus, properties of the calcium-containing cellular membranes can be measured independently of the structural change underlying the action potential. In addition to excitability phenomena the physical origins of rhythmically recurring impulses are found in the subthreshold polarization and impedance properties. More broadly considered, these studies bear upon the role of calcium in ionic exchanges across cell surfaces associated with nutrition and metabolism as well as with the special case of action potentials and after-potentials.

The potential difference measured across the surface of axons changes to a new steady value during current flow across the surface structures. The transition to the new steady-state potential difference may be characterized by damped oscillations about the new value (5, 30, 81, 111, 157a). The amplitude, frequency, and rate of damping of these oscillations are measurable properties of the processes involved. These processes are markedly affected by the concentration of calcium ions in the solution bathing the cells (5, 157a). An adequate interpretation of the origin of these rhythmic potential changes will thus exhibit one physical basis for the action of calcium on neuronal processes. At present such interpretation is, at best, in terms of electrical properties of the surface structures and the process of ion transport. Hence, the mechanism of action of calcium ions must necessarily be expressed in terms of changes in parameters characterizing such electrochemical processes.

The membrane potential changes during current flow have been measured in the giant axon of Squid between an internal electrode and an external one, with procedures to insure constant current density in the membrane (32, 83, 30). The specific resistance of the membrane of the giant axon of Squid increases when current flows inward and decreases when current flows outward. At first the rate of change in potential is the same during constant current flow inward or outward (83). For anodal currents, however, the resistance continues to rise along a smooth curve to a higher steady-state value. This is indicated by a final constant level of potential difference related to the imposed constant current density in the membrane. The fall of resistance for the same density of current flow outward is less. Consequently, the measured potential difference across the membrane is not a linear function of the membrane current density. In place of Ohm's law the relation is that described as rectification, i.e. the membrane resistance is different for inward and outward flow of current. However, this difference is established only after the constant currents flow for an appreciable time. For this reason the property is described as delayed rectification (83). Removal of calcium from the bathing solution reduces the degree of rectification across the surface of a Squid axon (83), so that the relation of current to voltage tends toward Ohm's law in calcium-deficient areas. Previously it had been established that the degree of rectification, measured in the giant axon of Squid, increased with increase in calcium ion concentration (164). However, Guttman observed no change in rectification in frog nerve exposed to high concentrations of calcium chloride (68).

When a constant outward current flow is begun the membrane potential falls with a time course that is at first similar to the rise of potential for an equal inward current. The depolarization is gradually arrested, reaches a maximum and declines part way toward its steady-state value. These physical phenomena are of direct physiological import for, if this initial peak of depolarization is of sufficient magnitude, a conducted action potential arises near its summit (5, 32, 30, 83).

The oscillatory transitions of the potential difference from its initial level to the final level during flow of a constant cathodal current may be markedly underdamped. Then the membrane potential difference exhibits several cycles of oscillation before approaching its final value. The damping of the oscillation depends upon the level of the membrane potential difference and upon the concentration of calcium ion with which the membrane is in equilibrium (5). The membrane potential difference of a nerve equilibrated with a high concentration of calcium ions shows little tendency to oscillate in response to cathodal current. In contrast a nerve membrane deficient in calcium ions shows a marked increase in potential oscillations during outward flow of a constant current. It has been suggested that electrical polarization and calcium ions have this action on the oscillatory response because these agents change the electrical resistance of the membrane (5, 30, 81, 125). This implies that high calcium concentration and high potential difference create a high membrane resistance while removal of calcium or depolarization have the opposite effect. In Squid axons the membrane resistance is approximately proportional to the external calcium chloride concentration (30).

It is important to note that this hypothesis says nothing about the mechanisms by which calcium ions and change of potential difference produce the change in resistance. Indeed, since the membrane conductance is a complicated set of processes the potentialities for modifying it are multiple. Hence the mode of action of an imposed potential difference might be quite different from the mechanism of calcium ion action. However, the electrolytic transfer of calcium into or out of the membrane may explain the effect of polarization and its simulation by higher or lower than normal concentrations of external calcium ions (56).

The oscillatory response to anodal and cathodal polarization is marked if the potential change is very small (81). Therefore, the rectification properties of the membrane conductance described above are not essential to these phenomena. Indeed, for larger inward currents the oscillations do not occur while for larger outward currents the amplitude increases so that a membrane action potential occurs.

The same electrical properties of the membrane can be exhibited by impedance measurements. In these experiments an increase in calcium concentration changed the low frequency impedance locus as though the resistance and low frequency capacitative reactance of the equivalent series circuit were increased (30). Lowering the calcium concentration caused changes in the membrane such that the equivalent circuit contains capacitance, resistance and inductance. Thus, the enhanced tendency for potential oscillations produced by low calcium is exhibited

in the impedance measured as a function of frequency, for frequencies below 500 cycles per second. From such data the apparent inductance is 0.2 Henrys (32), the capacitance is 1 microfarad (32), and the radial resistance about 1000 ohms per square centimeter in the Squid axon (30). From these data the underdamped oscillations of membrane potential would occur for $R < 2\sqrt{L/C}$ or for R less than 1000 ohms per square centimeter. As stated above it is probable that calcium ions react with the membrane structure to increase this resistance to ion transport, and deficiency of calcium ions is reflected in a lowering of the equivalent resistance below the critical value specified above. Further reduction of the resistance, as by further calcium depletion, would increase amplitude of the oscillatory potential change produced by a constant current. This has been observed (5). The data mentioned above, for the equivalent circuit that simulates the electrical properties of the nerve, lead to the prediction of a maximum undamped frequency $1/2\pi\sqrt{LC}$ or about 330 cycles per second (32). This is within the observed range though frequencies as high as 500 cycles have been noted (17, 32). Thus calcium ions affect the oscillatory properties of the cell membrane by changing the damping. This may be due solely to the change in membrane resistance that occurs. However, in a non-linear and non-conservative system like this damping can arise in other ways.

Some insight into the possible modes of action of calcium is obtained from the explanation (81) of the membrane resistance and its changes. The membrane current is divided into contributions from sodium, potassium and other ions. The conductance for sodium and potassium are functions of the potential difference across the membrane. Since the tendency to oscillate is related to the total effective resistance the required changes could be produced by calcium modifying at least one of these specific conductances or their relation to the potential difference across the membrane. This must occur without appreciable change in the resting potential and no marked change in spike configuration. It has been reported that removal of calcium reduces the electrical rectification by the membrane (83). This is some evidence that calcium ions act to change the potassium ion permeability because the rectification probably arises from the marked dependence of potassium ion permeability upon potential difference (30, 81).

All of this physical evidence adds up to the result that calcium ions modify the tendency of the membrane potential to oscillate during current flow, probably by action upon the resistance of the nerve surface to ion transport. In this way the physiological effects of calcium ions may be traced to these physical effects because the former are known to depend upon the electrical properties of the cell surface. It will be of interest to examine further whether the previously described actions of calcium on excitation processes can be understood in terms of its actions upon these electrical polarization properties of the membrane.

The polarization potential developed during flow of constant current in frog A-fibers (111) can exhibit damped oscillations like those for Squid or crab axons. The associated changes in excitability in frog fibers (111) and in Squid giant axons (107, 139) are correlated in time and intensity with these potential changes.

Thus, factors influencing damping of these electrical oscillations are those affecting Monnier's damping factor for the subthreshold irritability cycle (125). In this way, the action of calcium ions upon excitability cycle, subthreshold or after an impulse, can be traced to action upon the processes of membrane polarization changes during current flow. The observation that calcium depletion affects the membrane resistance (30) is thus a sufficient explanation of its action because the transient excitability changes are correlated with the expected transient electrical potential changes. Furthermore, the frequency of impulses initiated from calcium-deficient areas of nerve cells is correlated with the frequencies of potential oscillations expected from impedance and polarization characteristics of the membrane (30, 81, 17). These developments focus attention upon the polarizable structures and their modification by calcium binding (cf. Figure 1). In support of these ideas is the observation (70, 152) that the relation between duration and intensity of an imposed current required to produce a given magnitude of electrotonic potential is similar to that for threshold excitation. Furthermore, both relations change in a similar manner with changes in calcium ion concentration in the solution bathing the axons (9, 70).

Cole (30) reports approximate proportionality between membrane resistance and calcium ion concentration in the solution bathing Squid giant axons. An increase in membrane resistance (that shunting the capacitance) would tend to lower the rheobasic current (cf. 89). However, the latter rises markedly with increase in concentration of calcium in the external solution (17). Thus, the increase in resistance is not in itself a sufficient explanation for the rise in threshold. The associated increase in damping of the oscillatory polarization potential offers a ready explanation. The hypothesis is based upon two generalizations from experience (a) the critical state of excitation is a critical potential difference across the membrane and (b) the action potential develops from the first peak of depolarization for threshold excitation. Thus, any reduction in amplitude of this peak will require an increase in current density to produce the transient critical degree of depolarization. The change in damping is presumably a direct result of the change in electrical resistance.

In this discussion a terminology has been used that is suitable for linear equivalent circuits. Such circuits simulate, to some extent, the measured electrical properties of the membrane though the latter is probably a non-linear electrochemical system (30, 168). The potentialities for action of calcium are thus increased because it may affect the relation of resistance to membrane potential. This situation has been recognized in part in Karreman's theory of excitation (see Karreman and Landahl in 131) though the importance of sodium leakage in excitation processes of nerve (81) will require extensive modifications. Furthermore, the cellular system is not passive, and the energy sources may be directly concerned in the action of calcium ions upon excitation processes. Thus, Lorente de Nó (111) has suggested that oscillations of membrane potential are a resultant of the nerve reaction to imposed polarization potentials. The reaction develops as a change in the electromotive force arising in the intrinsic metabolic sources of energy. Shanes (157a) suggested oxidative uptake of ions that would cause

the membrane potential difference to oscillate. Studies of excitability of *Nitella* in relation to external calcium ion concentration (133, 134) suggested another relation to metabolism. A substance produced metabolically is supposed to leak from the cell at a rate controlled by the calcium content of the surface. The steady-state concentration of this substance determines the excitability. Thus, a minimum concentration of calcium is necessary for maintenance of the excitable state (cf. 156).

The terminology of equivalent circuits is appropriate to the physical operations and instruments employed in the measurements. The reasonable expectation that some detailed system of ionic or metabolic processes will account for these properties does not detract from the value of this provisional formal analysis. The latter is capable of exhibiting quantitative relations among the experimentally measured variables without regard to their detailed molecular-ionic origins. If the above analysis is valid the action of calcium upon the excitation properties of axons can be exhibited in terms of changes in membrane resistance. The remaining problem is to explain in physical terms how the calcium content of a membrane can change its resistance to current flow. These actions of calcium upon processes of ion transport in the membrane may account for all of the major effects upon excitation processes. This idea can be examined better when the relation of excitation processes to the polarization properties is better developed perhaps along the lines suggested by Teorell (168).

There are at least two general physical interpretations of the properties of the nerve membrane. In one instance the relation of current flow to membrane potential difference is described in terms of a non-linear resistance. The oscillations of potential are said to reflect interacting processes that determine the constraints on movements of ions across the membrane. The other terminology, based upon interacting components of potential difference and changing polarizability of structures within the membrane, accounts for the oscillations in terms of rhythmic variations of the intensity of electromotive forces. With the available knowledge there is no compelling reason for choosing between these systems of explanation (cf. 176). Indeed, more careful formulation of concepts may show that there is no difference except in terminology. For present purposes, it is merely necessary to be sure the actions of calcium are expressed in operationally significant terms regardless of the particular system of explanation under discussion.

b. Calcium and Membrane Potential. It is a rule for axonal excitability that an increase in the potential difference across the membrane raises the threshold for excitation while a moderate decrease, or depolarization, lowers this threshold. For example, there is the correlation of polarization potential changes and excitability just mentioned. The question arises, do changes in concentration of ionized calcium change the threshold for excitation in a resting nerve by changing the potential difference across the cell surface?

As previously described, the ultimate effect of calcium depletion is self-excitation in A-fibers in the frog sciatic nerve. This occurs with negligible change in demarcation potential (161). There is no evidence from measurements of demarcation potential for a decrease in the potential difference across the mem-

brane associated with the marked increase of excitability in calcium deficient axons. Thus, Lorente de Nó has stated his experience as follows: "Insofar as frog nerves are concerned, calcium ions at concentrations at which they have marked effects upon the properties of the nerve fibers, do not modify in a readily detectable manner the membrane potential of the nerve fibers", and Laget (103) reached the same conclusion. However, Shanes (158) reports that the demarcation potential of a frog sciatic nerve bathed in a solution containing some calcium chloride is somewhat higher than that of a nerve in a calcium-free medium. The difference recorded was about one millivolt and was the same for a nerve in 1.2 mM as for one exposed to 8.0 mM. This slight increase was with reference to a control nerve bathed in calcium-free medium, measuring the demarcation potential with respect to a potassium chloride treated end. Under these conditions the calcium deficient nerve would be spontaneously active and the average demarcation potential would be somewhat lower than that of an inactive nerve. Any amount of calcium above that required to suppress the spontaneous activity would produce the same slight increase in demarcation current, as observed. The experiments reported, therefore, do not indicate clearly an increase in membrane potential difference produced directly by increasing the concentration of calcium ions. Kuffler observed depolarization of as much as 8 millivolts in muscle membranes deprived of calcium (100). This potential change developed gradually over a period of 20 to 30 minutes and was maintained as long as 70 to 100 minutes. In some experiments the depolarization was followed by repolarization, and the calcium deficient area of muscle became positive to the part in Ringer's solution. These changes were independent of the presence of endplates and may, therefore, be compared with changes in electrical excitability observed in the nerve-free portion of the frog's sartorius by Chao (28). The time course for the change in excitability differs from that for change in membrane polarization. Furthermore, the changes in irritability are essentially completed in 10 minutes whereas the depolarization has just begun after 10 minutes of immersion in the test solution. This suggests that the primary changes in excitability produced by changes in concentration of calcium chloride are not developed as a result of the occasionally observed changes in membrane potential reported by Kuffler. More recent measurements on muscle, with one electrode inside, indicate an increase in membrane potential difference when the calcium concentration is increased from 1.3 to 10.4 mM (89). This contrasts with the lack of marked effect of calcium deficiency upon the demarcation potential (3). Perhaps in the latter measurements a decrease of membrane potential difference was obscured by a decrease in membrane resistance. The former would tend to decrease the demarcation current and the latter to increase it. Additional studies on nerve cells are needed since measurements of demarcation potential do not distinguish between changes in membrane resistance and membrane potential difference. It may be that increase in membrane potential difference of nerve, as for muscle, will only appear for increases in calcium concentration appreciably above Ringer's proportion. An increase in spike height in frog A-fibers occurs under such conditions (60).

