

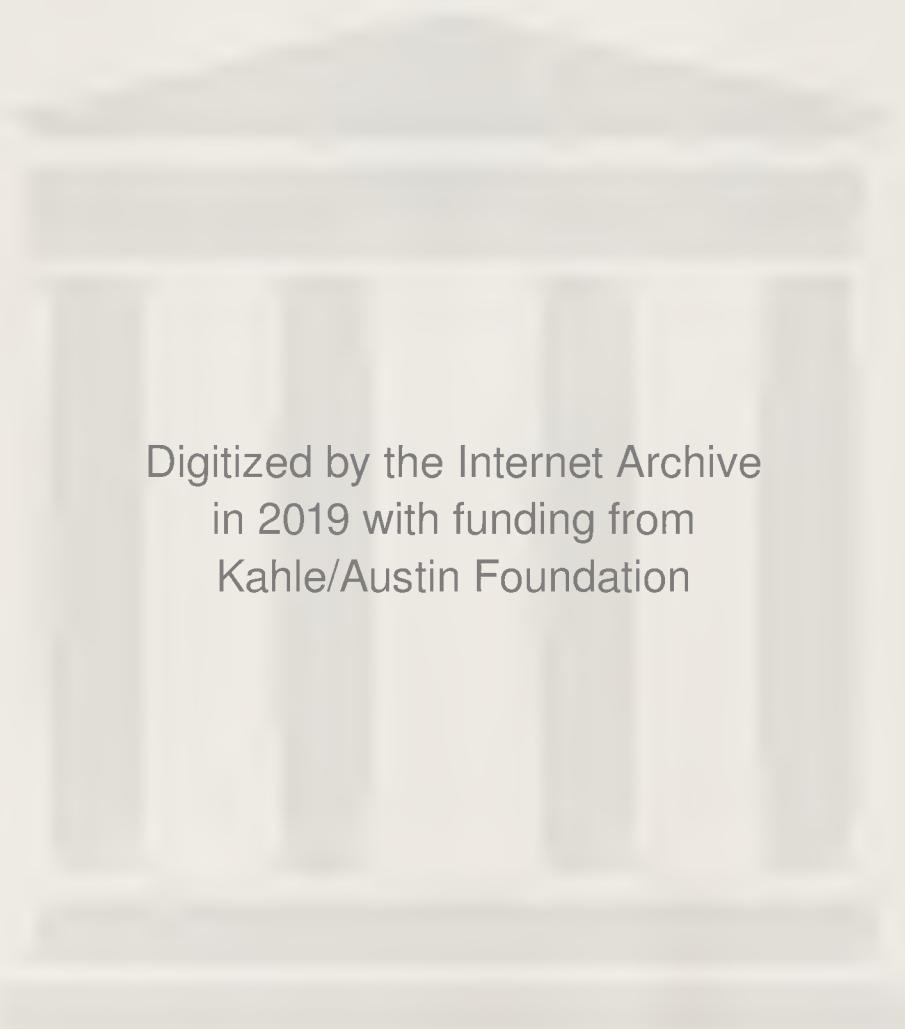


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NERVE EXCITATION
A Macromolecular Approach

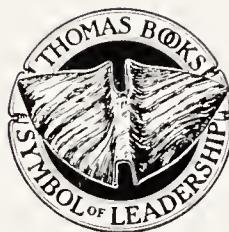
NERVE EXCITATION

A Macromolecular Approach

By

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PREFACE

In this monograph, the author attempts to describe and interpret the process of *nerve excitation* on the basis of physicochemical concepts and principles. Throughout this book, there is a conscious effort to avoid or reduce the use of expressions and ideas which are not familiar to students of physical chemistry or thermodynamics. This approach derives from the author's belief that some commonly accepted biological concepts are thermodynamically unsound and do not satisfy physicochemically oriented readers. It is hoped that a straightforward physicochemical approach will make this book acceptable to thoughtful and critical graduate and undergraduate students of physiology and biophysics.

Attempts to interpret excitability phenomena of nerve and muscle in terms of physicochemical concepts are not new. In fact, some of the great contributions to this particular field of biological science were made by physical chemists. The best known examples of this kind are determination of the velocity of the nerve impulse by Helmholtz (Ch. I) and analysis of nerve excitation by Nernst (1908). Nernst developed a theory of ion transport across a semi-permeable membrane and applied this concept to the problem of nerve excitation. It was W. Ostwald (see Heathcote, 1901 and 1907) who pointed out the similarity between the excitation taking place in nerve and the process of propagation of an "activation wave" on the surface of an iron wire in nitric acid. Their observations laid a physicochemical foundation for the "local circuit" interpretation of these processes. In the 1920's, Höber proposed that such approaches be applied to the problem of excitation; in his book, *Physikalische Chemie der Zelle und Gewebe* (1926), he developed his own "colloidal theory of nerve excitation." More recently, Segal (1958), Nasonov (1959), Ling (1962), Ungar (1963), Kavanau (1965) and others have made significant contributions toward placing the processes of nerve excitation and conduction on a sound physical chemical basis. However, it appears that these contributions have had

only limited influence on most investigators and writers in neurophysiology and biophysics.

Most investigators in the field of biophysics are experts in electronic engineering. Laboratories engaged in research in this field are usually equipped with a variety of elaborate electronic devices which enable biophysicists to carry out delicate electrical measurements on biological material such as squid giant axons. Perhaps because of their intimacy with electronic equipment, electrophysiologists have a strong tendency to interpret the results of their measurements in terms of capacitors, resistors and rectifiers in the nerve membrane rather than on the basis of ion selectivities, mobilities, or Gibbs free energy. It is entirely natural that the manner in which an investigator interprets some unknown phenomenon is strongly affected by his interest and his previous experience. This fact may account for the differences in the approaches used by different investigators for the study of nerve excitation.

Despite such trends in the investigation of excitation phenomena in nerve at present, there is little doubt that hypotheses which are physicochemically unsound will be superseded by more rigorous theories in the future. In recent years, geneticists, enzymologists and biochemists have offered ample evidence that labile macromolecules in cells and tissue obey the laws established for much smaller, more stable molecules and atoms. It is the purpose of this monograph to help advance physiology of the axon membrane to a level on which it can rightfully claim a seat in the assembly of various branches of molecular biology.

This monograph consists of eight chapters and three appendices. In Chapters I and II the general electrophysiological properties of the nerve and nerve fibers are described briefly; in these chapters, attempts to introduce biological terms, such as "nerve impulse," are made only following description of the experimental procedures needed to *define* the terms. Chapter III is prepared for biologically oriented readers as an introduction to electrochemistry of inanimate membranes; for example, this chapter might help unfamiliar readers appreciate the distinction between the *emf* and the potential difference between two plates of a charged dielectric condenser. In Chapters IV through VIII, the experimental data and interpretations established during the past decade, mainly from the giant axons of

the squid, are described. Readers who are accustomed to thinking in terms of batteries and resistances in the membrane might find these chapters difficult to reconcile with a purely electrical model of excitation; but it is important to realize that there are often several valid approaches to a problem which are not always mutually exclusive. The appendices, written in collaboration with Y. Kobatake, are prepared for physicochemically oriented readers who might want rigorous derivations of the equations used in the text.

The author is deeply indebted to Professor Aaron Katchalsky of the Weizmann Institute (Israel), Professor R. Schlögl of the Max Planck Institute (Germany), Professor Torsten Teorell of Uppsala University (Sweden), and to Dr. L. Nims of Brookhaven National Laboratory (New York), who have given the author many opportunities to discuss the problems described in this monograph. The author wishes to express his gratitude to his colleagues at the National Institutes of Health, Drs. Irwin Singer, Yonosuke Kobatake and Karl Sollner, without whose help the various ideas described in this book could not have been developed. The author is also grateful to Mrs. Zelda Wolk for typing and proofreading the manuscript, to Mrs. Nobuko Tasaki for preparing the figures, and to Dr. L. Lerman, Dr. A. Watanabe and Mrs. I. Zimmerman for their assistance. The author is again indebted to Charles C Thomas, Publisher for publication of this monograph approximately thirteen years after the publication of *Nervous Transmission*.

I. TASAKI

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NERVE EXCITATION
A Macromolecular Approach

Chapter I

HISTORICAL INTRODUCTION

DISCOVERY OF ACTION CURRENT

(1.1) When a pair of wires is placed in contact with a large nerve in the leg of a frog and then briefly connected to the terminals of a battery, the innervated muscles twitch strongly. This muscle twitch does not appear if the nerve is severed or ligated before entering the muscle, or if the nerve is treated with local anesthetics. From this simple experiment with a nerve-muscle preparation, it may be concluded that the nerve is able to carry some effect of applied electric pulses to the muscles. Although the muscle twitch is clearly visible, there is no detectable optical or mechanical sign of the process taking place in the nerve under these conditions; however, this process has a distinct *electrical sign*, which was discovered by Du Bois-Reymond in 1843.

Shortly before this discovery, Matteucci (1842) reported a phenomenon called *secondary twitch* of muscle at the Academy of Science in Paris. Matteucci arranged two frog nerve-muscle preparations in the following manner: the nerve of the first preparation was placed on a pair of metal electrodes; the nerve of the second preparation was placed in contact with the muscle of the first preparation. When pulses of electric current were delivered to the first nerve through the electrodes, he found that the muscles of both preparations twitched. Matteucci guessed that this secondary twitch was due to “stimulation” of the nerve of the second preparation by electric currents generated by the muscle of the first preparation, but he was not certain. However, this observation led Du Bois-Reymond to an exhaustive search for the electric currents generated by muscle and nerve.

At this time, the effect of electric current on a magnet was known (Oersted, 1820) and a galvanometer with astatic magnets had been invented (Ampère and Babinet, 1822). Du Bois-Reymond constructed a sensitive galvanometer (known as a “multiplicator” at that time)

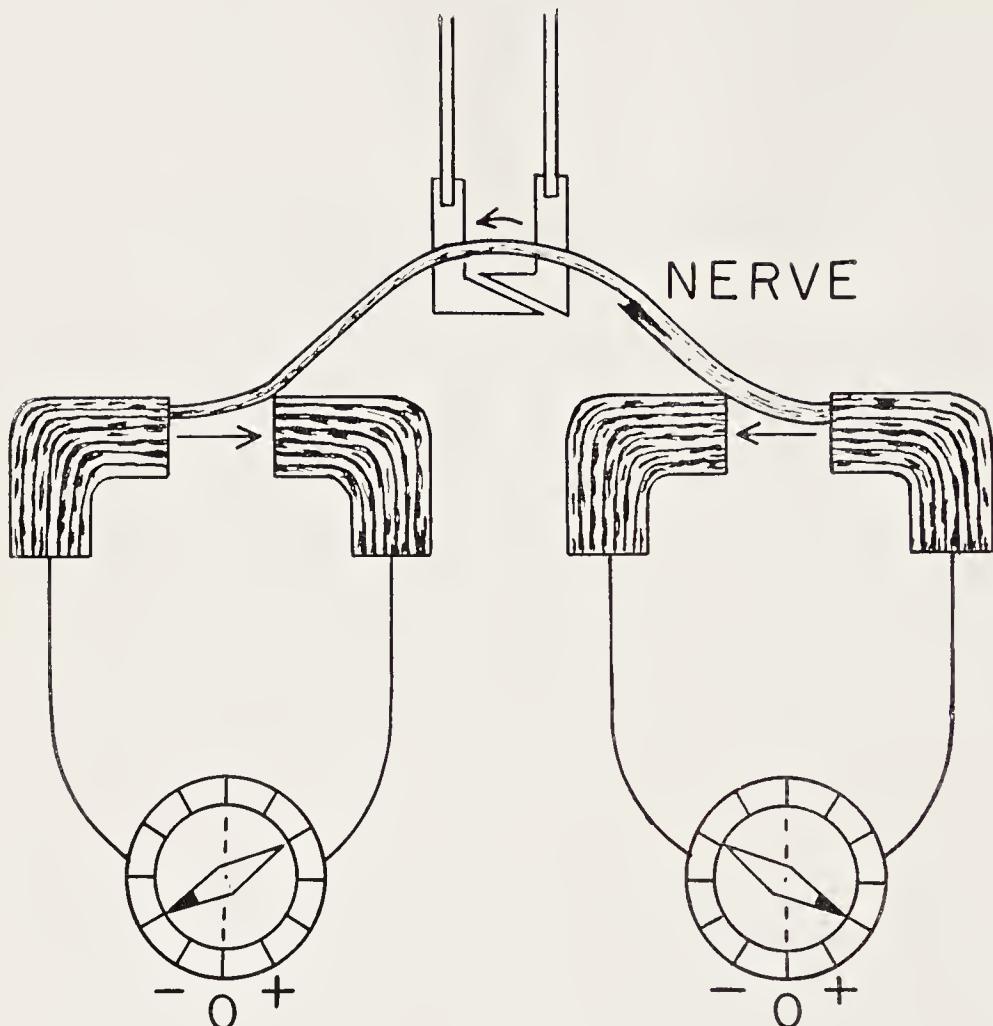


FIGURE 1. Schematic diagram showing one of the arrangements used by Du Bois-Reymond for demonstration of electric signs of the process which propagates along the nerve. The middle portion of a frog sciatic nerve was mounted on a pair of platinum electrodes; each end of the nerve was placed on a mass of blotting paper moistened with concentrated salt solution. The astatic galvanometers were connected to the blotting paper by means of platinum plates. (Adapted from Fig. 101, Du Bois-Reymond, E. Untersuchungen über thierische Elektricität, Berlin, Reimer, 1849).

which had a coil of nearly five thousand turns. The galvanometer was connected to the muscle or nerve with blotting paper (moistened with concentrated NaCl solution) and platinum plates. When the galvanometer was connected to two points along a muscle or nerve (one point at a cut end and the other at the intact surface), a steady

electric current was observed which is presently known as the *injury current* (see Fig. 1). When brief pulses of electric current (stimulating currents) were applied to the nerve, the intensity of the injury current was found to decrease. When the stimulating currents were delivered to the middle of a nerve (Fig. 1), the injury current was found to decrease at both ends of the nerve, which indicated that the *electric sign of nervous action* spreads toward both ends from the middle. Du Bois-Reymond invented a method of compensating for the injury current and obtained the pure *action current* of the nerve and muscle. A preliminary report of these findings was published in *Annalen der Physik und Chemie* in 1843, and the details were published in his two volumes: *Untersuchungen über thierische Elektricität* (1848–1849).

FIRST MEASUREMENT OF THE RATE OF PROPAGATION OF THE NERVE IMPULSE

(1.2) The demonstration of action currents in the nerve by Du Bois-Reymond did not indicate the mechanism or rate of propagation of the process along the nerve. Did the process travel at the speed of light or sound, or is the rate of propagation very slow? In 1849, Fiseau succeeded in measuring the velocity of light by using a cogwheel rotating at high speed; this was the first such laboratory measurement. At the same time, elaborate and accurate measurements of the velocity of sound were being carried out at several places in Europe. Under these circumstances, Hermann Helmholtz undertook the project of measuring the velocity of the unknown physicochemical process that travels along a nerve.

Again nerve-muscle preparations of the frog were used. At this time, induction coils became available for stimulation of the nerve. Measurements of short time intervals were made with a ballistic galvanometer: an electric current of known intensity was passed through a galvanometer at the moment when the stimulating induction current was delivered, and was interrupted at the moment when the muscle twitched. Since the deflection of the galvanometer was proportional to the duration of current flow, the time required for the process in the nerve to reach the muscle could be determined.

Helmholtz found that the measured time varied with the length of the nerve between the electrode and the muscle. By dividing the length of nerve between the two electrode positions by the observed

difference in time, values between 25 and 30 m/sec were observed at room temperature. These values are far smaller than the velocity of a sound wave in a comparable medium. This epoch-making discovery was communicated by Helmholtz in July of 1850 to the Physical Society of Berlin. The process that propagates along the nerve was subsequently referred to as the *nerve impulse*.

Later, Helmholtz (1850) succeeded in measuring the velocity of the nerve impulse by a different method. He registered the time course of the muscle twitches with a lever on a moving cylindrical surface. When an induction current was applied to the nerve where it enters the muscle, twitches started earlier than when the current was applied to the nerve far from the muscle. The results obtained by Helmholtz by this method were perfectly consistent with his previous results.

Nearly seventy-five years later, when cathode-ray oscilloscopes and high-gain vacuum tube amplifiers became available, these conclusions were confirmed by direct measurement with an oscilloscope (see Erlanger and Gasser, 1937; Schäfer, 1940).

CORE CONDUCTOR AND LOCAL CURRENTS

(1.3) After Helmholtz's determination of the velocity of nerve impulses, several attempts were made to explain the mechanism of propagation of the nerve impulse. Hermann (1872, 1879, 1905) explained the tendency of an electric current to spread along the nerve on the basis of a simple observation on the spread of electric current along a metal wire immersed in a salt solution. In his model, the nerve was thought to consist of a *core* and a *sheath*; the core is a good conductor of electricity and the sheath (or the boundary between the core and the sheath) is a poor electric conductor. Saline solution (a good conductor of electricity) is always present outside the nerve. When an electric current is applied to a portion of a nerve between a pair of electrodes, the current spreads along the nerve by a mechanism analogous to that in a submarine cable. At present, this behavior is referred to as the *cable properties* or *core-conductor properties* of the nerve (see Taylor, 1963).

Hermann (1879) was aware that the core-conductor property alone did not explain the process of *generation* of action currents by the nerve. Later he added another very important feature to his theory. He argued that a region of a nerve generating an action current can

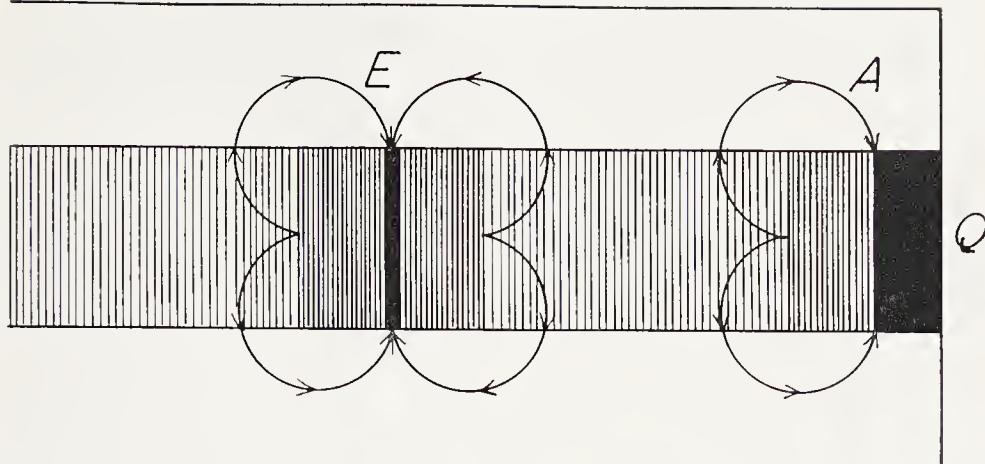


FIGURE 2. Diagram showing local currents. *E* represents an excited region of nerve; *A*, an injured portion of nerve and *Q*, the “core” of the nerve. (Redrawn from Fig. 26, Hermann’s *Handbuch der Physiologie*, Leipzig, Vogel, 1879.)

behave as a sink of current; consequently, there is a flow of electricity between the “excited” and “resting” regions of the nerve. In Figure 2, the shaded portions represent resting zones of a nerve, and the dark portion represents the active zone. The arrows in the figure represent the direction of the current between the two zones; such currents are now called *local currents*. Hermann proposed that the local current mediated spread of the physico-chemical process underlying propagation of nerve impulses.

The local current theory was strengthened considerably when Ostwald pointed out the similarity between the electrochemical process in an iron wire immersed in nitric acid and the nerve impulse. Under the supervision of Ostwald, Heathcote (1907) published an extensive study of the properties of the *passive iron wire*, i.e., an iron wire covered with a layer of iron oxide formed by immersion in concentrated nitric acid. This iron-iron oxide wire corresponds to Hermann’s core conductor. When a portion of the oxide layer is destroyed mechanically or reduced electrically, local currents are generated between the reduced (active) zone and neighboring oxidized (resting) zone. The local current tends to reduce the oxide layer. Under proper experimental conditions, the entire oxidized surface can be reduced by the continued spread of this “activation wave.” Much later, Lillie (1918, 1923) expanded this type of observa-

tion and confirmed the similarity between the passive iron wire and the nerve.

THE “ALL-OR-NONE” LAW

(1.4) The nerve discussed in the preceding pages is actually an assembly of a large number of *nerve fibers*, bound together by layers of connective tissue. The number of nerve fibers in a whole nerve varies with the size of the nerve; small nerves contain relatively few nerve fibers.

When a whole nerve is stimulated by an induction current, the amplitude of the action current increases as the strength of the stimulating current increases. This increase in the amplitude of the action current for the whole nerve can be attributed to either or both of the following factors: (a) the action current produced by each individual nerve fiber increases, or (b) the number of individual nerve fibers producing action currents increase. In 1902, Gotch analyzed the action currents of frog nerves with a sensitive capillary electrometer. He concluded that the major factor determining the amplitude of the action current of a whole nerve is (b), the number of nerve fibers involved. He suggested the important concept that individual nerve fibers develop action currents in an all-or-none manner: the amplitude of the action current of single nerve fiber is independent of the strength of the stimulating current, provided that the strength exceeds a certain critical level. When the stimulating current is below the critical level, there is no action current generated at all.

This all-or-none law was actually discovered in the heart muscle by Bowditch in 1871 and subsequently applied to the nerve by Gotch. The concept was given further support by Keith Lucas (1909), who found a stepwise increase in the strength of muscular contraction when a small skeletal muscle of the frog innervated by a small number of nerve fibers was stimulated through its nerve.

Anesthetics and narcotics have played an important historical role in the development of the all-or-none law. When an anesthetic, such as cocaine or ether, is applied to the portion of a nerve between the stimulating and recording electrodes, propagation of nerve impulses through the anesthetized zone is blocked. Fröhlich (1904) attributed this block of nerve impulses to a decrease in the “size of the nerve impulse” produced by the anesthetic. In the literature of the first

quarter of this century, the term “size of a nerve impulse” is found very frequently. This term is an expression of a vague notion that a “size” of a nerve impulse can be defined by its ability to stimulate the neighboring resting zone. (This term corresponds to the strength of the local current in modern neurophysiology, but the concept of local current was not generally accepted at that time.) In the anesthetized zone, the size of the nerve impulse was assumed to decrease progressively (i.e., undergo “decrement”), and propagation ceased when the size was reduced below a certain critical level.

In order to study the all-or-none nature of a nerve impulse, Adrian (1912) performed an ingenious experiment based on the decrement. He compared the time required to block nerve impulses in two groups of nerve-muscle preparations. The first group had an anesthetized zone 9 mm in length; the second had a zone 4.5 mm in length. It was found that nerves in the first group (9 mm zone) lost their ability to carry impulses sooner than those in the second group (4.5 mm zone). This finding supported the decrement concept, because as the length of the anesthetized zone increases, the size of the nerve impulse decreases and becomes too small to travel along the nerve. In another experiment, this anesthetic was applied to two separated 4.5 mm-long zones of the same nerve, leaving a short, unanesthetized zone in the middle (see Fig. 3, middle and bottom). The time required to block nerve impulses in these nerves was the same as in those with only one short anesthetized zone. It was found that the size of the nerve impulse, which had been reduced in the first anesthetized zone, recovered to its original (normal) size after entering the intermediate unanesthetized zone (Fig. 3, bottom). In other words, the normal zone of the nerve carried impulses of constant size, regardless of the condition of neighboring zones.

The all-or-none property of the nerve impulse travelling along an unanesthetized nerve fiber has not been questioned since that time. However, the mode of decrement in the anesthetized zone has been the subject of much discussion and of considerable controversy. Kato (1924, 1926) argued that the size of the nerve impulse decreased abruptly at the entrance of the anesthetized zone. Davis *et al.* (1926) believed that the decrement was limited to the short portion of nerve near the entrance to the anesthetized zone. Others supported the original concept of Fröhlich and Adrian. These arguments over

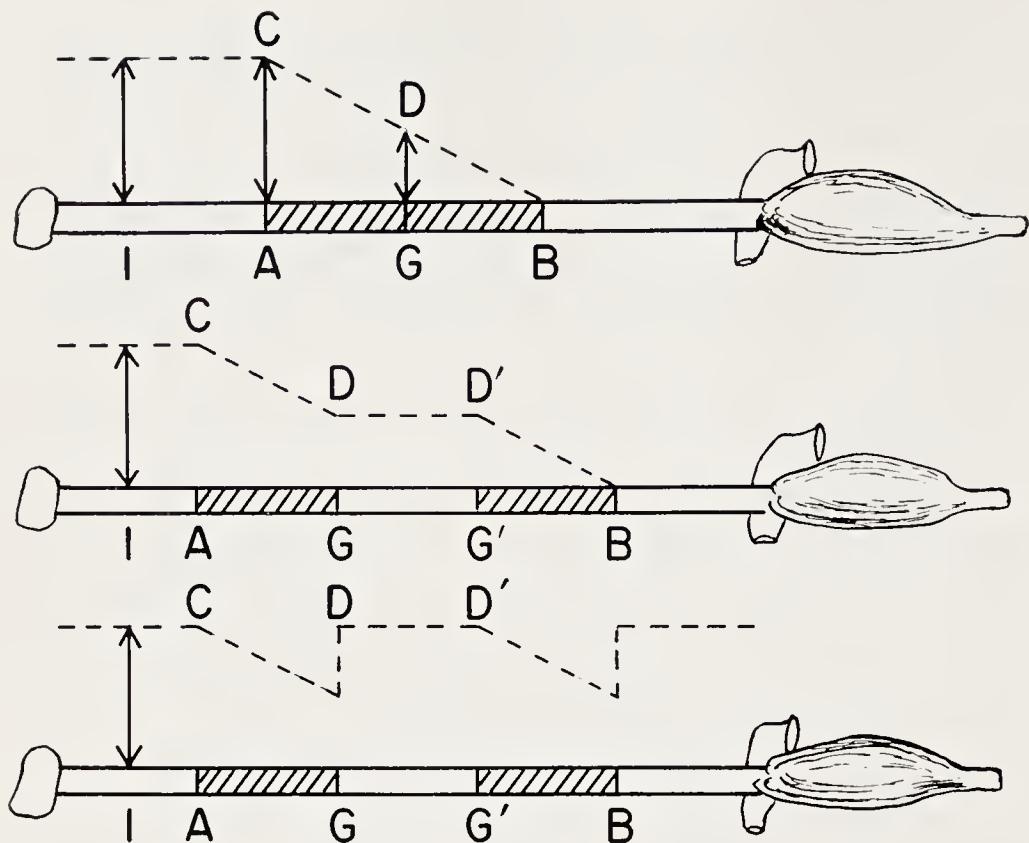


FIGURE 3. Diagram used by Lucas to illustrate “decrement” of the nerve impulse in the narcotized region of nerve. Top diagram shows that the normal “size” of the nerve impulse AC is reduced to DG at G and to zero at B ; the shaded area AGB indicates the narcotized region. Middle and bottom diagrams show two alternative modes of propagation of the nerve impulse in the non-narcotized zone GG' intervening between two narcotized zones, AG and $G'B$. (Adapted from Lucas, K. The Conduction of the Nervous Impulse, London, Longmans, 1917).

decrement subsided soon after physiologists started to investigate properties of isolated single nerve fibers and to discover the significance of the microscopic structures known as *nodes of Ranvier* (see Ch. II).

THE METABOLISM OF NERVE

(1.5) One question that has been asked repeatedly in the past is whether propagation of a nerve impulse is a physical process or a chemical process. This question does not have a definite answer,

since there are many natural processes which cannot be classified as purely physical or purely chemical; however, some of the attempts made to answer this question resulted in important observations of the metabolic processes which take place in nerves.

In 1913, Tashiro found that the rate of CO₂ production by a nerve is increased during repetitive stimulation. Later, Tashiro (1917) found that NH₃ production by nerves is increased by repetitive stimulation, and Fenn (1927) established that O₂ consumption in the frog nerve increased during stimulation. In 1927, Gerard published the results of an extensive study of nerve metabolism.

Most chemical reactions taking place in biological systems are exothermic. If nerve stimulation is associated with chemical reactions, heat production during repetitive stimulation of a nerve might be expected. In Heilbrunn's textbook (Heilbrunn, 1952), it is stated that Claude Bernard was in the habit of demonstrating to his students heat production by rabbit nerve on stimulation. However, in a well-publicized article, Hill (1912) concluded that there was no such heat production in the frog nerve; consequently, there was widespread skepticism concerning the reliability of previous heat measurements. Some textbooks published at that time even questioned the reliability of Tashiro's results because those results were thought to be inconsistent with Hill's conclusion (e.g., Bayliss, 1924, p. 379). Later, Downing, Gerard and Hill (1926) established that there is definite heat production associated with the propagation of nerve impulses.

It is evident that there are a number of chemical reactions (metabolism) which take place in the resting nerve, and that repetitive stimulation increases the rate of these chemical reactions. However, at present most investigators in this field of research do not believe that these chemical reactions are directly related to the process of generation of the action current.

Chapter II

ISOLATED SINGLE NERVE FIBERS

ISOLATION OF SINGLE MYELINATED NERVE FIBERS

(2.1) The electric currents generated by individual fibers in a nerve trunk must flow through the interstitial fluid and other nerve fibers. The electric conductivity of the nerve trunk is highly *anisotropic*; the conductivity in the longitudinal direction is very different from that in the radial direction. The conductivity in the longitudinal direction is nearly ohmic, whereas the conductivity in the radial direction is time dependent: a rectangular pulse of electric current directed radially through the nerve trunk produces a nearly exponential potential variation in the nerve (cf., e.g., Tasaki, 1964). For this reason, it is extremely difficult to determine the time course of the current generated by an individual nerve fiber from the records obtained with electrodes placed on the surface of the whole nerve trunk.

Neurophysiology took a great step forward when it became possible to carry out direct observations of isolated single nerve fibers. In 1928, Adrian and Bronk made the first successful attempt to obtain "single nerve fiber responses." In their study of the nerve impulses regulating respiratory movements in the rabbit, they were able to record electric signals from single nerve fibers after surgical reduction of the number of active nerve fibers. During the period from 1927 to 1934, a group of investigators working under Kato (1934) developed and perfected a microtechnique for isolating single nerve fibers from nerve-muscle preparations of the frog and toad. This technique made it possible to investigate the properties of isolated single nerve fibers without interference from surrounding nerve fibers and connective tissue.

Since the time of Du Bois-Reymond, Helmholtz and Gotch (see Ch. I), many neurophysiological studies have been made with whole nerve trunks, and the results obtained have been confirmed by in-

vestigations made with single fibers. Since the observations made with isolated nerve fibers yield more direct results than the early studies, only single fiber results relevant for later discussions are summarized below.