At this point a provisional summary of calcium action on excitability seems

desirable. Reference to Figure 1 will facilitate the résumé. The reversible effects of changes in calcium on the excitability and polarizability of frog nerve fibers and of Squid giant axons take place with little or no change in the demarcation potential difference. The excitation processes that are sensitive to changes in calcium ion concentration, below Ringer's proportion, are correlated with changes in properties of polarization potentials developed during or after current flow. The primary action of calcium may be upon the polarizable structures in the nerve surface. Thus, these effects are more strikingly manifested in transient polarization potentials related to current flow than in the initial membrane potential difference. The effects on amplitude and damping of rapid oscillatory potential changes and upon impedance properties suggest that decrease in calcium lowers the electrical resistance of the membrane while increase in calcium raises it. This resistance change has been measured in Squid axon. These phenomena are prominent in excised Squid nerve and in frog A-fibers when the calcium-ion concentration is varied in the range below normal for each of these axons. The suggested mechanism of calcium action is in agreement with much of the earlier evidence indicating that calcium ion concentration in the bathing solutions affects the permeability of cells to ions. Recent studies of permeability of crustacean nerve to sodium chloride indicate an increase in cells deprived of calcium ions and a decrease for those exposed to high concentration of these ions (79). The question of changes produced by calcium ions in the intrinsic stability of the metastable structure remains unanswered. To examine this properly requires a measurement (with one electrode inside the axon and uniform immersion in the bathing medium) of the threshold degree of depolarization at several concentrations of calcium ion.

c. Calcium and Negative After-potentials. The action of calcium ions (in the concentration range above Ringer's proportion) upon A-fibers of frogs and of mammals involves an additional electrochemical process not mentioned heretofore. In many kinds of nerve fibers the initial descending phase of the action potential is followed by a slower component of repolarization, the negative after-potential. Thus the total membrane potential difference is composed of a large part that is re-established quickly and a smaller fraction developed at a slower rate. The magnitude of the component of potential difference produced by the slow process is very sensitive to physical and chemical agents to which the nerve is exposed. As will be discussed later, an increase in calcium above Ringer's proportion will markedly increase the amplitude and duration of this negative after-potential (58, 59, 60, 76, 108a, 108b). Because of the slowness of this phase of repolarization the magnitude of residual depolarization can be increased by repetitive stimulation. This increase reaches a limit, however, indicating that this component of the potential difference has a maximum value that is much less than the total potential difference across the membrane (111). Lorente de Nó named the part of the membrane potential difference, thus defined, the L-fraction because of its lability with respect to environmental variables. This component of the total potential difference across the surface of a resting axon thus measures a charge separation that occurs by a process that is different from that

identified with the "spike" process. It is important to note that the latter process can take place without measurable trace of the former, *i.e.*, the L-fraction is not indispensable for impulse conduction and recovery therefrom. Indeed, it is not a prominent part of the membrane phenomena previously described for the Squid giant axon.

The L-fraction of the membrane potential can also be measured as the slow component of depolarization during flow of constant current outward across the axon surface. The maximum value of this slow component of the electrotonic potential change at a cathodal region is about equal to the maximum negative after-potential resulting from repetitive action potentials. The common origin of these two phenomena was experimentally established by Lorente de Nó (111).

The L-fraction of the membrane potential difference can be directly measured as a change in demarcation potential (111). The demarcation potential of a sciatic nerve excised from a frog is measured first with the nerve in moist oxygen. The maximum negative after-potential and the maximum slow component of depolarization are small or absent in such a nerve. Substitution of 5 per cent carbon dioxide plus 95 per cent oxygen for the pure oxygen causes a slow increase in the potential difference across the membrane. This reaches a constant level after some time. The nerve now has a prominent maximum negative after-potential and a slow component of depolarization during outward current flow across

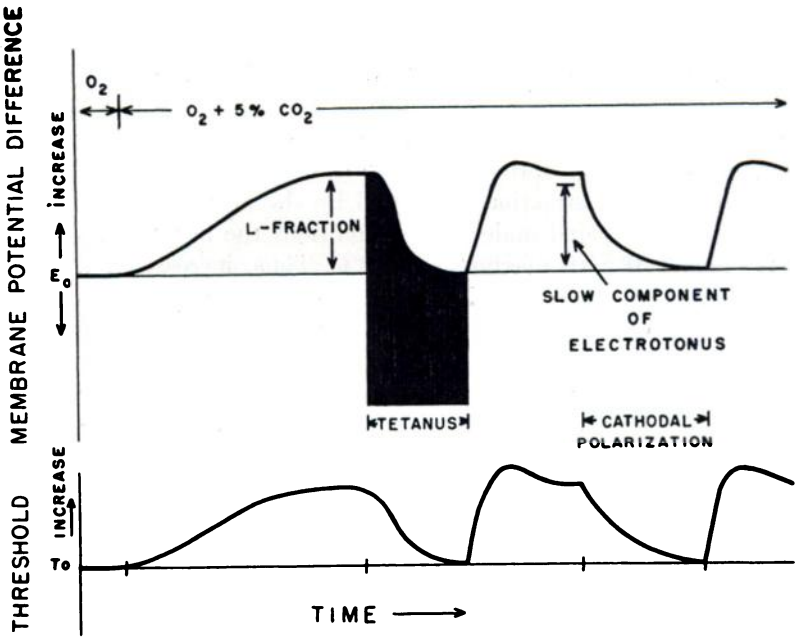

FIG. 2. *Diagrammatic construction of relations among L-fraction of membrane potential, negative after-potential and slow component of electrotonus.* Similar changes have been described for increase of concentration of calcium ion above Ringer's proportion. See text.

the axon surface. Furthermore, the magnitude of the maximum negative after-potential, the maximum slow component of depolarization, and the increment in demarcation potential produced by the carbon dioxide are approximately equal (111). With some liberty with the details these relations are shown diagrammatically in Figure 2. The corresponding changes in excitability are indicated. This slowly changing component of the membrane potential difference is the L-fraction.

A concentration of calcium that is higher than Ringer's proportion changes the axonal processes in frog A-fibers so that the negative after-potential is increased in magnitude. If a nerve (from frog or cat) is equilibrated in a solution with a lowered calcium concentration the negative after-potential is attenuated (76, 108). The changes in after-potentials are not prominent except at concentrations of calcium about four times greater than Ringer's proportion (60). These observations reveal a process that is sensitive to the calcium ion concentration at the nerve surface and that is related to a component of the potential difference across this surface. In this sense, it can be said that calcium ion acts upon the nerve by affecting intrinsic processes that establish part of the potential difference measured across the surface structures. Laget has extended this system of relations by correlating increase of the damping factor with increase of negative after-potential (103).

Observations on mammalian nerves support this system of correlations (112). The reaction of mammalian nerves to increased calcium ion concentration is different from that of frog nerve. A modest increase in calcium ion concentration tends to decrease the damping factor and thus enhance the phase of lowered excitability following brief subthreshold depolarization (the post-cathodal depression). This has been related to the displacement by increased calcium chloride of the thermal optimum of the membrane potential (112). In Krebs' solution the optimum is 37°C for L-fraction. Increasing the concentration of calcium chloride abolishes the L-fraction, measured by the negative after-potential. If the temperature is lowered under these conditions the negative after-potential reappears (Figure 3) with maximum at 32°C. Thus, increase in L-fraction of membrane potential and of damping go together, as in frog A-fibers, but at 37° the direction of change produced by increasing concentration of calcium ions is opposite from that of frog fibers at 20°C. The additional action of calcium ion on the temperature optimum is a new fact that explains this reversal of the usual relations of L-fraction of potential to increase in calcium-ion concentration (Figure 3).

Thus, the temperature for maximum negative after-potential is shifted from 37° to 32° by increasing calcium chloride in the Krebs' solution (112). This is interpreted to mean that the maximum value of the L-fraction of the membrane potential occurs at 32° in nerves exposed to high calcium chloride concentrations and that in such nerves this fraction is zero at 37°C. The total membrane potential (demarcation potential) also has its maximum shifted from 37° to a temperature near 30°C by an increase in concentration of calcium chloride in the bathing solution (Figure 3). In addition, the removal of calcium ions by

diffusion into the bathing solution increases the sensitivity to block at low temperatures. In such a calcium deficient nerve the spontaneous activity is a maximum at 27.5°C and is absent at 37° and at 15°C. These extremely interesting actions of calcium upon mammalian axons are illustrated in Figure 3. This system of correlations cannot be generally true because increasing the calcium ion concentration bathing Squid axons will increase threshold (17), increase the damping factor (157a), and decrease the negative after-potential (157a).

The excitability changes associated with a 50-millisecond period of depolarization have been studied (33). The initial maximum excitability change at 2 milliseconds (frog) is followed by a minimum at 4 milliseconds with a subsequent slow rise, tending to level off at 40–50 milliseconds. This delayed hyperexcitability is enhanced by high calcium concentrations and also develops progressively with time in excised frog axons. The authors explain the slow depolarization and related excitability change in terms of the L-fraction of the membrane potential. Thus, high calcium concentrations favor retention of this delayed catelectrotonic increase of excitability, as observed, because the L-fraction is large in such fibers. There is a correlation with large negative after-potentials.

In this way, an interpretation of a considerable range of phenomena is based upon the idea that the membrane potential is compounded from two principal internal sources of potential difference. One is particularly related to the stability of the excitable system (L-fraction), while the other underlies the conducted action current (111). These separate charge distributions, whose totality is measured by the potential difference across the membrane of the resting cell,

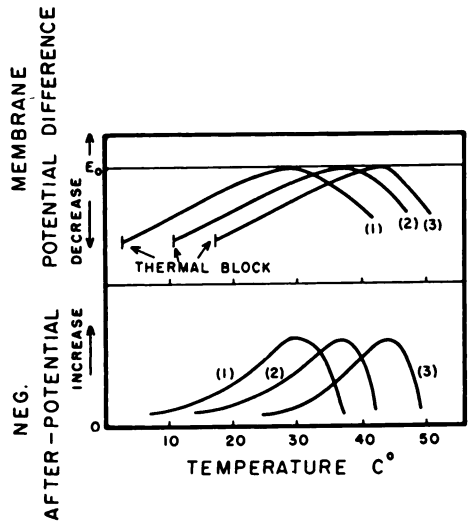

FIG. 3. *Diagrammatic construction of potential changes in mammalian nerve in relation to temperature and calcium ion concentration.* Upper chart, demarcation potential. Lower chart, negative after-potential. Curve 2, Ringer's proportion of calcium chloride in bathing solution; Curve 1, calcium concentration higher than for Curve 2; Curve 3, calcium concentration lower than for Curve 2. Arrows indicate temperature for block of conduction. Constructed from information given in reference (112).

are different in that different processes are involved in maintaining the average spatial separation of ions from which the electric potential gradients arise. These several components are recognized by their differential susceptibility to modification by outside influences. The L-fraction is measured by the size of the negative after potential, by the size of the slow component polarization, or by the magnitude of post cathodal depression of excitability. Agents which affect these are said to do so because they primarily have changed the L-fraction of the membrane potential. Because this set of properties (L-fraction, post-cathodal depression, negative after-potential, and slow component of polarization) retain definite relationships to each other under a variety of conditions (anoxia, ion changes, temperature, electrical polarization, carbon dioxide action) they may be considered to be properties of a particular organized structure in the nerve. The state of this system, it is proposed, is determined by specifying the value of the L-fraction of the membrane potential. The state of this system is changed by changes in concentration of calcium ions in the external solution. These observations suggest that if the L-fraction of the membrane potential is specified then the rhythmic excitability cycle, negative after-potential and the changed excitability are all determined. The implication is that the only primary action of calcium ion is on the L-fraction of the membrane potential (111, 112).

If this hypothesis is valid then a variety of individual facts regarding the action of calcium ions will be explained provided their action upon the L-fraction of the membrane potential is explained. This is a very important idea because to reduce a problem of explaining stability to one of explaining a potential difference is an accomplishment. One reason for this is that the physical basis for a potential difference is known while the physical basis for excitability or metastability is poorly understood. Therefore, it is important to examine the meaning of the statement that increase in calcium ions acts upon axons by increasing the L-fraction of the membrane potential.

It must be emphasized that the marked changes in excitability of calcium deficient frog axons have not been experimentally correlated with a decrease in membrane potential as expected if the L-fraction decreased. However, the proponents of this theory point out that the L-fraction for normal frog nerve may be 6–7 per cent of the total potential difference across the membrane. Its changes are therefore not easily measurable by the usual demarcation current method. This explanation and the theory will be subjected to more searching tests when the membrane potential is measured directly as a function of calcium ion concentration by means of an inside electrode. It is possible that the steady-state value of the membrane potential difference is determined by factors other than the kinetics of the reaction pathways for its establishment. The latter may have slow and fast components revealed in different proportions during transient changes in potential difference without the final level of potential difference being affected. This kind of interpretation could be related to the ideas inherent in the view that independently variable resistances to movements of specific ions across the membrane underlies transient and steady state values of the potential difference.

d. Inter-relations of Processes Affected by Calcium Ions. Two prominent actions of calcium ions on the excitable properties of nerve have been mentioned. One is related to slow components of potential change and the other to damping of rapid oscillatory potential changes. Frog nerves with negligible L-fraction of the membrane potential have a marked tendency toward oscillatory potential changes during current flow (111). This state of the frog nerve is favored by low carbon dioxide concentration (111) and by depletion of ionized calcium in the bathing solution (5, 17, 125). Thus, for frog A-fibers, as for the giant axon of Squid, it has been established that depletion of ionized calcium favors appearance of oscillatory transitions of membrane potential during current flow. Increasing the calcium concentration above Ringer's proportion for frog nerves causes another change in the membrane, measured by the L-fraction of the potential difference, which obscures (see, however, 33) the fast oscillations of polarization potential previously described for outward current flow (111). The increase in excitability, paralleling the changes in membrane potential difference, now exhibits a rapid component followed only by a superimposed slow component. There has been described a single rapid oscillation of excitability and polarization potential preceding the slow component (33). These observations are considered to be evidence for an appreciable L-fraction of membrane potential. Associated with this state of the membrane is a high threshold for excitation. The increase of threshold by increase of calcium concentration is attributed to the stabilizing effect of the L-fraction of the membrane potential. A subthreshold cathodal current merely reduces this L-fraction which now dominates the excitation properties. However, a frog axon without an L-fraction of membrane potential (as in absence of carbon dioxide) has a finite threshold that decreases as the concentration of ionized calcium decreases (17). Furthermore, the tendency to rhythmic activity during current flow increases after such depletion of calcium. These changes in excitability occur with negligible change in demarcation potential. They are actions of calcium on nerve processes that seem to be independent of its action upon the L-component of potential difference.

Perhaps calcium has two ranges of action on frog A-fibers. From 0–5 mM the tendency toward oscillatory changes of potential during current flow with concomitant oscillatory changes of excitability are affected by calcium ions through changes in electrical resistance, as manifested by the degree of damping. In the range above 5 mM the L-fraction of membrane potential, manifested in slow polarization transients and large negative after-potentials is the predominant factor raising the threshold for excitation. For example, Graham and Blair (60) report little change in recovery processes, including negative after-potentials of frog nerve, unless the calcium concentration is greater than four times Ringer's proportion (60). The idea of two ranges of calcium action is merely a suggestion permitting one to understand much of what has been written on these subjects.

Changes in calcium concentration are known to affect many properties of nerve cells. Any one of these can be used as a measure of the changes wrought by changes in calcium. However, these properties are not independent, and until

their relationships to each other are worked out it will not be clear what are the basic properties modified by calcium. The pattern of research in this area may be formalized as follows. Experience shows that the stability of the membrane depends upon many variables:

$$E = E(\text{Pd, L-fraction, } CO_2, K^+, Ca^{++}, H^+, T, \text{ etc.}) \qquad (a)$$

This equation thus expresses the facts of the problem. One area of research is concerned with discovering and adding new variables to the expression. A second is concerned with finding relations among the variables, as for example that the action of potassium ions or carbon dioxide is mediated through change in membrane potential. Thus

$$\begin{aligned} \text{Pd.} &= P(CO_2, K^+ \text{ etc.}) \\ \text{L-fraction} &= L(Ca^{++}, CO_2, \text{ etc.}) \end{aligned} \qquad (b)$$

This kind of investigation will reduce the expression (a) so that E is eventually expressed in terms of a set of independent variables. A third type of study is the quantitative variation of E when one of the variables is made experimentally to be a principal variable. Ideally, all other variables would be held constant. Actually, this is only assumed to be the case because subsidiary relations like (b) preclude this unless such related variables are held constant by definite constraints. This is seldom done. If this condition prevails then the partial differential coefficients of E can be determined subject to the constraints such as (b). The physical problem is then to give a molecular mechanical explanation of the origin and magnitude of these coefficients. Actually, all phases of this problem are being examined. However, the physical analysis will go forward satisfactorily only after the form of equation (a) and the subsidiary relation (b) are all known. Only then will we know what are the basic processes for which we seek a physical explanation. Two prominent possibilities are that calcium acts on surface structures to 1) change resistance to current flow, and 2) change the L-fraction of the potential difference across the membrane. Changes in excitability and in the damping of the rhythmic self-excitation processes, it is proposed, are a consequence of these two primary physical effects.

III. SYNAPTIC TRANSMISSION

In section I the effects of calcium ions upon properties of the excitable structure of axons and of muscle cells have been discussed. On the basis of this information about the individual cells one might have expected that the effect of varying the calcium concentration upon the process of excitation of a muscle by a nerve could be readily understood. The same expectation could arise in considering transynaptic excitation in multineuronal systems. In some degree this is correct. When a synaptic region such as a stellate ganglion is perfused with a calcium-free Ringer's solution both pre- and postganglionic cells become hyperexcitable. The direct evidence for this is spontaneous activity in these two types of cell (17, 19). If a neuromuscular junction is similarly treated spon-

taneous muscle twitches occur, and the motor nerves are active (99, 100, 120). These are signs of the hyperexcitability of excitable elements on both sides of the cellular junctions. If the calcium ion concentration is increased sufficiently transynpatic excitation is prevented, and this seems to be a result of the expected increased threshold of the post-junctional unit. These actions of calcium agree with previous studies on axons and on muscles. They reveal a considerable range of variation of concentration that is compatible with effective transjunctional excitation, a low concentration range associated with phenomena of heightened excitability and a high range correlated with inexcitability. However, the earliest investigations of neuromuscular excitation revealed that removal of calcium from the extra-cellular fluid bathing a nerve muscle preparation leads to block of transjunctional excitation. This occurs in conjunction with the evidence of hyperexcitability of pre- and postjunctional cells.

It is now clear that the role of calcium in transynaptic excitation is a much more complicated process than could have been anticipated from studies of axons and may be different at neural and at neuromuscular junctions. Indeed, the slow and fast neuromuscular systems actuating a crayfish claw are modified in opposite directions by changes in concentration of potassium ion and, perhaps of calcium ion (175). The inherent spontaneous activity of the isolated nerve cord of crayfish was maximal for a one-to-one ratio of calcium and potassium in the bathing solution (141). This activity, presumably involving self-excitation of cells and transynaptic excitation, was stopped by a twofold increase in potassium or a corresponding decrease in calcium. The activity in this preparation showed an unusually marked dependence upon potassium-calcium interaction. In contrast, similar activity in isolated nerve cord of the cockroach was markedly insensitive to changes in concentrations of either of these ions (142). Such information is a strong deterrent to generalizing about ionic mechanisms at synapses and re-emphasizes the necessity for quantitative evaluation of the effect of each variable by experiment (14, 19). Such data will permit a useful classification of types of synaptic mechanisms.

A mechanistic description of nervous action is usually an account of the relations between a stimulus imposed at one part of the nervous system and a response detected in other parts of the nervous system or in effector organs such as muscles. The simplest experimental system of this kind is a single neuron in which the stimulus is imposed at one part and the response is detected there or at a remote part of the same cell. Regardless of the anatomical complexity of the system the process can be thought of as a sequence of excitation and response that is traceable in detail from the point of initiation to the point of detection.

The process of excitation of a muscle by means of a stimulus applied to a nerve may be described in terms of a sequence of changes of potential difference across cellular membranes. The action currents spread along the motor axons and their terminal branches as each successive part of the membrane responds to the stimulating action of the current flow across it. This neuronal process is terminated with the response and recovery of the terminal parts of the axon branches. Within 0.5 milliseconds after this response at the axonal terminations

the potential difference across the muscle membrane (frog) begins to change appreciably (101, 102). This is a focal change localized to regions of the muscle in the neighborhood of nerve terminations. The electrical sign of this localized change in the endplate region of the muscle membrane is called the endplate potential (44). When this localized change of impedance and polarization is sufficient the current flow through adjacent areas of muscle membrane produces sufficient depolarization, and a response occurs therein. Thereafter, the action currents spread along the muscle fiber and the contractile process occurs. Experimental observations have suggested that an additional process intervenes between the response of the nerve terminals and the response of the muscle membrane at the endplate. There is evidence that this process outlasts the action currents in the nerve terminals. It is given the noncommittal name of the transmitter process. The transmitter process is the mechanism by which the stimulus is imposed upon the endplate region of the muscle membrane as a result of the activity in motor nerve terminals (101, 102). To some investigators the transmitter is a prejunctional product that acts upon the muscle membrane. To others it is a chemical product of a process in the muscle membrane that leads to the observed changes in impedance and potential difference. From both viewpoints the transmitter action is a result of motor nerve impulses and has an independently variable intensity. Thus, the process can be diagrammed as follows:

Nerve impulse → transmitter action
→ endplate response → muscle impulse (cf. 88)

Our problem is to understand the role of calcium ions as an environmental variable that modifies this sequence of excitation and response.

In any process of excitation and response there are two conceptually and operationally separable aspects. First, there is the intensity of the stimulus developed by the active cell and second, there is the sensitivity to the stimulus of the cell being acted upon. The same ideas apply to such a relation between two adjacent regions of the same cell. Thus, in general, an agent such as calcium chloride may be expected to affect the overall process of excitation and response in three general ways. (a) It acts only on the intensity of the stimulus developed by the active cell. (b) It acts only upon the excitability of the cell to be excited. (c) It does both, either at the same concentration or at different concentrations. The pattern of experimentation and thinking in this area of research can be understood in terms of these ideas plus the hypothesis that the time course and intensity of the physiologically significant events is reflected in the changes of electrical potential across cellular membranes.

In the transjunctional process there are clearly at least three sets of the causal relation, stimulus-response. They are:

(a) Neural membrane → neural membrane, (b) neural membrane
→ endplate membrane, (c) endplate membrane → muscle membrane

An agent, like calcium chloride, can potentially influence each of them in the three ways previously outlined. One purpose of the experimental study of the

role of calcium ions in the process of transynaptic excitation is to locate the principal sites of action of calcium salts. For the purposes of this review we may ask whether the experimental facts about the role of calcium chloride in neuromuscular excitation can be understood in terms of the described conceptual mechanism for this type of intercellular excitation and response?

Locke (110) showed that excitation of the muscle by impulses in the motor nerves in frogs was blocked if the concentration of calcium chloride was either too high or too low. In other words, there was an optimal range of concentrations compatible with the adequate physiological functioning of this process of excitation of one cell by another. The prevailing explanation of the action of high concentrations of calcium ions (above 10 mM for frogs) is that the intensity of the membrane current developed by depolarization at the endplate is insufficient to excite the adjacent areas of muscle membrane. This explanation is suggested by the following experimental facts. The threshold for direct excitation of a muscle by imposed electrical currents is raised by increasing the concentration of ionized calcium in the environment of the cells (23, 28, 89, 120). At the concentration that blocks neuromuscular excitation the endplate potential change is present (88). This testifies to the adequacy of every step in the process from excitation of the nerve to depolarization of the muscle membrane at the endplate. This explanation hinges upon several unexpressed postulates that have been more or less clearly exhibited by careful studies of the neuromuscular excitation process to be described.

There is evidence that the transmitter process acts to short circuit the sensitive areas of the endplate membrane (91). Adjacent areas of muscle membrane, that are capable of an active electrical response, then begin to discharge through these depolarized areas of low resistance. The average potential change, between an inside and outside electrode, as recorded initially from the end-plate region, is the rising phase of the endplate potential. When the potential across the surrounding muscle membrane has thus fallen to a critical level the usual active change in sodium permeability occurs, and a reversed membrane potential develops. This structural alteration propagates away from the endplate region by the mechanism of successive excitations of adjacent areas. The advent of the response in the muscle membrane surrounding the endplate region is signaled by a rapid increase in the rate of change of the average potential difference recorded between an inside and outside electrode (48, 130). This notch in the potential recorded by a locally placed external electrode had been previously observed and interpreted (44). This "step" in the rising phase of the total potential change recorded at the endplate is a measure of the excitability of the muscle membrane adjoining that region because it indicates the average potential difference (44, 48, 130) at which the muscle membrane becomes unstable and responds by a rapid development of a reversed potential difference.

In 3.6 mM calcium chloride the average value of this critical depolarization was 41 millivolts whereas for a muscle in 1.8 mM calcium chloride the value was 33 millivolts (48). Thus, this measure of the threshold for response of the muscle membrane is increased 25 per cent by increasing the calcium chloride content of Ringer's solution twofold. However, the observations of Jenerick

and Gerard suggest that the critical potential for electrical excitation of muscle does not change with calcium ion concentration (89). Only the initial membrane potential changed. The two sets of data are not incompatible but raise an interesting question. Is the critical endplate potential higher in presence of extra calcium ions merely because the muscle membrane potential difference is higher?

A comparable measure of the stability of the muscle membrane can be obtained when the depolarization is produced by an imposed electrical stimulus. This stimulus can be imposed upon the muscle membrane by outward flow of current between an internal electrode and an external one. The experiments show that the critical degree of focal depolarization produced is about equal to that developed at the endplate following arrival of a nerve impulse (48). Furthermore, the stability of the muscle membrane was shown by this test to be the same near or far from the endpalte. It is this latter fact that permits a comparison between the excitability of a muscle, as measured by an imposed electrical current, and the excitability as tested by responsiveness to endplate depolarization.

The increased stability of the muscle membrane exposed to higher than normal calcium concentrations could lead to inexcitability. However increase in endplate potential under these ionic conditions (39) tends to maintain transmission. It is assumed that the increase in stability of the muscle membrane is so great that the maximum degree of focal depolarization produced by one nerve impulse at the endplate is subthreshold. Thus, the muscle membrane is depolarized at the endplate by a nerve impulse but the action potential is not initiated in the muscle.

Precise quantitative evidence for the adequacy of this explanation is not available. In the recent paper by Fatt and Katz (48) some of the experiments were made in solutions containing 9 mM calcium chloride. The junctions under observation were not blocked. Kuffler states (100) that junctions may be blocked by solutions containing five times the usual amount of calcium. We may guess that the critical concentration is around 10 mM. Coppée (34) indicates the blocking concentration to be 16 times Ringer's proportion. If the latter be taken even as low as 1 mM calcium chloride then this would make 16 mM calcium chloride the blocking concentration, according to Coppée. On the basis of facts reported for nerve and muscle this seems impossible. Thus, Mines (120) states that 10 mM calcium chloride may make some muscles inexcitable as tested by electrical stimuli. Furthermore, Misske (121) states that 15 mM calcium chloride produces irreversible inexcitability in motor nerves of frogs. These various experiments have not been done under comparable conditions or on identical species. This may explain the divergent results. However, it remains to be demonstrated quantitatively that a specified liminal increase in calcium chloride has: (a) raised the threshold to electrical excitation of the muscle fiber a specified amount and (b) left an end-plate potential which is, quantitatively, insufficient to excite a membrane with the specified stability with respect to electrical depolarization. At present, only the methods introduced by Fatt and Katz (48) seem adequate for such a precise test.