All or None Law

When the action currents generated by a single nerve fiber are recorded at some distance away from the site of stimulation, it is found that (1) both the size and the time-course of the action currents are completely independent of the strength of the stimulating currents (see Fig. 4), and (2) no action current is observed with stimuli below a certain critical level or *threshold*. When a nerve fiber possesses

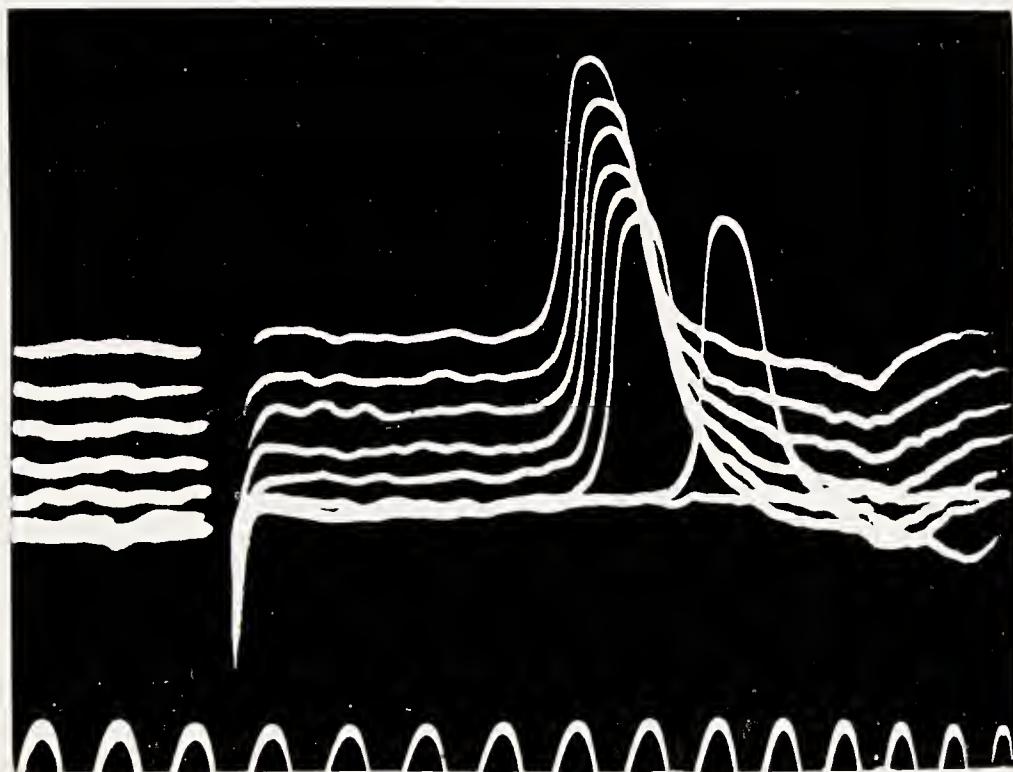


FIGURE 4. All-or-none property of the action current associated with a nerve impulse propagating along a single frog nerve fiber. The relative strengths of the applied electric current, from the bottom upward: 100, 105, 150, 200, 250, 300 and 350, respectively. (The base line of the oscillograph beam was shifted in proportion to the strength of the applied current.) The two records at the bottom, one with and the other without action current, were taken at the same strengths. Time marker, 5000 cps. (From Tasaki, Ichiji, *Nervous Transmission*, Springfield, Thomas, 1953).

these properties, it is said to obey the all-or-none law. The threshold strength of a stimulating current for a given nerve fiber is dependent on the arrangement of the stimulating electrodes in relation to that particular fiber.

Approximate Constancy of the Velocity of the Nerve Impulse

The velocity of a nerve impulse can be determined accurately by recording all-or-none action currents propagating along a single nerve fiber. The velocity is approximately constant along a single nerve fiber, but varies greatly with the diameter of the fiber. Larger nerve fibers tend to show much higher impulse velocities than small fibers (see Erlanger and Gasser, 1937). The velocities of frog nerve fibers are from 0.1 to 30 m/sec at room temperature.

Collision of Two Nerve Impulses

When a single nerve impulse is generated at each end of a nerve fiber at the same time, each impulse is propagated toward the middle of the nerve fiber. The two nerve impulses collide after travelling about the same distance from the ends. After collision both impulses disappear; i.e., there is no propagation beyond the site of collision (see Tasaki, 1949). In this respect, the process of propagation of a nerve impulse is very different from propagation of sound or light waves.

Effect of Two Stimuli Applied at a Short Interval

When two stimuli separated by a relatively long time interval are delivered to a nerve fiber, the impulse evoked by the second stimulus travels at the same velocity as the first impulse. If the time interval is short, the second stimulus evokes either an impulse that travels at a much slower rate, or no impulse at all—the fiber is *refractory*. Immediately following stimulation, a nerve fiber is in an *absolutely refractory period* during which no impulse can be evoked, no matter what the strength of the stimulus. This period is followed by a *relatively refractory period*, during which the threshold stimulus intensity is markedly increased and only a slowly propagated impulse can be evoked.

SALTATORY THEORY

(2.2) In classical neurophysiology, propagation of a nerve impulse was frequently compared to the spread of combustion of gunpowder

(Hermann, 1879; Lucas, 1917; Kato, 1924). Although some properties of the nerve fiber (all-or-none property, refractoriness) can be reproduced by the gunpowder model, the *iron wire model* (see Ch. I) is far superior.

In one version of this model, an iron wire is covered with segments of glass tubing spaced at regular intervals and is immersed in concentrated nitric or sulfuric acid. Under these circumstances, the wave of chemical reaction spreads from exposed segment to exposed segment by jumping across the glass-covered segments. This mode of spread of a chemical wave in an iron wire was called *saltatory transmission* by Lillie (1925); this saltatory transmission is the consequence of the great reduction in the intensity of the local current through the glass-covered segments of wire (Franck, 1955).

It has long been known that the axoplasm of the large vertebrate nerve fiber is covered by a lipid layer called the *myelin sheath*. The myelin sheath is regularly interrupted by short gaps known as *nodes of Ranvier*. In large frog nerve fibers, nodes of Ranvier are roughly $1\text{ }\mu$ long and the distance between nodes is about 2 mm. These conditions, together with the high electrical resistance of myelin (see below), suggest that the propagation of a nerve impulse may be similar to Lillie's saltatory transmission in an iron wire covered with segments of glass tubing (Tasaki, 1939; Tasaki and Takeuchi, 1941, 1942; Tasaki and Tasaki, 1950; Huxley and Stämpfli, 1949).

The first experimental evidence of the decisive role of the myelin sheath and its interruptions (nodes of Ranvier) in electric stimulation of a nerve fiber was obtained by Kubo, Ono and Yuge (see Kato, 1934, 1936). In a single nerve fiber-muscle preparation immersed in Ringer's solution, it was found that the threshold strength of a stimulating current varies with the distance between the stimulating microelectrode and the nodes of Ranvier (see Fig. 5). The threshold is lowest when the microelectrode is placed directly on the node and rises as the stimulating electrode is moved away from the node.

There are two possible interpretations that can account for the observed variation in threshold along the nerve fiber: (a) some unknown property of the nerve fiber varies along the fiber; or (b) the myelin sheath is a good electrical insulator and stimulating currents can leave and enter the fiber only through the nodes. In the latter case, the strength of the applied current needed to produce the same effect in the nerve fiber should vary with the position of the stimulat-

ing microelectrode relative to the nodes of Ranvier. Based on the assumption that the myelin sheath is a good insulator, a quantitative theory was developed to account for the variation of threshold along the nerve fiber (see Tasaki, 1950).

When a sink and a source of electric current exist in a conducting fluid medium, a field of potential is generated in the fluid medium. In

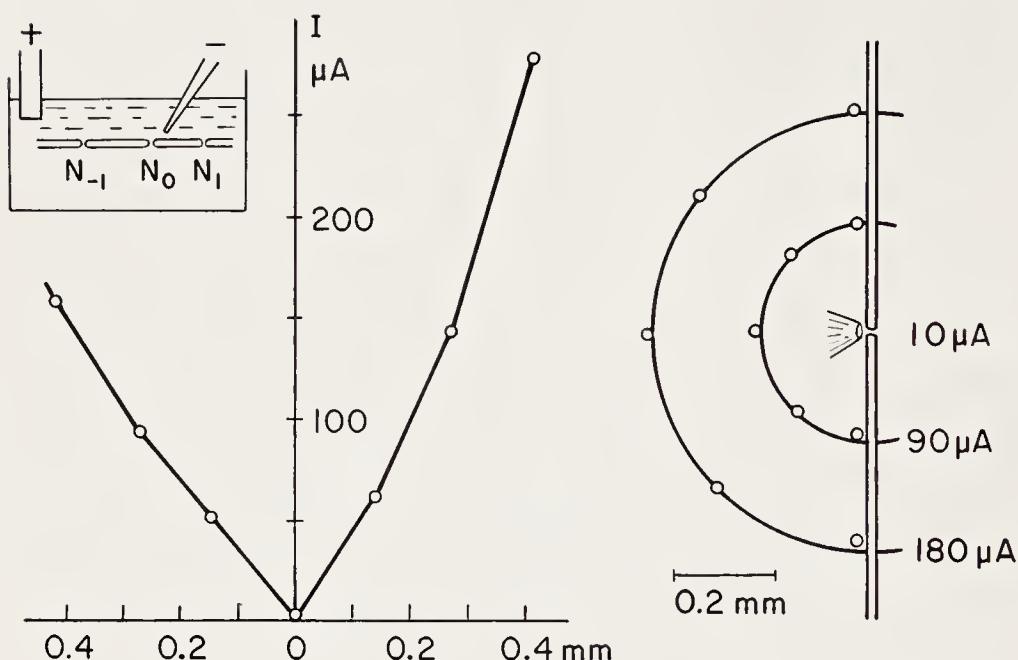


FIGURE 5. The strength of a rectangular current pulse (10 msec in duration) required to excite a single nerve fiber, plotted against the distance between a node of Ranvier and the stimulating microelectrode. A single nerve fiber was suspended in a large pool of Ringer's solution; a twitch of the innervated muscle was taken as an index of excitation. The right-hand diagram shows that the threshold strengths do not depend on the direction in which the stimulating electrode is moved away from the node. (From Tasaki, *Jap J Physiol*, 1:7-15, 1950.

the vicinity of a microelectrode (acting as a sink of electric current) placed in a three-dimensional fluid medium, the potential φ varies inversely with the distance from the microelectrode. (In a two-dimensional medium, φ varies with the logarithm of the distance.) The potentials of three successive nodes N_{-1} , N_0 , and N_1 (see Fig. 5), shall be denoted by φ_{-1} , φ_0 and φ_1 , respectively. If the myelin sheath is a perfect insulator, the electric current which flows through the

axon interior from N_1 to N_0 is proportional to $(\varphi_1 - \varphi_0)$. Similarly, the current between N_{-1} and N_0 is proportional to $(\varphi_{-1} - \varphi_0)$. Node N_0 should then be traversed by a current which is proportional to the sum $[(\varphi_1 - \varphi_0) + (\varphi_{-1} - \varphi_0)]$. When the microelectrode is moved in the vicinity of node N_0 , the current through N_0 varies with the position of the microelectrode. In the experiment shown in Figure 5, the current delivered through the microelectrode was adjusted to obtain a constant threshold intensity through the node.

If the effective resistance between two neighboring nodes (approximately 20 megohms) is denoted by ω , the current flowing outward through node N_0 , i , is given by

$$(2.2.1) \quad i = \frac{1}{\omega} \{(\varphi_{-1} - \varphi_0) + (\varphi_1 - \varphi_0)\} = \frac{-1}{\omega} \{2\varphi_0 - \varphi_1 - \varphi_{-1}\}$$

The distances between the microelectrode and the nodes N_0 , N_1 and N_{-1} shall be denoted by x_0 , x_1 , x_{-1} respectively. In the vicinity of a microelectrode acting as a sink of current I , the three-dimensional potential field is given by

$$(2.2.2) \quad \varphi = - I \frac{\rho}{4\pi x}$$

where ρ is the resistivity of the medium (approximately 100 ohm·cm for Ringer's solution) and x the distance from the microelectrode. By the use of equation 2.2.1 it is found that the outward current through N_0 , i , is given by

$$(2.2.3) \quad i = I \frac{\rho}{4\pi\omega} \left\{ \frac{2}{x_0} - \frac{1}{x_1} - \frac{1}{x_{-1}} \right\}$$

In the case where the microelectrode is very close to N_0 , namely, when $x_1 \gg x_0$ and $x_{-1} \gg x_0$, the following approximate relation is obtained:

$$(2.2.4) \quad i = I \frac{\rho}{2\pi\omega} \frac{1}{x_0}$$

In the experiment shown in Figure 5, the distance x_0 was varied, and at each x_0 , the value of I which gives rise to the critical value, i_c , of current i was determined. The relationship between I and x_0 is then given approximately by

$$(2.2.5) \quad I = \frac{2\pi\omega i_0}{\rho} x_0$$

The experiment shown in Figure 5 demonstrates that the threshold strength, I , does increase with the distance, x_0 (as expected from equation 2.2.5). It is important to note that the threshold is practically independent of the direction in which the microelectrode is moved away from the node. This and other experiments have established that the myelin sheath is a good electric insulator for a direct current, and that stimulating currents produce effects only at the nodes of Ranvier. (For further details, see Tasaki, 1950, 1953.)

Later, it was found that the myelin sheath is not a perfect insulator, and that there is a measurable leak of current when there is a rapid variation in the potential difference across the myelin sheath (Tasaki, 1940; Tasaki and Takeuchi, 1942; Huxley and Stämpfli, 1949). There are three possibilities to account for this leak of current: (1) the myelin sheath behaves as an ohmic conductor with a high resistance; (2) it behaves like an ideal capacitor; and (3) it has properties of a leaky capacitor. If the myelin sheath behaves as an ohmic conductor, the current I_m through the myelin sheath should be proportional to the deviation of the membrane potential from its resting level; i.e., $I_m = (1/R_m)V$, where R_m is the resistance of the myelin-covered portion of the membrane and V the deviation of the membrane potential. If the myelin sheath behaves like an ideal capacitor, the current I_m should be proportional to the time-derivative of the intracellular potential V , namely $I_m = C_m(dV/dt)$, where C_m is the effective capacity. If the myelin sheath behaves like a leaky capacitor, the current should be given by

$$(2.2.6) \quad I_m = C_m \frac{dV}{dt} + \frac{1}{R_m} V$$

The oscillograph records presented in Figure 6 show the difference between the node and the myelinated portion of a nerve fiber. Under the conditions of experiment A, records were obtained from a one millimeter-long portion of the fiber without nodes of Ranvier.

This current through the myelin sheath has two peaks separated by an interval of approximately 0.1 msec; this interval corresponds to the time required for a nerve impulse to travel from the node on one side

of the pool (N_1 in Fig. 6) to the next node (N_2). The intensity of current through the myelin sheath is totally insensitive to anesthetics introduced into the small middle pool of Ringer's solution. When anesthetic is introduced into the distal pool where node N_2 is immersed, the second peak in the current disappears immediately. A small amount of anesthetic introduced into the proximal pool (where node N_1 is immersed) reduces the size of the first peak of the current

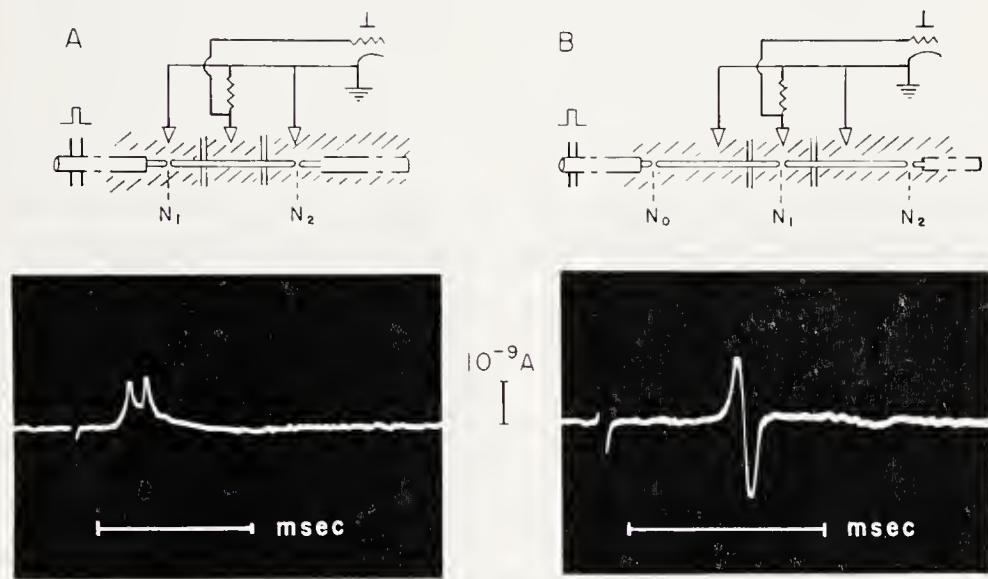


FIGURE 6. A: Electric current recorded through a 1 mm-long myelin-covered portion of a single nerve fiber.

B: Similar to A but with a single node in the middle pool of Ringer's solution. The nerve fiber was stimulated by a brief current pulse applied at one end of the fiber. (From Tasaki, *Handbook of Physiology*, (I), Field, J. (ed), Amer Physiol Soc, 1958).

(see Tasaki and Takeuchi, 1942). From these observations, it is concluded that these peaks represent the onset of the excitation processes at nodes N_1 and N_2 . An extensive analysis of the currents through the myelin sheath has shown that equation 2.2.6 does describe the time courses adequately (Tasaki, 1955).

Record B in Figure 6 was obtained from a one millimeter-long myelin-covered portion of a nerve fiber including one node of Ranvier. The membrane current shown in this record is very different from that in A; this distinct difference is due to the existence of a single node of Ranvier, about 1μ in length, in the middle pool.

The current flowing through the myelinated portion of the nerve fiber is mainly directed outward. With a node in the middle pool, the observed current is roughly biphasic: the first phase is outward-directed and the second is inward-directed with respect to the surface of the nerve fiber. Since the strong inward current traverses only the small surface area of the node, the surface density of this current is approximately two thousand times as large as that of the outward current flowing through the myelinated region. The inward current at the node is very sensitive to anesthetics.

Based on this finding and on many other experimental results described elsewhere (Tasaki, 1950b, 1953) the following conclusions were reached:

- A. The leak of current through the myelinated portion of the nerve fiber is mainly capacitative.
- B. The *emf* variation in the nerve membrane which is responsible for generation of all-or-none action currents is localized at the nodes of Ranvier.

C. When a node is traversed by an outward current greater than its threshold (approximately $4 \times 10^{-10} A$), an approximately all-or-none change in the *emf* takes place in the nodal membrane. This change generates an action current of about five times the threshold which flows outward through the neighboring node and stimulates it. By repetition of this process of restimulation by action currents, the process of excitation of the nodes spreads along the nerve fiber.

Readers interested in the details of this saltatory theory and its application to nerve fibers under various experimental conditions are referred to reviews written by Tasaki (1953, 1959) and by Stämpfli (1954).

ADVENT OF SQUID GIANT AXONS

(2.3) The vertebrate nerve fibers discussed in previous sections are usually less than fifteen microns in diameter. There are much larger nerve fibers in the nervous systems of various invertebrates. For example: the earthworm has giant nerve fibers (axons) about 100μ in diameter (see Rushton and Barlow, 1943; Bullock, 1945); lobsters have several giant axons measuring up to 100μ in diameter (see Wiersma, 1961); Chilean squid have axons as large as 1.5 mm in diameter; North Atlantic squid, available both in North America and Great Britain, have giant axons measuring between 400 and 800 μ .

With these giant axons, it is possible to make various measurements which are impossible with small vertebrate nerve fibers.

A glass micropipette electrode can be inserted longitudinally into a squid giant axon to record potential variations (Hodgkin and Huxley, 1939; Curtis and Cole, 1940). The use of internal wire elec-

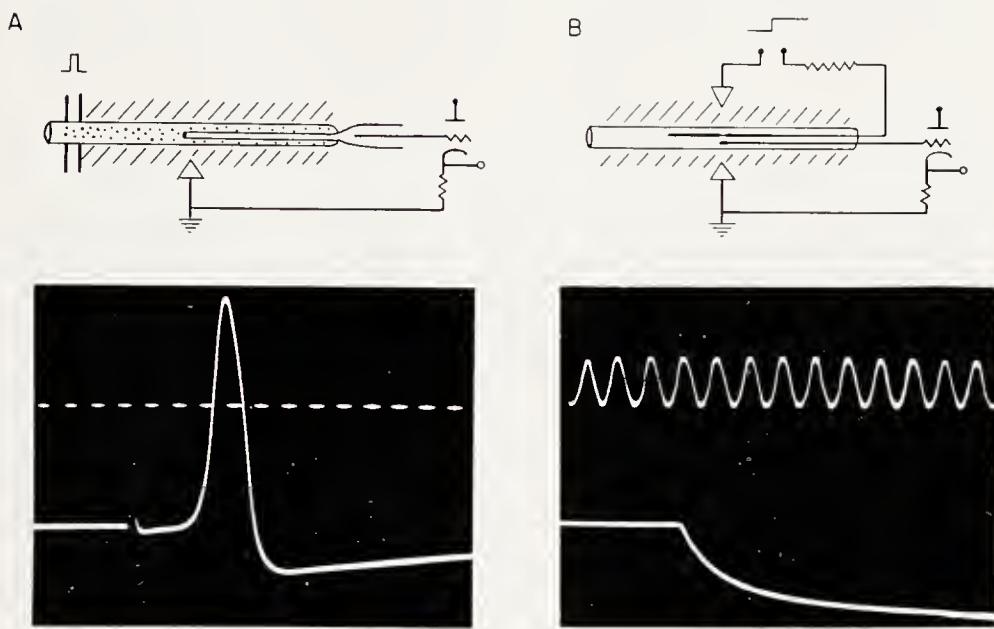


FIGURE 7. A: Resting and action potential of a squid giant axon recorded with an intracellular glass pipette electrode. The time markers (0.5 msec apart) indicate the potential level recorded with the tip of the electrode immersed in the surrounding sea water.

B: Change in the membrane potential produced by a rectangular pulse of inward current applied through an internal wire electrode; recording was made with another metal electrode situated near the middle of the current electrode; the sinusoidal wave is 1000 cps. (Schematic diagrams above are not to scale.) (From Tasaki, *Handbook of Physiology*, vol. I, Fied, J. (ed), Amer Physiol Soc, 1958).

trodes is one of the most important techniques applicable to squid giant axons (Marmont, 1949). This technique makes it possible to stimulate a large portion of an axon almost uniformly, and to record the potential variations inside that region without drawing appreciable current.

Examples of records obtained by the use of these techniques are shown in Figure 7. A giant axon, approximately 500μ in diameter and 40 mm in length, was mounted on a horizontal Lucite chamber filled with natural sea water. The sea water was grounded by means

of a coil of “chloridized” silver wire. In the experiment presented in Figure 7A, a glass-pipette electrode was introduced into the axon. The electrode was filled with 600 mM KCl solution and was connected to an oscilloscope through a high input-impedance amplifier. As the electrode was slowly advanced into the axon through an incision in the axon membrane, the magnitude of the recorded potential (referred to the ground in the external sea water) continuously increased. When the tip of the electrode was about 5 mm beyond the incision, the recorded potential reached a steady level of about -50 mV relative to the level observed when the electrode was in the surrounding sea water. The potential difference recorded in this manner is known as the *resting potential* of the axon. [The resting potential is the intracellular counterpart of the process maintaining the *injury current* (see Fig. 1) of the axon.]

When an axon was stimulated by a brief current pulse, a transient rise and then a fall of the internal potential was observed with an internal glass-pipette electrode (see record A). The potential variation recorded in this manner is called the *action potential* of the axon. There is an intimate relationship between the action potential and the *action current* discussed in Chapter I.

There are no structural discontinuities such as nodes of Ranvier in the invertebrate giant axons. Nerve impulses travel along these axons *continuously*. Since the velocity of propagation of the nerve impulse is finite, the time interval from delivery of the stimulus to the beginning of the action potential—the *latency*—varies with the distance along the axon. The time required for the impulse to travel distance x is x/v , where v is the velocity. The action potentials recorded at two points separated by a distance x differ in latency by x/v .

Both the amplitude and the shape of the action potential are practically independent of x . Then, the intracellularly recorded action potential V , expressed as a function of space coordinate x and time t , can be expressed by the following formula:

$$(2.3.1) \quad V(x, t) = f(t - x/v) + E_r$$

where $f(t)$ represents the time course of the potential variation recorded at $x=0$, and E_r represents the resting potential.

Equation 2.3.1 describes the spatial distribution of the electric potential along the axon at given moment t . The gradient of the internal

potential in the longitudinal direction is accompanied by an electric current in that direction (Ohm's law). The internal longitudinal current, I_i , is expressed by

$$(2.3.2) \quad I_i = -\frac{1}{\omega} \frac{\partial V(x, t)}{\partial x} = -\frac{1}{\omega} \frac{\partial f(t - x/v)}{\partial x} = \frac{1}{\omega v} \frac{\partial f(t - x/v)}{\partial t}$$

$$= \frac{1}{\omega v} \frac{\partial V(x, t)}{\partial t}$$

where ω denotes the resistance of a unit length of axoplasm. Equation 2.3.2 shows that the longitudinal current is a function of x and t and is proportional to the first derivative of the internally recorded action potential. The spatial variation of the internal longitudinal current is directly related to the membrane current I_m by Kirchhoff's law. The spatial and temporal distribution is given by

$$(2.3.3) \quad I_m = \frac{-\partial I_i}{\partial x} = \frac{1}{\omega} \frac{\partial^2 V(x, t)}{\partial x^2} = \frac{1}{\omega v^2} \frac{\partial^2 V(x, t)}{\partial t^2}$$

The membrane current described by equation 2.3.3 generates a weak potential variation in the fluid medium outside the axon. This is the extracellularly recorded action potential of classical neurophysiology. It is important to note that the amplitudes of electric signals recorded intracellularly are usually one hundred to ten thousand times as large as those recorded extracellularly.

In record B of Figure 7, a long (3 m sec) rectangular pulse of electric current was applied to the axon via a long internal wire electrode. This current is directed inward through the axon membrane almost uniformly. The potential variation, $V(t)$, produced by the transmembrane current was recorded with a short intracellular wire electrode, and was found to be approximately exponential. The resistance of the axon membrane (usually between 1,000 and 3,000 ohm · cm²) will be denoted by R_m and the applied current (of the order of 1 μA/cm²) by I . Then, the observed exponential potential variation may be described by

$$(2.3.4) \quad V(t) = IR_m(1 - e^{-t/\tau}),$$

where τ is the time constant of this potential variation. The *apparent membrane capacity* is determined by dividing this time constant by the membrane resistance, and is of the order of 1 μF/cm² (see Cole, 1955).

INTRACELLULAR PERfusion OF SQUID GIANT AXONS

(2.4) From a physicochemical point of view, frog nerve fibers and squid giant axons are extremely complicated systems. The axoplasm and axon membrane contain both organic and inorganic cations and anions, as well as various proteins (polyelectrolytes) (Koechlin, 1955; Davison and Taylor, 1960; Deffner, 1961) and enzymes (see Nachmansohn, 1959; Skou, 1957). Furthermore, even in the resting state there are continuous fluxes of metabolites across the axon membrane. (Most of these fluxes are increased during and after repetitive stimulation of the axon.)

Many biologists add various *metabolic inhibitors* to the external fluid medium of the axon in an effort to simplify these discouragingly complicated systems; yet the technique of using inhibitors does not seem to simplify the problem. If these inhibitor molecules are to modify the metabolism, they must enter the membrane; the existence of these molecules in the membrane should then affect the mobilities and concentrations of various ions in the membrane. Moreover, metabolic inhibitors not only reduce the fluxes of normal metabolites, but may also create fluxes of new, abnormal metabolites and lead to a gradual accumulation of new chemical substances in the axon interior. Physicochemical studies of systems in such a nonstationary state are extremely difficult.

Quite recently, physicochemical studies of the squid giant axon were greatly simplified by removing the axoplasm partially or completely and perfusing the axon interior with artificial salt solutions (Oikawa, Spyropoulos, Tasaki and Teorell, 1961; Baker, Hodgkin and Shaw, 1961). With proper intracellular perfusion solutions, the axon remains excitable for hours. Under these experimental conditions, the chemical composition of the solutions on each side of the axon membrane is well defined and time independent. In other words, the system consisting of the axon membrane and the internal and external fluid media is in a *nonequilibrium, stationary state*. The time required for attaining this stationary state in internally perfused squid giant axons is of the order of one minute.

The experimental arrangement developed by Tasaki, Watanabe and Takenaka (1962) for internal perfusion of the squid giant axon is shown in Figure 8. A photomicrograph of a squid giant axon internally perfused with a dilute salt solution containing a mild proteolytic

enzyme is shown in Figure 9A. A picture of an unperfused control is shown in Figure 9B. As shown by Tasaki, Singer and Takenaka (1965), and by Takenaka and Yamagishi (1966), squid giant axons

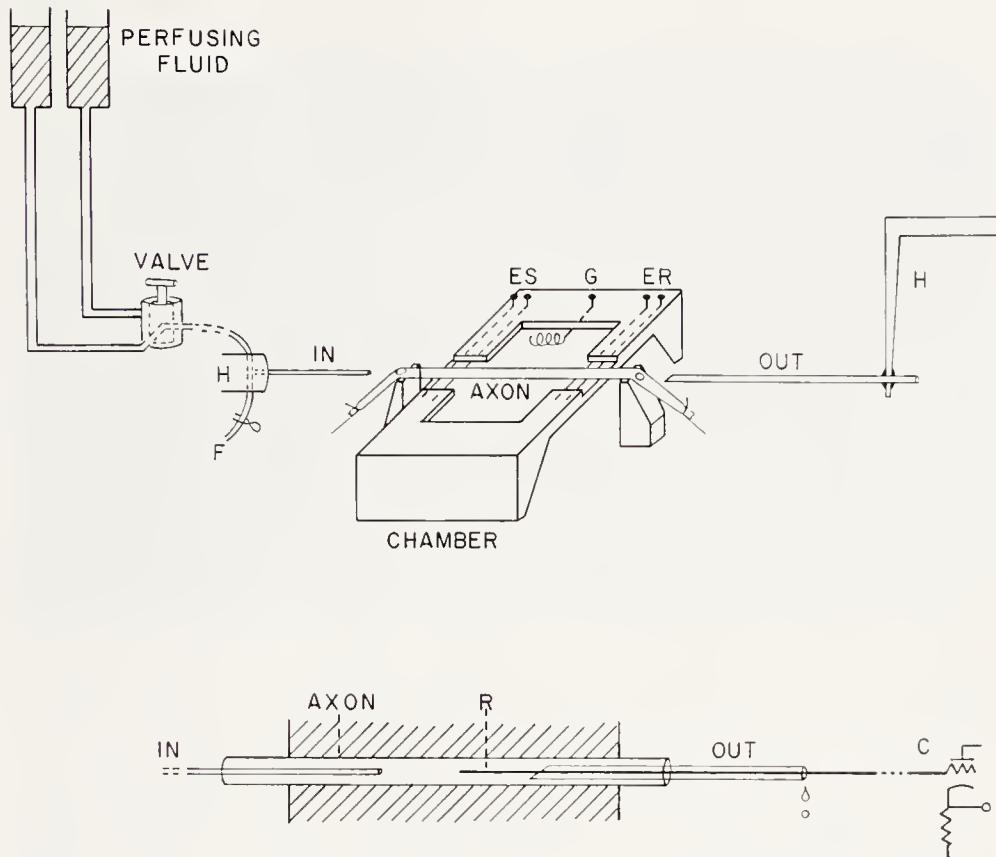


FIGURE 8. Schematic diagram illustrating the experimental setup used for intracellular perfusion of squid giant axon. The inlet cannula (*IN*) is connected to two reservoirs of the perfusion fluid through a valve. The outlet cannula (*OUT*), the internal recording electrode (*R*) and the inlet cannula are held with separate micromanipulators. *ES* represents a pair of stimulating electrodes; *ER*, external recording electrodes; *G*, ground electrode; *H*, holders held by micro-manipulators; *C*, cathode-follower.

are capable of maintaining excitability after complete removal of the axoplasm.