The magnitude of the endplate potential decreases with decrease in concentration of ionized calcium in the bathing solution (39). Thus, the intensity of the stimulus delivered to the muscle membrane decreases under these circumstances. However, the threshold for excitation also decreases under these conditions. As long as the intensity of the stimulus to the muscle membrane developed by the endplate depolarization is above threshold neuromuscular excitation occurs. However, it is known that if the calcium ion concentration is below one-fourth of normal in the aqueous environment neuromuscular excitation is blocked (49b, 88, 100). The prevailing explanation is that the stimulatory effectiveness of the endplate potential falls below that required to excite the muscle membrane even though this is hyperexcitable in calcium-deficient Ringer's solution. There

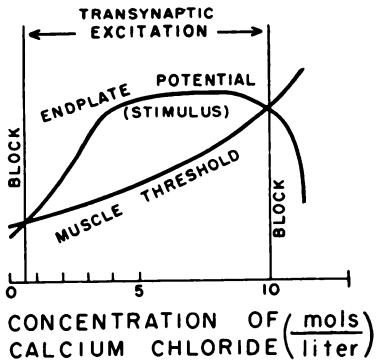

FIG. 4. *Diagrammatic construction of relations of endplate potential and excitability of muscle to concentration of calcium chloride in solution bathing a myoneural junction (frog).* The excitation of the muscle by motor nerve impulses is blocked if the concentration is too high or too low. Suppression of transjunctional excitation, as measured by endplate potential, does not occur at either blocking concentration. Modified from Coppée (34).

is direct evidence that block of transjunctional excitation is caused by a subthreshold intensity of endplate potential. Thus, the endplate potential from one volley may be added to that of a second volley, and this summated potential change in the muscle membrane can initiate a muscle impulse (34, 44). The implied relations between magnitude of endplate potential and sensitivity of the muscle membrane are shown diagrammatically in Figure 4.

There arises the problem of explaining the mechanism by which the endplate potential developed in response to a motor nerve stimulation decreases with decrease in concentration of ionized calcium at the endplate region. Two general possibilities appear within the framework of the present analysis. First, the responsiveness of the endplate membrane is reduced by calcium deficiency so that the transmitter action is less effective. Second, the transmitter action is reduced in intensity so that the stimulus imposed upon the endplate membrane is less intense. The latter explanation could involve a decrease in intensity of transmitter action developed by impulses in each nerve termination or it could arise as the average effect of the failure of impulses to be conducted to the end of

terminal branches of the axons (49b). The experimental evidence bearing upon the significance of these possible mechanisms will be examined.

The first mechanism mentioned above is equivalent to saying that removal of calcium from the endplate membrane changes it as does addition of curare. Curare is known to reduce the magnitude of the endplate potential developed by a motor nerve volley. At sufficient concentration the endplate potential is lowered to 30 per cent of its initial magnitude and then neuromuscular excitation fails. It is on this basis that the stimulating effectiveness of such an endplate potential is estimated to be three times the threshold value for exciting the adjacent muscle membrane (44). Curare reduces the endplate potential by making the endplate membrane less responsive to transmitter action. The type of experiment used to demonstrate this has been used to demonstrate that low calcium acts by reducing the intensity of the transmitter but leaves the responsiveness of the endplate either unchanged or enhanced. This amounts to a demonstration that calcium deficiency and curare act differently even though both environmental variables reduce the size of the endplate potential and thus prevent neuromuscular excitation. It is a remarkable fact that magnesium ions act at the endplate like curarine rather than like a deficiency of calcium ions (45, 88).

In the absence of calcium in the bathing solution muscle impulses arise spontaneously at the endplate (99, 100). This exhibits the state of hyperexcitability at the endplate. This spontaneous activity occurs in curarized calcium-deficient muscles and is therefore of postjunctional origin. It probably represents periodic depolarizations of the endplate regions analogous to that described for focal calcium deficiency in nerves. This hyperexcitability of the endplate is reflected in response to other stimuli. The region is hyperexcitable to electrical currents, to topical application of potassium chloride, acetylcholine, and to several other chemicals (71, 88). In brief, there is no evidence that the endplate membrane is less responsive to any effective stimulus. For this reason alone one would expect that neuromuscular block at low calcium levels arises from attenuation of the intensity of stimulus delivered to the endplate membrane by a motor nerve volley. These observations are in sharp contrast to the action of curare. At concentrations of curare which reduce the endplate potential to subthreshold intensity the responsiveness of the endplate to depolarization by topically applied acetylcholine is lost (44).

Until now the hypothesis that the transmitter process is the release from a precursor of free acetylcholine has not been mentioned. This was an attempt to exhibit in how far the role of calcium ions could be analyzed without entering into chemical details. Since the action of low calcium in blocking transmission is traceable to attenuation of the transmitter process further analysis must depend upon more specific knowledge of this process. Thus, the acetylcholine mechanism will be introduced at this point.

The acetylcholine hypothesis states that the transmitter process at neuromuscular junctions of twitch-producing skeletal muscles is the release of acetylcholine from a bound form followed by its depolarizing action upon the muscle membrane at the endplate. This focal decrease in membrane potential is asso-

ciated with a focal decrease in electrical impedance (91). There seems to be general agreement that acetylcholine is the active depolarizing agent at endplates on twitch-producing skeletal muscles of frogs and mammals (47, 49b, 67). However, some investigators believe this substance is released from a bound form in the nerve endings (44, 48, 49b, 101) while others believe it is released from a bound form within the muscle membrane at the endplate (67, 128). For purposes of discussing the action of calcium ions this distinction seems to be of secondary importance. The problem can be stated as follows. Do low concentrations of calcium reduce the endplate potential by making the membrane less responsive to acetylcholine or by decreasing the amount of this substance released for action on the membrane? Since, as stated above, the endplate in a calcium deficient muscle is more responsive to topically applied acetylcholine the block of neuromuscular excitation seems to be traceable to a reduced amount of acetylcholine per nerve volley acting upon the endplate. This could arise because the number of active nerve terminals was reduced or because the production per active terminal was reduced (49b). Until recently it was supposed that low calcium acted by a graded decrease in the mobilization of acetylcholine from its precursor. The new evidence suggests that reduction in endplate potential occurs in a quantized manner when this potential change is attenuated by calcium depletion. The spontaneous miniature endplate potentials described by Fatt and Katz (49a, 49b) are not reduced in size by reduction in calcium ion concentration. However, the endplate potential evoked by nerve stimulation is reduced in size in relation to the decrease of calcium concentration. At one-fourth the Ringer's proportion of calcium an endplate potential evoked by nerve is only several times larger than the spontaneous potentials (*i.e.*, about 3–4 millivolts instead of 30 millivolts). Repeated nerve volleys now elicit various amplitudes of endplate potential which vary stepwise. The stepwise deficits or increases are of the order of magnitude of the spontaneous potentials. Thus, it appears that the endplate potential evoked by nerve under these conditions is the summation of several miniature contributions. The magnitude of the miniature contributions is not dependent upon calcium ion concentration, but the number of these processes created by a nerve impulse is dependent upon calcium ion concentration. A normal endplate potential evoked by a nerve impulse in presence of 1.8 mM calcium chloride in the bathing solution would need to be integrated from about 100 such miniature endplate potentials. Fatt and Katz suggest that this signifies either 100 separate nerve branches and terminations or 100 different active areas for release of acetylcholine at each junction. According to this view the reduced calcium chloride concentration acts by either blocking individual axon branches or by inactivating individual areas for acetylcholine production (49a, 49b). The analysis of the mechanism has not been carried beyond this stage.

The observed changes in endplate response and muscle excitability produced by variation in concentration of calcium chloride can explain several previously established properties of the neuromuscular junction. Thus, increase in calcium favors more rapid development of block during high frequency stimulation of

motor nerve (35). This is supposed to occur because more acetylcholine is released per volley and blocking concentrations of acetylcholine accumulate at the endplate. Furthermore, the enhancement of response by high concentration of calcium ions in a partially curarized preparation is explained by a larger output of acetylcholine. The quantitative relation between magnitude of endplate potential and increase in calcium chloride between 1 and 7.5 mM calcium chloride is such (39) that one would expect a liminally curarized junction to become functional if the calcium chloride concentration is increased. This de-curarizing action of calcium chloride has been described (34, 51). Thus, in many respects, the observed action of changes in concentration of calcium chloride upon neuromuscular excitation, on changed sensitivity to drugs, on fibrillation and on fasciculation can be understood in terms of demonstrated actions upon the endplate potential, muscle membrane or nerve membrane stability. There seems to be only two fundamentally different mechanisms that need to be invoked (a) the action of calcium ions in stabilizing excitable membrane structures (b) the role of calcium ions in controlling the release of acetylcholine. The latter may be a direct chemical effect or secondary to an interference with nervous conduction in fine axon terminations. This circumstance sharply defines the kind of molecular studies of calcium action that are most likely to aid in further studies of these cellular phenomena: (a) the role of calcium as a stabilizing agent in protein and phospholipid structures and (b) the role of calcium ions as a co-factor in a specific enzyme system.

As previously stated, in a curarized preparation, the amplitude of endplate depolarization that just failed to excite the muscle was about one-third the initial value. However, a more extensive survey by Fatt and Katz (48) indicates that this safety factor may be smaller or larger, and, indeed, some endplate potentials developed by a single nerve volley may be of subthreshold intensity. Though such junctions are rarely observed in frogs this condition is produced by calcium deficiency as described previously. Thus, in a calcium-deficient preparation a subthreshold endplate depolarization produced by one volley may be added to that of a second to produce a response in the muscle fiber (34, 35). Presumably this is the mechanism of the recruitment of muscle responses observed at the beginning of a train of motor nerve impulses in other studies of calcium-deficient animals.

It has been observed that the action potential recorded from a muscle in such animals increases with each successive volley of impulses produced by maximal stimulation of the motor nerve (34, 35, 100). This is a normal property of neuromuscular junction in the chicken (20) and crab (10, 177) and has been studied in goats following removal of the parathyroid glands. In the latter instance the effect is a result of calcium deficiency in the extracellular fluids and can be removed by added calcium chloride. In the chicken the property is not affected by added calcium but is enhanced by calcium deficiency. In the crab the phenomenon is related to the high magnesium concentrations in the tissue fluids. In chronically calcium-deficient animals it is probable that this recruitment of muscle fibers by temporal summation of effects of nerve impulses is actually a

temporal summation of endplate potentials as observed in frogs. It is medically significant, for the understanding of symptoms, that calcium deficiencies compatible with continued life of a mammal are of sufficient degree to block neuromuscular excitation by one or more volleys of nerve impulses at some junctions. The junctions most susceptible to calcium deficiency are presumably those with the lowest safety factor.

The effects of calcium ions upon transynaptic excitation processes in sympathetic ganglia are superficially similar to those described for neuromuscular junctions. If calcium chloride is absent from the perfusion fluid ganglionic transmission becomes blocked (17, 19, 72). Larrabee has shown that such a block occurs in the stellate ganglion of the cat at a time when pre- and postsynaptic cells are self-excited (14, 17, 19). A further indication of the enhanced excitability of the postsynaptic cells is the increased frequency of impulses elicited by perfusion with acetylcholine in absence of calcium ion. Correspondingly, the frequency of impulses elicited by acetylcholine is reduced during perfusion with a solution containing higher than normal concentration of calcium chloride.

The output of acetylcholine from perfused stimulated ganglia is suppressed if calcium chloride is omitted from perfusion fluid (72). The postganglionic cells are more responsive to injected potassium chloride under these conditions but there is no output of acetylcholine, as in normal ganglia similarly injected. Harvey and MacIntosh suggest that in absence of calcium ions preganglionic impulses cannot liberate acetylcholine from its precursors.

These observations on neural synapses are comparable to those on neuromuscular junctions with one remarkable exception. Harvey and MacIntosh (72) reported that the above actions occurred during perfusion with calcium-deficient Locke's solution. However, if the ganglion was perfused with 0.9 per cent sodium chloride (plus eserine) there was no effect upon transynaptic excitation nor any spontaneous activity of the postsynpatic neurons. It seems that simultaneous omission of calcium chloride and potassium chloride is quite different from omission of calcium chloride only. This is in sharp contrast to the original observations on the neuromuscular junction of the frog. Locke (110) observed block during perfusion with sodium chloride solution which was eliminated by adding some calcium chloride. The observation was confirmed by Cushing in the perfused frog (37). The observations upon ganglia focus attention upon the phenomenon of calcium-potassium interaction which is not so prominent a part of the story for calcium-deficient frog A-fibers or for neuromuscular transmission in the frog. As far as the present author is aware this contrast has never been discussed before. Further information regarding the action of calcium ions at neural synapses is needed. This is particularly apparent in those situations where the chemical nature of the transmitter is unknown (18).

Saunders and Sinclair observed in the sympathetic ganglion of the frog an interaction between high pH and high calcium ion concentration. The transynaptic block produced by the former was counteracted by a sufficient increase in calcium chloride concentration in the perfusion fluid (148).

In résumé, it has been experimentally established that transynaptic excitation contributed by a presynaptic impulse is modified by the local chemical environment of cells and terminations in synaptic regions. When calcium ion concentration is the variable the changes of excitability are analogous to those produced in single axons, as tested by electric current. Thus, the higher the calcium the lower the excitability. This is generally interpreted to mean that the cell structure that alters during an impulse is made more stable by calcium. There is an important difference in the observations of transynaptic excitability. When the calcium ion concentration tends to low values the pre- and postsynpatic cells become more and more excitable, as expected. The end result is repetitive self-excitation in each cell. However, at low concentrations of ionized calcium transynaptic excitation by previously effective presynaptic impulses becomes blocked. There are two prevailing explanations, (a) the presynaptic impulses no longer reach the termination for some reason and (b) these impulses cause less of a stimulus to be imposed upon the postsynaptic unit.

It is interesting to see in how far observations in more complex neuronal systems can be understood in terms of these principles without invoking anything new. An increase in calcium ion concentration in the perfused ventricles of a dog's brain will decrease the systemic blood pressure and reduce the intensity of the reflexly-produced change in blood pressure from a standard stimulus to the central end of the severed vagus or from the pressure receptors of the carotid sinus. Lowering the ionized calcium increases the blood pressure and increases the response to these afferent stimuli. The author (104) believes that there was no depression of reflex activity even at the lowest calcium ion concentration. Consequently, these observations on the vasomotor system can be understood solely in terms of the usual effects of calcium changes upon excitability of cell membranes. The intravenous injection of calcium chloride had no effect upon the electrical activity of the cortex in cats (145). Under the same conditions there was an effect of magnesium chloride. In contrast, the spontaneous rhythmic potentials of the lateral geniculate of the cat (43) and of the excised frog brain were decreased by added calcium or magnesium chloride (109). In the latter studies the frequency of the rhythmic potential changes decreased with increase in concentration of calcium chloride from 0–7 mM. A discontinuous further decrease was observed at higher concentrations, about 18 mM. There was a marked antagonism between potassium and calcium ions (as observed in the stellate ganglion but not in the neuromuscular junctions). Obviously these properties are like those of single calcium-deficient axons and do not seem related to specific synaptic phenomena.

IV. NEURONAL METABOLISM

The oxygen uptake of frog nerve increases in absence of calcium ions in the external solution (17, 26, 55). Under these circumstances many of the axons are self-excited and conducting impulses. A more extensive study of the dependence of the rate of oxygen uptake upon concentration of calcium ions indicated an increase in the concentration range where the spontaneous activity was neg-

ligible but the A-fibers were more excitable. Indeed, there was observed a correlation between the decrease in threshold of frog A-fibers and increase in rate of oxidation when calcium ion concentration was changed in the range below Ringer's proportion (17). When these properties are measured as a function of calcium ion concentration in the presence of 2 per cent carbon dioxide the same relation holds. Under these conditions the spontaneous activity is moderate and appears at lower calcium ion concentrations. This evidence indicates that the rate of oxygen uptake of frog nerve increases with decrease in calcium ion concentration even in the absence of spontaneously initiated conducted impulses. The increased rate of oxidation is a property of the state of the resting nerve that occurs in the same concentration range within which the decrease in threshold occurs. At very low concentrations of calcium ion part of the increase may arise from spontaneously initiated impulses.

The earlier observations indicated that the maximum increase of the rate of oxygen uptake by the nerve during conduction of impulses (17) was less than the maximum increase after deprivation of calcium ions. In the latter experiment all of the tissue is involved while in the former only the A-fibers take part. Thus, the observations are not inconsistent with the idea that the component of oxygen uptake that is sensitive to calcium ion concentration arises from the same oxidative processes set into increased action by conduction of impulses. A further indication of this is that axons having an increased rate of oxidation during calcium deprivation will conduct impulses at high frequencies without a further increase in rate of oxygen uptake. Perhaps calcium ions are part of the mechanism linking the electrochemical processes of conduction to the rate of oxidative metabolism, as suggested by Gerard on the basis of other evidence (55). The increase in rate of oxidation in frog nerve deprived of calcium is through a reaction pathway that is inhibited by azide and chloretone. In these respects the reaction pathway resembles that of nerves in Ringer's solution. This suggests that the increased uptake of oxygen in calcium deficient axons measures an increased rate in a chemical reaction system normally present in the nerves.

The removal of calcium ions from the solution bathing slices of mammalian cortex causes an increase in the rates of oxidation and of glycolysis by the cells (57). The creatine phosphate content falls, too. As in frog nerve the rate of oxidation in cortex slices is not changed by so much as a threefold increase in calcium ion concentration.

The control of the rate of oxidation in nerve cells by the concentration of calcium ions in the bathing fluid focuses attention upon ionic requirements for enzymatic activity. The possibility arises that the effects of calcium upon other axonal properties, previously described, are a result of a primary action on some enzyme system. A review of the role of metal ions in enzyme catalysis was reported recently by Lardy (106) for phosphorylating enzymes, and by Najjar (129) for other enzyme systems. Phosphorylations involving adenosinetriphosphate (ATP) usually require magnesium for full activity. Some of these are inhibited by calcium ions, pyruvate phosphoferase for example. Others are not inhibited by calcium, such as creatine phosphoferase and some are activated by

either magnesium or calcium, adenosinetriphosphatase (ATP-ase) for instance. The calcium sensitive adenosinetriphosphatase of nerve cells is located in mitochondria [cf. (2) for references]. Since calcium acts to increase or decrease the rate of phosphate transfer anything could happen in the cell where some unknown combination of such processes occurs. Perhaps the important fact to remember is that several steps in the phosphate transfer system are sensitive to calcium ion concentration. This is perhaps the principal system of chemical processes that makes oxidative energy available for maintenance of structure and function.

The metabolic system for oxidative phosphorylation is present in mitochondria derived from cerebral gray matter, spinal cord white matter, and peripheral nerve (2). It is probable, therefore, that the increased rate of oxygen uptake of calcium deficient axons reflects increased rates of oxidation processes in the mitochondria. The mechanism, however, is not apparent. If low calcium produced phosphate acceptors somewhere in the cell this could initiate higher rates of oxidation in mitochondria (138, 159). If calcium deprivation changed the ease of access of substrate to mitochondria the observed increase could occur. In addition it is quite possible that calcium deficiency may affect the mitochondria directly. For example, the oxygen uptake of liver mitochondria increases with decrease in calcium ion concentration over about the same range of concentration as does that of frog nerve (90). The decrease in rate of oxidation below 0.3 mM calcium chloride is not observed in frog nerve. The effect is supposed to arise from activity of ATP-ase in the mitochondria. Perhaps the same mechanism occurs in axonal mitochondria in situ. However, this is not a property of all mitochondria for ATP-ase of insect mitochondria is not activated by calcium chloride but its activity is depressed at concentrations above 6×10^{-3}M (146). Low concentrations of calcium ions reduce the rate of oxygen uptake of insect mitochondria and prevent oxidative phosphorylation (147). Because of all the suggestive possibilities it would be a useful experimental accomplishment to demonstrate which of the possible metabolic actions is enhanced in nerve cells deprived of calcium and to show whether or not the mitochondria are involved directly.

In addition to these generally important systems of chemical reactions, oxidative phosphorylations and phosphate transfer, the acetylcholine system of reactions is considered especially important for nerve function. Calcium ions may be involved in the activity of choline acetylase (50) and they have been implicated in the release of acetylcholine from cells (113). The synthesis of acetylcholine by cell-free brain extracts is inhibited 50 per cent by 3–5 mM concentration of calcium chloride (50). This list of possibilities for explaining the observed effects of calcium ions upon metabolism and function, as studied on neurons and axons, is by no means exhaustive. For example Buffa and Peters have suggested that the intracellular level of citrate, as determined by rates of processes in the Krebs cycle, may influence excitability of nerve cells through its calcium-binding properties (21). Thus, citrate accumulates in the axons and neurons of animals poisoned with sodium fluoroacetate. These animals are hyperexcitable and may die in convulsions. There is evidence that the citrate content of the blood can increase enough to produce symptoms of tetany (169).

The structural detail of specific proteins derived from a precursor can be determined by the concentration of calcium ions in the solution. The enzymatic formation of trypsin from trypsinogen (autocatalytic) is accompanied by the formation of inert protein. The rate of the latter reactions is depressed by calcium ions in the solution. If the concentration of calcium chloride is greater than 20 mM there is 100 per cent conversion of trypsinogen to trypsin (117). Accessory tests suggest that calcium ion reaction with the trypsinogen is the important process. Thus, bound calcium ions can influence the direction of reaction in enzymatically controlled changes of protein structure. Recent studies of trypsin activity revealed the remarkable fact that calcium binding can enhance its esterase activity without changing its proteolytic activity (61).

Many years ago Professor Heilbrunn described an action of calcium ion upon pigmented cytoplasmic particles in arbacia eggs (74, 75). When such cells are injured by needle puncture the hole is repaired if the outside solution contains calcium ions. In a series of direct experiments it was demonstrated that the red-colored cytoplasmic particles were disintegrated in presence of calcium ions. If this reaction was prevented, by absence of calcium ions or by absence of the particles at the site of needle puncture, then the injury was not repaired and cytolysis followed. The same reaction has been studied more recently in egg homogenates (86, 87). There, it is accompanied by acid production and an increase in viscosity similar to the changes produced by added trypsin. The primary action of calcium ions is supposed to be with a soluble cytoplasmic factor which is activated and then acts to break up the cytoplasmic particles. This suggestion arose from the observation that well-washed particles are insensitive to added calcium ions. This observation has been confirmed (66). The action of calcium ions upon the egg homogenates is accompanied by an increase in oxygen uptake that is prevented by added monoiodoacetate. The author explains this metabolic effect as secondary to unmasking of SH groups by the cytoplasmic changes and a subsequent oxidation to S–S linkages, supposedly causing the associated increase of viscosity.

Heilbrunn (75) has elaborated a theory of excitation and response with the surface precipitation reaction as an important component. Definitive connection of this theory to neural excitation and response has not been forthcoming. However, these are demonstrated relations of cytoplasmic processes to calcium ion concentration that are comparable to the activation of specific enzymatic reaction systems and must be considered in interpreting observations of calcium action on neurons and axons. Obviously, it is necessary to develop experiments that distinguish between these potentially significant processes if they occur in the nerve cell following changes in external calcium-ion concentration.

The extruded cytoplasm from a giant axon of Squid is dispersed in presence of ionized calcium but remains as a coherent rod-like structure in calcium-free sea water (84). The time required for dispersal increases in a regular manner with decrease in concentration of calcium chloride in the external medium. This action seems rather specific in that it could not be duplicated by magnesium chloride, strontium chloride or barium chloride. The authors compare this phenomenon with the liquifying action of calcium ions on protoplasm of Stentor

and contrast it with the surface precipitation reaction described above (cf. 75). The studies suggest that the axoplasm of Squid, normally gelatinous (171), must contain less than 500 μ mol/liter of ionized calcium. [This evidence in conjunction with the reversible and rapid (see later) effects on excitability of Squid axons following changes in external calcium ion concentration (5–70 mM) indicate a primary reaction of calcium with the external cell surface.] Since there is some evidence (143) that the total calcium content of Squid axoplasm is much higher than 0.5×10^{-3} mols/liter most of the internal calcium is bound.

At first sight the experimental sorting of the numerous possibilities for action of calcium ions on cellular processes seems too complicated for analysis by available indirect experimental methods. Calcium ions can act to either disperse cytoplasmic structure or coagulate it and they can act, at different steps in the chain, to enhance or depress rates of metabolic reactions. Perhaps some progress in understanding and analysis of the data can be developed from the following suggestions. A prototype of the first contrasting action is known. Mercuric ion is bound firmly by some serum proteins. If the metal ion concentration is low it is shared by two molecules thus tending to form agregates by means of a salt link (85). If the mercuric-ion concentration is higher each protein molecule is independently saturated and dispersion is favored. Another approach is to study the specificity of the several actions of calcium with respect to magnesium ions. In enzyme systems described above calcium ions may substitute for or antagonize magnesium. In the studies of cortical rhythms or excitability of excised axons calcium ions can replace magnesium and the latter is not needed in the external medium (17, 109). Thus, in assessing the importance of the possible mechanisms for action of calcium on excitability of axons those in which calcium ions and magnesium ions act alike should receive particular attention. Such a reaction is the activation of ATP-ase (106, 146) but not the lytic action of calcium on the pigment granules of Arbacia eggs or the dispersal of Squid axoplasm. However, rectification of current flow across nerve membranes is increased by calcium ions and decreased by magnesium ions (164). Block of neuromuscular transmission occurs with magnesium ions (45) in a concentration range (4–8 mM) where calcium ions facilitate transynaptic excitation (39). Here, one recalls a similar interaction of these two ions on pyruvate phosphoferase (106) and in magnesium narcosis (45).

Recently, the rate of loss of radioactive calcium from crab nerve has been studied (163). The process was characterized by two time-constants. Obviously, a simultaneous measurement of the kinetics of the change in metabolism may be a powerful means of relating it to particular fractions of the exchangeable calcium.

At this point a brief digression will serve to mention important facts that would require another review if discussed adequately. Every property of a neuron that changes with the concentration of calcium ions is a function of other independent variables such as potassium concentration, temperature, and narcotics. The interaction between two such variables may be a simple addition of their independent effects upon the cellular property being measured. As a striking

example, the external concentrations of calcium and potassium ions act as independent variables that together determine the excitability of muscle cells (89). However, the interaction can be more complicated. For example, the relation of membrane potential of mammalian nerve to temperature is shifted by a change in calcium ion concentration. As further examples, the depolarization of nerve by potassium chloride is opposed by increase in calcium ion concentration (69), and anoxic depolarization is slowed under these conditions (111, 156). Such interactions, measured in terms of a complex cellular property could arise from primary interactions of the agents at a common molecular site. Thus, the enzyme system pyruvate phosphoferase requires potassium ions and magnesium ions for full activity, and its activity is inhibited by calcium ions. The relation of calcium to magnesium in this relatively simple system suggests a competition for a common reaction sited on the enzyme. The relation of calcium inhibition to potassium-ion enhancement of activity suggests additions of two opposing effects but not competitive interaction. The reporting and analysis of such interactions would require another full-scale review, especially if the relations of calcium to adrenaline, acetylcholine, histamine (75) and ascorbic acid (140) were considered. In addition, agents like quinoline or DDT excite crayfish nerves, and the action is opposed by calcium (56). Anaesthetics like ether, chloroform and cocaine depress excitability of muscle and, at the same time, calcium is released into the bathing solution (7). The molecular basis of such interactions will be clarified when the primary reactions of calcium with cellular components are known. Their orderly exposition here would double the length of this review.

V. PROCESSES OF ION BINDING AND ION EXCHANGE

a. Calcium Binding by Organic Molecules. Previously, the effects of calcium ions upon a variety of neural and neuronal processes have been reviewed. The treatment was descriptive for the purpose of clearly delineating the experimental facts. Some of the implications of these facts for the operation of the nervous system in the animal were considered for these are, to some extent, independent of the explanation of the physical mechanism by which the ions act upon the nerve cells. Now, will be considered the mechanism by which calcium ions react physically with neuronal structures to cause the various changes in measured properties. As a preliminary to this discussion, the interactions between calcium ions and biologically significant organic molecules and molecular aggregates will be reviewed. The latter information is an indispensable minimum amount of the kind that will be required for a molecular description of the role of calcium in nervous processes.