It is important to note that there are no significant metabolic reactions in this system during intra- and extracellular perfusion with artificial saline solutions. There are no metabolic substrates provided in the two fluid media, and all of the water-soluble, diffusible com-

pounds in the system are removed by the continuously flowing perfusion solutions. Therefore, physicochemical investigation of internally perfused squid giant axons is far simpler and more direct than investigation of ordinary excitable tissues. For this reason, the physicochemical analysis of nerve excitation presented in this monograph

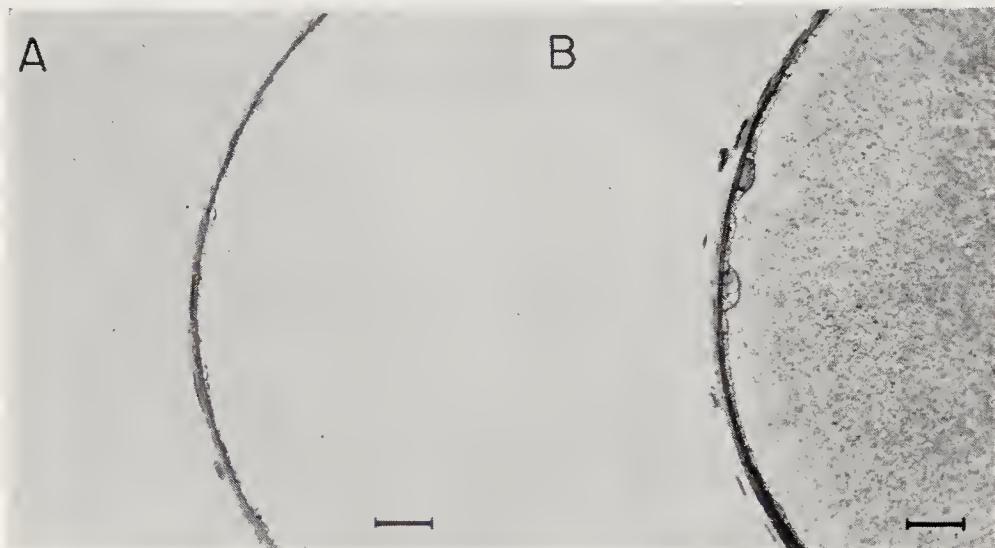


FIGURE 9. A: Photomicrograph of a squid giant axon (a part of cross-section) which had been internally perfused with a dilute solution of KF containing pronase; the axon was fixed with glutaraldehyde while still excitable.

B: A squid giant axon fixed without internal perfusion. Note that the axon is filled with protoplasm in B, whereas protoplasm is completely absent in A. The bars represent $25\ \mu$. It should be noted that in A, only the Schwann-cell and connective tissue sheath can be visualized. The axonal membrane itself (about 0.01μ in thickness) cannot be seen at this magnification.

is largely derived from data obtained with axons under continuous internal perfusion.

Although such perfused axons are far less complicated than other excitable tissues, they still are not simple physicochemical systems to which the various principles established by membrane physical chemists can be applied without reservation. To a great extent, interpretations of the physiological data depend on what approximations and assumptions are adopted. Therefore, in the following chapter several properties of inanimate membranes, and various approximations which may have to be adopted in later discussions of the squid axon membrane will be discussed.

Chapter III

PHYSICAL CHEMISTRY OF INANIMATE MEMBRANES

IN this chapter, several basic properties of inanimate membranes are described. Sections 3.1 and 3.2 deal with the electromotive forces (*emf*'s) of electrochemical cells in which there is equilibrium across a membrane. In Section 3.3 a membrane with continuously diffusing cations is discussed. Section 3.4 is concerned with the difficulties of treating the membrane as a structureless boundary between two solution phases. The relationship between membrane resistance and ion flux is described in Section 3.5. Section 3.6 deals with properties of a composite membrane consisting of two different layers. The final section, 3.7, considers the distribution of anions and cations across the interface between the membrane and the solutions.

An adequate knowledge of the properties of inanimate membranes is essential for proper understanding of the process of nerve excitation. It is hoped that this chapter will serve to clarify the physicochemical foundation on which the interpretations of bioelectric phenomena described in this monograph are built. Readers who are familiar with the classical physicochemical treatments of inanimate membranes may find this chapter superfluous. On the other hand, readers who may have difficulty with an involved mathematical treatment of synthetic membranes will not find their understanding of subsequent material seriously impaired; the qualitative aspects of the arguments are usually repeated in later chapters.

ELECTROCHEMICAL POTENTIAL

(3.1) The electrochemical potential of a particular ion species is derived from the Gibbs' free energy by differentiation with respect to the number of moles of that species at constant pressure, temperature and concentration of all other species (see Kirkwood and Oppenheim, 1961, p. 9). The electrochemical potential plays an essential role in determining the condition of equilibrium and the

rate of ion transfer between two phases; this role is analogous to that of temperature in heat transfer between systems.

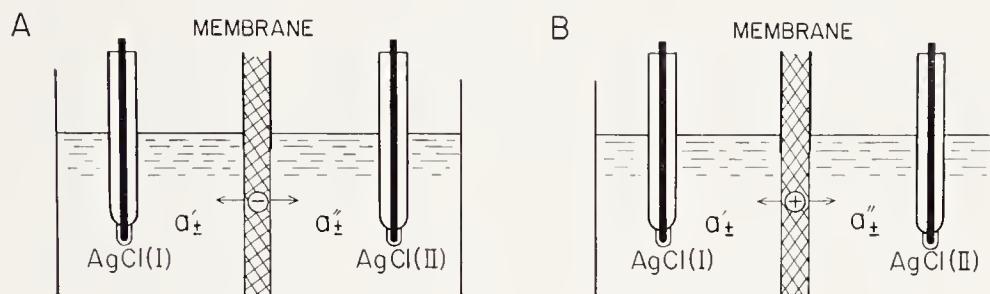
When two phases at different temperatures are brought into contact, heat flows between the two phases as long as there is a temperature difference; provided that there is no transfer of matter, the magnitude of the heat flow is simply proportional to the temperature difference between the two phases. In a similar manner, transfer of matter takes place when two phases in contact are different in chemical composition but have the same temperature and pressure. For example, when a solvent containing a solute is brought into contact with a different solvent (which is immiscible with the first solvent), transfer of the solute from one phase to the other continues until the chemical potential of the solute in the first phase becomes equal to that in the second phase. When ion-exchanger material is immersed in an aqueous solution of several electrolytes, the ion exchange process ceases and equilibrium is reached when the electrochemical potential of every mobile ion in the ion exchanger becomes equal to that in the aqueous phase.

The electrochemical potentials of mobile ions play essential roles in physicochemical treatments of membrane phenomena. Analysis of membrane phenomena is relatively simple when the membrane is "ideally permselective," namely, when the membrane is permeable to ions with one kind of electric charge but completely impermeable to ions with the opposite charge. Two examples of equilibrium across ideally permselective membranes will be considered in this section (see Fig. 10).

In system A, the membrane is assumed to be permeable only to anions, and in B, only to cations. In these cases, the concentrations of the dilute NaCl solutions in the two compartments are different; the mean activities of the electrolyte on two sides of the membrane are denoted by a_{\pm}' and a_{\pm}'' , respectively. (For definition of activities cf. Lewis and Randall, 1961.) A silver-silver chloride electrode is immersed in each of the aqueous solutions in order to measure the potential difference. In case A, the *emf* of the cell is zero; but in case B, the *emf* is given by $2(RT/F) \ln(a_{\pm}''/a_{\pm}')$; when the concentration ratio is 10:1, a potential difference of approximately 116 mV is observed (see Lakshminarayanaiah and Subrahmanyam, 1964).

In general, the *emf* of a cell can be calculated from the chemical

change associated with a virtual transfer of electricity through the cell (see Guggenheim, 1957, p. 386). In case A, transfer of electricity from the left to the right produces (1) the reactions $\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + e^-$ at the left electrode, and (2) the reverse reaction at the right electrode. Since chloride ions carry the electric charge across all the phase boundaries, there are no changes in the salt concentrations of the system. Therefore, there is no difference in the free energy of the system before and after the charge transfer. Hence, the *emf* of cell



$$\bar{\mu}_{\text{Cl}}^{(I)} = \bar{\mu}_{\text{Cl}}' = \bar{\mu}_{\text{Cl}}'' = \bar{\mu}_{\text{Cl}}^{(II)}$$

$$\bar{\mu}_{\text{Cl}}^{(I)}, \quad \bar{\mu}_{\text{Na}}' = \bar{\mu}_{\text{Na}}'', \quad \bar{\mu}_{\text{Cl}}^{(II)}$$

FIGURE 10. A: Ideally permselective anion-exchanger membrane separating two solutions of sodium chloride.

B: Ideally permselective cation-exchanger membrane separating two NaCl solutions. In both cases silver-silver chloride electrodes were used to measure the *emf*. The equilibrium conditions in these systems are indicated.

A should be zero. In case B, however, the situation is very different; sodium ions carry the charge across the membrane, and chloride ions transfer the charge across the boundary between each solution and the electrode. When one faraday of electricity is transferred from left to right in the cell, there is transfer of one mole of NaCl from the left compartment to the right. If the salt concentration on the left is higher than that on the right (i.e., $\alpha_{\pm}' > \alpha_{\pm}''$), there is a decrease in the free energy of the system; therefore, electrical work is done by the system, and the *emf* can be calculated from the free energy change associated with the transfer of NaCl.

Now the *emf*'s of the systems shown in Figure 10 will be discussed on the basis of the electrochemical potentials. Note that the difference in electrochemical potential of an ion between two phases with identical pressure, temperature and chemical composition is propor-

tional to the electrical potential difference between the two phases (see Guggenheim, 1957, p. 374; Katchalsky and Curran, 1965, p. 135). There is no difference in chemical composition between the two AgCl phases (I) and (II) of the system. Hence,

$$(3.1.1) \quad \bar{\mu}_{\text{Cl}^{\text{(II)}}} - \bar{\mu}_{\text{Cl}^{\text{(I)}}} = z_{\text{Cl}} F \Delta\varphi$$

where $\bar{\mu}_{\text{Cl}^{\text{(II)}}}$ and $\bar{\mu}_{\text{Cl}^{\text{(I)}}}$ are the electrochemical potentials of chloride ion in phase (II) and (I), respectively, z_{Cl} is the charge number of chloride (i.e., -1), F the Faraday constant, and $\Delta\varphi$ the electrical potential of phase (II) referred to that of phase (I) (see Guggenheim, *loc cit*). Since each of the AgCl phases is in equilibrium with the Ag (metal) phase, the potential difference between the two metal phases is equal to $\Delta\varphi$.

In Case A of Figure 10, the electrochemical potential of chloride ion in each of the solution phases is equal to that in the membrane, because these phases are in equilibrium. There is also equilibrium between the AgCl phase and the surrounding solution phase with respect to chloride ion. Therefore, the electrochemical potential of chloride ion in one AgCl phase is equal to that of the other AgCl phase; namely $\bar{\mu}_{\text{Cl}^{\text{(II)}}} = \bar{\mu}_{\text{Cl}^{\text{(I)}}}$. Consequently, on the basis of equation 3.1.1, the electrical potential difference between the two electrodes must be zero.

In case B, the electrochemical potential of chloride ion in the solution is equal to that in the AgCl phase of the immersed electrode: namely, $\bar{\mu}_{\text{Cl}'} = \bar{\mu}_{\text{Cl}^{\text{(I)}}}$ and $\bar{\mu}_{\text{Cl}''} = \bar{\mu}_{\text{Cl}^{\text{(II)}}}$, where $\bar{\mu}_{\text{Cl}'}$ and $\bar{\mu}_{\text{Cl}''}$ are the electrochemical potentials of chloride ion in the two bulk phases (see Fig. 10B). From these conditions, it follows that

$$(3.1.2) \quad -F\Delta\varphi = \bar{\mu}_{\text{Cl}^{\text{(II)}}} - \bar{\mu}_{\text{Cl}^{\text{(I)}}} = \bar{\mu}_{\text{Cl}''} - \bar{\mu}_{\text{Cl}'}$$

The electrochemical potentials of sodium ion in the left and right aqueous phase are denoted by $\bar{\mu}_{\text{Na}'}$ and $\bar{\mu}_{\text{Na}''}$. The condition of equilibrium across the cation-exchanger membrane is then given by

$$(3.1.3) \quad \bar{\mu}_{\text{Na}''} = \bar{\mu}_{\text{Na}'}$$

The chemical potential of NaCl is defined as the sum of the electrochemical potentials of the two constituent ions:

$$(3.1.4a) \quad \mu_{\text{NaCl}'}' = \bar{\mu}_{\text{Na}'} + \bar{\mu}_{\text{Cl}'}' = \mu_{\text{NaCl}^{(0)}} + 2RT \ln a_{\pm}'$$

and

$$(3.1.4b) \quad \mu_{\text{NaCl}}'' = \bar{\mu}_{\text{Na}}'' + \bar{\mu}_{\text{Cl}}'' = \mu_{\text{NaCl}^{(0)}} + 2RT \ln a_{\pm}'$$

where $\mu_{\text{NaCl}^{(0)}}$ is the reference value of the chemical potential of NaCl in aqueous solutions (see Robinson and Stokes, 1959, p. 28; Kirkwood and Oppenheim, 1961, p. 191). From equations 3.1.2, 3.1.3 and 3.1.4, it follows that the *emf* in system B is given by

$$(3.1.5) \quad \Delta\varphi = 2 \frac{RT}{F} \ln \frac{a_{\pm}'}{a_{\pm}''}$$

If a high resistance is connected between the two electrodes in case B of Figure 10, electricity flows in such a direction that the free energy of the system is decreased by the current. In accordance with Ohm's law, the current intensity is given by $\Delta\varphi/r$, where r is the resistance in the circuit. In case A, no electric current can be drawn from the system.

"SALT-BRIDGES" AND THE SUSPENSION EFFECT

(3.2) If a membrane separating two dilute electrolyte solutions of different concentrations has a high density of fixed negative charges, the membrane will be permeable to cations and practically impermeable to anions. The potential difference across such a cation-exchanger membrane can be measured with various types of electrodes. The observed potential difference varies, depending on the type of electrodes immersed in the two electrolyte solutions.

In the experimental setups illustrated in Figure 11, the electrolyte used is NaCl. In case A (left), a pair of sodium-sensitive glass electrodes is employed to measure the potential difference. In case B (middle), calomel electrodes combined with salt-bridges are immersed in the solutions. In case C (right), there is a Ag-AgCl electrode in each of the bulk solutions.

From the discussion in the preceding section, it can be seen that the potential difference in case A is zero. All the phase boundaries in this system are permeable to Na^+ and impermeable to Cl^- ; therefore, the situation is analogous to that described in Figure 10A, except that the mobile ion species has the opposite charge. Since the electrochemical potential of sodium ion is constant throughout the system from the surface of one electrode to the other, no electric energy can be drawn from this system.

The arrangement of Figure 11C, where two Ag-AgCl electrodes are used, has already been discussed in the preceding section: the *emf* of this system is given by $2(RT/F) \ln (a_{\pm}'/a_{\pm}'')$.

It is important to note that the salt-bridges are not in equilibrium with the aqueous solutions; there are continuous fluxes of ions between the KCl solution in the bridges and the dilute salt solutions of the system. However, the potential difference across the liquid junction between a concentrated KCl solution and a very dilute solution of uni-univalent salts has been calculated to be quite small (see Teorell, 1936; MacInnes, 1939, p. 226).

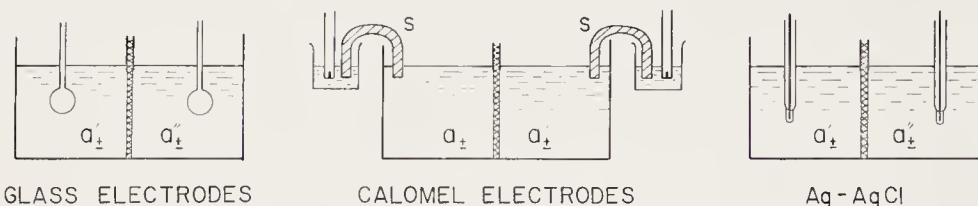


FIGURE 11. Systems of two solutions of NaCl separated by ideally permselective cation-exchanger membrane. The electrodes for measuring *emf*'s are (left) sodium-sensitive glass electrodes, (middle) calomel electrodes with salt-bridges *S*, or (right) silver-silver chloride electrodes.

The electrochemical potentials of sodium ion in the two aqueous solutions are given by

$$(3.2.1) \quad \bar{\mu}_{\text{Na}}' = \mu_{\text{Na}}^{(0)} + RT \ln a_{\text{Na}}' + F\varphi'$$

and

$$(3.2.2) \quad \bar{\mu}_{\text{Na}}'' = \mu_{\text{Na}}^{(0)} + RT \ln a_{\text{Na}}'' + F\varphi''$$

where φ' and φ'' are the electrical potentials of the two phases, and $\mu_{\text{Na}}^{(0)}$ is the reference value of the chemical potential. Since the two solutions are in equilibrium across a membrane permeable to Na ions, it is clear that $\bar{\mu}_{\text{Na}}' = \bar{\mu}_{\text{Na}}''$. From this condition, it follows that

$$(3.2.3a) \quad \varphi'' - \varphi' = - \frac{RT}{F} \ln \frac{a_{\text{Na}}''}{a_{\text{Na}}'}$$

or

$$(3.2.3b) \quad \Delta\varphi = - \frac{RT}{F} \ln \frac{a_{\pm}''}{a_{\pm}'}$$

This is the electrical potential difference that is expected with calomel electrodes under the assumption of negligible liquid junction potentials.

The liquid junction potentials in case B may become significant when relatively concentrated solutions of NaCl are used. In addition, it is almost impossible to evaluate the magnitude of the liquid junction potentials when multivalent ions are added to the system. The inability to evaluate liquid junction potentials creates serious difficulties in measuring membrane potentials with KCl-bridges. For this reason, most physical chemists avoid the use of salt-bridges in their potential measurements. However, the use of salt-bridges is unavoidable in biology. Consequently, there is always considerable uncertainty in the absolute values of membrane potentials in biological systems.

Another important factor to be considered when electrodes with liquid junctions are used is the *suspension effect* or the *Pallmann effect* (Pallmann, 1930; Overbeek, 1952; Sollner, 1953; and others). When there is a suspension (or emulsion) of colloidal material in a salt solution, considerable uncertainty arises in determining the potential of the suspension relative to that of an aqueous solution of inorganic salts. This phenomenon is illustrated in Figure 12 (Tasaki and Singer, 1967). A beaker containing finely powdered cation-exchanger was equilibrated with a dilute NaCl solution. Since the cation-exchanger material has a greater density than the NaCl solution, the suspension separates from the supernatant. When the potential difference between the suspension and the supernatant is measured with a pair of sodium-sensitive glass electrodes, there is no observable *emf*. (This is expected because the whole system is in equilibrium.) But when saturated KCl-calomel electrodes are employed for measurement, a sizeable potential difference may be observed.

The suspension effect also appears when an emulsion of the sodium salt of polyglutamic acid is used instead of sulfonic resin (see right diagram). The polarity of the potential difference is such that the calomel electrode in the cation-exchanger is negative with respect to that in the supernatant. The polarity is reversed when anion-exchanger is used. The Pallmann effect is more pronounced when the concentration of the particles is great, and the electrolyte concentration in the aqueous phase is low. The effect is decreased when the concentra-

tion of the KCl in the liquid junction of the calomel electrode is lowered. It is important to note that there is no membrane between the supernatant phase and the emulsion or suspension phase, and that this phenomenon appears very clearly even when the boundary between the emulsion and the supernatant is diffuse.

In the arrangement of Figure 12, the following phases are present

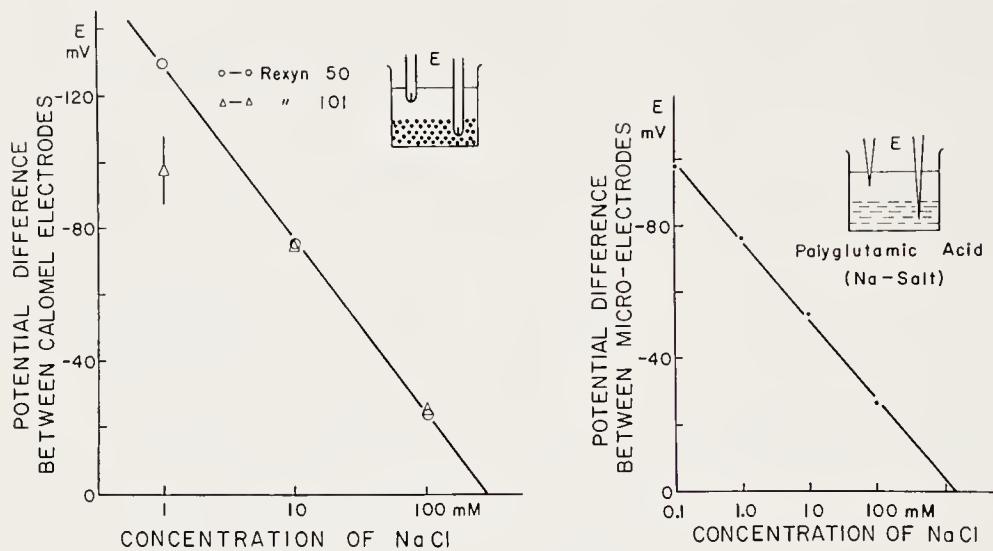


FIGURE 12. Demonstration of the suspension effect.

Left: the electric potential of the calomel electrode in the layer of cation-exchange resin particles, referred to the potential of the electrode in the supernatant, plotted against the concentration of the NaCl solution which is in equilibrium with the resin particles.

Right: Similar observation made with polyglutamic acid (Na-salt) and micro-electrodes. (From Tasaki and Singer, *Ann NY Acad Sci*, 1967, in press.)

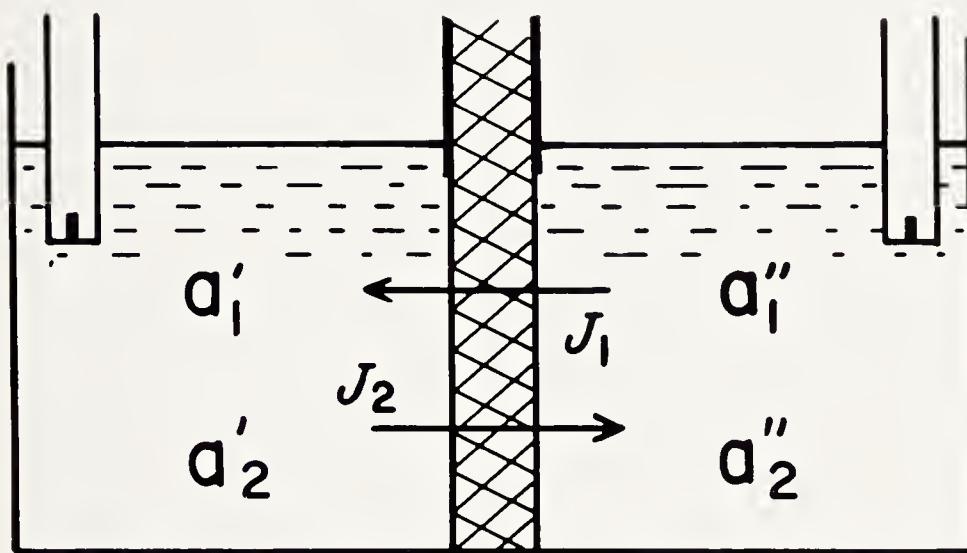
between the two calomel electrodes (from the left electrode to the right): (1) KCl solution; (2) dilute NaCl solution; (3) loose layer of cation-exchanger material; and (4) KCl solution. If the entire layer of the suspension is regarded as a loose cation-exchanger membrane, the polarity and concentration dependence of the observed potential can be understood qualitatively. (Overbeek, 1952, has discussed the relationship between this effect and the Donnan potential.)

The Pallmann effect creates enormous uncertainty in determining the resting potential of many biological cells, particularly of those cells living in fresh water (e.g., fresh water amoebae, *Nitella*). Only

under the conditions of intracellular perfusion can this effect be completely eliminated.

INTERDIFFUSION OF IONS ACROSS A MEMBRANE

(3.3) In the systems discussed in the preceding sections, there was no ion flux unless there was an electric current across the membrane. In this section, systems with continuous transmembrane ion fluxes



$$a'_1 < a''_1$$

$$a'_2 > a''_2$$

FIGURE 13. Cation-exchanger membrane separating mixtures of salts of two univalent cations, species 1 and 2. Fluxes of the two species, J_1 and J_2 , are indicated by the arrows.

(in the absence of electric currents) will be considered. A uniform, ideally permselective cation-exchanger membrane separating two different mixtures of two dilute uni-univalent salt solutions (e.g., solutions of NaCl and KCl) will be used as a representative example (Fig. 13). Under these conditions, there is continuous interdiffusion of cations across the membrane. (In Ch. IV and V it will be shown that consideration of such interdiffusion flux is essential for analysis of the behavior of the axon membrane, both in the resting and excited states.)

In commercially available cation-exchanger membranes, the con-

centration of fixed charges is roughly two to three equivalents per liter of exchanger water content. When the salt concentrations in the aqueous solution are relatively low, the total concentration of cations in such a cation-exchanger is practically equal to the fixed charge concentration of the exchanger; anions are almost completely excluded from the exchanger under these conditions. If the concentration of the fixed charges is denoted by \bar{X} , and the concentrations of the two cations in the exchanger by \bar{C}_1 and \bar{C}_2 , then

$$(3.3.1) \quad \bar{C}_1 + \bar{C}_2 = \bar{X}$$

Both \bar{C}_1 and \bar{C}_2 vary with the coordinate x , normal to the surface of the membrane and equation 3.3.1 is satisfied at every point x in the membrane.

When a salt solution in contact with a cation-exchanger membrane is vigorously stirred, the surface layer of the membrane can be regarded as being in equilibrium with the contiguous solution. Therefore, the electrochemical potential of each of the two cations in the exchanger is equal to that in the surrounding aqueous solution. From these conditions, the following relationship between the concentrations of the two cations in each superficial layer of the exchanger and the activities of these cations in the solution can be obtained:

$$(3.3.2) \quad \frac{\bar{C}_2(0)}{\bar{C}_1(0)} = K_1^2 \frac{a_2'}{a_1'} ; \quad \frac{\bar{C}_2(\delta)}{\bar{C}_1(\delta)} = K_1^2 \frac{a_2''}{a_1''}$$

where the two surfaces of the membrane are denoted by $x=0$ and $x=\delta$. K_1^2 is known as the selectivity coefficient of ion species 2 over 1. If species 2 is preferred by the exchanger, K_1^2 is greater than unity; if species 1 is preferred, K_1^2 is less than unity. To some extent the selectivity coefficient varies with the fraction $a_1/(a_1+a_2)$. (For further information concerning the selectivity coefficient, the readers are referred to books by Calmon and Kressman, 1957, and by Helferich, 1962.)

The driving force within the membrane matrix which causes the flux of an ion is the negative gradient of its electrochemical potential. The flux of cation species 1 shall be denoted by J_1 and the flux of species 2 by J_2 ; the dimensions of flux are $\text{mole} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$. The following equations describe the fluxes of cations through the membrane:

$$(3.3.3a) \quad J_1 = -\bar{u}_1 \bar{C}_1 \frac{d\bar{\mu}_1}{dx}$$

and

$$(3.3.3b) \quad J_2 = -\bar{u}_2 \bar{C}_2 \frac{d\bar{\mu}_2}{dx}$$

where \bar{u}_1 and \bar{u}_2 are the mobilities, \bar{C}_1 and \bar{C}_2 are the concentrations of species 1 and 2 at point x in the membrane. If the electrochemical potentials are expressed in terms of the ion activities, \bar{a}_1 and \bar{a}_2 , and the electrical potential, $\bar{\varphi}$, in the membrane, then

$$(3.3.4a) \quad J_1 = -\bar{u}_1 \bar{C}_1 \left[RT \frac{d \ln \bar{a}_1}{dx} + F \frac{d\bar{\varphi}}{dx} \right],$$

and

$$(3.3.4b) \quad J_2 = -\bar{u}_2 \bar{C}_2 \left[RT \frac{d \ln \bar{a}_2}{dx} + F \frac{d\bar{\varphi}}{dx} \right].$$

It is important to recognize that these two fluxes are not independent; they are bound by the condition of zero electric current:

$$(3.3.5) \quad J_1 + J_2 = 0$$

When the cation mobilities are unequal, i.e., $\bar{u}_1 \neq \bar{u}_2$, the field of electrical potential and the variation in the ratio \bar{C}_1/\bar{C}_2 should be such that equations 3.3.1 and 3.3.5 are satisfied at every position x within the membrane.* When the membrane is uniform, it is possible to solve equations 3.3.4 under the condition that the fluxes do not vary with x . Teorell (1951) solved the simultaneous equations 3.3.4 under the condition that the activity coefficients in the membrane are unity. A more general method of solving these equations is given in the Appendix. The results of these mathematical analyses are presented below.

* The existence of an electrical field within the membrane is, in this treatment, a consequence of the electroneutrality requirement (see p. 368 in Helfferich, 1962). An extremely small deviation from condition 3.3.1 may exist in the system, but the information about such a deviation is not needed for calculation of the membrane potential, and the membrane potential does not give any information about the magnitude of such a deviation.

The interdiffusion flux $J (= J_2 = -J_1)$ is given by

$$(3.3.6) \quad J = \frac{RT\bar{X}\bar{u}_1\bar{u}_2}{(\bar{u}_1 - \bar{u}_2)\delta} \ln \frac{(a_1'' + Q_1^2 a_2'')(a_1' + K_1^2 a_2')}{(a_1' + Q_1^2 a_2')(a_1'' + K_1^2 a_2'')}$$

where $Q_1^2 = K_1^2 \bar{u}_2 / \bar{u}_1$. In deriving equation 3.3.6, the mobilities (\bar{u}_1 and \bar{u}_2) and the selectivity, K_1^2 , are assumed to be constant throughout the membrane.

The activities of individual cations in the two bulk solutions, a_1' , a_1'' , a_2' and a_2'' , may be expressed as products of the concentrations and the activity coefficients. It is well known that activity coefficients of single ions in a mixed salt solution are quantities that are inaccessible to thermodynamic measurements (see Guggenheim, 1957; Helfferich, 1962, p. 371). However, equation 3.3.6 is useful when the salt solutions are dilute, namely, when the activity coefficients can be taken as unity.

The special case in which $a_1' = 0$ and $a_2'' = 0$ is very important in later analyses of ion fluxes across the axon membrane. In this "bionic" case, equation 3.3.6 reduces to

$$(3.3.7) \quad J = \frac{RT\bar{X}\bar{u}_1\bar{u}_2}{(\bar{u}_1 - \bar{u}_2)\delta} \ln \frac{\bar{u}_1}{\bar{u}_2}$$

Equation 3.3.7 contains no single ion activity. The validity of the relationship between the mobilities and the ion flux in equation 3.3.7 has been demonstrated by Helfferich and Ocker (1957).

The membrane potential, as measured with a pair of calomel electrodes, is generally non-zero under these conditions. According to Teorell's (1935, 1951, 1953) theory of charged membranes, the membrane potential is divided into three components: two phase-boundary potentials and an intramembrane diffusion potential. The equation for the membrane potential under the conditions of Figure 13 has been derived by Wyllie and others (see Wyllie, 1954; Helfferich and Schlögl, 1956; Helfferich, 1962, p. 384; Eisenman, 1965):

$$(3.3.8) \quad \Delta\varphi = \varphi'' - \varphi' = -\frac{RT}{F} \ln \frac{a_1'' + Q_1^2 a_2''}{a_1' + Q_1^2 a_2'}$$

This equation has some resemblance to that derived previously by Planck (1890), by Goldman (1943) and by Hodgkin and Katz (1949) for an uncharged diffusion-barrier.

In the special case where $a_1' = a_2'' = 0$, equation 3.3.8 reduces to

$$(3.3.9) \quad \Delta\varphi = \frac{RT}{F} \ln \frac{Q_1^2 a_2'}{a_1''}$$

Equation 3.3.9 was obtained by Wyllie (1954) and describes the membrane potential for a cation-exchanger membrane separating two different salt solutions. When the salt concentrations on both sides of the membrane are the same, this equation becomes

$$(3.3.10) \quad \Delta\varphi = \frac{RT}{F} \ln Q_1^2$$

where Q_1^2 is the product of the selectivity and the mobility ratio. The membrane potentials observed under these circumstances are called *bi-ionic potentials*. Sollner and his colleagues have studied such bi-ionic potentials extensively (see Sollner, Dray, Grim and Neihof, 1955).

DISCONTINUOUS TREATMENT OF MEMBRANE PHENOMENA

(3.4) In the thermodynamics of irreversible processes, the equations for describing transmembrane ion fluxes under constant temperature and pressure are often written in the following form:

$$(3.4.1) \quad -J_i = \sum_{j=1}^n L_{ij} \Delta\bar{\mu}_j = L_{i1} \Delta\bar{\mu}_1 + L_{i2} \Delta\bar{\mu}_2 + L_{i3} \Delta\bar{\mu}_3 \\ + \cdots + L_{in} \Delta\bar{\mu}_n \quad (i, j = 1, 2, \dots, n)$$

where $\Delta\bar{\mu}_j$ represents the difference in the electrochemical potential of the j -th ion species in the two bulk solutions; L_{ij} represents the phenomenological coefficient, and n is the number of the mobile species in the system (see Kirkwood, 1954, p. 122). This formulation differs from the treatment in the preceding section (3.3) in that the *difference in $\bar{\mu}_j$ (across the membrane)*, instead of the *gradient of $\bar{\mu}_j$ (within the membrane)*, is taken as the driving force. In the thermodynamics of irreversible processes, analyses of membrane phenomena based on flux equations of the form 3.4.1 are generally called *discontinuous treatments*, as opposed to the *continuous treatment* or the *kinetic treatment* adopted in the preceding section.

A discontinuous treatment of membrane phenomena has an ad-

vantage over a continuous treatment in that no detailed knowledge of the membrane properties or of the distribution of the mobile species in the membrane is needed. As Kirkwood (1954) has shown, the two treatments are mathematically interconnected and equations of the form 3.4.1 can be derived from the continuous form:

$$(3.4.2) \quad -J_i = \sum_{j=1}^n \omega_{ij} \frac{d\bar{\mu}_j}{dx}$$

where ω_{ij} are the phenomenological coefficients of the continuous treatment.

In spite of the apparent simplicity of equation 3.4.1, analysis of certain aspects of membrane phenomena by discontinuous treatment leads to results without practical value (see Schlägl, 1956, pp. 48 and 118; Lorimer, Boterenbrood and Hermans, 1956, p. 142). The following discussion will clarify this point.

With an ideally permselective membrane, equation 3.4.1 has the following form for the interdiffusion of cations species 1 and 2:

$$(3.4.3) \quad -J_1 = L_{11}\Delta\bar{\mu}_1 + L_{12}\Delta\bar{\mu}_2$$

and

$$-J_2 = L_{21}\Delta\bar{\mu}_1 + L_{22}\Delta\bar{\mu}_2$$

In the absence of an electric current through the membrane, the fluxes of the two (univalent) cations are related by

$$(3.4.4) \quad J_1 + J_2 = 0$$

From equations 3.4.3 and 3.4.4, it follows that

$$(3.4.5) \quad \begin{aligned} -J_1 &= g_1\Delta\bar{\mu}_1 = g_1 \left(RT \ln \frac{a_1''}{a_1'} + F\Delta\varphi \right) \\ &\text{and} \end{aligned}$$

$$-J_2 = g_2\Delta\bar{\mu}_2 = g_2 \left(RT \ln \frac{a_2''}{a_2'} + F\Delta\varphi \right),$$

where g_1 and g_2 are given by

$$g_1 = L_{11} - \frac{L_{11} + L_{21}}{L_{22} + L_{12}} L_{12}$$

(3.4.6)

and

$$g_2 = L_{22} - \frac{L_{22} + L_{12}}{L_{11} + L_{21}} L_{21}$$

(The equations of the form 3.4.5 have been used by Hodgkin and Huxley (1952) to describe the fluxes of sodium and potassium ions across the squid axon membrane.) Equations 3.4.5 may be solved for the interdiffusion flux, $J (= J_2 = -J_1)$:

$$(3.4.7) \quad J = \frac{RT g_1 g_2}{g_1 + g_2} \ln \frac{a_1'' a_2'}{a_1' a_2''}$$

and for the membrane potential, $\Delta\varphi$

$$(3.4.8) \quad \Delta\varphi = \frac{RT}{F(g_1 + g_2)} \left\{ g_1 \ln \frac{a_1'}{a_1''} + g_2 \ln \frac{a_2'}{a_2''} \right\}$$

These equations are very different from equations 3.3.6 and 3.3.8 described in the preceding section. The difference becomes very clear when one of the ion activities in the bulk solution is vanishingly small. When a_1'' approaches zero, $-\ln a_1''$ approaches infinity. Consequently, equations 3.4.7 and 3.4.8 are totally useless under these circumstances. However, equations 3.3.6 and 3.3.8 in the preceding section are quite useful under these circumstances.

As has been pointed out by Kirkwood (1954), the phenomenological coefficients (L_{11} , L_{12} , L_{21} and L_{22}) are functions of all the variables (a_1' , a_1'' , a_2' , a_2'' and $\Delta\varphi$). Conductances g_1 and g_2 are related to L 's by equation 3.4.6, and vary markedly when ion activities on either side of the membrane are altered. When $a_1' \ll a_2'$, for example, the level of a_1' has practically no effect on the membrane potential, the resistance or the ion fluxes across the membrane. This is clearly seen in equations 3.3.6 and 3.3.9 in the preceding section and in the Appendix. But, even under these circumstances, g_1 and g_2 , defined by equation 3.4.6, are markedly influenced by how small the ratio $a_1':a_2'$ is (e.g., by whether the ratio is 1:100 or 1:1000). In biological cells, it frequently happens that $a_1' \ll a_2'$ on one side of the membrane (e.g., a high Na ion and a low K ion concentration outside) and $a_1'' \gg a_2''$ on the other side. For this reason, equations 3.4.5 have only limited value when applied to biological membranes.

Equations 3.4.5 represent the relationship between the ion fluxes and the driving forces, and have the form of Ohm's law. However, it is important to note that the relationship is only apparently linear, because the coefficients (g_1 and g_2) in these equations are dependent on the driving forces. This dependence deprives these equations of their practical value (Schlögl, 1956, p. 118).

Other aspects of the problems associated with application of equations 3.4.5 to nerve membrane have recently been discussed by Finkelstein and Mauro (1963). The discontinuous treatment of membrane phenomena leads to useful relations when applied to calculation of free energy dissipation associated with ion fluxes (see Kedem and Katchalsky, 1961; Nims, 1961).

MEMBRANE RESISTANCE

(3.5) The membrane resistance is determined by applying a current and dividing the measured change in the membrane potential by the intensity of the current. When the current is small, the potential change in the steady state is proportional to the applied current. The resistance is defined as the limiting value of the ratio when the current intensity approaches zero. This value gives important information concerning the concentration and the mobility of the ions present in the membrane.

In a membrane in which two different univalent cations carry charge, the resistance dr of a layer of thickness dx in the membrane is given by

$$(3.5.1) \quad dr = \frac{dx}{(\bar{u}_1\bar{C}_1 + \bar{u}_2\bar{C}_2)F^2}$$

(see Appendix). If the mobilities and concentrations are known as functions of x , the membrane resistance can then be calculated. When the mobilities, fixed charge density, and selectivity within the membrane are independent of x , equation 3.5.1 can be integrated. When only cation species 1 is present on one side of the membrane and only species 2 on the other side, integration yields

$$(3.5.2) \quad r = \frac{(\bar{u}_1 - \bar{u}_2)\delta}{F^2\bar{X}\bar{u}_1\bar{u}_2 \ln (\bar{u}_1/\bar{u}_2)}$$

where \bar{X} is the fixed charge density (Teorell, 1951, p. 466; see also Appendix of this book).

When equation 3.5.2 for the membrane resistance is compared with equation 3.3.7 for the interdiffusion flux under the same conditions, it is found that the resistance-flux product is equal to a constant. This constant is independent of the membrane properties and of the chemical species of the ions concerned:

$$(3.5.3) \quad rJ = \frac{RT}{F^2} = 2.62 \times 10^{-7} \text{ equiv} \cdot \text{sec}^{-1} \cdot \text{ohm at } 20^\circ\text{C}$$

When cation species 2 is an isotope of species 1, measurements of r and J are relatively simple; Spiegler and Coryell (1953) and Gottlieb and Sollner (personal communication) showed that the relationship 3.5.3 is obeyed within an accuracy of about 10 per cent in this case.

In the case where the two cation species are not related isotopes, the validity of this conclusion has been tested by direct experiments (see Appendix). Oxidized collodion and sulfonated polystyrene membranes, with resistances varying between 10 and 10,000 ohm · cm², were used to measure interdiffusion fluxes of cations under various experimental conditions. The values observed for the resistance-flux product were found to agree with the constant RT/F^2 within 10 per cent.

It is possible to expand this argument to the case where the fixed charge density of the membrane varies with the coordinate x normal to the surface of the membrane. Even in this case, if the mobility ratio (\bar{u}_2/\bar{u}_1) and the ion selectivity (K_1^2) do not vary with x , the rJ -product can be shown to be equal to RT/F^2 (see Appendix).

The mobility ratio of the ions in the highly hydrated membrane matrix is known to be similar to that in the aqueous media (see Robinson and Stokes, 1959, pp. 130 and 308; Helfferich, 1962, p. 302; Kobatake *et al.*, 1965). But, if the membrane matrix is very compact or nonaqueous, the mobility ratio in the membrane can be very different from that in water. Therefore, it is possible that there is a wide variation in the mobility ratio within biological membranes; in such cases the rJ -product may deviate significantly from RT/F^2 . In a membrane consisting of two layers which markedly differ from each other, the rJ -product may approach (but does not exceed) $2RT/F^2$.

The rJ -product in the axon membrane provides a deep insight into the mechanism of excitation and will be discussed in the following chapters for both the resting and excited states.

THE DOUBLE LAYER MEMBRANE AND UNSTIRRED LAYER

(3.6) Until quite recently, physical chemists were only interested in uniform membranes with which they could carry out relatively

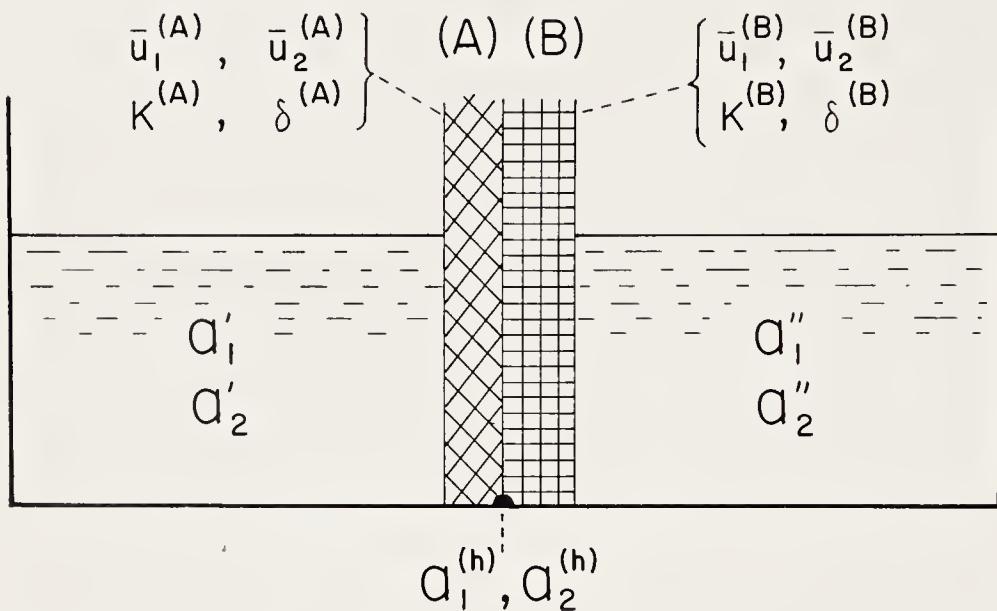


FIGURE 14. Diagram showing a double-layer membrane separating mixtures of salts of two univalent cations, species 1 and 2. The mobilities, selectivities and thickness of the two layers, (A) and (B), are indicated.

simple measurements. Since biological membranes are far from being uniform, it seems worthwhile to investigate the properties of a membrane consisting of more than one layer with different properties (see Kedem and Katchalsky, 1963).

The case to be considered in this section is a composite two-layer membrane separating two different salt solutions. It is assumed that at least one of the two layers possesses a high density of negative fixed charges, so that the membrane as a whole is impermeable to anions (see Fig. 14). The mobilities of the two interdiffusing cations in layer A are denoted by $\bar{u}_1^{(A)}$ and $\bar{u}_2^{(A)}$, and those in layer B by $\bar{u}_1^{(B)}$ and $\bar{u}_2^{(B)}$. The ion selectivities in the two layers are designated

$K^{(A)}$ and $K^{(B)}$. (Since only two cations are considered, the super- and subscripts 1 and 2 for constant K are omitted in this section.) The system is considered to be in a stationary (time-independent) state.

An imaginary experiment can now be carried out in which a droplet of aqueous solution is brought into contact with the composite membrane at the boundary between the two layers. When a stationary state is reached, the *imaginary droplet* should be in equilibrium with the surface of each layer at the boundary; hence, the chemical composition of the droplet should be uniquely determined by the equilibrium conditions.

Under these circumstances, the membrane potential can be expressed as the sum of (1) the potential difference between one bulk solution and the imaginary droplet, and (2) the difference between the droplet and the other bulk solution. The activities of the two cations in the imaginary droplet will be denoted by $a_1^{(h)}$ and $a_2^{(h)}$. The membrane potential can then be expressed as

$$(3.6.1) \quad -\Delta\varphi = \frac{RT}{F} \ln \frac{a_1'' + Q^{(B)}a_2''}{a_1^{(h)} + Q^{(B)}a_2^{(h)}} + \frac{RT}{F} \ln \frac{a_1^{(h)} + Q^{(A)}a_2^{(h)}}{a_1' + Q^{(A)}a_2'}$$

where

$$(3.6.2) \quad Q^{(B)} = K^{(B)} \frac{\bar{u}_2^{(B)}}{\bar{u}_1^{(B)}} \quad \text{and} \quad Q^{(A)} = K^{(A)} \frac{\bar{u}_2^{(A)}}{\bar{u}_1^{(A)}}$$

By rearranging the terms, $\Delta\varphi$ can be written in the form

$$(3.6.3) \quad -\Delta\varphi = \frac{RT}{F} \ln \frac{a_1'' + Q^{(B)}a_2''}{a_1' + Q^{(A)}a_2'} + \Delta\varphi^{(h)}$$

where $\Delta\varphi^{(h)}$ is the contribution of the junction of the two layers to the overall membrane potential:

$$(3.6.4) \quad \begin{aligned} \Delta\varphi^{(h)} &= \frac{RT}{F} \ln \frac{a_1^{(h)} + Q^{(A)}a_2^{(h)}}{a_1^{(h)} + Q^{(B)}a_2^{(h)}} \\ &= \frac{RT}{F} \ln \frac{\{\bar{u}_1^{(A)}\bar{C}_1^{(A)} + \bar{u}_2^{(A)}\bar{C}_2^{(A)}\}\bar{u}_1^{(B)}\bar{C}_1^{(B)}}{\{\bar{u}_1^{(B)}\bar{C}_1^{(B)} + \bar{u}_2^{(B)}\bar{C}_2^{(B)}\}\bar{u}_1^{(A)}\bar{C}_1^{(A)}} \end{aligned}$$

where \bar{C}_1 's and \bar{C}_2 's are the cation concentrations in the membrane at the junction. (The second equality in equation 3.6.4. derives from the definition of $Q^{(A)}$ and $Q^{(B)}$, equations 3.6.2 and 3.3.2.)

On the basis of equations 3.6.1, 3.6.3 and 3.6.4, several simple cases with relatively clear results can now be discussed. In the first case, the major diffusion barrier is assumed to reside exclusively in one of two layers. When the mobilities of layer A are far smaller than those in layer B of a comparable thickness, layer A behaves as the major diffusion barrier between the two bulk solutions. In this case, $a_1^{(h)} \approx a_1''$ and $a_2^{(h)} \approx a_2''$: consequently the first term of equation 3.6.1 vanishes, and the membrane reduces to a single uniform membrane.

In the second case, $Q^{(A)}$ is assumed to be equal to $Q^{(B)}$; then $\Delta\varphi^{(h)}$ vanishes and the whole membrane behaves as a single uniform layer, even when the two layers have different fixed charge densities.

In all other cases, the contribution of $\Delta\varphi^{(h)}$ to the membrane potential remains finite. Furthermore, since $Q^{(A)}$ and $Q^{(B)}$ are different, the two surfaces of the membrane behave very differently in response to a variation in the ratio of cation activities.

When the thickness of one layer (e.g., $\delta^{(B)}$), is overwhelmingly greater than the thickness of the other layer, $\delta^{(A)}$, the effect of layer B becomes important even when the ion mobilities in layer B are far larger than those in layer A. The most important example of this kind is a thin cation-exchanger membrane (A) in contact with a relatively thick unstirred layer (B) of salt solution. In this situation, the membrane behaves very differently from a simple, uniform membrane. The existence of such an unstirred layer was first suggested by Nernst (1904), and quantitative studies of unstirred layer effects have been carried out by many investigators (see Schulman and Teorell, 1938; Mackay and Mears, 1959; Helfferich, 1962; p. 383).

The effect of an unstirred layer on the membrane potential is very pronounced when multivalent ions are introduced into the bulk solutions (Scatchard and Helfferich, 1956). Unfortunately, the mathematical expression describing the membrane potential is very complex when the salt solutions contain mixtures of uni- and divalent cations (see Appendix). Since accurate computation of the unstirred layer effect is very difficult in this case, the effect will be illustrated by describing some qualitative results obtained in this laboratory.

Figure 15 shows the potential difference across a cation-exchanger membrane plotted against the concentration of KCl in the medium on one side of the membrane. Throughout the experiment a 50 mM

CaCl_2 solution was present on the other side. When the fluid on both sides of the membrane was stirred vigorously, the potential of the calomel electrode in the KCl solution (relative to that in the CaCl_2 solution) rose with dilution. When the two surfaces of the cation-

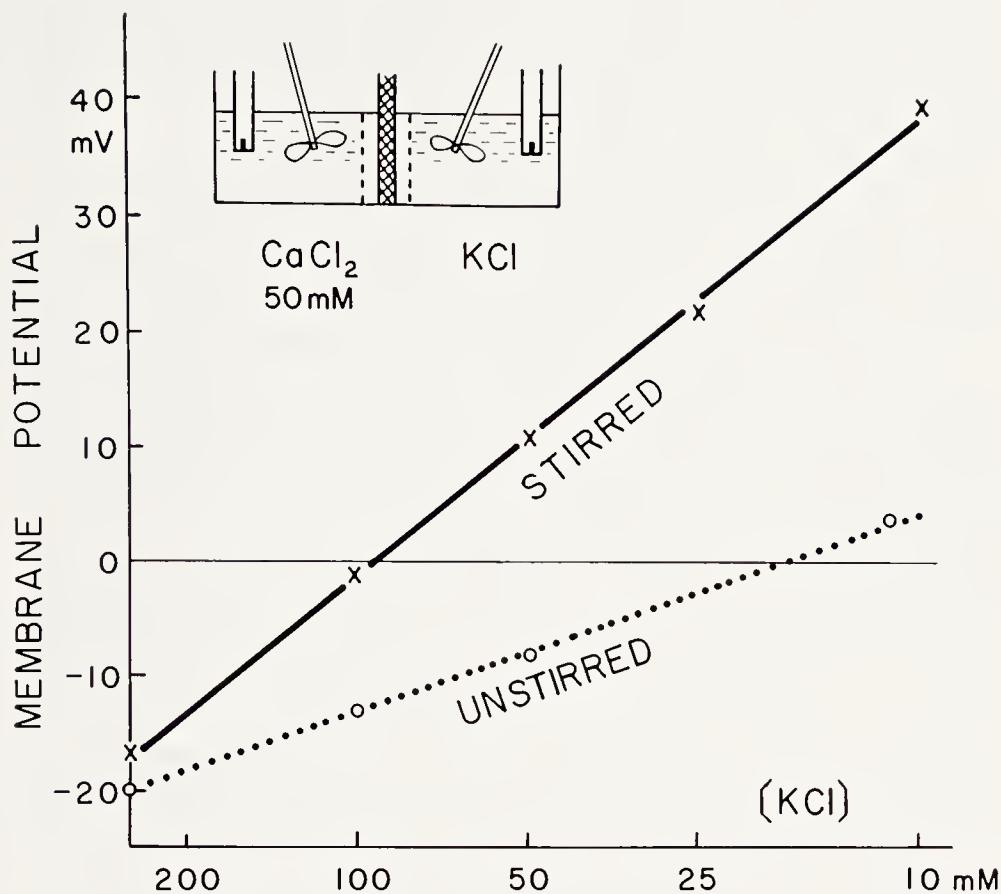


FIGURE 15. Potential difference across a cation-exchanger membrane (measured with a pair of calomel electrodes), plotted against the salt (KCl) concentration on one side of the membrane. The concentration of the salt (CaCl_2) on the other side was held constant (50 mM). The data marked "stirred" were obtained with strong stirring on two sides of the membrane. The data marked "unstirred" were obtained after coating the surface of the membrane with a thin layer of agar. The membrane used was of the sulfonated polystyrene type.

exchanger membrane were covered with thin layers of agar gel, creating well-defined unstirred layers on both sides of the membrane, the concentration dependence of the membrane potential was markedly reduced. Over a wide range of KCl concentrations, the observed membrane potential in the unstirred situation had a po-

larity opposite to that in the well-stirred situation. Similar reversals of the membrane potential by cessation or resumption of stirring have been reported by Scatchard and Helfferich (1956).

It is important to note that the effect of stirring increases with increasing dilution of the salt solution and with decreasing thickness of the membrane for a given membrane material. Since biological membranes are extremely thin, the effect of unstirred layers is expected to be significant (see Ch. IV).

EFFECTS OF CO-IONS AND OTHER FACTORS

(3.7) When a cation-exchanger membrane does not completely exclude co-ions, there is an equilibrium distribution of co-ions across the boundary between the bulk solution and the membrane phase. The best known treatment of this problem is to assume a Donnan distribution across the boundary (Teorell, 1935; Meyer and Sievers, 1936). When applied to a system containing a single uni-univalent electrolyte, this distribution is described by

$$(3.7.1) \quad \bar{C}_+ \bar{C}_- = c^2$$

where \bar{C}_+ and \bar{C}_- represent the intramembrane concentrations of the cations and the anions, respectively, and c represents the concentration of the salt in the bulk solution.

The Donnan distribution 3.7.1 can be derived from the continuity of the electrochemical potentials of the two ions across the boundary. If the solution phase is denoted by the superscript $(')$ and the membrane phase by (m) , then

$$(3.7.2) \quad \bar{\mu}_+^{(0)} = \mu_+^{(0)} + RT \ln a_+^{(0)} + F\varphi'$$

and

$$(3.7.3) \quad \bar{\mu}_+^{(m)} = \mu_+^{(0)} + RT \ln a_+^{(m)} + F\varphi^{(m)}$$

describe the electrochemical potential for the cation;

$$(3.7.4) \quad \bar{\mu}_-^{(0)} = \mu_-^{(0)} + RT \ln a_-^{(0)} - F\varphi'$$

and

$$(3.7.5) \quad \bar{\mu}_-^{(m)} = \mu_-^{(0)} + RT \ln a_-^{(m)} - F\varphi^{(m)}$$

describe the electrochemical potential for the anion. The chemical potentials in the reference state, $\mu^{(0)}$, are assumed to be identical in

the membrane phase and in the solution. (It is not permissible to make this assumption when the membrane is nonaqueous.) By introducing equations 3.7.2 through 3.7.5 into

$$(3.7.6) \quad \bar{\mu}_+ ' = \bar{\mu}_+^{(m)}, \quad \text{and} \quad \bar{\mu}_- ' = \bar{\mu}_-^{(m)}$$

and using the relation $a_+ ' a_- ' = (a_{\pm} ')^2$, it is found that

$$(3.7.7) \quad a_+^{(m)} a_-^{(m)} = (a_{\pm} ')^2.$$

If an additional assumption is made that the activity coefficients of all the ions are unity, equation 3.7.7 is transformed into 3.7.1.

Under the assumption that the ions behave ideally in the membrane phase, Teorell (1935), Meyer and Sievers (1936) and Schlögl (1954) derived mathematical expressions describing potential differences and ion fluxes across a membrane with imperfect co-ion exclusion (see Appendix). Some of the equations derived by these authors have been examined experimentally (see Teorell, 1953). Qualitatively, the experimental data agreed with the theory, which suggests that the theory is basically sound; quantitatively, however, there are some discrepancies between the theory and the experimental data. At present, these discrepancies are attributed to the nonideal behavior of ions in the membrane (see Hills *et al.*, 1961).

In aqueous solutions of polyelectrolytes, the behavior of the counter-ions is known to be far from ideal (Katchalsky, 1954; Rice and Nagasawa, 1961; Katchalsky and Alexandrowicz, 1963). In such solutions, the counter-ions tend to stay close to the charged sites of the polyelectrolyte, while the co-ions remain relatively far from the sites. For these reasons, Kobatake *et al.* (1965, 1966) have expressed the condition at the boundary between the bulk solution and the charged membrane in the following form:

$$(3.7.8) \quad \gamma_{\pm}^{(m)} = \frac{1}{1 + \bar{X}/c}$$

where c is the salt concentration in the bulk solution, \bar{X} is the fixed charge density in the membrane and $\gamma_{\pm}^{(m)}$ is the mean activity coefficient in the membrane. When this boundary condition was used instead of $\gamma_{\pm}^{(m)} = 1$, good agreement was obtained between the observed and calculated membrane potentials over a wide range of salt concentrations.

At present, there is no general analytical solution of the Nernst-Planck flux equations describing the nonideal behavior of three ions (e.g., Na, K and Cl) moving across a charged membrane. In the special case where the co-ion concentration is constant throughout the membrane, it is possible to solve the flux equations.

The constant-field equation derived by Goldman (1943) and by Hodgkin and Katz (1945) has been used by many biologists to describe the potential difference across membranes where three mobile ions are present (Na^+ , K^+ and Cl^-). This equation has the following form:

$$(3.7.9) \quad \Delta\varphi = \frac{RT}{F} \ln \frac{P_{\text{Na}}C_{\text{Na}}' + P_{\text{K}}C_{\text{K}}' + P_{\text{Cl}}C_{\text{Cl}}''}{P_{\text{Na}}C_{\text{Na}}'' + P_{\text{K}}C_{\text{K}}'' + P_{\text{Cl}}C_{\text{Cl}}'}$$

where P_i ($i = \text{Na, K or Cl}$) is the "permeability constant" of species i . Planck (1890, p. 571) previously obtained an equation of this form using mobilities instead of permeability constants.

It must be realized that the constant field equation has been derived without explicitly considering the possibility that φ , $\mu_i^{(0)}$ and a_i may undergo discontinuous changes at the boundary between the bulk solution and the membrane phase. Therefore, it is difficult to apply equation 3.7.9 to charged or nonaqueous membranes. In order to justify the use of this equation in biological membranes, it is highly desirable that the permeability constants be determined by independent measurements. No direct determinations of these constants have yet been made by nonpotentiometric means. In the practical application of this equation, it is important to note that the permeability constant may vary with the ionic composition of the bulk solutions. Furthermore, the existence of a multilayer structure and the presence of divalent cations in the medium produce important effects which cannot be treated by the constant field equation.

In concluding this chapter on physical chemistry of inanimate membranes, it should be emphasized that there has been substantial progress in membrane theories during the past two decades. The progress has been brought about primarily by advances in thermodynamics of irreversible processes, and in the synthesis of ion-exchange resins and polyelectrolytes. Membrane potentials and ion fluxes have been measured under various experimental conditions;

the results obtained have been compared with equations obtained by integration of the Nernst-Planck flux equations or of the basic flux equations in thermodynamics of irreversible processes. Readers who are interested in further details of these recent advances are referred to the reviews written by Teorell (1953), Helfferich (1962), Lakshminarayanaiah (1965) and Schlögl (1964).

Chapter IV

PROPERTIES OF THE AXON MEMBRANE IN THE RESTING STATE

ION FLUXES ACROSS AN AXON MEMBRANE AT REST

(4.1) During the past fifteen years, a number of investigators have examined ion fluxes across the squid axon membrane, both in the resting and excited states (Hodgkin and Keynes, 1955, 1957; Shanes and Berman, 1955; Caldwell and Keynes, 1960; Tasaki, Teorell and Spyropoulos, 1961; Brinley and Mullins, 1965; etc.). These investigations were carried out on excised axons without intracellular perfusion. In these radio-tracer experiments, the concentrations of the nonradioactive ions in the axon interior were not controlled, and intracellular metabolic processes were not eliminated. Therefore, it was difficult to estimate the magnitudes of ion fluxes (see Ch. II).