Several very general suggestions about the physical mechanism of calcium action upon cells have been made. Some of these involve the idea that calcium compounds control the permeability of the cell surface to other substances. It has been observed that the leakage of potassium from frog muscle requires a minimum outside concentration of calcium chloride (173b). This was interpreted as a change in permeability to potassium of the muscle surface. In contrast, in concentrated calcium chloride solutions erythrocytes lose potassium and gain

calcium as a simple anion exchange process (137). Alexander and Teorell suggest that calcium binding to cephalin molecules makes a tight molecular structure and thus reduces permeability (4). Martin (114) points out that calcium binding regulates the degree of coiling of chondroitinsulphuric acid and could thus control permeability by altering pore size in a structure. Hodgkin, Huxley and Katz invoke the idea that calcium compounds, when dissociated, act as carriers for transport of sodium across the membrane (83). These ideas were preceded by the suggestion that calcium compounds release calcium ions during excitation to invoke a response in the form of a colloidal change in deeper regions of the cell (75). The presence of a layer of ribose nucleic acid at the surface of some cells, the marked calcium-binding properties of nucleic acids and the deleterious effect of ribonuclease on calcium uptake in *Elodea* cells have lead to the idea (105) that nucleic acid regulates cellular permeability to calcium. The latest suggestion is that calcium ions (and other divalent ions) may act, indirectly, by modifying specific enzymatic activities or by combining with metabolic intermediates (6, 21, 106, 124, 144). These various ideas are, obviously, not mutually exclusive. Each of these very general suggestions implies a primary reaction of calcium ions with molecular constituents of the cells. The subsequent causal relations are shown diagrammatically in Figure 5. Some trace of the properties of such primary reactions should appear in the relation of the measured cellular property to the calcium ion concentration, regardless of the intervening mechanism. It is physically significant that ATP-ase activation (94),

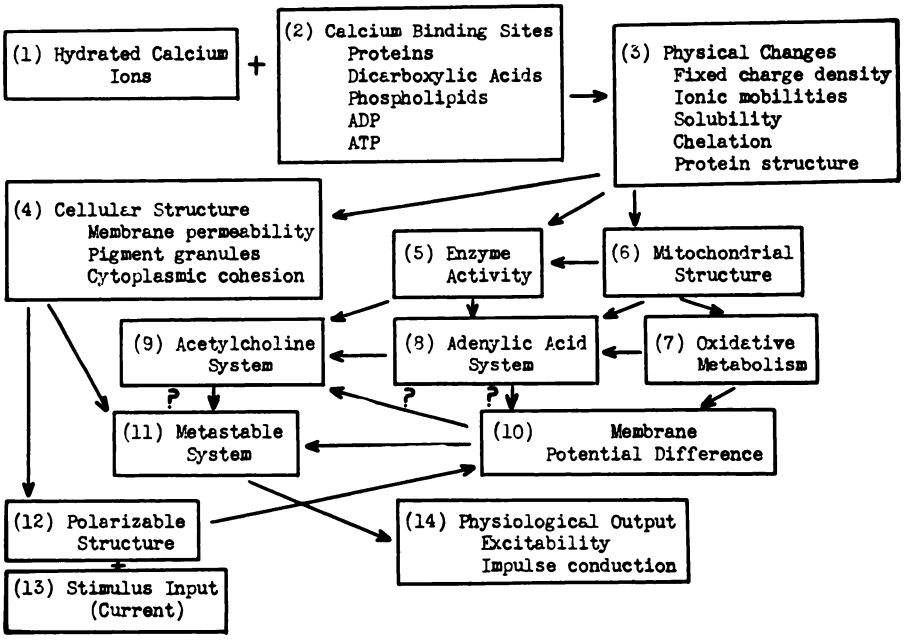

FIG. 5. *Diagram of some possible relations among properties of nerve cells, suggesting the role of calcium in neuronal processes.*

pyruvate phosphoferase inactivation (90), rate of oxygen uptake of liver mitochondria (90) suppression of acetylcholine synthesis (50), change in excitation threshold and rate of oxygen uptake of frog A-fibers (17) all change markedly between 0.4 and 3.0 mM concentration of calcium ions.

The potentialities for the physical interaction of calcium ions in the aqueous environment with constituents of living cells are not numerous. Changing the concentration of calcium ions will change the ionic strength of the external aqueous solution and that of any solvent phase in equilibrium with the external one. Properties of cell structures and of dissolved components that are influenced by electrostatic forces between charged chemical groups will be modified by such a change in the electrolyte content of the aqueous environment. For example, the degree of dissociation of ionizable groups on the cell surface could be affected in this way and the electrostatic interaction of neighboring charged groups could be modified. Such non-specific effects of calcium ions can be initiated by changes in ionic strength produced by other ions. A second kind of interaction of calcium ions with cells arises if there exist cell structures with fixed charged groups and with interstices filled with aqueous solution, after the manner of ion exchange structures and of aqueous phases enclosed by selectively permeable membranes. Such interactions are describable only in terms of equilibrium constants that characterize the calcium content of the cell phase in relation to the calcium ion concentration of the aqueous environment. The processes involved may be the reversible transfer of calcium ions from the aqueous environment to an aqueous phase inside the cell, to aqueous pockets within some solid structure, or to binding sites in the solid phase. The general thermodynamic treatment for equilibrium states of ion-exchange systems can represent all of these special instances. The exchange of calcium ion for sodium ion, for instance, can be written

$$Ca_0^{++} + 2Na_i^{+} \rightleftarrows Ca_i^{++} + 2Na_0^{+} \qquad 1$$

For such a process the Gibbs theory for equilibrium of heterogenous substances can be used in the form given by Gaines and Thomas (54). Thus,

$$K = \frac{(Ca_i)(Na_0)^2}{(Ca_0)(Na_i)^2} \qquad 2$$

In the special case that K is unity the equilibrium is described by the Donnan ratio r defined by

$$\frac{(Ca_0)}{(Ca_i)} = \frac{(Na_0)^2}{(Na_i)^2} = r^2 \qquad 3$$

This occurs when the process is completely described by Equation 1 and the standard state of each kind of ion is the same in both phases. These two conditions are obviously met in experiments involving exchange between an inner and outer aqueous solution. In this case the symbols Ca^{++} and Na^{+} refer to fully hydrated ions like those of the outer phase. However, if the inner phase is a porous solid with fixed charges and an experimentally inaccessible aqueous

phase the physical state of the ions in the inner phase cannot be studied directly. Only the total content of each ion per unit weight of material can be measured for each equilibrium state. The activity coefficients for each ion in the inner phase must then be calculated from these data and knowledge of the ion activities in the external phase. If the system is in thermodynamic equilibrium with respect to ion distributions this can be done (54). For practical purposes, however, the important fact is that the exchange process is characterized by an equilibrium constant different from unity (cf. 12).

It is known that calcium ions can be bound by some anions to form salts in dissociation equilibrium with the hydrated calcium ions. In such a compound the bound calcium is not a hydrated ion. Such equilibria within one phase are described by

$$CaX \rightleftarrows Ca^{++} + X^{=} \qquad 4$$

For calcium citrate the reaction at low calcium ion concentrations is

$$CaCit^{-} \rightleftarrows Ca^{++} + Cit^{\equiv} \qquad 5$$

Two or more molecules may be held together by calcium

$$CaX^{+} + Y' \rightleftarrows Y\text{-}Ca\text{-}X \qquad 6$$

Polyvalent electrolytes like proteins can bind more than one calcium atom,

$$Ca_nP \rightleftarrows Ca_{n-1}P^{=} + Ca^{++} \qquad 7$$

In a thermodynamic sense all these interactions are the same in that the equilibrium states are determined by constants related to the energy and entropy differences between products and reactants in their standard states.

As previously mentioned calcium can exist in two distinct states in aqueous solutions of certain organic salts. Thus, in a solution of sodium citrate and calcium chloride part of the calcium reacts with trivalent citrate ions to form monovalent calcium citrate anions and part is in the form of hydrated divalent calcium cations (73). The reaction is apparently that of chemical Equation 5, and is characterized by a dissociation constant (73, 118).

$$K = \frac{(Ca^{++})(Cit^{\equiv})}{(CaCit^{-})} \qquad 8$$

Proteins also bind calcium by a reaction that is characterized by a monomolecular dissociation constant (118, 119). Thus, the reaction behaves as though

$$CaP \rightleftarrows Ca^{++} + P^{=} \qquad 9$$

where

$$K = \frac{(Ca^{++})(P^{=})}{(CaP)} \qquad 10$$

TABLE I

Dissociation Constants for Organic Salts of Calcium in Aqueous Solutions

Compound	Dissociation Constant $\frac{\text{Mols}}{\text{Liter}}$	Reference
Proteins		
Serum	6.1×10^{-3}	(119)
Albumin	3.7×10^{-3}	(25)
Euglobulin I	2×10^{-3}	(41)
Euglobulin II	1×10^{-2}	(41)
Euglobulin III	7.4×10^{-4}	(41)
Pseudoglobulin $-\gamma$	1.1×10^{-3}	(41)
Globulin (α and β)	4.0×10^{-4}	(42)
Casein (in NaCl)	4.32×10^{-3}	(27)
Cephalin (in Ringer's sol.)	1.27×10^{-3} (mixed)	(42)
Aminoethanol	1×10^{-5}	(42)
Serine	4×10^{-2}	(42)
Phosphate	1.5×10^{-2} ($\mu = 0.100$)	(65)
Polyphosphates	1×10^{-3} (Coordination 2)	(174)
Adenosine triphosphate	8.7×10^{-5} ($\mu = 0.1$)	(40)
Adenosine diphosphate	1.8×10^{-4} ($\mu = 0.1$)	(40)
Citrate	6.1×10^{-4} ($\mu = 0.155$)	(73)
Oxalate*	1×10^{-3}	(122)
Succinate	6.3×10^{-2} ($\mu = 0.2$)	(22)
Malonate*	3.5×10^{-2} ($\mu = 0.2$)	(22)
Bicarbonate	0.15	(64)
Carbonate	1×10^{-3}	(64)
Sulfate	5.3×10^{-3}	(122)

* Compare values in (63) and (64).

Thus each binding site on the protein appears to act independently even though each protein molecule may bind many calcium ions.

In 1944 Greenberg (62) said of this simple empirical law (Equation 10) "that the mass law equation fits the dissociation of calcium proteinate probably has an at present unrealized significance". This significance was available in the analysis of protein titration curves (29, pp. 449–453) but was not generally recognized until Klotz (95) made the explicit connection to data on the dissociation of calcium proteinates. Proteins seem to have a limited number of ion binding sites (m) for a particular kind of ion. If these sites act independently of each other and are characterized by the same equilibrium constant K then the expression for the multiple equilibria reduces to the observed simple hyperbolic form, Equation 10. Each molecule of protein with m binding sites behaves like m separate molecules with one binding site. This has been a familiar phenomenon from experiments on titration of proteins by acids and bases and is evidence for independent binding sites and a single intrinsic dissociation constant characteristic of the ion-binding group (95).

The intrinsic equilibrium constant is a measure of the strength of the binding at each site. When the concentration of binding molecules is known it is possible to experimentally estimate the maximum number of calcium-binding sites per

molecule (96). For proteins this number is different for different cations, and the information can be used in conjunction with other structural data to help define chemically and spatially the concept of a binding site (62, 96).

The calcium is bound to these multiple-charged anions in such a way that some of it is carried toward the anode during electrolytic conduction of current through such a solution. This is evidence for binding through calcium salt formation, involving the dehydration and electrostatic binding of the small ion. As in monovalent calcium citrate anion the charge of the bound calcium is neutralized by close electrostatic binding to anionic sites on the binding molecules. Such a binding may involve formation of closed rings within a molecule (62), dimerization by formation of double salts of calcium, or a variety of chelate forms with different coordination numbers (174). The term calcium complex can be used to designate all of these kinds of binding in which the calcium ion is dehydrated and a part of the larger anion. Such binding, in which the calcium migrates in an electric field with the multiple charged anion, must effectively reduce the anionic charge of the binding anion by two units per atom of calcium in the complex.