In recent years radiotracer measurements have been carried out during continuous intracellular perfusion with salt solutions of controlled chemical composition. When the internal perfusion fluid contains only potassium salts and the external medium is potassium free, the efflux of K ion can be faithfully traced by adding radioactive K^{42} ion to the perfusion fluid and measuring the flux of K^{42} across the axon membrane. Similarly, the fluxes of other ions in the system can be determined without ambiguity when there is no cation species common to both the internal and external media.

One of the simplest combinations of internal and external media for squid axons is an approximately 0.5 M solution of KF (inside) and a mixture of 0.4 M NaCl and 0.08 M CaCl₂ (outside). If the pH of the internal fluid is maintained at 7.3 ± 0.1 with a trace of phosphate buffer [and if a small amount of glycerol is added to maintain tonicity], the axon is able to produce action potentials for many hours. Radiotracer determinations of ion fluxes across the squid giant axon under these experimental conditions were performed quite recently in this laboratory (Tasaki, Singer and Watanabe, 1967).

The ion fluxes in the resting state of the membrane will be discussed in this section. (In this monograph the term *resting state* is used to designate the "unstimulated" state of *excitable* axons.)

The efflux of K ion under these conditions is estimated to be 1.5 to 2×10^{-10} moles per cm^2 of the membrane per sec, or 150 to 200 pmole· $\text{cm}^{-2} \cdot \text{sec}^{-1}$. The influx of Na ion is of the same magnitude as the efflux of K ion within the accuracy of present measurements. The influx of Ca ion (expressed in pmole· $\text{cm}^{-2} \cdot \text{sec}^{-1}$) is less than 1/20 of that of Na ion. At present, the influx of Cl ion is estimated to be roughly 1/10 of the univalent cation fluxes. When there is no electric current through the membrane,

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

Hence, it is inferred that the efflux of F ion is less than 1/10 of the univalent cation fluxes.

These results show that there is a large difference in cation and anion fluxes at comparable concentrations; cation fluxes are much greater than anion fluxes. This finding suggests that there is a layer which has a relatively high density of fixed negative charges in the axon membrane (Tasaki, Teorell and Spyropoulos, 1961). The existence of such a layer should reduce fluxes of anions by exclusion of co-ions and confer cation-exchanger properties on the membrane.

Michaelis (1933) was the first to suggest that biological membranes might contain fixed charges, and Teorell (1935) was the first to propose a quantitative theory for the charged membrane. Steinbach and Spiegelman (1943) were the first to demonstrate the approximate equality between the Na influx and K efflux in (unperfused) squid giant axons.

MEMBRANE RESISTANCE AND APPARENT CAPACITY

(4.2) When a rectangular pulse of electric current is passed through the membrane of an intracellularly perfused squid giant axon, a variation in the membrane potential is produced by the current. When the density of the current applied over a wide area of the membrane is small and uniform, the potential variation follows an approximately exponential time course described by

$$(4.2.1) \quad V(t) = IR_m(1 - e^{-t/R_m C_m})$$

$V(t)$ is the change in the membrane potential (expressed in volts) generated by a current of density I (expressed in amp/cm²); R_m and C_m are constants required to describe the time course (see Sect. 2.3). The constant R_m represents the resistance of a unit area of membrane; the constant C_m is called the “apparent membrane capacity.” When the applied current is suddenly interrupted, the membrane potential $V(t)$ approaches zero with an exponential time course given by

$$(4.2.2) \quad V(t) = IR_m e^{-t/R_m C_m}$$

At present, the physicochemical nature of the membrane capacity is obscure. It is usually assumed to be dielectric in nature (see Hodgkin and Huxley, 1952b). Although this assumption is reasonable, there are other possibilities which may account for at least part of the observed potential variations. Nernst (1908) pointed out that a current passed through a semipermeable membrane produces a capacitative effect, due to accumulation and depletion of electrolytes at and near the two membrane surfaces. Mauro (1962) suggested that the observed potential variation could be attributed to the existence of a layer with fixed negative charges in contact with an oppositely charged layer in the membrane. An electric current through such a double-layer membrane should produce a time-dependent potential change as the consequence of depletion or accumulation of ions in the junctional zone between the layers. Teorell (1960) considers the membrane capacity as arising from a transient change in the membrane resistance.

It is interesting that when weak alternating current is used to measure the membrane capacity, the observed value of the capacity varies with the frequency of the measuring a.c. Such a frequency dependence may be explained in terms of the multilayered structure of the axon membrane; when two leaky capacitors are connected in series, the resultant capacity is frequency dependent (see Schwan, 1957).

The apparent membrane capacity may be treated as an expression of a general relaxation phenomenon associated with variations of the “transference numbers” of the counter-ions and other mobile charges in the membrane. When a rectangular current pulse (I) traverses the membrane, the ion concentrations are altered at and near all the interfaces of the system where there are abrupt changes in the value of $z_i J_i / I$. (In this case the flux J_i of charge species i with valence z_i

is regarded as a function of time and space coordinates.) When a constant current is maintained, the system consisting of the membrane and the two aqueous solutions approaches a new stationary state. If the change in the system caused by the current is small, the time course of the approach to the new stationary state may be exponential. This treatment appears to be general enough to include all the possible mechanisms suggested above.

When a squid giant axon is immersed in a solution containing 0.4 M NaCl and 0.08 M CaCl₂ and internally perfused with 0.5 M KF solution, the excitable axon membrane shows a resistance of 1 to 2 kilohm·cm². Under these conditions the relaxation time, $R_m C_m$ is approximately 1 msec. These values are not different from those obtained from unperfused axons immersed in natural sea water (Cole, 1955; Taylor, 1963).

The membrane resistance is closely related to the interdiffusion flux, since both are determined by the mobilities and concentrations of ions in the main diffusion-barrier of the membrane (see Sect. 3.5 and Appendix). When the present estimate of the interdiffusion flux is combined with the membrane resistance stated above, the resistance flux product may be calculated.

The present experimental estimate of the rJ -product is

$$rJ = (2 - 4) \cdot 10^{-7} \text{ mole} \cdot \text{sec}^{-1} \cdot \text{ohm}.$$

This figure agrees fairly well with the expected rJ -product in a simple bi-ionic situation; i.e., RT/F^2 , or 2.62×10^{-7} mole·sec⁻¹·ohm. This close agreement strongly suggests that the axon membrane has a major diffusion barrier containing a relatively high density of negative fixed charges.

DILUTION OF ELECTROLYTES IN THE EXTERNAL MEDIUM

(4.3) In inanimate membranes, the effect of dilution of the electrolyte solutions on the bi-ionic (membrane) potential is relatively well understood (see Sollner *et al.*, 1954; Bergsma and Staverman, 1956). When the membrane consists of a layer of cation-exchanger with a uniform “selectivity-mobility ratio” product (but with a variable fixed charge density), the bi-ionic potential for two univalent counter-ions (see equation 3.3.9) is given by

$$(4.3.1) \quad \varphi'' - \varphi' = \frac{RT}{F} \ln \frac{a_2'}{a_1''} + \frac{RT}{F} \ln \frac{K_1^2 \bar{u}_2}{\bar{u}_1}$$

where the left-hand term represents the potential of the medium containing species 1 (with activity a_1'') referred to the fluid containing species 2 (with activity a_2'). The last term in equation 4.3.1 arises from the selectivity, K_1^2 , and the mobility ratio, \bar{u}_2/\bar{u}_1 , of the two univalent cations. A large number of experiments using various cation-exchanger membranes have fully demonstrated the validity of equation 4.3.1. It has been shown that dilution of the solution on one side of the membrane (without changing the fluid on the other side) does raise the potential of the diluted solution by about 58 mV for a ten fold reduction in the concentration (see Bergsma and Staverman, 1956).

When cation species 1 is univalent and species 2 is divalent, integration of the Nernst-Planck flux equations for a uniform cation-exchanger membrane yields the following equation (see Helfferich and Ocker, 1957; Appendix):

$$(4.3.2) \quad \begin{aligned} \varphi'' - \varphi' &= \frac{RT}{2F} \ln \frac{a_2'}{(a_1'')^2} + \frac{RT}{2F} \ln (2K\bar{X}) \\ &\quad + \frac{RT}{F} \frac{\bar{u}_1 - \bar{u}_2}{\bar{u}_1 - 2\bar{u}_2} \ln \frac{2\bar{u}_2}{\bar{u}_1} \end{aligned}$$

where K is the selectivity of species 2 over species 1, \bar{X} is the fixed charge density, and \bar{u} 's are the mobilities of the two counter-ions. (In the derivation of 4.3.2, K , \bar{X} and \bar{u}_2/\bar{u}_1 are assumed to be constant throughout the membrane.) The validity of equation 4.3.2 was examined by Helfferich and Ocker (1957), who used a sulfonic resin membrane separating solutions of strontium and sodium salts. Both the cation fluxes and the membrane potential were determined under these conditions, and the results obtained agreed with the theory. According to equation 4.3.2, dilution of the solution containing univalent cation (of activity a_1'') raises the membrane potential ($\varphi'' - \varphi'$) by about 58 mV for a ten fold reduction of the concentration. In contrast, dilution of the divalent cation salt solution lowers the potential by about 29 mV for a ten fold reduction in a_2' .

Compared to the dilution experiments using inanimate membranes, the effect of electrolyte dilution in squid giant axons is somewhat

complex. First of all, dilution must be performed by mixing the electrolyte solution with isotonic solutions of some nonelectrolyte (e.g., sucrose or glycerol). This procedure is essential to obtain reproducible results; the reason for this requirement appears to be that dilution with distilled water creates a large swelling pressure within the membrane, and permanently disrupts the normal membrane structure.

The effect of dilution of several external media (sea water, isotonic NaCl solution, etc.) was studied some years ago by Teorell and Spyropoulos (unpublished). Dilution by a factor of 10 lowered the potential inside the axon referred to the external medium by 30 to 40 mV.* This finding is consistent with the view that the squid axon membrane contains a layer with negative fixed charges. (Note that if the axon membrane behaved as an anion-exchanger, dilution of the external medium would decrease the intracellular negativity.)

Quite recently, the effect of dilution of the external medium was examined by using internally perfused squid giant axons (Tasaki, Watanabe and Lerman, 1967). An example of the results is shown in Figure 16. An extensively cleaned axon was internally perfused with a solution containing 300 mM KF and 30 mM K-phosphate (pH 7.3). The external medium was a rapidly flowing solution containing 200 mM MgCl₂. (This medium was prepared by mixing a 400 mM MgCl₂ solution with a 12 volume-percent glycerol solution, and subsequently adjusting its pH to 7.9 with a trace of "tris" buffer.) The external medium was then switched to a rapidly flowing 50 mM MgCl₂ (glycerol) solution. As can be seen in the figure, there was an immediate fall (increased negativity) in the intracellular potential referred to the potential of the external medium (measured with a saturated KCl-calomel electrode). When the original, concentrated MgCl₂ solution was reintroduced, there was a prompt rise in the intracellular potential. In most cases, the effect of dilution was completely reversible.

The magnitude of the potential change observed was 14 to 18 mV

* In this and following chapters, the quantities of the extracellular phase are denoted by a single prime ('') and those of the intracellular phase by double primes (''). The membrane potential $\Delta\varphi$ represents $\varphi'' - \varphi'$; i.e., the potential of the electrode in the intracellular fluid referred to that in the extracellular fluid. Ordinarily, a fall in the intracellular potential indicates an increase in the absolute value of the membrane potential, because $\Delta\varphi$ is usually negative.

for a four fold change in the concentration. This observed value may be compared with the value expected from equation 4.3.2, under the assumption that only a_2' is altered by the experimental procedure. The mean activity coefficient of 200 mM MgCl₂ solution is 0.49 and that of 50 mM MgCl₂ solution is estimated to be approximately 0.61

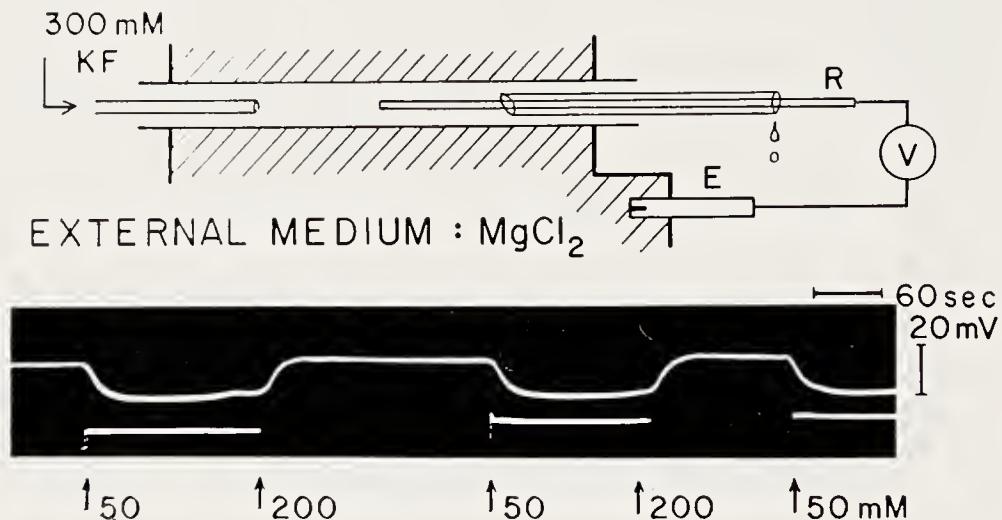


FIGURE 16. Dependence of the membrane potential of an internally perfused squid giant axon on the concentration of the external MgCl₂ solution. The rapidly flowing external solution was alternated between 200 mM and 50 mM. An upward deflection of the oscillograph beam indicates a rise (i.e., a decreased negativity) of the intracellular potential. (From Tasaki, Watanabe and Lerman, 1967).

(see Stokes and Robinson, 1948). The potential change (in mV) expected from equation 4.3.2. is approximately

$$\frac{25.2}{2} \ln \frac{200 \times 0.49}{50 \times 0.61} = 14.6$$

at 19°C. For this type of biological measurement, the agreement between the observed and theoretical values may be regarded as very good. This agreement strongly suggests that the chloride ion in the external medium is effectively excluded from the axon membrane. Co-ion exclusion is effective only when the density of the fixed charge in the membrane is high compared to the electrolyte concentration in the bulk solution (see equation 3.7.1). The anion con-

centrations in the experiment cited in Figure 16 were 0.1 and 0.4 equiv/l. The density of the fixed charge in the outer layer of the squid axon membrane appears to be high enough to exclude these moderately concentrated anions.

Similar results were obtained when CaCl_2 was used instead of MgCl_2 in the external medium. In the concentration range between 100 and 400 mM, replacement of Mg ion with Ca ion usually produced little or no change in the membrane potential. At lower concentrations, however, the membrane potential frequently became unsteady; the fluctuation of the membrane potential under these conditions was probably related to the process of action potential production (see Ch. V).

DILUTION OF ELECTROLYTES IN THE INTERNAL MEDIUM

(4.4) Dilution of the internal fluid medium was first examined by Tasaki, Watanabe and Takenaka (1962), and by Baker, Hodgkin and Shaw (1962). The original internal medium was a 400 to 500 mequiv/l K-salt; when this solution was diluted by a factor of ten, there was a fall in the resting potential of only about 10 mV instead of 58 mV. An example of the dilution experiments of this type is shown in Figure 17. The external medium used was natural sea water. The internal medium was a 1:10 mixture of sodium sulfate and potassium sulfate. Dilution was performed by addition of isotonic sucrose solution to the internal perfusion fluid. As can be seen in the lower part of the figure, the concentration dependence of the resting potential (i.e., the slope of the broken line) is far smaller than the Nernst slope (shown by the solid line). The axon remained excitable over the entire concentration range.

Hodgkin and Chandler (1965) explained this unexpectedly small change in the resting potential in terms of a variation in the ionic double layer on the inner surface of the axon membrane. However, the effect of such an ionic double layer was taken into consideration when the condition of continuity of the electrochemical potential at the surface of the charged membrane was introduced in deriving equations 4.3.1 and 4.3.2. It should also be noted that the equilibrium distribution of ions across a double layer does not contribute to

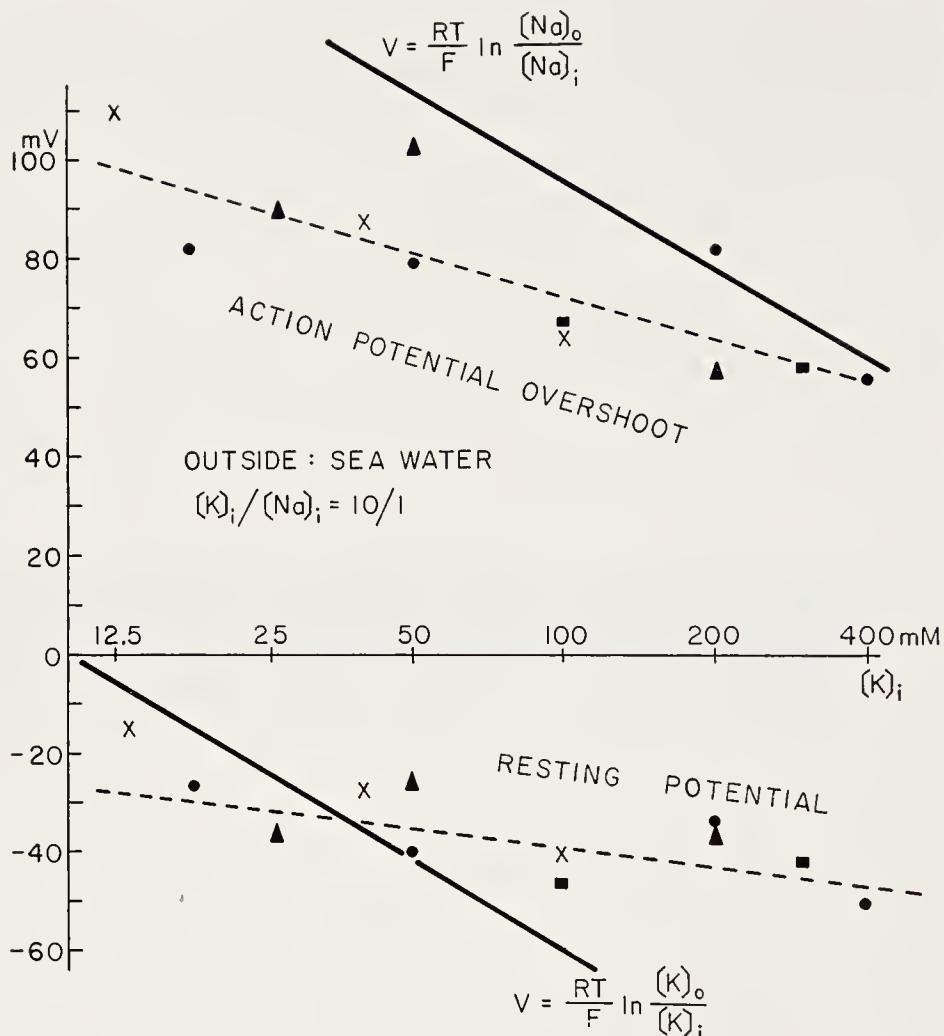


FIGURE 17. Resting and action potentials plotted against the salt concentration of the internal perfusion fluid. The ratio of potassium to sodium ion concentration inside was kept at 10:1. The concentration was varied with isotonic sucrose solution. The theoretical values of the membrane potential calculated by the two equations indicated are shown by the thick straight lines. [From Tasaki and Takenaka, *The Cellular Functions of Membrane Transport*, J. F. Hoffman (Ed.), 1964, reprinted by permission of Prentice-Hall, Inc., Englewood Cliffs, N. J.]

dissipation of the free energy associated with transfer of charges across the membrane (see Sect. 3.1).

The observed small potential change may be attributed to the effect of interdiffusing cations and to the existence of an additional unstirred layer adjacent to the major diffusion barrier with a high

fixed charge density. If there is a layer of colloidal material with a relatively low fixed charge density on the inner side of the major diffusion barrier, the effect of dilution of the internal medium should be far smaller than the effect of external dilution (see Fig. 15).

EFFECTS OF SUBSTITUTION OF ANIONS AND CATIONS IN THE MEDIA

(4.5) Under ordinary experimental conditions, the major anion in the external fluid medium for squid giant axons is chloride. Replacement of the chloride ions with bromide or sulfate does not usually change the resting membrane potential; the excitability of the axon also remains practically unaffected by this replacement. These findings again support the view that the major diffusion barrier in the axon membrane has cation-exchanger properties and excludes anions. Anions which precipitate the divalent cations (e.g., fluoride, phosphate, citrate) reduce both the resting and action potentials of the axon. Divalent cations in the external medium are indispensable for the maintenance of excitability; this point will be discussed in Chapters V and VII.

Substitution of one anion for another in the internal perfusion fluid often produces complex, delayed effects. Substitution generally produces very little or no immediate change in the resting potential or membrane resistance in freshly excised axons, but the survival time of the perfused axon is affected enormously by the nature of the internal anion (see Sect. 6.2).

The effect of replacement of the K ion in the internal fluid medium with Na ion was investigated at an early stage in the development of the perfusion technique (Baker, Hodgkin and Shaw, 1962; Tasaki, Watanabe and Takenaka, 1962). An increase in the internal Na concentration often leads to a rapid loss of excitability; this loss can largely be prevented by increasing the divalent cation concentration in the external medium (Tasaki and Shimamura, 1962) and/or by using Na- and K-salts of a favorable anion (i.e., fluoride, glutamate, etc.) in the internal medium (see Sect. 6.3). Under these favorable conditions, substitution of Na ion for internal K ion produces little or no change in the resting potential (Tasaki and Takenaka, 1963; Tasaki, Luxoro and Ruarte, 1964). This finding indicates that $Q_{K^{Na}}$, the product of the selectivity coefficient and the mobility ratio,

is close to unity for the inner surface of the axon membrane. (Under unfavorable conditions, $Q_{K^{Na}}$ can not be determined because of rapid deterioration of the axon.)

The effect of replacement of the internal K ion with Rb or Cs ion varies with the divalent cation concentration in the external medium. If isotonic solutions of RbF or CsF are used for internal perfusion of axons, action potentials are often generated without stimulation (i.e., without electric currents from external sources); this "spontaneous" production of action potentials can be prevented either by raising the divalent cation concentration in the external fluid medium, and/or by dilution of the internal RbF or CsF solution. Internal perfusion solutions of dilute CsF or RbF are extremely favorable for maintaining the excitability of the axons; under these conditions the membrane resistance is high and the magnitude of the resting potential is relatively small (Tasaki, Singer and Watanabe, 1965).

Addition of various divalent cations to the internal fluid medium frequently leads to rapid and irreversible loss of excitability (Grundfest, Kao and Altamirano, 1954; Tasaki, Watanabe and Takenaka, 1962). In the axon interior, divalent cations have a strong influence on the labile structure of the excitable membrane (see Sect. 6.3).

The effect of replacement of external Na ions with K ions has been the subject of repeated investigation since the time of Biedermann (1895, p. 303). Hodgkin (1951) summarized the results of such replacement in unperfused axons and other excitable tissues. This effect has also been studied in internally perfused axons, and the results obtained are similar to those in unperfused axons. An experimental example is shown in Figure 18; the concentration ratio (KCl:NaCl) in the external medium was varied, while the sum of the two salt concentrations was held constant at 400 mM. In addition to NaCl and KCl, the external medium contained 150 mM MgCl₂ and 50 mM CaCl₂. The internal medium was a 400 mM KF solution (also containing phosphate buffer and glycerol).

As can be seen in the figure, the membrane potential of the squid giant axon is not appreciably affected by an increase in the external K-concentration from zero to about 30 mM. At about 50 mM KCl outside, there was a slight rise in the intracellular potential and the ability to develop action potentials was lost. When the external K-concentration was increased further, the membrane potential was

found to vary with the logarithm of the external K ion concentration; for a ten fold increase in the external K-concentration there was approximately a 50 mV rise in the intracellular potential.

It is important to note that the membrane potential varies with

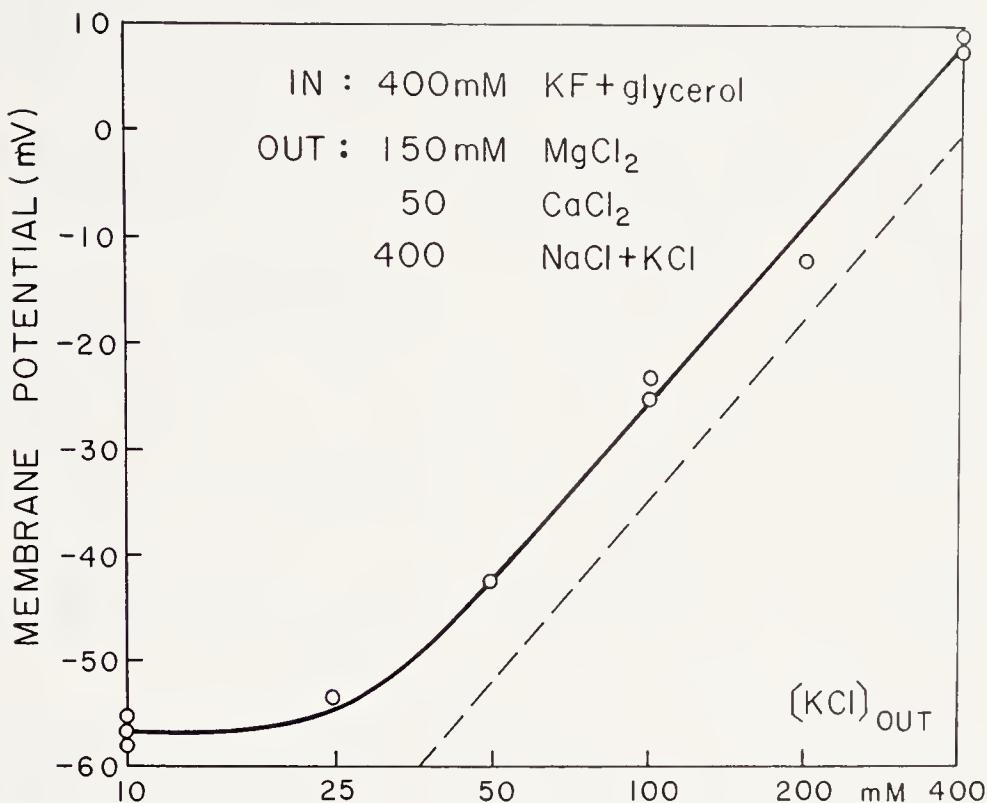


FIGURE 18. Membrane-potential of a squid giant axon plotted against the KCl concentration in the external fluid medium. The axon was internally perfused with 400 mM KF solution. The composition of the external fluid medium is given. The broken line indicates the value described by

$$V = (RT/F) \ln [K]_{\text{out}}/[K]_{\text{in}}$$

the external potassium concentration logarithmically only when the axon is rendered inexcitable by the increased potassium concentration. Axons immersed in potassium-rich media have the following properties: (1) the magnitude of the membrane potential is decreased, (2) the membrane resistance is low, and (3) the excitability is depressed. An axon is said to be steadily *depolarized* when it has these properties. *Depolarization* can be produced by external application of cesium, rubidium, or ammonium salts also. In axons immersed

in a solution of sodium and calcium salts, depolarization can be produced by decreasing the calcium ion concentration. The depolarizing action of univalent cations can be counteracted by increasing the divalent cation concentration in the external medium.

In Section 4.3, evidence for the existence of negative fixed charge in the axon membrane was presented. Under the conditions of the experiments shown in Figure 18, the negative sites in the axon membrane are occupied by Na, Ca and K ions. The fraction of the sites occupied by K ion increases when a portion of the Na ion is replaced with K ion. Therefore, depolarization of the membrane by K-ion can be attributed to an increase in the fraction of the negative sites occupied by K ion (see Sect. 5.1). The fact that the membrane potential is dominated by K ion in the depolarized state strongly suggests a high selectivity (see Sect. 3.3) of the membrane for K ion in this state.

As stated above, the resting state is defined as the unstimulated state of an axon membrane capable of producing action potentials. According to this definition, a depolarized membrane is not in its resting state. Analysis of the process of transition from the resting state to the depolarized state is one of the major problems discussed in this monograph (see also Ch. VI).

Depolarized axons are inexcitable in that they are incapable of producing action potentials when stimulated by an outward membrane current. However, depolarized axons can produce physiological responses when stimulated by inward currents (Stämpfli, 1958; Segal, 1958; Tasaki, 1959). The effect of such inward membrane current will be discussed briefly in the following section.

OBSERVATION OF ABRUPT DEPOLARIZATION

(4.6) In this section, several observations will be described to demonstrate that the process of depolarization by external application of some univalent cations (e.g., potassium) is often extremely rapid. The first observation of this phenomenon was reported by Hill and Osterhout (1938) and by Osterhout and Hill (1938), who investigated the effect of K ion on the membrane potential of a freshwater alga, *Nitella*. The top record in Figure 19 shows the effect of a stepwise increase of the external K ion concentration on the membrane potential of this excitable plant.

When the K ion concentration outside a *Nitella* cell was raised from 0.1 mM to 0.316 mM, there was a transient rise in the intracellular potential (relative to the potential of the external medium) of about 7 mV. When the concentration was again raised by a factor of 3.16, the intracellular potential initially rose by about 15 mV,

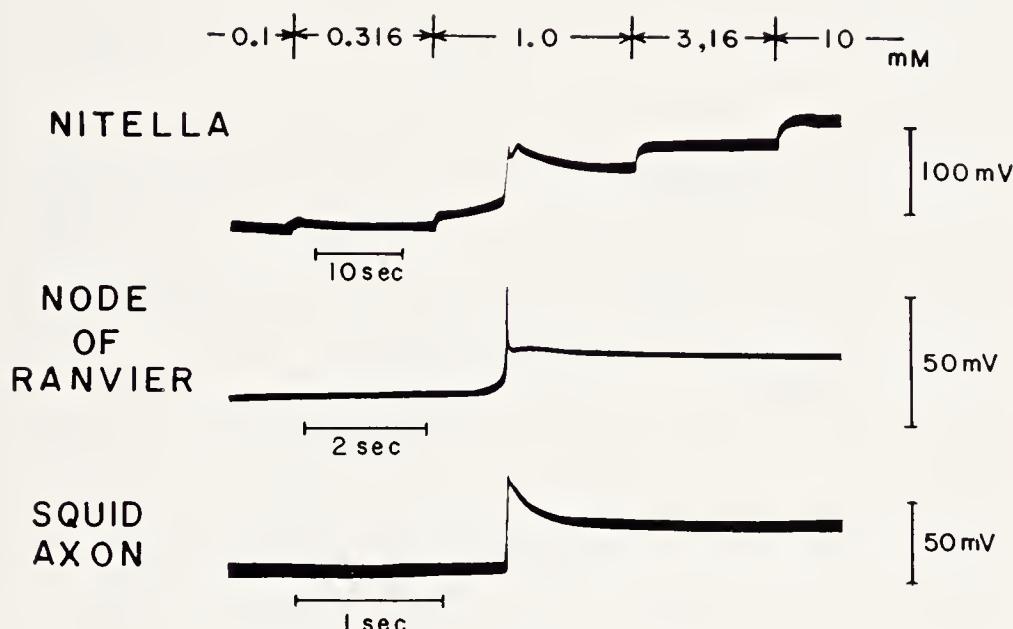


FIGURE 19. Abrupt depolarization by external application of KCl solution.