Deamination of crystalline egg albumin did not change the capacity to bind calcium ions (1). Acetylation, which blocks hydroxyl and amino groups abolished the calcium binding. This implicates the hydroxyl groups in some way. Reasoning from calcium-binding properties of organic acids Greenberg points out spatial and electrostatic reasons why an hydroxyl group, as in citrate ion, should enhance calcium-binding by the neighboring carboxyl groups. Thus, citrate has a somewhat stronger calcium-binding site than does oxalate ion (cf. 62). The suggested reason is the spatial formation of a six-membered ring through calcium linking the carboxyls and a five-membered ring through linking carboxyl to hydroxyl. The hydroxyl group has a residual negative charge on the oxygen which affords an attractive force for calcium ions. The dissociation constant for calcium citrate is pK = 3.22 or K = 0.6×10^{-3} (73). The estimated values for protein binding sites range from pK = 2.0 to a pK of 3.13 for different proteins, Figure 6 (1, 24, 27, 41, 119). These data suggest the binding sites to be paired carboxyl groups with the binding strength enhanced in some proteins by adjacent hydroxyl groups, as it is in citrate ions.

b. Calcium binding in Complex Molecular Systems. The potentialities for modification of cellular properties by calcium ions are manifold, though presumably arising from these few kinds of primary molecular-ionic interactions. For example, calcium ions affect the catalytic activity of certain enzyme systems. Therefore, it is natural to guess that the increase in oxygen uptake of a nerve when transferred from Ringer's solution to one of lower calcium ion concentration is related to changes in enzyme activity produced by calcium binding. However, in such an experiment the axons also become more sensitive to excitation by electrical currents, and it is less evident, though not inconceivable that enzymatic action is involved. The controlled association of ideas, in this instance, leads equally easily toward notions of structural arrangement of surface constituents (cf. 56, 76). For example one could suggest as a working hypothesis that excita-

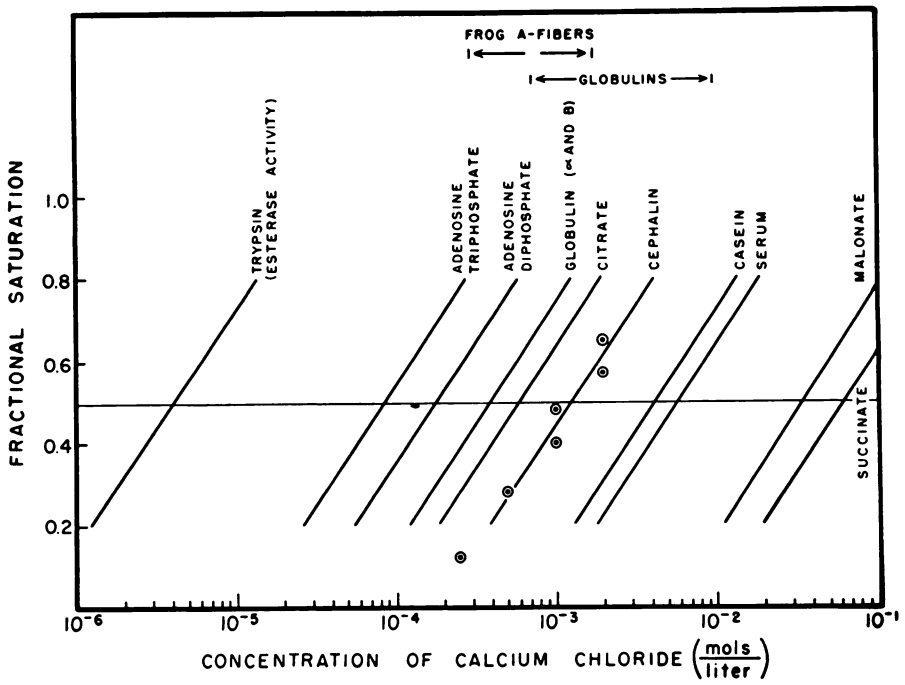

FIG. 6. *Relation of binding of calcium ions by various substances to concentration of ionized calcium in the solution.* Ordinate, fractional saturation of available binding sites. Abscissae, concentration of calcium chloride on a logarithmic scale. Lines drawn between twenty and eighty per cent saturation to indicate range of concentration of calcium in which marked changes occur in binding by one substance. The intersections with the horizontal line indicate the values of the dissociation constants (on abscissae) of the corresponding compounds, see Table and references in text.⊙ Ratio of measured rheobase to maximum rheobase at each indicated concentration of calcium chloride (frog A-fiber). Arrows indicate range of dissociation constants reported for various globulin fractions and for "dissociation constants" estimated from dependence of rheobase of frog A-fibers upon calcium chloride concentration.

tion processes are connected with electrical polarization of surface structures in the axon because such surface structures have protein and phospholipids as prominent constituents (150), and the structure of kephalin layers or suspensions show great sensitivity to calcium ion concentration (4, 135, 136). Obviously, the mechanism by which a change in concentration of calcium ion in the aqueous environment affects a cellular property may be quite indirect and complicated. Some prominent possibilities are outlined in Figure 5. The existing data are sufficient to formulate the experimental problems rather clearly but not adequate for constructing convincing physical explanations of the mechanisms of calcium action.

Available data can be used to discard some of the physically possible mechanisms. The excitability of axons in frog nerves changes markedly with calcium concentration in the range 0–2 mM in a solution containing 120 mM sodium

chloride. At constant calcium concentration (2 mM for frog axons) an equivalent change in ionic strength produced by increasing sodium chloride concentration has no such effect on excitability. This action of calcium ion does not arise merely because of a change in ionic strength of the aqueous solution. Therefore, the mechanism involves the kind of primary molecular-ionic interaction which is characterized by an equilibrium constant, like process 2 or 3. More generally, effects of calcium ions upon any cell property probably develop from these few, rather simple, molecular-ionic interactions or some combination of them. Effects arising from changes in ionic strength can be simulated by suitable concentrations of other ions. Effects of calcium, arising from ionic exchange processes (cf. Equation 1) are subject to modification by changes in the external concentration of the other exchanging ion (12, 115a). This suggests a possible explanation of calcium and potassium antagonisms frequently observed. However, effects arising from ion-binding can be more specific or may have a limited specificity in that only certain other divalent ions may be substituted for calcium. Particularly, ion-binding by linking two different anions may be very specific because of spatial requirements. Of course, the above statements refer only to equilibrium distributions of calcium through all phases of the cell capable of exchanging calcium with the external bathing solution. It is worth mentioning that exchanges may take place more rapidly in some cell structures than it does in others (163). Thus, some properties may reach new equilibrium values more rapidly than do others.

Nerve cells and their axons contain numerous molecules capable of binding calcium to form compounds. Among these are the proteins (62), phospholipids (25, 42), the dicarboxylic acids (22, 122, 124), the adenylic acid derivatives (40), and ribonucleic acid (180). These substances are considered important as constituents of cell structures, as metabolic intermediates or as enzymes. Whenever there exists an equilibrium with respect to the process of calcium exchange between the cell system and the external aqueous phase some calcium is bound to each of these constituents in accordance with the concentration and intrinsic binding constant for each. The expected degree of saturation of each type of binding site over a wide range of concentration of calcium ion is readily estimated from Figure 6. In a mixture of constituents there is, in addition, the possibility of a new compound formation by linking one kind of anion to another. The consequence of this is the formation of molecular aggregates. In such systems low concentrations of calcium may favor such aggregation or sharing of the ions among the polyvalent anions while high concentrations favor dispersal by saturating the binding sites of each anion independently. Internal salt formation, linking anionic groups to cationic groups, can be dissociated by competitive calcium binding to the anionic groups. Changes in molecular structure can occur in this manner. A complication in the experimental problem is that mixtures of calcium-binding molecules do not necessarily add their binding capacity. Thus, in the cephalin and protein solution studied by Drinker and Zinsser (42) only the cephalin appeared to be in equilibrium with the calcium ion in the solution. The protein seemed inaccessible to the calcium ions. This was explained on the basis

that the protein was on the inside of micelles with a cephalin layer on the outside. Thus, one may expect the precise organization of the cell structure to exert a modifying influence upon the equilibria since thermodynamic activities of the anionic sites can reflect structurally imposed concentration patterns and activity coefficients for each binding site. Such effects may be different in different cell types.

Only part of the calcium of a frog sciatic nerve, about 40 per cent, is in diffusion equilibrium with the external solution (170). All of the measured changes in cellular properties must be related to this freely exchangeable fraction. It may be that the remaining 60 per cent is bound by protein that is coated with lipids in the manner described above so that ionic exchange with the outside solution is prevented. Of course, one must assume that in both instances the apparent absence of interaction merely reflects a vast difference in the rate of approach to equilibrium. It is preferable, therefore, to think of a part of the cell structures that readily exchange calcium so that they come into equilibrium with the outside solutions. Equilibrium with the second part is achieved so slowly that it appears unchanged during 24–36 hours of equilibration. Thus, for many experimental situations 60 per cent of the calcium content of frog axons may be described as non-exchangeable with the external bathing solutions. Some reagents that bind calcium ions also fail to reach this residual calcium (173a). A similar non-exchangeable calcium fraction was described in *Elodea* leaves (115b) and in nuclei of thymus cells (180). In the latter the nucleic acids seem to bind calcium so tightly that dissociation would be difficult to demonstrate in a physiological experiment. Only the freely exchangeable fraction is of interest in experimental changes produced in nerves by changing the concentration of calcium in the external bathing solutions.

Recent experiments upon crab nerve, using tracer calcium indicate two processes of exchange with different rate constants (163). If a cellular property depends upon the average amount of calcium bound by a particular cellular constituent in the presence of many others the fractional change in the property as a function of calcium concentration will reflect the dissociation properties of the significant binding agent. However, the rate of change of the property from one equilibrium state to another and the total amount of bound calcium will reflect the presence of other binding sites. Experiments upon cleanly dissected giant fibers of the Squid were designed to exhibit the presence of such calcium binding sites that might have to be saturated before the change in threshold reached an equilibrium value after a change in outside calcium concentration. The onset of impulse discharge during a first topical application of a solution of isotonic sodium citrate occurred in about one minute. The activity ceased abruptly on return of the axon to sea water. The time of onset for subsequent tests averaged 10 seconds and was reproducible from test to test and from axon to axon. Thus, the first removal of calcium seemed to require the depletion of some reservoir of calcium that was not refilled upon reapplication of the sea water. The rapid onset of activity and its rapid cessation suggest that excitability is controlled by a very superficial calcium binding cell structure that is rapidly saturated from an

outside source of calcium, the cell surface for example. In contrast, the development and cessation of activity in a single fiber in a frog's sciatic nerve is relatively slow (76). This probably represents the time to saturate or desaturate calcium binding components other than those at the nodal regions of the axon surface. Such surrounding binding sites serve as a slowly drained supply of calcium during removal and a slowly filling sink for calcium during resaturation of the cellular binding sites. At least this seems to be a reasonable way to understand these observations. The suggestion is, therefore, that the excitability of a bare axon changes in a few seconds to a new value following each change in calcium concentration at the cell surface because the significant sites of interaction with calcium are in the cell surface. A discussion of other evidence for the binding of exchangeable calcium at cell surfaces has been given by Mazia (116). Uranyl ions seem to control oxygen metabolism through binding at cell surfaces (6, 144). Hertz considers the slow onset of effects of calcium deprivation in frog single fibers to indicate an intracellular site of action (76).

VI. PHYSICAL MECHANISM OF CALCIUM ACTION ON CELLS

It is reasonable to expect that the physical laws for calcium binding by cellular constituents will be reflected to some extent in the dependence of cellular properties upon external calcium ion concentration. For example, if some property of a cell were proportional to the average density of charge in a protein structure the law of variation of the property when calcium concentration is the only variable should be like that of Equation 1. The degree of change in the property would correspond to the degree of change in the bound calcium. If the laws of association between cellular molecules and ionized calcium are thus related to a cell property then certain qualitative phenomena should appear, even though exact matching to Equation 1 may not be observed for a variety of reasons. These necessary relations are:

1. The magnitude of a cellular property, such as the threshold for excitation, should change with the external calcium concentration over some definite range of concentration.

2. The magnitude of the property should become independent of external changes at sufficiently high concentrations, where the calcium-binding sites of a particular kind are all covered.

3. If the property is linearly related to the concentration of bound calcium then there should be a hyperbolic relation as in Equation 1. The estimated dissociation constant should correspond to that of a calcium-binding component in the cell.

4. The relation between change in calcium concentration and change in cellular property should be related to properties of extractable constituents.

The earliest studies of the relation of excitability of axons to calcium chloride content of the bathing solution (121) in the concentration range below Ringer's proportion fit with expectation 1 and 2. In this sense these data seem to reflect the underlying process of calcium binding by those molecular constituents that are important to excitation processes. However, the suggestion is even stronger

because recent studies on single fibers of frog and of Squid (13, 17) indicate a hyperbolic relation between threshold and calcium ion concentration, as required by the third expectation. This evidence is in accord with the idea that the threshold increases in proportion to the density of calcium-occupied binding sites in some axon structure. These data may be quantitatively compared with dissociations of calcium proteinates and of other anions that bind calcium, Figures 6 and 7.

Ion-binding molecules seem to have a maximum number of binding sites for a particular kind of ion. Casein, for example, can bind sixteen calcium ions per molecule (27, 96), and albumin can bind eight (24). Consequently, a given amount of casein becomes saturated with calcium as the concentration of free calcium ions is increased. At saturation the maximum quantity of bound calcium is obviously the product of the mols of protein and the number of binding sites per mol. In complicated ion-binding structures only this maximum amount of bound ion can be estimated since the molecular constitution is unknown. The maximum amount of bound ion per unit weight of material is the average density of ion-binding sites in the structure (cf. 12, 54). In a similar manner when the threshold for excitation of a nerve ceases to increase with further increase in ionized calcium some structure in the nerve is presumably saturated with calcium. In order to compare direct studies of ion-binding with those in which bound calcium is indirectly measured it is convenient to express the amount of bound calcium per unit weight of material as a fraction of the estimated value at saturation. Thus, the threshold for excitation, by hypothesis a measure of calcium bound at particular sites in the nerve, can be expressed as a fraction of the maximum value approached at high concentrations of ionized calcium. In Figure 7 such normalized quantities are represented on the ordinate as a function of the logarithm of the calcium concentration. In this way the dependence of nerve properties upon calcium ion concentration can be compared with each other and with calcium-binding properties of various known substances. To the extent that the relations are hyperbolic (Equation 10) each is characterized by a single constant which is numerically equal to the calcium concentration for half-saturation of that kind of site, Figures 6 and 7.

The straight lines in Figures 6 and 7 are fitted to the points with a slope of 2.303/4. This is the theoretical slope at the midpoint of these curves if there is a hyperbolic relation between degree of saturation and calcium concentration. Since the points are distributed satisfactorily along these lines the graphs show that the data are represented by the mass law relation of Equation 10. The value of the calcium ion concentration for the 50 per cent saturation point is equal to the "dissociation" constant K. Thus, the K for frog nerve is about 10^{-3} mols/liter and that for Squid giant axon is 30 times greater.