Top: Effect of stepwise increase in the external KCl concentration on the membrane potential of *Nitella*; the KCl concentrations used are given at the top of the figure (redrawn from Hill and Osterhout, *J Gen Physiol* 22:139-146, 1938). *Center:* Abrupt depolarization in the node of Ranvier, treated with 0.4 mM NiCl₂, brought about by a gradual increase in the external KCl concentration from 15 mM to 30 mM (adapted from Tasaki, *J Physiol* (London), 148:306-331, 1959).

Bottom: Abrupt depolarization caused by external application of KCl in squid giant axon intracellularly perfused with 0.1 M CsF solution. The external KCl concentration was raised slowly from approximately 30 mM to 60 mM.

followed by a large and abrupt rise in the intracellular potential. A further increase in the K ion concentration changed the membrane potential without any discontinuous jump; the change was approximately 29 mV for 3.16-fold change in the K-concentration. Blinks (1933) had observed a similar phenomenon in a large seawater plant cell, *Halicystis*, treated with NH₄ ion. These two findings appeared

to create serious problems for the investigators who interpreted the bioelectricity in plant cells on the basis of the classical membrane theory.

According to Bernstein's membrane theory (Bernstein, 1912), the membrane potential, E_r , across a biological cell membrane at rest is given by

$$(4.6.1) \quad E_r = \frac{RT}{F} \ln \frac{[K]_e}{[K]_i}$$

where the bracketed quantities represent the intra- and extracellular K ion concentrations. This formula predicts a 29 mV change for a 3.16-fold increase in the extracellular concentration; experimentally, a change ranging roughly from 75 mV (steady level) to 110 mV (at the peak) was actually observed when $[K]_e$ was raised from 0.316 mM to 1 mM. The relationship between the membrane potential and the external potassium ion concentration for *Nitella* is shown in Figure 20. The slope of the curve is close to the theoretical value in the range above 1 mM. Between 0.316 and 1 mM, however, the observed slope exceeded the theoretical slope given by (4.6.1); this indicates that the phenomenon of abrupt depolarization cannot be explained in terms of the classical membrane theory.

The second report of *abrupt depolarization* by external application of K-salts came from investigations of the node of Ranvier treated with traces of nickel or cobalt salts (Tasaki, 1959). An example of the records obtained from the node is reproduced in the middle of Figure 19. Initially, the node under study was immersed in a Ringer's solution containing 15 mM KCl (and 0.4 mM NiCl_2). Then the KCl concentration in the medium was raised slowly (and continuously) by introducing a new Ringer's solution containing 30 mM KCl (and 0.4 mM NiCl_2) at a corner of the pool in which the node was immersed. Equation 4.6.1 predicts no discontinuous change in the membrane potential when the potassium concentration is increased continuously; but, actually a potential jump of the order of 30 mV was observed under these conditions. After this sudden jump in the membrane potential, a further increase in the K ion concentration never produced another sudden change in potential. If the external divalent cation (e.g., Ca) concentration was increased immediately after this jump, the membrane potential often returned discontinuously to the original level.

The most recent report of abrupt depolarization by univalent cations (K, Rb, or NH₄) came from investigations of the squid giant axon internally perfused with favorable salt solutions (e.g., 100 mM solutions of RbF or CsF; Tasaki, Singer and Watanabe, 1966). The external solution was usually a mixture of 100 mM CaCl₂ and 450

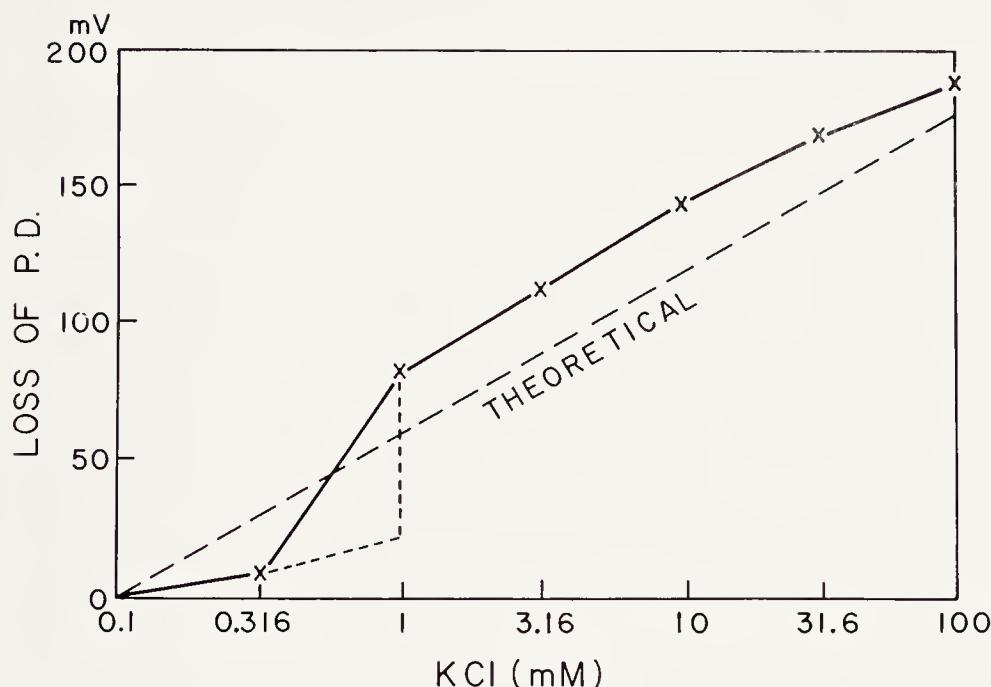


FIGURE 20. Effect of KCl on the membrane potential of *Nitella*. The broken line represents the Nernst slope for the external K ion. Note that between 0.316 and 1 mM the change in membrane potential exceeds the theoretical slope. Note that the relationship between the KCl concentration and the membrane potential is given by two separate, approximately straight lines of which the slopes do not exceed the theoretical value. (Hill and Osterhout, *J Gen Physiol*, 22:139-146, 1938.)

mM tetrathylammonium chloride at the onset of the experiment. A 600 mM solution of KCl was added to the medium in very small aliquots. When the K ion concentration reached a level between 50 and 100 mM, a sudden 30 to 70 mV rise in membrane potential was observed. An example of the records obtained by this method is presented in Figure 19, bottom. As in the cases of *Nitella* and nodes of Ranvier, once this abrupt change occurred, a further increase in the external KCl concentration did not produce another abrupt potential change. When the KCl concentration was lowered imm-

diately after this abrupt change had occurred, the membrane potential often returned abruptly to the original level.

As originally suggested by Hill and Osterhout (1938), this abrupt depolarization is comparable to the process of initiation of an action potential. The major difference between an ordinary action potential and the potential jump discussed in this section is that the potential level reached by an abrupt depolarization is usually permanent. If the ratio of the uni- and divalent cation concentrations in the external medium is lowered, or if inward membrane current is passed, the potential level may return abruptly to the resting level. The physiological response produced in a potassium-treated axon by an inward current is an expression of this abrupt return of the membrane potential (see Tasaki, 1959). In Chapter VII, the phenomenon discussed in this section will be interpreted in terms of a "phase transition" of the membrane macromolecules.

Finally, a comment should be made on the continuous curve relating the membrane potential to the external K ion concentration (Fig. 18). It is reasonable to assume that the properties of a giant axon membrane are not perfectly uniform over the entire membrane, and that different parts of the membrane may become depolarized at different K ion concentrations. When this nonuniform depolarization takes place, the axon membrane can be said to be in a *mixed state* which consists of small areas or patches of depolarized membrane surrounded by resting areas.

The mixed state is schematically illustrated in Figure 21, left. As the external K ion concentration is increased, the depolarized fraction of the membrane area is expected to increase. At a sufficiently high potassium concentration, the membrane eventually becomes uniformly depolarized. In the absence of depolarizing univalent cations (or in the presence of a high concentration of Mg or Ca ion) in the medium, the membrane may be considered to be in a uniform resting state. The difference between axons showing abrupt depolarization and those giving rise to no distinct discontinuity may derive from the following two factors: (1) the difference in the degree of membrane uniformity; and (2) the difference in the strength of interactions between different parts of the axon membrane. It is extremely difficult to demonstrate abrupt depolarization in partially injured axons; this fact indicates the importance of the spatial uni-

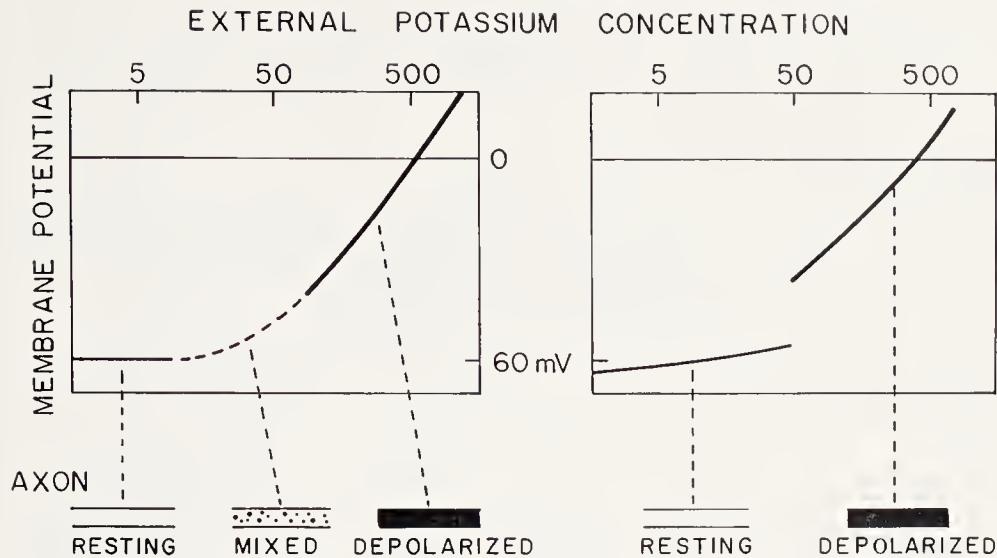


FIGURE 21. Schematic diagram showing different states of the squid axon membrane (bottom) for an axon which gives no discontinuous potential change (left) and for an axon showing abrupt depolarization by KCl (right). The relationship between the membrane potential and the external K-concentration for two kinds of axons are given. The uniformly depolarized state is shown by black, the resting state by white, and the mixed state by black (depolarized) spots surrounded by the white (resting) zones. The right diagram in this figure corresponds to Figure 20 for *Nitella*.

formity of the axon membrane, although no membrane is perfectly uniform. The importance of the short- and long-range interactions in the axon membrane will be discussed in detail in Chapter VIII; the relationship between abrupt depolarization and the process of excitation will be discussed in the following chapter.

Chapter V

THE AXON MEMBRANE IN THE EXCITED STATE

IN THE preceding chapter, evidence for the existence of negative fixed charges in the axon membrane was presented. The term *depolarization* was introduced to designate transformation of the axon membrane caused by various univalent cations. In a depolarized state, the axon has (1) a relatively small resting potential, (2) a small membrane resistance and (3) depressed excitability. It was shown that the process of depolarization is very abrupt under certain experimental conditions. Abrupt depolarization was interpreted as representing initiation of an action potential. When a potassium salt is applied externally, an ion-exchange process involving potassium ion and the external cations (Na and Ca) takes place in the axon membrane. Thus, depolarization by potassium salts was attributed to an increase of the fraction of the negative sites in the axon membrane occupied by potassium ion.

The relationship between abrupt depolarization and the normal action potential will be discussed in this chapter. It will be recalled that the potassium salt normally present in the axon interior is a strong depolarizing agent. It will be shown that an ion-exchange process involving the internal cation (K) and the external cations (Ca and Na) can take place when a pulse of outward-directed current is applied to the axon membrane. The relationship between the membrane resistance and ion fluxes in the excited state will be discussed in the later sections of this chapter.

ELECTRIC STIMULATION OF THE SQUID GIANT AXON

(5.1) The excitability of a squid giant axon can be maintained for hours with an external medium of 400 to 500 mM NaCl and about 70 mM CaCl₂, and an internal perfusion fluid consisting of 400 to 500 mM KF. Delivery of an outward electric current through the axon membrane can produce a large transient potential variation called

the *action potential*. The action potentials produced under these conditions closely resemble those observed in unperfused squid giant axons.

Figure 22, left, shows a typical tracing of the action potential evoked by an outward membrane current. When a rectangular current, $I(t)$, is applied, the membrane potential, $V(t)$, begins to change along an approximately exponential time course. This exponential potential change is the consequence of the resistance and apparent capacity of the membrane in the resting state (see Sect. 2.3 and

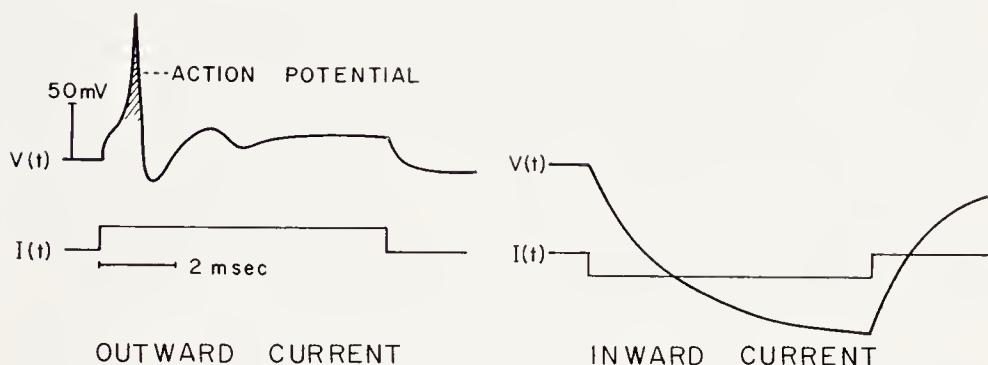


FIGURE 22. Effects of rectangular pulses of outward (left) and inward (right) membrane current of the same intensity on the intracellular potential in the squid giant axon. $I(t)$ represents the time course of the membrane current and $V(t)$ the change in the membrane potential induced by the current pulses.

Sect. 4.2). When the potential of the intracellular fluid (relative to that of the external medium) is raised 20 to 30 mV above the resting level by the applied current, an action potential is initiated. Figure 22, right, shows that an inward membrane current only produces an approximately exponential potential change.

The process of initiation of an action potential in these axons can now be discussed from a physicochemical viewpoint. In the preceding chapter, it was concluded that the major diffusion barrier in the axon membrane has cation-exchanger properties and that the fixed charge density is high enough to effectively exclude 400 mM of anions in the medium (Sect. 4.3). In a cation-exchanger with a high charge density, divalent cations are known to be preferentially absorbed at the negative sites (see Appendix). Thus, the major barrier contains a relatively high concentration of divalent cations. [The preference of the negatively charged sites of a cation-exchanger for

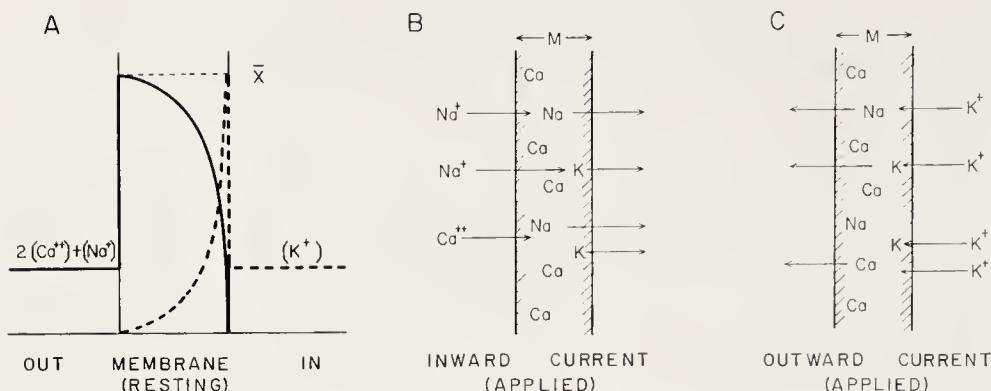


FIGURE 23. A: Schematic diagram showing concentration profiles of cations in the membrane; the axon membrane is assumed to have a macroscopic thickness and a uniform fixed-charge density in the resting state.

B: Diagram showing that an inward-directed membrane current tends to increase the concentrations of the external cations (Na and Ca ions in this case) in the membrane.

C: Diagram showing replacement of the external cations in the membrane with K ion by an outward-directed membrane current.

divalent cations over univalent cations is often called *electroselectivity* (see Helfferich, 1962, p. 156).]

The distribution of the cations in the critical layer of the membrane is shown schematically in Figure 23A. This diagram was constructed under the assumption that the density of the fixed charges is uniform and the layer with negative fixed charges is macroscopic in size. In diagrams B and C, the layer is assumed to be of molecular dimensions. (For the present discussion the thickness of the layer is unimportant.) In these diagrams, the negative sites in the membrane are assumed to be occupied by Na and Ca ions. It can be inferred from the experiment described in the preceding section (4.5) that the major counter ion species in the critical layer of an excitable membrane are Ca and Na ions rather than K ions. It was shown that in a K-rich external medium, the axon membrane is incapable of developing action potentials. In such an inexcitable state, the membrane potential is dominated by the K ion (see Fig. 18); this indicates that the negative sites in the axon membrane are occupied predominantly by K ion because of the high selectivity (see Sect. 3.3) of the membrane for K ion. Since the axon becomes inexcitable

when the negative sites in the membrane are occupied predominantly by K ion, the major cations in an *excitable* axon membrane must be either Na or Ca ions.

Further evidence that the negative sites in the excitable membrane are occupied mainly by the external cations is presented in Figure 23B. An inward current drives external cations (Na and Ca) into the membrane and tends to increase the fraction of the negative sites occupied by the external cations (see Fig. 23B). When most sites are occupied by these cations before application of the current, the effect of an applied inward current on the concentration profile is small. Under these conditions (the concentration profile remaining practically unaffected by the applied current), the relationship between the membrane potential and the current is expected to obey Ohm's law; this is actually the case when currents are passed inward through the membrane (see Fig. 25 in Sect. 5.3).

A strong current directed outward through the membrane carries the internal cations (K ions in this case) into the membrane (see Fig. 23C). Simultaneously, Ca and Na ions occupying the negative sites in the membrane are transferred to the external fluid medium. If the current is maintained, the membrane should eventually become rich in K ions and become depolarized.

It will be recalled that under favorable conditions, an increase in the fraction of the negative sites in the membrane occupied by K ions evokes an abrupt variation in the membrane potential (Sect. 4.6). When the K ion concentration in the membrane is increased by external application of potassium salts, the potential of the axon interior (relative to that of the external medium) is often found to rise abruptly. It is evident that a similar increase in the intramembrane K ion concentration can be brought about electrophoretically by passage of an outward membrane current. An electric current can raise the intramembrane K ion concentration more rapidly (and uniformly) than external application of a K rich solution. Therefore, delivery of an outward current is expected to be a much more effective means of inducing an abrupt depolarization. In Chapter IV it was argued that an abrupt potential change is nothing but the beginning of an action potential. Thus, initiation of an action potential by an electric stimulus may be regarded as an abrupt depolarization

resulting from replacement of some fraction of the external cations (Na and Ca) at the negative sites in the membrane by the internal cation (K). A further discussion of this point will be postponed until the role of divalent cations in excitation is clarified in Chapter VII.

In contrast to rectangular current pulses, when the applied current increases slowly, the K ion concentration in the membrane increases slowly. So far as the ion-exchanger process in the membrane is concerned, this situation is analogous to a slow, gradual increase in the external K ion concentration. Under ordinary experimental conditions, the latter procedure depolarizes the axon without producing an action potential (see Fig. 18). Similarly, a slowly increasing outward current usually depolarizes the axon without evoking an action potential. In classical neurophysiology (see Nernst, 1908), the fact that a slowly increasing electric current fails to evoke an action potential is known as *accommodation* (see Tasaki, 1958).

REDUCTION OF MEMBRANE IMPEDANCE DURING EXCITATION

(5.2) To demonstrate changes in the membrane impedance during excitation, two different electrode arrangements have been used. One arrangement used small platinized platinum electrodes pressing the surface of the axon membrane (Cole and Curtis, 1939). The other electrode arrangement is illustrated in Figure 24, top. A long intracellular wire electrode and a large extracellular wire electrode were connected to an impedance bridge, operated at an a.c. frequency of about 20 kc/sec. The short internal wire electrode was used to record the action potentials of the axon.

No Na ions were present in the experiments shown in Figure 24; the external medium contained a mixture of 300 mM hydrazinium chloride and 200 mM CaCl_2 in the experiment shown on the left, and a mixture of guanidinium chloride, tetramethylammonium chloride, and CaCl_2 in the experiment shown on the right. The K-free internal perfusion fluid contained 100 mM RbF (glycerol) solution. In these Na-free, K-free media, the squid axon membrane maintained its ability to produce all-or-none action potentials for more than one hour (Tasaki, Singer and Watanabe, 1965).

Initially, the impedance bridge was balanced for the membrane in

the resting state. When a stimulating electric shock was delivered to the axon under these conditions, an action potential and a simultaneous transient unbalance of the impedance bridge were observed (Fig. 24, left records). Then, the impedance bridge was adjusted to obtain the best balance at the peak of the action potential (right records).

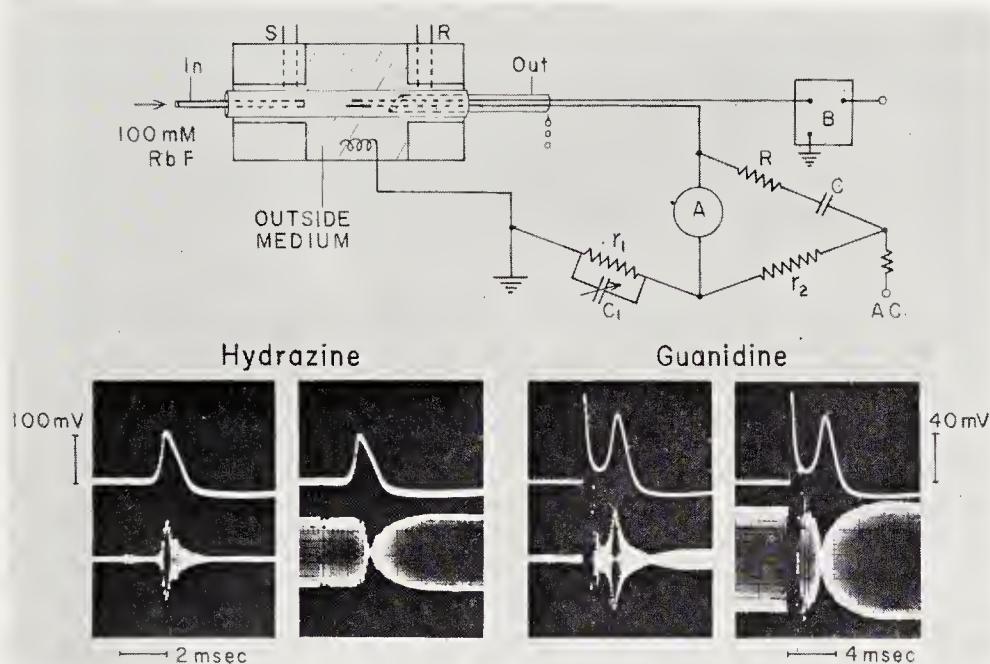


FIGURE 24. Changes in the membrane impedance during excitation. Squid giant axons were internally perfused with a 100 mM RbF solution (containing a small amount of phosphate buffer). The external medium contained hydrazinium chloride and CaCl_2 (left), or guanidinium chloride and CaCl_2 (right). The impedance bridge was balanced either in the resting state or at the peak of excitation. (From Tasaki, Singer and Watanabe, *Amer J Physiol*, 211:746-754, 1966.)

By this method, it was found that production of an action potential is invariably associated with a reduction in the membrane impedance. The impedance loss is largest at the peak of the action potential. There is no simple relationship between the shape of the action potential and the time course of the impedance bridge unbalance. When the action potential duration is relatively short (Fig. 24, left), the impedance loss outlasts the action potential. When the

action potential shows a long, slowly declining plateau, however, the unbalance of the impedance bridge may diminish before the end of the plateau of the action potential (Tasaki and Hagiwara, 1957).

The significance of these observations will be discussed in Chapter VII.

OHM'S LAW APPLIED TO AN AXON MEMBRANE AT THE PEAK OF EXCITATION

(5.3) The production of an action potential is a time-dependent process in a multi-ionic system. Therefore, it is difficult to speak of the ohmic resistance of the membrane at the peak of excitation. Measurement of the ohmic resistance may be justifiable if the membrane potential varies very slowly compared to the time required for measurement. Note that during excitation there is a profound reduction in the membrane impedance and consequently that the relaxation time (time constant) of the membrane is far shorter than that in the resting state. Under ordinary experimental conditions, the duration of the action potential is approximately 1 msec, and the time constant is about 0.01 msec. Therefore, the ohmic resistance of the membrane during excitation may be determined by using weak rectangular current pulses with a duration of about 0.1 msec (which is much longer than the time constant, but significantly shorter than the action potential duration).

An alternative means of measuring the membrane resistance frequently used in recent years is the voltage clamp method (Hodgkin, Huxley and Katz, 1952; Cole and Moore, 1960). With this device, the intracellular potential (referred to the potential of the external medium) is suddenly shifted from the resting level to a preselected level, and the membrane current is recorded as a function of time. It is relatively easy to measure the membrane resistance at the peak of excitation by these means.

The membrane resistance at the peak of excitation will be denoted by r_a , and the intracellular potential for zero membrane current at this moment by E_a . Then, Ohm's law applied at the peak of excitation is

$$(5.3.1) \quad I = \frac{1}{r_a} (V - E_a).$$

When the potential V , at which the intracellular potential is clamped, is smaller than E_a ($V < E_a$), I is negative; i.e., the current is directed inward through the membrane. Since the resistance, r_a , reaches a minimum at the moment when the *emf*, E_a , reaches a maximum (see

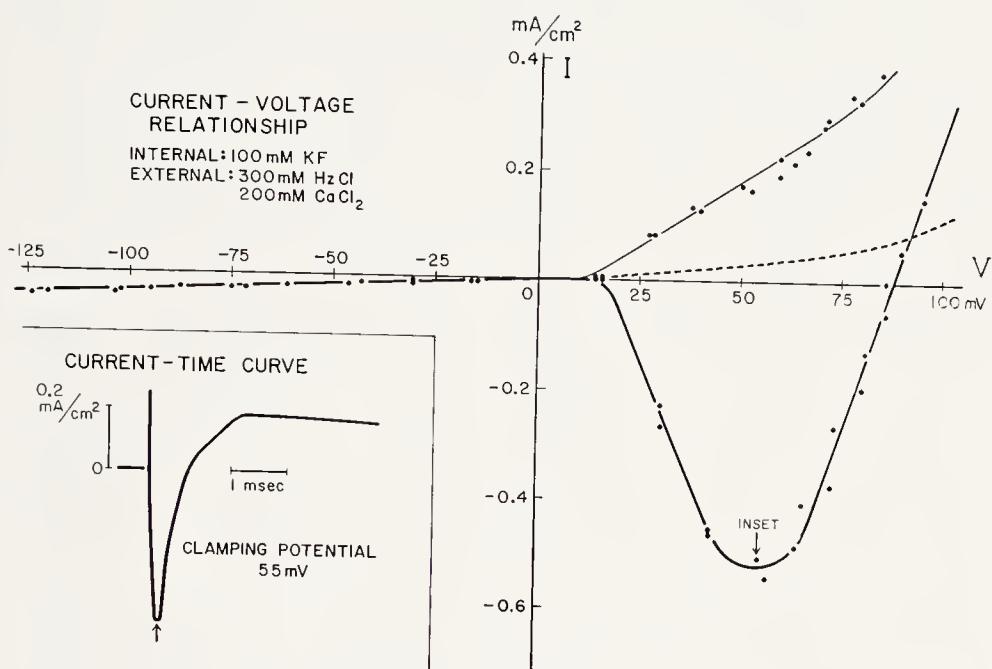


FIGURE 25. Current-voltage relationship for the squid axon membrane determined by the voltage clamp technique. The compositions of the internal and external media are indicated. The thick line indicates the relationship at the peak of excitation. The thin, continuous line represents the I - V relationship determined 4 msec after the onset of the clamping pulse. The broken line shows the I - V relationship after application of tetrodotoxin (measured at the moments when the maxima of inward currents were reached previously).

Inset: Tracing of the membrane current at a clamping potential of 55 mV. (From Tasaki and Singer, *Ann NY Acad Sci*, 137:792-807, 1966.)

Fig. 25), the inward current, $-I$, attains a maximum at the peak of excitation. Therefore, the validity of equation 5.3.1 can be examined by plotting the maximum value of the inward current as a function of the clamping potential, V .

An example of the experiments carried out by this method is presented in Figure 25. The external medium contained a Na-free

mixture of 300 mM hydrazinium chloride and 200 mM CaCl_2 ; the internal medium was a 100 mM KF solution. When the maximum value of the inward current was plotted against the clamping potential V , an approximately linear relationship was obtained near $I=0$. The results obtained with Na-free media are very similar to the previous results using Na-containing media (see Hodgkin, Huxley and Katz, 1952).

In the biological literature, the term *sodium current* is used almost synonymously with the inward current. Although the major cation species in the external medium is usually Na ion, the inward current cannot be regarded as being carried solely by sodium. There is significant interdiffusion of cations across the membrane in the absence of electric current (see Sect. 5.4). Therefore, a (net) inward current through the membrane represents one of the following three alternatives: (1) an increase in the influx of the external cations; (2) a decrease in the efflux of the internal cations; or (3) a combination of both (1) and (2). At present, it seems most probable that both decreased cation-effluxes and increased cation-influxes are involved (Tasaki and Singer, 1966). A further discussion of this point will be presented in Chapter VII.

The voltage clamp method for determining the membrane resistance at the 'peak of excitation' is far more reliable than the a.c. impedance method described in the preceding section. In axons immersed in a solution containing Na- and Ca-salts and internally perfused with a K-containing solution, the membrane resistance often falls by a factor of 100 to 200 at the peak of excitation. In the experiment shown in Figure 25, the membrane resistance fell from a resting state value of 1,500 ohm · cm² to about 17 ohm · cm².

When the membrane resistance is drastically reduced during excitation, the resistance of the layer of perfusion fluid near the membrane is no longer negligible as compared with the observed overall membrane resistance. With the electrode arrangement shown in Figure 7B, the resistance of the axoplasm, or perfusion fluid, is included in the measured value of the membrane resistance; this series resistance is estimated to be of the order of 4 ohm · cm² when 100 mM KF solution is used as perfusion fluid.

CATION FLUXES DURING EXCITATION

(5.4) Under continuous internal perfusion with a solution containing no ions present in the external medium, measurements of ion effluxes by the radio-tracer technique is direct and unambiguous. However, this method is limited by poor time resolution when applied to the axon membrane in the excited state. The time resolution in efflux measurements is determined by the process of ion diffusion across the layers of connective tissue and Schwann cells outside the axon membrane proper.

Spyropoulos, Tasaki and Hayward (1961) attempted to increase

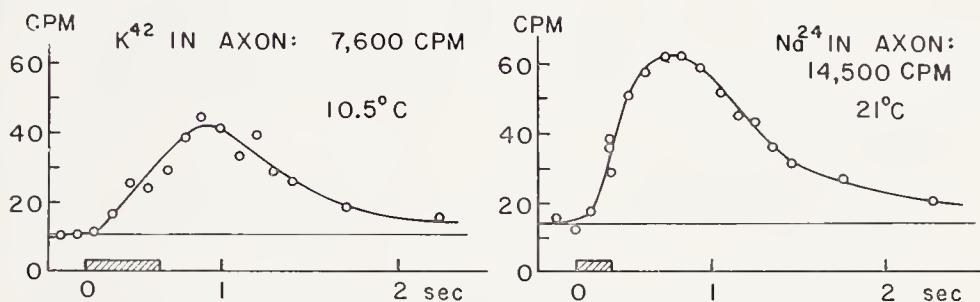


FIGURE 26. Effluxes of K⁴² (left) and Na²⁴ (right) through the axon membrane. The radioactive tracers were injected into cleaned axons. The sea water running outside the axon was collected in approximately forty small chambers. The shaded area on the base line indicated the period during which fourteen electric shocks were delivered to the axons.

the time resolution of efflux studies. With a rapidly flowing external medium (sea water), radioactive samples of the external medium were collected in a large number of small chambers moving at a constant speed. Stimulating current pulses were initiated by a photo-electric cell rotating with the collecting chambers. Figure 26 shows two examples of the results obtained by this method.

Radioactive solutions containing either K⁴² or Na²⁴ were injected into extensively cleaned axons. Since the effect of a single train of nerve impulses could not be detected, the effects of multiple trains were superposed until detectable levels of radioactivity were reached. There was a gradual increase in the effluxes of radioactive cations during repetitive stimulation (Fig. 26). When stimulation was termi-

nated, the efflux of radioactive cations gradually fell. The observed delay in the efflux of radio tracers may be attributed to the time required for diffusion through an unstirred layer of about 50μ in thickness. There was no significant difference between efflux time courses of Na^{24} and K^{42} .

In spite of the limitations of the radio tracer technique, the amount of extra cation flux associated with production of a single action potential can be determined unambiguously by the following standard technique (Fig. 27). The average efflux of K ion during a collection period of 5 min increases with the frequency of stimulation. If the extra K-efflux (i.e., the efflux above the resting level) is divided by the number of nerve impulses elicited during one collection period, the quotient obtained is roughly independent of the frequency of stimulation (up to about 100 stimuli/sec); this quotient is the extra K-efflux per impulse.

Under the conditions of the experiment shown in Figure 27, left, the extra efflux was 10 to 17 pmole $\cdot\text{cm}^{-2}$ per impulse. This value is close to that estimated before the development of the intracellular perfusion technique (see Caldwell and Keynes, 1960; Hodgkin, 1964). The fluxes of various anions are not affected by repetitive stimulation (Caldwell and Keynes, 1960; Tasaki, Teorell and Spyropoulos, 1961). Although there is a definite increase in the Ca influx during repetitive stimulation, the Ca ion influx is estimated to be less than 0.5 pmole $\cdot\text{cm}^{-2}$ per impulse.

Under these experimental conditions (Fig. 27), a flow of local current through the axon membrane is associated with the propagation of nerve impulses. Based on the cable property of the axon, the quantity of electricity carried by the local current can be estimated in the following manner. As a nerve impulse travels along the axon, the intracellular potential in the resting area in front of the excited zone rises rapidly. This potential rise is associated with a current, I , which can be described by $I = C_m dV(t)/dt$, where C_m is the apparent membrane capacity. In this approximation, the resistive component of the current (see equation 2.2.6) can be neglected because the potential rise is rapid. The total quantity of electricity transferred across the membrane by this process does not exceed $C_m E_a$, where E_a is approximately 100 mV. The product $C_m E_a$ is approximately 10^{-7} coulomb/cm 2 . When this value is divided by the Faraday

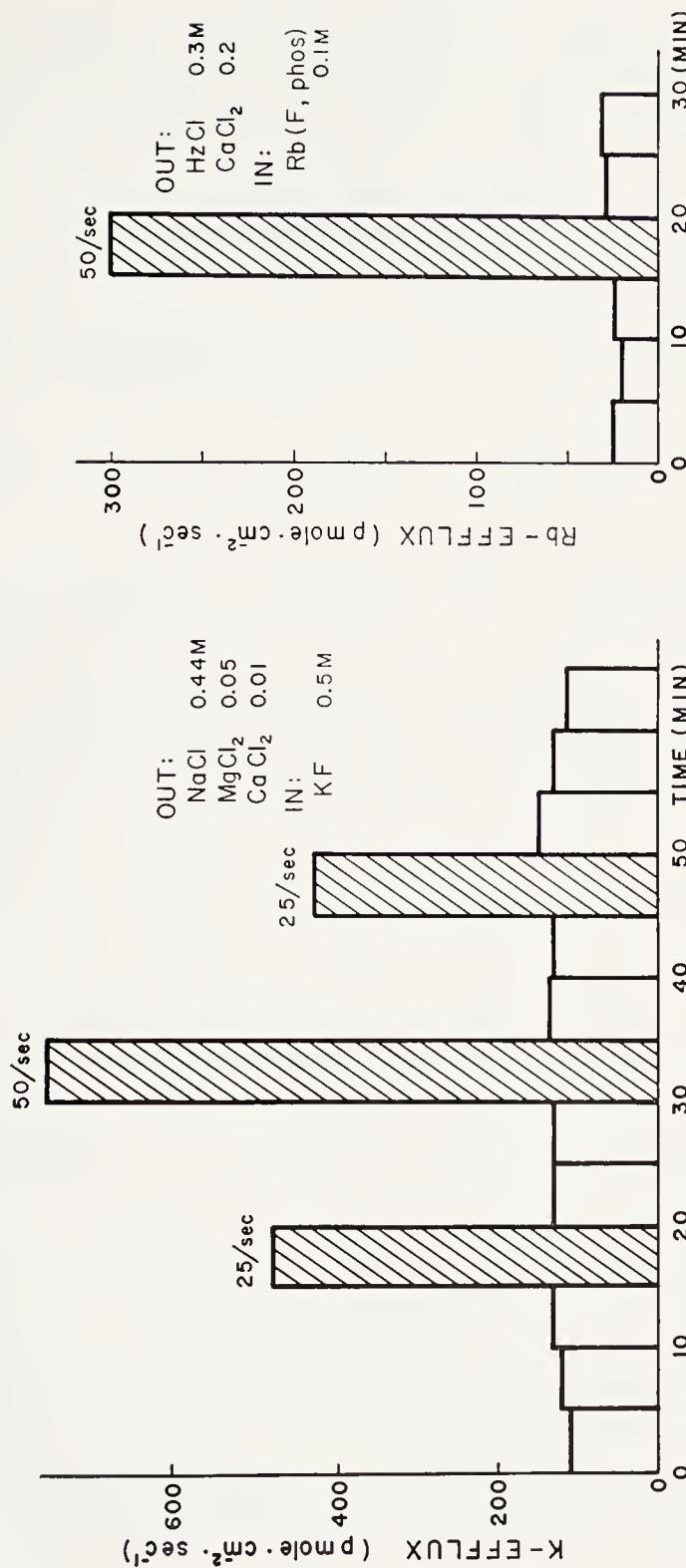


FIGURE 27. *Left:* Efflux of K^+ ion at rest and during repetitive stimulation. The compositions of the external and internal solutions and the frequency of stimulation are given.

Right: Efflux of Rb^+ ion through squid axon membrane at rest and during repetitive stimulation in sodium-free, potassium-free fluid media. (Tasaki, Singer and Watanabe, *J Gen Physiol*, 50:988-1007, 1967.)

constant, the extra cation flux associated with the local current is approximately 1 pmole/cm² per impulse. Since this quantity is less than 1/10 of the observed value of the K eflux during excitation (i.e., 10 to 17 pmole·cm⁻² per impulse), it will be ignored in the following discussion.

It should be noted that the external fluid medium must always satisfy the condition of electroneutrality. Therefore, any gain of K ion by the external fluid medium must be accompanied by the loss of an equivalent amount of Na ion. (Note that no distinct increase in anion fluxes during excitation has been detected.) The results of direct measurements of the Na influx are consistent with this argument. Repetitive stimulation of an axon immersed in Na containing medium (tagged with Na²² ions) produces a large increase in the Na influx during excitation.

The relaxation time (time constant) of the axon membrane is on the order of 0.01 msec at the peak of excitation (see Sect. 5.3). Therefore, when the cation fluxes through the axon are suddenly altered, approximately 0.01 msec is needed before new (approximately stationary) concentration profiles are established for moving cations in the system (see Sect. 4.2). This relaxation time is far shorter than the duration of the action potential (about 1 msec). Therefore, one may assume that the membrane *slowly* changes from one nearly stationary state to another during excitation. If the axon is assumed to be in such a quasi-stationary state, the behavior of the cation fluxes during excitation can be analyzed further. In this case, ion fluxes are directly related to the membrane resistance, since both are determined by the concentrations and mobilities of the interdiffusing cations in the membrane. The magnitude of the cation interdiffusion fluxes should then rise and fall along the same time course as the reduction in the membrane impedance.

In the experiment shown in Figure 27, left, the impedance loss lasts for about 1 msec. The average extra flux in this brief period is (10 to 17) · 10³ pmole·cm⁻²·sec⁻¹. Since the membrane impedance decreases and increases along a roughly triangular time course (see Fig. 24), the flux is expected to rise and fall along a similar triangular time course. Then the peak value of the flux is approximately twice the average flux. The peak value of the cation flux estimated in this manner is (2.0 to 3.4) · 10⁻⁸ mole·cm⁻²·sec⁻¹. This value is 150 to 200 times the

resting level of the interdiffusion fluxes. Thus, the ratio of the interdiffusion flux in the resting state (J) to that at the peak of excitation (J_a) is roughly equal to the ratio of the membrane resistance in the two states (at rest and at the peak of excitation) r and r_a . In other words, the resistance-flux product at the peak of excitation is roughly equal to the product for the membrane in the resting state, or $rJ \approx r_a J_a$.

In the resting state the rJ product is close to the theoretically most probable value RT/F^2 (see Chs. III and IV); hence, it may be concluded that

$$(5.4.1) \quad r_a J_a \approx rJ \approx \frac{RT}{F^2}$$

This approximate equality provides deep insight into the process of nerve excitation, and will be discussed in the next section.

CHANGES IN IONIC MOBILITIES DURING EXCITATION

(5.5) The membrane conductance is defined as the reciprocal of the membrane resistance. Under the conditions of the experiments shown in Figure 27, left, the conductance rises at the peak of excitation to a level approximately two hundred times that in the resting state. Simultaneously, there is an equally large increase in the interdiffusion flux (see equation 5.4.1). According to the physicochemical considerations of membrane phenomena discussed in Chapter III, this enormous increase in membrane conductance and interdiffusion flux reflects the large increase in the products of mobilities and concentrations of the interdiffusing cations in the membrane. The following discussion is designed to analyze the nature of this profound physicochemical change in the membrane.

In order to simplify the discussion at this moment, the major diffusion barrier in the squid axon membrane may be assumed to be perfectly permselective and uniform. In the case where ion species 1 exists only on one side of the membrane, and species 2 on the other side, the ion selectivity K_1^2 affects neither the membrane conductance nor the interdiffusion flux. Both membrane conductance g and the interdiffusion flux J are given by the following equation (see equations 3.3.7 and 5.4.1):

$$(5.5.1) \quad g = \frac{F^2}{RT} J = \frac{F^2 \bar{X} \bar{u}_1 \bar{u}_2}{(\bar{u}_1 - \bar{u}_2) \delta} \ln \frac{\bar{u}_1}{\bar{u}_2}$$

where \bar{u}_1 and \bar{u}_2 are the intramembrane mobilities of the two interdiffusing univalent cations; \bar{X} is the density of the negative charges in the membrane, and δ is the membrane thickness. Equation 5.5.1 indicates that when all other factors are kept constant, the membrane conductance is a monotonic function of \bar{u}_1 .

This equation, 5.5.1, can be rewritten in the following form:

$$(5.5.2) \quad g \frac{\delta}{F^2 \bar{X} \bar{u}_2} = \frac{\ln (\bar{u}_1 / \bar{u}_2)}{1 - (\bar{u}_2 / \bar{u}_1)}$$

Figure 28 shows the dependence of the left-hand term of this equation on the mobility ratio \bar{u}_1 / \bar{u}_2 ; the membrane conductance in bi-ionic situations is plotted against the mobility of one ion species \bar{u}_1 , while the other parameters (\bar{u}_2 , \bar{X} and δ) are held constant. For example, when the mobility ratio \bar{u}_1 / \bar{u}_2 is increased from unity to twenty (i.e., \bar{u}_1 is increased from \bar{u}_2 to twenty times \bar{u}_2), g increases by a factor of 3.6. When the mobility ratio changes from unity to one hundred, the value of g only increases by a factor of 4.6. An increase in \bar{u}_1 from $\bar{u}_2 / 20$ to twenty times \bar{u}_2 (i.e., a four hundred-fold increase in \bar{u}_1) increases the membrane conductance by a factor of twenty.

The membrane conductance and ion flux observed at the peak of excitation is about two hundred times that of the resting level. In the classical sodium theory (see Hodgkin, 1952), it is assumed that this large increase in g is caused by a large increase in membrane "permeability" specifically for the external cation; i.e., for sodium ions. If the external cation cation species is denoted by subscript 1 and the internal cation species by 2, the sodium theory proposes that at the peak of excitation $\bar{u}_1 \gg \bar{u}_2$. Although the rise in the intracellular potential during excitation can be explained in terms of this increase in \bar{u}_1 (see equation 3.3.9), the observed rise in membrane conductance and interdiffusion flux cannot be attributed to a large increase in mobility of only the external cation. As can be seen in Figure 28, a large change in the mobility of only *one* of the two interdiffusing cations does not affect either g or J appreciably. (Note the difference in scale between the abscissa and ordinate in Fig. 28.)

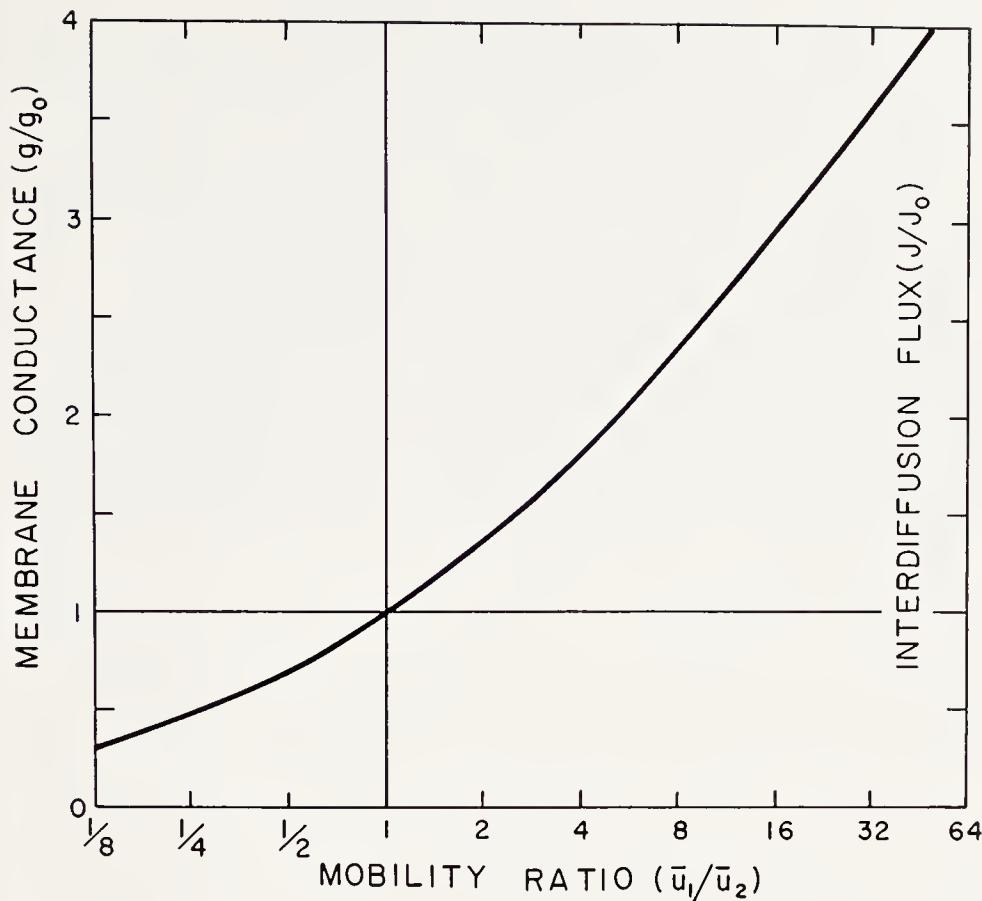


FIGURE 28. Theoretical curve relating the membrane conductance and interdiffusion flux to the mobility of one of the two interdiffusing univalent cations. Abscissa represents the mobility of cation species 1, \bar{u}_1 , expressed in the unit of the mobility of the other cation, \bar{u}_2 . In calculation, it was assumed that the flux of anions are negligible, that the ion selectivity does not vary within the membrane, and that cation species 1 exists only on one side and species 2 only on the other side of the membrane. J_0 and g_0 indicate the flux and the conductance, respectively, for $\bar{u}_1 = \bar{u}_2$, (see equation 5.5.2).

There are three alternatives to account for the increase in flux and conductance at the peak of excitation: (1) both \bar{u}_1 and \bar{u}_2 increase simultaneously; (2) the density of the fixed charge in the membrane, \bar{X} , increases, or (3) all of the three quantities, \bar{u}_1 , \bar{u}_2 and \bar{X} , increase simultaneously. Of these possibilities (3) appears to be the most probable (see Ch. VIII).

In the derivation of equation 5.5.1, the membrane was assumed to

be uniform and ideally permselective. But, uniformity of the fixed charge density in the membrane is not essential to arrive at the conclusion stated above. Even when the density, \bar{X} , is assumed to be a function of the coordinate x normal to the surface of the membrane, an equation of the form of 5.5.2 can be derived (see Appendix). (In this case, the product $\bar{X}\bar{u}_2$ in the left-hand member of the equation is regarded as an “average” membrane quantity.)

Equation 5.5.1 shows that in charged membranes, both the conductance and the interdiffusion fluxes are determined predominantly by the *slower* counter-ion species in the membrane. This property of charged membranes is associated with accumulation of the slower counter-ion in the membrane and will be discussed further in Appendices II and III.

Chapter VI

MACROMOLECULAR STATE OF AXON MEMBRANE

THE EFFECT OF ENZYMES ON AXON MEMBRANE

(6.1) It is generally believed that the excitable membrane of the squid giant axon is 50 to 100 Å in thickness. This estimation was originally made on the assumptions that the apparent membrane capacity (approximately $1 \mu\text{F}/\text{cm}^2$) is dielectric in nature and that the dielectric constant of the membrane material is between five and ten. More recently, electron-microscopic studies of the membrane have demonstrated two electron-dense layers separated by a distance of about 100 Å (see Robertson, 1960). These morphological studies support the view that the axon membrane is a multilayered structure rather than a single uniform layer, and that the excitable membrane is extremely thin. The demonstration of axon excitability after complete removal of the axoplasm (see Fig. 9) indicated that the process of action potential production can take place in this thin, multilayered structure.

The fact that the excitable membrane is extremely thin creates various problems in physicochemical analysis of the process of nerve excitation. Although thermodynamic concepts (e.g. chemical potentials) are known to be applicable to layers less than 100 Å in thickness (see Guggenheim, 1957, p. 267, 367), it is certainly impossible to determine directly the concentration profiles of various ions within the membrane. Furthermore, it is extremely difficult to obtain reliable information concerning the chemical composition of the excitable membrane by direct chemical analysis, because the adherent tissues (basement membrane, connective tissue fibers, Schwann cells, etc.) occupy an overwhelmingly large proportion of any sample of axon membrane. The difficulty of conducting direct chemical analyses of the excitable membrane has led to more indirect methods for examining the macromolecular state of the membrane. A very prom-

ising method of investigation by immunological techniques has been developed by several investigators at MIT and several interesting results have been obtained (Huneeus-Cox, personal communication). Treatment of the axon membrane with enzymes is another indirect method for studying the chemical composition of the membrane. Several of the results obtained by the latter method will be summarized below.

External applications of various protease solutions, e.g., trypsin, chymotrypsin, or ficin (at concentrations of about 1 mg/ml sea water), have no clear effect on the excitability of squid giant axons (Tasaki and Takenaka, 1964). These observations are consistent with those made by Tobias (1955) using lobster and crayfish axons. It is evident that these enzyme molecules do not reach the critical layer of the membrane when applied externally. Internal application of these proteases has a profound effect on the ability of the axon to produce action potentials.

Intracellular injection of trypsin is known to suppress the excitability of squid axons (Rojas and Luxoro, 1963). Since the degradation products of proteins (see Katchalski, *et al.*, 1965, p. 577) may suppress excitability, interpretation of the effect of trypsin injection is not simple. Recently, the effects of several enzymes were examined by the method of internal perfusion (Tasaki and Takenaka, 1964).

Trypsin

Internal perfusion with trypsin produces a fall in the resting potential followed by a gradual decline in the action potential amplitude, and eventually, by a complete loss of excitability. With 1 mg trypsin dissolved in 1 ml of 300 mM K-aspartate (glycerol) solution, irreversible loss of excitability occurs in 2.5 to 6 minutes. Since trypsin acts only at peptide bonds adjacent to basic amino acids (lysine, arginine), positively charged groups must exist in the axon membrane at physiological pH.

Chymotrypsin

Alpha- or gamma-chymotrypsin produces a gradual decline in the resting potential, often accompanied by repetitive firing of nerve impulses. With a perfusion fluid containing 1 mg/ml of either enzyme, excitability is eliminated three to six minutes after the onset

of perfusion. Note that the disruption of peptide bonds by these endopeptidases creates free carboxyl and amino groups. The lowering of the membrane resistance by enzymatic digestion of the axon interior may be attributed to an increase in the concentrations of mobile ions in the membrane, caused by breaking the macromolecular cross-linking in the membrane matrix.

Papain and Ficin

The effects of the proteases papain and ficin are interesting because they produce a "flip-flop" phenomenon. During the action of

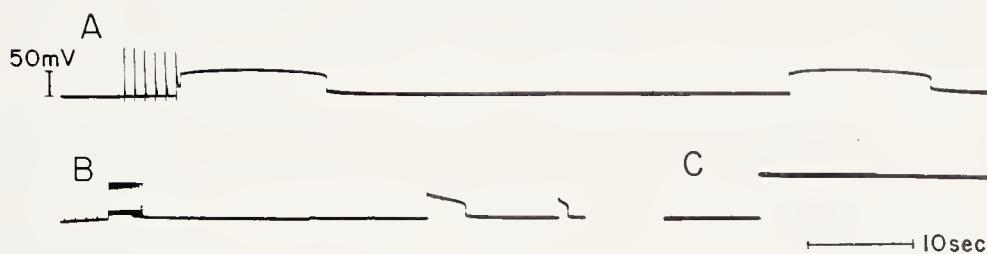


FIGURE 29. Prolonged action potentials produced by intracellular perfusion with solutions containing ficin.

In A the protease was dissolved in 400 mM potassium aspartate solution; six electric shocks were delivered before a prolonged action potential was elicited.

In B and C, 50 mM sodium glutamate (glycerol) solution was used as solvent. The external medium was natural sea water in A and a 300 mM NaCl and 50 mM MgCl₂ solution in B and C. (Tasaki and Takenaka, *Proc Nat Acad Sci USA*, 52:804-810, 1964.)

these enzymes on the inner surface of the membrane, the internal potential of the axon often rises suddenly by 20 to 50 mV. This sudden rise is often triggered by repetitive stimulation of the axon or spontaneous firing of impulses. The sudden rise (flip) is generally followed by a sudden fall (flop) in the potential. Three records showing this phenomenon are presented in Figure 29. This phenomenon is similar to abrupt depolarization by externally applied K ions (Sect. 4.6). A further discussion of these and similar phenomena will be found in Chapter VII. It is important to note that these protease effects can not be attributed to nonspecific damage of the axon membrane; simple mechanical injuries of axons never produce such potential jumps as shown in Figure 29.

Other Peptidases

Carboxypeptidase, leucine aminopeptidase, and l-amino acid oxidase also have definite, suppressive effects on the excitability of the axon membrane. These results indicate that there are N-terminal amino groups and C-terminal carboxyl groups in the inner layer of the membrane.

Lipases

The pancreatic lipases, and phospholipases-C and D bring about a slow reduction in the potential difference across the membrane and subsequent loss of excitability. This finding suggests the presence of lipids and phospholipids in the critical layer of the axon membrane.

Nucleases

Intracellular applications of desoxyribonuclease or ribonuclease (DNase or RNase) have no clear effects.

In summary, the enzyme experiments offer strong support to the idea that the excitable membrane is composed of protein, lipids and phospholipids. These experiments show that there are side-chain and terminal amino and carboxyl groups, phosphate groups, etc., in the membrane. The demonstration of the flip-flop phenomenon under the action of papain and ficin suggests that polypeptide molecules play an essential role in the process of production of membrane potential variation.

THE ORDER OF FAVORABILITY OF INTERNAL ANIONS

(6.2) In the early stage of development of the perfusion technique, it was recognized that squid giant axons survived for only thirty to forty minutes under continuous internal perfusion with isotonic KCl solution (Oikawa *et al.*, 1961). Later, it was found that isotonic solutions of K-aspartate, K-glutamate, K-phosphate, or KF are far more favorable than KCl solutions for maintaining the excitability of internally perfused axons (Tasaki and Takenaka, 1964; Tasaki, Singer and Takenaka, 1965). Particularly, perfusion with KF salt is so beneficial that it often restores excitability after suppression by injury from surgery, by fatigue from prolonged stimulation, or by unfavorable perfusion solutions.

In one series of experiments, the order of favorability of various

potassium salts was determined on the basis of survival time; i.e., the time required to produce irreversible loss of excitability under continuous intracellular perfusion. The results obtained for 400 mM potassium halides by this method are reproduced in Figure 30. Iodide has the shortest survival time, followed by bromide, chloride and fluoride. The survival time with KF is much longer than three hours for axons immersed in sea water.

The right-hand diagram in the figure shows the relationship between the survival time and the lyotropic number for the halides. This number has been defined by colloid chemists as a measure of the relative abilities of different neutral salts to precipitate protein emulsions (Bruins, 1932, Voët, 1937). The number is determined by the nature of interaction between the added anions and the charged group of the macromolecules (and intramembrane water). The figure demonstrates the linear relationship between lyotropic number and survival time. Many physicochemical properties, including the hydration energy (see Fig. 30, inset), are known to vary with the lyotropic number.

The order of favorability was also established by determining the ability of the potassium salt of one anion to restore excitability, after internal perfusion with a potassium salt of a different anion had suppressed excitability. An example of the results obtained by this method is shown in Figure 31. Under continuous internal perfusion with a 400 mM KCl solution, the amplitude of the action potential remained almost constant for about twenty minutes. Following this period there was a gradual decline in the resting and action potentials. At the moment when the axon became incapable of carrying nerve impulses across the perfusion zone, the perfusion fluid was switched to a 400 mM KF solution. The ability of the axon to produce action potentials was immediately restored. This recovery was taken as an indication that the second anion was more favorable than the first anion.

The order of favorability established by these methods is as follows:



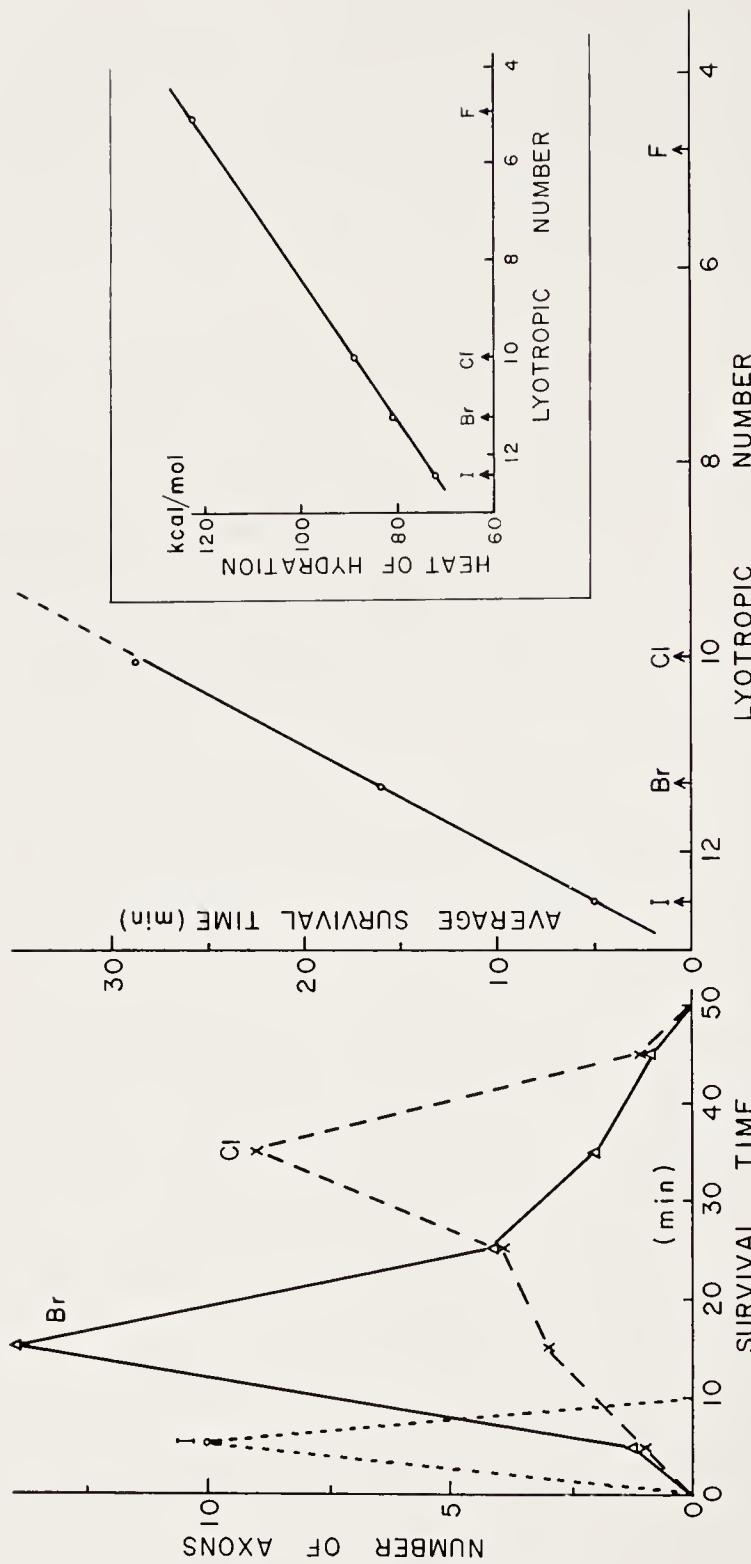


FIGURE 30. *Left:* Survival times for 400 mM potassium halide perfusion solutions. The average survival times were 4.9 minutes for iodide (ten axons), 16.9 minutes for bromide (twenty-six axons), and 27.5 minutes for chloride (eighteen axons). The data for fluoride are not included because the survival time is generally longer than two hours.