Thus, the empirical constant for the hyperbolic relation of excitation threshold to calcium concentration in frog axons differs from that for Squid nerve. If the abscissae were calcium ion activity instead of molarity the difference in ionic strength of the two bathing solutions would decrease this difference in K values by a small amount. The pH of the external solution for frog nerve is about one

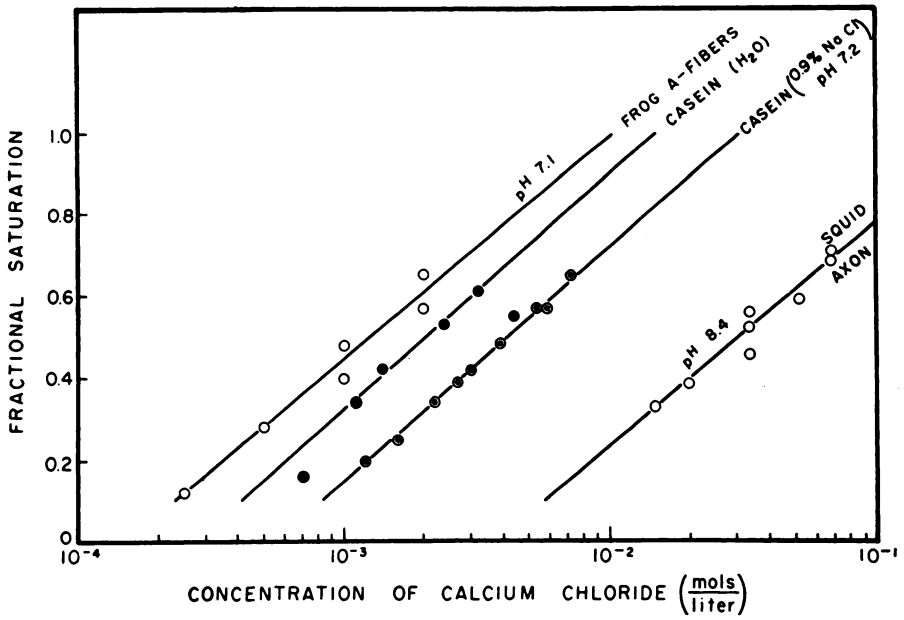

FIG. 7. *Relation of binding of calcium ions by various substances to concentration of ionized calcium in the solution.* The fractional saturation of binding sites in casein was estimated from data in reference (27). The fractional saturation for the nerves refers to the ratio of measured rheobase to maximum rheobase, on the supposition that the data conform to the hyperbolic form of the mass law. In all cases the lines are drawn with the theoretical slope 2.30/4, indicating that each set of data is adequately described by a hyperbola.

unit lower than that for Squid (8.4). However, the experimental effect of pH on frog nerve does not indicate a sufficient change in the empirical K arising from this source. These data and the model of ion binding suggest that the dissociation constant for certain calcium-binding sites in Squid axons is about 25–30 times larger than that for certain sites in frog nerves.

The empirical constants for calcium binding to proteins range from 0.4×10^{-3} mols/liter for γ-globulin to 6×10^{-3} mols/liter for casein, Figure 6. This is not as great as the apparent difference in K's for frog and Squid. However, there are many other constituents of cells that bind calcium and may influence cellular properties (Figure 6).

The sodium chloride content of sea water is about five times greater than that of Ringer's solution for frog nerves. The difference in the concentration ranges within which calcium ions change the excitability of frog A-fibers and of Squid giant axons may be related to this great difference of total cation content of the external solution. The following facts about calcium binding by other cells and tissues strongly suggest further study of nerve cells from this point of view. The amount of calcium bound by cartilage depends upon the sodium ion content of the bathing solution (12). The process is an ion exchange reaction like Equation 1 and is characterized by a mass law constant given by Equation 2. Previously

Mazia demonstrated (115a) that the calcium binding by *Elodea* leaf cells varied with sodium ion content of the medium. He concluded that the same amount of calcium was bound by the cells if the ratio of calcium ion concentration to that of the sodium ion were held constant. However, since the sodium content of the leaves was not measured it is possible that an interpretation according to Equation 2 would also fit his data. Wilbrandt reported that dilution of Ringer's solution had the same effect on properties of frog heart as increasing the calcium ion concentration (179). He explained the action in terms of a Donnan distribution of sodium and calcium ions between the solution and a surface phase containing fixed anionic groups. In this theory the cellular properties are related to the calcium and sodium concentration at the surface rather than in the solution. The dilution effect arises because the surface concentrations are determined by the Donnan ratio, r, given by Equation 3. However as pointed out previously the Donnan distribution may be regarded as a special case of the general process of ion exchange described by Equation 2. Further study may show that the relation given in Equation 2 is more suitable to explain quantitatively the interactions of calcium and sodium ions on properties of frog hearts.

The interaction of these ions in the studies of cartilage (12, 114) were traced to binding sites on chondroitinsulfuric acid in the structure. Perhaps the postulated binding sites in *Elodea* leaves, heart muscle and nerve cells may become better identified in further experiments, using similar methods. This advance will require extensive studies of the calcium exchanges measured directly for each kind of cell. A start in this direction has been made in a study of the release of Ca^{45} from stimulated muscle (181) and from crayfish nerve treated in various ways (163).

The effect of changes of pH upon excitability of frog A-fibers is largely independent of the effect of changes in calcium ion concentration. These phenomena have not been studied thoroughly but it is easily demonstrated that the effect on rheobase of increased acidity and increased calcium concentration are approximately additive (however, see 89). The apparent dissociation constant for the significant calcium compounds in frog axon, if it changes at all, seems to decrease slightly i.e. the postulated calcium complexes hold calcium more firmly. These observations are in accord with the idea that protein-binding of calcium is involved. Thus, the dissociation constant for calcium-binding by casein is independent pH from about 6.3–8.0 (27).

In résumé, the molecular-ionic interactions between hydrated calcium ions and cellular anionic sites are thus pictured as governed by a set of intrinsic mass-law constants. If this view be correct then one might expect that these regular relations to the ion activity in the external solution would be reflected in some measure in the dependence of even complex cellular properties upon the calcium ion concentration of the aqueous environment. In the simplest instance, if the property were proportionally related to the density of binding sites occupied by calcium (or to the number of such free sites) then the property would be related to the ionic calcium concentration by the mass law itself. On the other hand, if the property is rather indirectly related to these specific anionic sites in the cellu-

lar structure then a less clear correlation with the mass law of interaction would be mirrored by an overall measurement such as excitability. The remaining evidences of the relation would be as follows. (a). The property changes with concentration of calcium ions only over the range in which the change in occupation of the critical binding sites is marked. (b). Since the number of such sites is limited and saturable the property should become independent of further changes in calcium ion at some higher concentration range. Even these rudimentary signs of the basic mechanism of molecular-ionic interaction may be obscured if there are several kinds of binding sites (with different intrinsic dissociation constants) contributing to the change in the measured property or if factors other than the calcium concentration are changing secondarily and affect the measured property.

We may hope such confusing interactions of processes will not completely prevent the analysis. Unfortunately, the recognition of such possibilities has the effect of offering a plausible explanation for all deviations from hypothesis. Under such circumstances plausible explanations tend to remain plausible rather than convincing. We have exhibited what are perhaps authentic instances where the variations of a cellular property with changes in ionized calcium concentration of the aqueous environment do reflect the primary equilibria set up with calcium-binding anionic sites within (or on) the axonal structures of frog and Squid. Whenever such relations do emerge in the presence of the equilibria with many different calcium-binding constituents of the cell it may be supposed that members of one class of binding sites are of primary importance to the particular property, excitability for instance. Conversely, if a property does not change with changes in concentration of calcium over the same range we may suppose that these anionic sites are of no significance to the property in question. There is always the possibility that in the latter instance the property arises in a structure which is inaccessible to calcium ions in the aqueous environment.

It seems desirable to close this phase of the discussion with the reminder that this analysis is based upon the idea that the physiologically significant interactions of calcium ions with the cellular constituents are at equilibrium. However, the measurements of the cellular properties employed as indices of the state of the cell do not need to be equilibrium measurements.

It is not obvious how to make tests of these suggestions by direct measurement of bound calcium in a cell. One inherent difficulty is that many equilibria of this kind may exist in the cell and have no bearing upon the properties under investigation. Any form of quantitative analysis of bound calcium would fail to make such distinctions. When proteins and other calcium binding agents are buried in cell structures such as surface membranes the idea of binding sites per mol of protein becomes nebulous. Only the idea of sites per unit area or per gram of cell is of operational significance and is like the unit employed in the theory of solid phase ion exchangers (54). It seems possible at present to measure the maximum overall calcium-binding capacity of each kind of nerve cell and axon and to determine the change with external concentration as has been done for cartilage (12). With this information and data about composition of cell struc-

tures perhaps we could obtain an understanding of the significance for cellular function of the multiple equilibria that must be present between structural components and calcium ions. Some experimental approach may be developed by analogy with studies of ion exchange systems. For here, too, the inner phases are inaccessible. However, the questions asked about ion exchangers are far less specific than those that are asked about cellular properties in relation to calcium-binding.

There is little information about nerve cells on this aspect of the problem. As previously mentioned, Tipton (170) demonstrated that only a part of the total calcium content of frog sciatic nerve is in diffusion equilibrium with the external solution. The total content was 7.35 milliequivalents per 1000 grams of tissue (cf. 160). The range was 5.6–9.05 meq/1000 gr. About 5 meq/1000 gr. of moist tissue could not be removed by diffusion. Two of his published curves suggest that part of the diffusible calcium comes from the intracellular portion. However, available information is insufficient to establish the quantity of cellular calcium that is in equilibrium with the external concentration of calcium ion. Such information is needed for further physical explanation of the mechanism of action of calcium on nerve cells.

The considerations of Section V suggest that the primary physical reaction of calcium ions with cellular constituents will impose certain inevitable characteristics upon the relation between a cellular property and the environmental concentration of calcium ions. Beyond this process lies the far more complex one of establishing the physical basis for the dependence of a cellular property upon the calcium-containing cellular structures. For example, an effect may be traceable to change in density of surface charge or to breaking of an internal salt link in a protein thereby changing the molecular structure. Obviously, these are some expected physical effects, following from the primary binding of a calcium ion in a complex protein and phospholipid structure. The problem is to exhibit a relation between rate of oxygen uptake, negative after-potential, and threshold, on the one hand, and items in a list of possible physical effects of calcium binding, on the other hand. This area of the science is poorly developed though the suggestiveness of the insufficient evidence has led to numerous proposals. These may be enumerated and classified as follows. The most prominent suggestion is that there is an inverse relation between extent of calcium-binding and permeability to potassium. The changes in cellular properties are thus referred to the potassium permeability. An extensive physical theory based upon this idea has yielded equations relating excitability, polarization potentials, potassium concentration and calcium concentration (see Karreman and Landahl in 131). The ancillary evidence is extensive. The "swinging door" version of calcium-controlled potassium permeability is a mechanistically attractive version of this idea (76). There is another proposal in which the calcium-binding molecules control the permeability to sodium ions. No extensive theory has been developed in this instance, but the idea is seriously considered (80, 83). Another version of this general idea is that the ion permeability of the membrane is controlled by its bound calcium because this influences the degree of hydration of the structure. Another theory

emphasizes the cross-linking of condensed anionic molecules by calcium with a cycle of dissociation and binding during activity (56). Gordon and Welsh suggest that the degree of damping of electrical transients may be related to the degree of cross-linking of structural molecules by calcium (56). An alternative proposal is that an enzyme like ATP-ase regulates ion movements across the membrane (38). If the latter kind of suggestion could be scientifically established the problem considered here would resolve itself into ascertaining the physical mechanism for enzyme activation by calcium. Finally, the Meyer-Sievers-Teorell model (cf. 168 for references) of a membrane suggests that calcium binding would change the charge density and therefore the permeability and polarizable properties of the cell surface. Obviously, at this stage of development more definitive experiments are needed, preferably ones that measure directly some physical state of the membrane that is then related to calcium content on the one hand and to a cellular property such as threshold, on the other. The recent work of D. K. Hill (79) and of Tobias (171) is a start in this direction.

In the previous analysis the change in excitability was used as a measure of the changes in the cell produced by changes in calcium content of the cellular phase. If the dependence of a cellular property upon the state of those molecular constituents which bind calcium were known the problem would be resolved. Under such circumstances a change in the property such as excitability would be related to a specifiable change in the amount of bound calcium in a known molecular structure. The equilibrium of the latter with various concentrations of calcium could be determined independently. Thus, the dependence of the property upon external calcium concentration would be explained. Unfortunately, only the changes in excitability and the changes in external calcium ion concentration are known. The intervening processes involving ion-binding sites on particular cellular constituents can only be surmised. It would seem that the sequence of experimental analysis has been and must continue to be:

1. Determination of cellular constituents that interact with calcium ions,
2. Determination of constants for equilibrium of calcium ions with these constituents or combinations of them,
3. Study of properties of parts of cells in relation to calcium ion concentration, such an activity of enzyme systems, mitochondrial metabolism or structural organization of extracted parts,
4. Determination of dependence of cellular properties upon calcium concentration over wide ranges, and interaction with other ionic variables, and
5. Construction of theoretical molecular models that account for observation 4 in terms of data from investigations 1, 2, 3.

This program seems the best exploitation of available methods and data. It may suggest ways to directly observe postulated processes created in program, step 5. If the theoretical analysis merely exhibits clearly the kind of information needed then it would be serving its most useful function, a guide to further significant experimental analysis.

In this review attention has been focused upon the physical explanation of the role of calcium in simple neural structures. The information has a broader sig-

nificance in relation to the physiological mechanisms that regulate the ionic content of the fluids bathing the cells of organisms. The absorption, storage, and excretion of calcium is through processes controlled by parathormone and vitamin D (132). The cellular surfaces and their cohesion in tissues are in turn related to the level of ionized calcium and the ascorbic acid intake (140). In deficiency diseases involving the latter processes the symptoms are high basal metabolic rate, muscular weakness, and paralysis (11, 140). All of these are suggestive of facts about isolated nerve and muscle, as discussed earlier. The symptoms of parathyroid deficiency apparently arise from chemical lesions along the nerve fibers and at the synapses that resemble those produced experimentally by localized calcium deficiency (98). More general metabolic disturbances leading to high levels of citrate ion in the serum produce symptoms of tetany (169) presumably by binding calcium ions and thus making nerve cells deficient. These diseases related to calcium metabolism have symptoms of neuronal origin. However, they seem to arise primarily from defects in the mechanisms for regulating the ionic environment of the nerve cells rather than from diseases originating within the neurons. Thus, tetany is controlled if the concentration of ionized calcium bathing the nerve cells is raised by any means. This can be accomplished even though the defect in the organism, such as hypoparathyroidism, remains. There seems to be no evidence for a direct action of parathormone in the calcium metabolism of nerve cells. There is some evidence in man for a partial adjustment of the excitation properties of neurons chronically exposed to a low concentration of serum calcium (178). However, insofar as the neurological symptoms of defects in the internal ionic environment lead an animal to correction of the defect (as in medication or altered diet) they serve the same adaptive purpose as a pain fiber stimulated by something in injured tissue. On an even broader basis these studies have a bearing upon the evolution of specialized cells that must be geared to the evolution of mechanisms for controlling the internal environment within certain limits of variation.

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