Right: Relationship between survival time and lyotropic number. The lyotropic numbers of the halides are indicated below the abscissa.

Inset: Relationship between heat of hydration and lyotropic number (Voët, 1937). The exceptionally long survival time found for fluoride might be predicted from this diagram. (Tasaki, Singer and Takenaka, *J Gen Physiol*, 48: 1095-1123, 1965.)

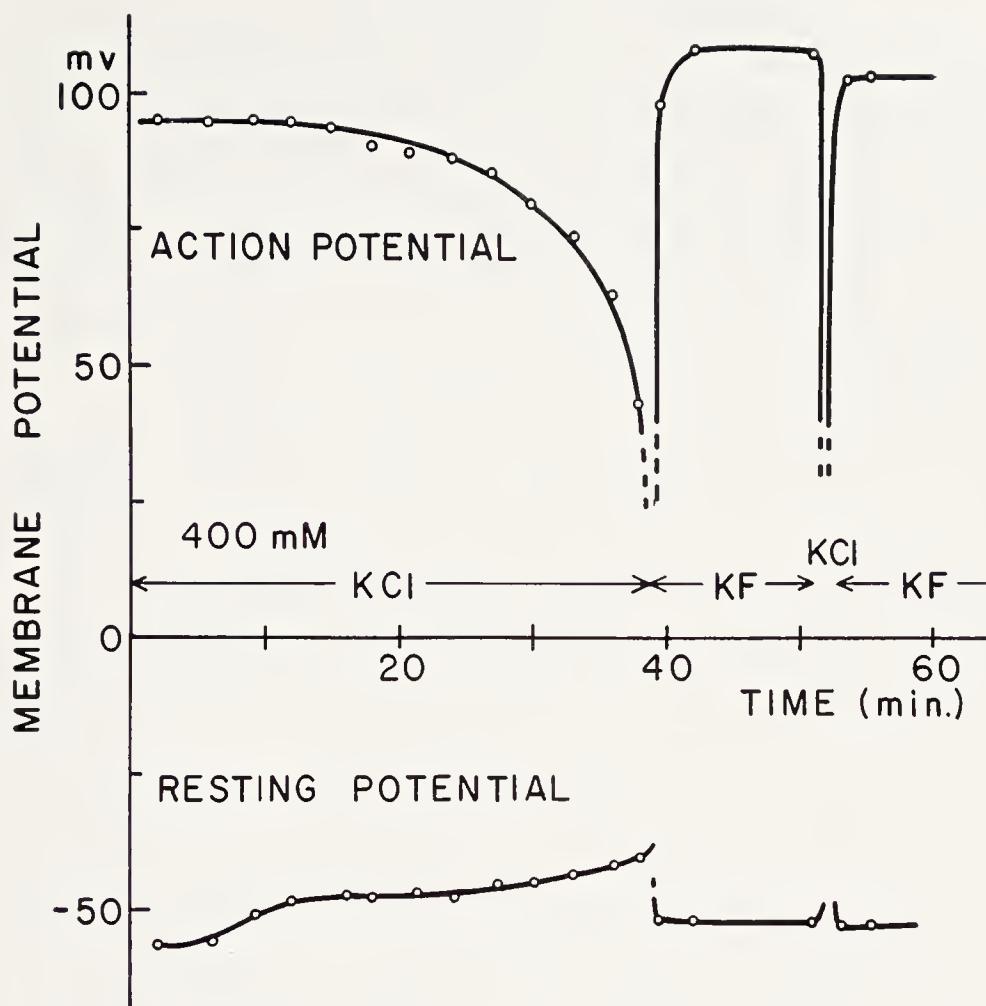


FIGURE 31. Effect of intracellular anions as examined by the restoration technique. The resting and action potentials (obtained with 1 shock/sec stimuli) are indicated by the lower and upper curves, respectively. The break in each curve represents the time during which internal perfusion solutions (indicated just above the time scale) were changed. (Tasaki, Singer and Takenaka, *J Gen Physiol*, 48:1095-1123, 1965.)

where F^- is most favorable, and SCN^- is the most unfavorable anion. This order is identical with the anion sequence arranged according to the lyotropic number (see Tasaki, Singer and Takenaka, 1965).*

The finding that anions affect the excitability of the axon in ac-

* Although the most favorable anions, F and phosphate, are strong Ca-precipitating agents, the entire anion sequence cannot be explained in terms of this effect. The difference among I, Br, and Cl is marked, despite the high solubility of their Ca salts.

cordance with their lyotropic number is consistent with the fact that polypeptides are major constituents of the excitable membrane. The most unfavorable anion in this series, SCN⁻, has a strong tendency to "reverse the charge" of positively charged colloidal particles, but has the weakest "salting-out" effect on proteins (see Overbeek and Bungenberg de Jong, 1949, p. 208; von Hippel and Wong, 1964). In fact, SCN⁻ antagonizes the salting-out effect of more favorable anions. On the other hand, the most favorable anions, e.g., fluoride, have a weak reversal of charge effect, but a strong salting-out effect. There seems little doubt that these effects play decisive roles in the maintenance of excitability in internally perfused squid giant axons.

It is important to observe that the deterioration of the resting and action potential is usually accompanied by a simultaneous increase in the membrane conductance. In Chapter III it was noted that the membrane conductance is determined by the mobilities and concentrations of the ions in the membrane. Both the intramembrane ion mobilities and concentrations could be increased if salt-linkages between membrane macromolecules were disrupted by added neutral salts. Disruption of salt-linkages takes place more readily if the added ions have strong affinities to oppositely charged groups of membrane macromolecules (see Overbeek and Bungenberg de Jong, 1949, p. 227, 229). In ion-exchange resins, the ion mobilities are known to vary with the degree of cross-linking (see Soldano, 1953); the disruption of salt-linkages in the axon membrane is considered to be analogous to a decrease in the cross-linking in resins and to increased intramembrane ion mobilities.

EFFECTS OF INTERNAL CATIONS

(6.3) Replacement of one cation in the internal perfusion medium with another cation affects the function of the axon in two distinct phases: an initial, immediate effect and a later, gradual effect. Immediately after replacement, there is a change in the amplitude of the action potential. After this immediate change, both the resting and action potentials may remain almost constant for a certain period of time; finally, excitability may gradually deteriorate. The rapidity with which the immediate effect develops is determined by the diffusion process between the membrane and the perfusion fluid. The slow, gradual effect is considered to reflect alterations of the membrane macromolecules.

When cation species A is a substitute for cation species B, A is designated more favorable than B if there is an increase in action potential amplitude. Conversely, if there is an immediate or gradual decrease in the size of the action potential following substitution of A for B, cation species A is called less favorable or more unfavorable than B. In most cases studied thus far, an unfavorable immediate effect was followed by an unfavorable later, gradual effect.

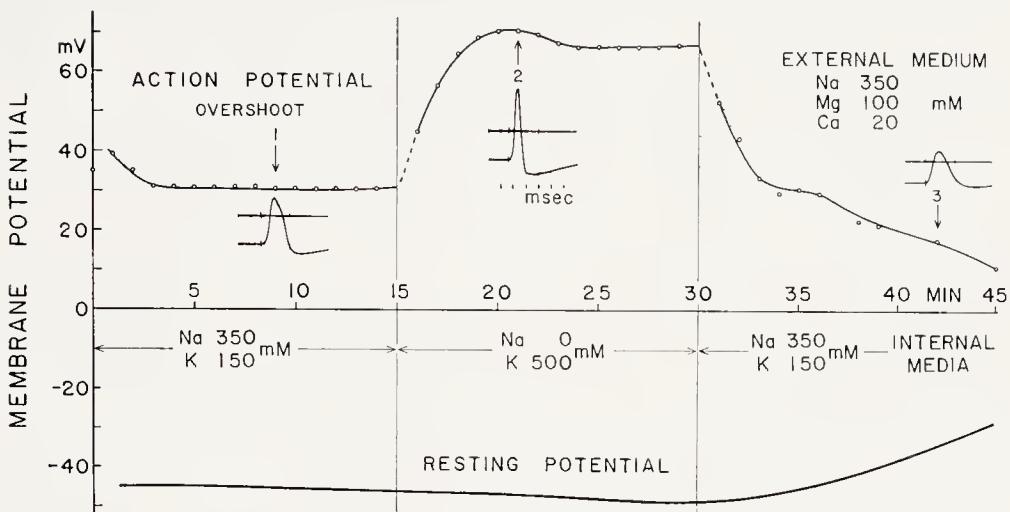


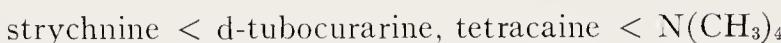
FIGURE 32. Resting and action potential of an axon intracellularly perfused first with a solution high in sodium, then with a pure potassium solution, and finally with a sodium-rich medium. A calomel electrode (0.6 megohm resistance) was used for recording. The diameter of the axon was 0.94 mm. (Tasaki, Luxoro and Ruarte: *Science*, 150:899-901, 1965; copyright by the American Association for the Advancement of Science.)

An example of intracellular perfusion experiments comparing the effects of internal Na ion with internal K ion is shown in Figure 32 for a Chilean squid axon (approximately 1 mm in diameter). The axon was internally perfused first with a mixture of 350 mM Na-glutamate and 150 mM K-glutamate. After the initial fall in the action potential amplitude, both the resting and action potentials remained constant. When the internal fluid was replaced with pure 500 mM K-glutamate solution, the action potential amplitude increased immediately. The perfusion fluid was then changed back to the original mixture of 350 mM Na-glutamate and 150 mM K-glutamate. This substitution of Na ion resulted in an immediate unfavorable effect, followed by a gradual unfavorable effect.

The order of favorability found by this method for alkali metal and ammonium ions was



where Li ion was most unfavorable. The unfavorable effects of intracellular divalent cations had been studied previously by Tasaki, Watanabe and Takenaka (1962). Ca ion was shown to be less favorable than Mg ion; both divalent cations were far more unfavorable than any alkali metal ion. The following order was found for several organic cations (Tasaki, Singer and Takenaka, 1965):



These polyatomic cations are all more unfavorable than the alkali metal ions.

Cation sequences of this type are encountered very frequently in colloid chemistry. An example is shown in Figure 33 (from Bungenberg de Jong, 1949), where the reversal of charge spectra of four different phosphate colloids and two carboxyl colloids are indicated for cations of biological importance. These sequences reflect the relative tendency for ion-binding (or ion-fixation) at the negatively charged groups of these colloids. The order of favorability of cations in the internal perfusion fluid agrees remarkably well with the order for phosphate colloids, but not with the order for carboxyl colloids (Fig. 33). (Note that the relative positions of univalent and divalent cations are opposite in these two groups of colloids.)

According to Bank and Hoskam (1940), the cation sequence for disruption of salt-linkages between macromolecules has diagnostic value; a close agreement between the cation sequence for an unknown biocolloid and a given sequence in Figure 33 can be taken as a reasonable indication that the majority of negatively charged groups are identical in the two colloids. On this basis, the negatively charged groups in the inner layer of the axon membrane are likely to be phosphate, rather than carboxyl, in nature.

EFFECT OF DILUTION OF INTERNAL PERFUSION FLUID

(6.4) In Section 4.4, the effect of dilution of the internal perfusion fluid on the resting potential of the axon was discussed. In this sec-

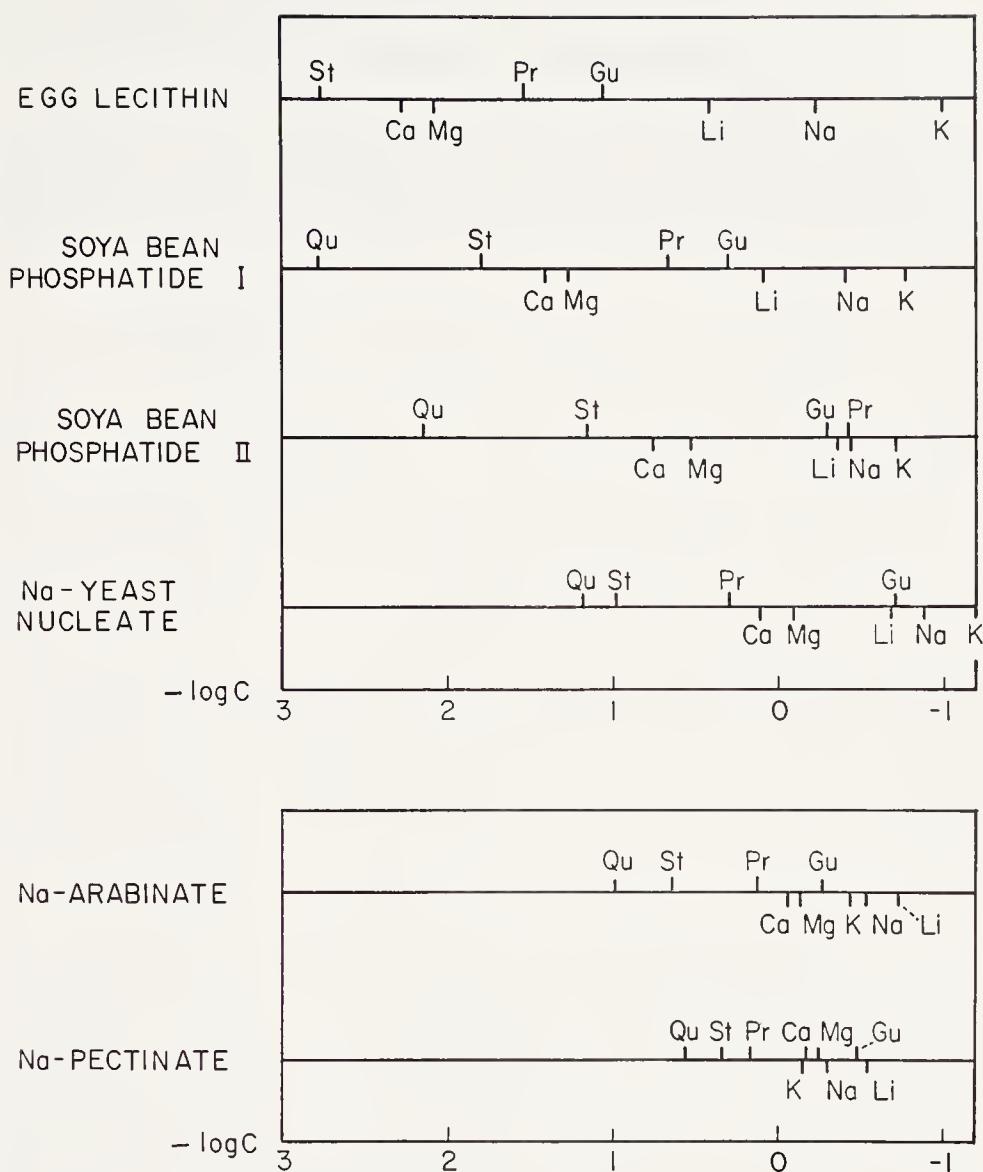


FIGURE 33. Concentrations (in logarithmic scale) of chloride salts of various cations required to reverse the charge of four different phosphate colloids (top) and two carboxyl colloids (bottom). *Gu* stands for guanidinium, *Pr* for procaine, *St* for strychnine and *Qu* for quinine. The charge-reversal concentrations were determined from the relationship between the electrophoretic velocities and the salt concentrations. Cations on the right-hand side of the diagram have weaker "reversal-of-charge" effect. (Adapted from Bungenberg de Jong, in Kruyt, H. R. (ed.), *Colloid Science*, Elsevier, Amsterdam, 1949, p. 301 and 302).

tion the effect of dilution on the ability of the axon membrane to develop action potentials will be examined.

Under continuous internal perfusion with a 500 mM KCl solution, the axon loses its ability to carry nerve impulses approximately thirty-five minutes after the onset of perfusion. The time course of the changes in the resting and action potentials are shown in Figure

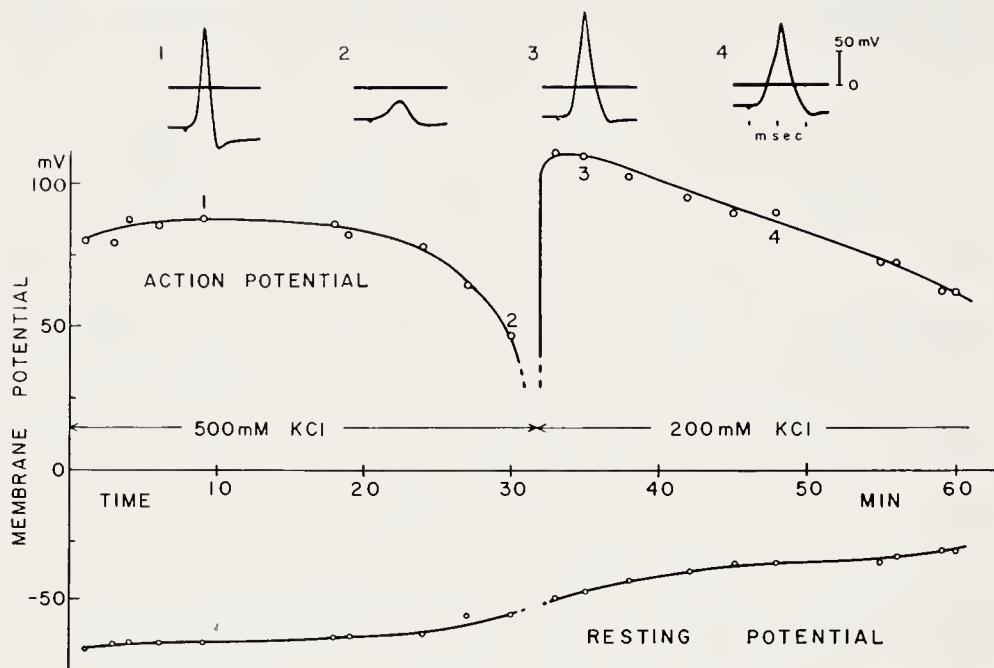


FIGURE 34. Effect of dilution of intracellular electrolyte. The time courses of the resting potential and action potential overshoot are indicated by the lower and upper curves, respectively. The break in each curve represents the time during which the perfusion solution was switched from 500 mM to 200 mM KCl. Four representative oscillosgraph records are shown at the top. The numbers refer to their respective positions in time along the action potential curve. (Tasaki, Singer and Takenaka, *J Gen Physiol*, 48:1095-1123, 1965.)

34 for internal perfusion with this isotonic KCl solution. After an initial period during which the action potential amplitude remained almost constant, there was a gradual decline in the magnitudes of both the resting and action potential. At the moment when the axon lost its ability to carry nerve impulses, the internal solution was replaced with a 200 mM KCl solution (prepared by diluting the original perfusion with a 12 percent glycerol solution). As can be seen in the figure, this dilution immediately restored excitability. At this time

the action potential amplitude was even larger than during the initial period. Later, there was a gradual decline in the resting and action potentials.

The sudden increase in the action potential amplitude observed in this experiment indicates that a dilute KCl solution is more favorable than an isotonic KCl solution. Similarly, dilute solutions of KBr, NaCl, Na₂SO₄, (CH₃)₄NCl, etc., are more favorable than the isotonic solutions of the corresponding salts.

It is assumed that these changes in axon excitability are due to alterations in the state of macromolecules in the membrane. The basis for analysis of the underlying mechanism derives from studies of the effects of neutral salts on charged colloidal macromolecules. According to Bungenberg de Jong (1949), the effects are divided into the following three categories: (a) simple screening effect; (b) ion-binding effect; and (c) salting-out effect (see also von Hippel and Wong 1963).

The simple screening effect, (a), results from formation of an ionic atmosphere around charged groups of macromolecules by the added salt of a low ionic strength. Since this effect is rather insensitive to differences in chemical properties of ions of the same charge, the effects of different neutral salts on the axon membrane cannot be attributed to simple screening.

Ion-binding at the charged sites of macromolecules, (b), is considered to be the basis of reversal of charge phenomena in colloids (see Kruyt, 1949, p. 259), of lowering the ion activity (Rice and Nagasawa, 1961), and of ion selectivity in membranes (e.g., Helfferich, 1962). Ion-binding does depend on the specific properties of ions added and the nature of the charged groups of macromolecules. When oppositely charged macromolecules (e.g., positive protein and negative phosphate colloids) form salt-linkages, addition of neutral salts tends to break the linkages. The tendency to break salt-linkages is strong for ions which have strong affinities to oppositely charged sites. Furthermore, the effect of anions and cations tend to be additive; when both the anion and cation of a given salt show a strong tendency to be fixed to oppositely charged macromolecular sites, that salt shows a very strong tendency to break salt-linkages. It is important to note that the effects of anions and cations on the squid axon membrane show similar additivity; a salt with both a favorable

cation and anion is more favorable than a salt with only one favorable ion. Since the tendency to break salt-linkages decreases with dilution of the added neutral salts, the favorable results observed with dilution can be interpreted in terms of the ion-binding effect.

The salting-out effect, (c), refers to the mechanism of precipitation of dissolved colloidal particles, with or without charges, by the addition of neutral salts. (In the actual process of protein precipitation, both the salting-out and the ion binding effects play important roles.) At very high ionic strength, this effect is difficult to differentiate from the ion-binding effect.

Klotz (1958), Kauzmann (1959), Scheraga (1963) and others have suggested that hydrophobic bonds play an important role in macromolecular interaction. When the nonpolar groups of two macromolecules approach each other, the entropy of the whole system increases; this entropy change tends to keep these macromolecules close together. It has been shown that this entropy change is related to alterations of the ice-like structure of water molecules surrounding the macromolecules. Hydrophobic bonds are strengthened by an increase and weakened by a decrease in the salt concentration (Kauzmann, 1959). If it is assumed that the macromolecules in the axon membrane are held together by hydrophobic bonds, dilution of neutral salts would be expected to disrupt the membrane structure more rapidly. Therefore, alteration of hydrophobic bonds is not likely to account for the dilution effect (Fig. 34).

Hydrogen bonds may also play a role in macromolecular interaction. The neutral salts used in the present experiment are not known as hydrogen bond breakers, but since hydrogen bonds are essentially electrostatic in nature, the salts in the perfusion fluid may affect these bonds.

PORES IN THE AXON MEMBRANE

(6.5) With a view toward interpreting excitation phenomena in squid axons, Kuhn and Ramel (1959) suggested a special mechanism of ion transport across the axon membrane (see Fig. 35A). They postulated the existence of pores in the membrane of such small dimensions that no permutation of positions of ions within one pore was possible ("single-file theory"). This suggestion was made to replace the assumptions of specific permeability changes made by

Hodgkin and Huxley (1952) with a postulate which appeared to be more reasonable to some physical chemists. However, there are serious difficulties with a single-file transport mechanism when theory and experiment are compared.

According to this postulate, the membrane potential is uniquely determined by the population of ions filling the pores. The membrane resistance may change when the population of ions in the pores is altered by an electric current through the membrane. However, there

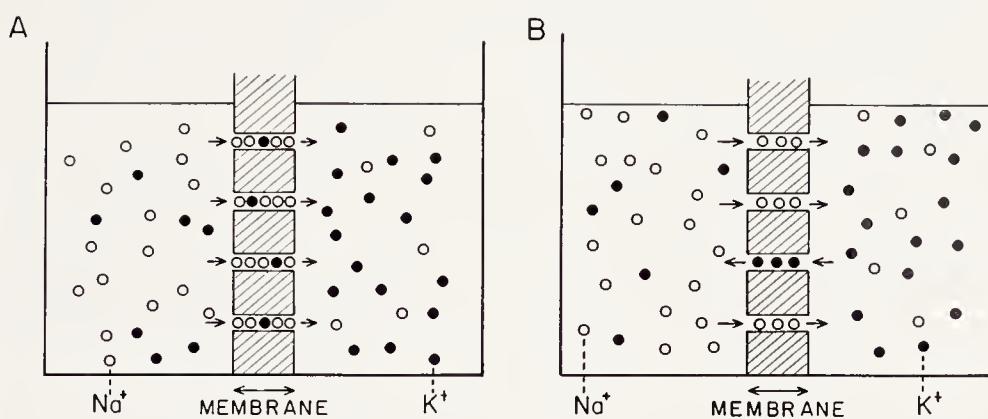


FIGURE 35. A: Single-file transport mechanism for squid axon membrane suggested by Kuhn and Ramel (1959). The pores in the membrane are assumed not to allow any permutation between Na and K ions. An inward (from left to right) current is carried predominantly by Na ions as shown in the figure.

B: Membrane provided with two kinds of pores, one specific for Na ions and the other for K ions. The arrows indicate the direction of the electric current carried by the ions in the pores.

is no way to increase the interdiffusion flux if only one kind of pore is assumed to exist in the membrane, and the measured fluxes of cations during excitation are far greater than those calculated from the passage of electric currents (see Fig. 27). In the excited state of the membrane, the postulated pores are thought to be filled predominantly with Na ions, as shown in the figure. This state can only be produced by passing an inward-directed membrane current. Therefore, this model is stimulated by an inward current rather than by the outward current required in a real axon (compare with Figs. 22 and 23). Thus, the excitation process in the squid giant axon cannot be explained by a single-file, one-pore mechanism.

If one assumes that there are two kinds of pores in the axon mem-

brane, one specific for Na ion and the other specific for K ion, it would be possible to account for the observed cation fluxes. In a recent article, Baker (1966) presented a detailed mechanism of nerve excitation based on two kinds of specific pores (see Fig. 35B). This pore model of the axon membrane was introduced as a morphological counterpart of the equivalent circuit model proposed by Hodgkin and Huxley (1952). The mechanism proposed for action potential production assumes that the Na pores open first, followed by opening of the K pores and almost simultaneous closing of the Na pores. This sequential process can account for the rise and fall in the membrane potential during excitation.

However, the assumption of two specific kinds of pores cannot explain the enhanced interdiffusion which occurs during excitation. In the absence of membrane current, significant cation interdiffusion fluxes can only take place during the period when both pores are open (electroneutrality requirement), and this period is generally assumed to be much shorter than the duration of the action potential. Therefore, it is difficult, if not impossible, to account for the observed value of the interdiffusion fluxes during excitation.

There is another set of experimental observations which cannot be accounted for by this model of the excitation process. According to the specific two-pore model, the Na pores are traversed by files of inward-moving Na ions. When radioactive Na^{24} ions are introduced into the axon interior, these radioactive ions should not be able to pass through the Na pores, because there is no permutation permitted in the pores. These sodium radiotracers should not be able to pass through the specific K pores. Therefore, there should be no increase in efflux of this radiotracer during excitation. Actually, Na^{24} in the axon interior traverses the membrane more rapidly than either K^{42} or Rb^{86} (Tasaki, 1963).

The effluxes of radiotracers injected into the axon interior are shown in Figure 36. For the same level of radioactivity, the effluxes of the radiotracers of alkali metal ions were found to decrease as atomic number increased. The efflux of Cs^{134} (not shown) was slower than those shown in the figure. These results indicate that the assumption of two kinds of ion pores in the axon membrane does not solve any of the problems of nerve excitation. It should be noted in this connection that a single-file transport mechanism has never been

suggested to account for any physicochemical properties of inanimate membranes.

Finally, a biochemical aspect of the difficulty in the concept of ion pores will be discussed briefly. According to Chapman, Byrne and Shipley (1966, p. 140), phospholipids in biological membranes appear to be in a fluidal state. If this is the case, it is unlikely that small and

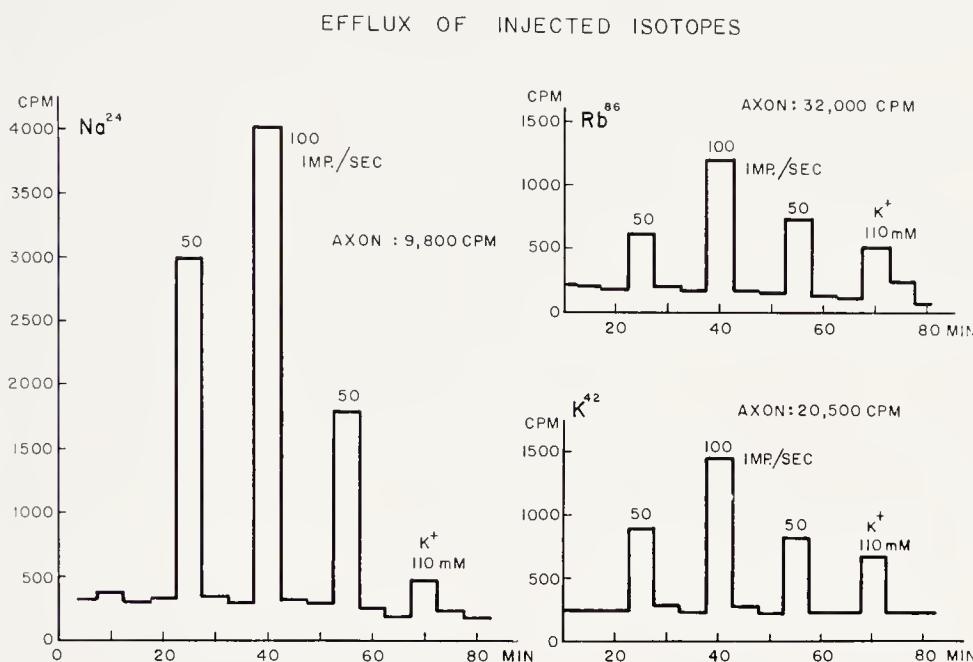


FIGURE 36. Effluxes of radioisotopes of three different alkali metal ions injected into squid giant axons. *Ordinate:* radioactivity in counts per minute in sea water collected in each collection period of five minutes. *Abscissa:* time after intracellular injection of radio-tracers. Radioactivity remaining in axons at end of experiment is given. The effects of repetitive stimulation at the rate of 50, 100 and 50 impulses per sec and of potassium depolarization are shown. (Tasaki, *J Gen Physiol*, 46: 755-722, 1963.)

rigid pores exist in the phospholipid layer of the membrane. Similarly, the (secondary and tertiary) structures of protein molecules are labile, because they are stabilized by weak noncovalent bonds such as salt-linkages, dipole-dipole interactions, hydrogen bonds, hydrophobic bonds, etc. (see Steiner, 1965, p. 131). It is well known that the complex structures formed by these macromolecules are strongly affected by temperature changes, pH, and the concentrations of various inorganic ions in the medium (see Bungenberg de

Jong, 1949, p. 438). Therefore, it seems more reasonable to regard the pores in the membrane as dynamic, time-dependent structures, rather than as rigid, static holes.

The cation distribution in the ion pores shown in Figure 35 is energetically very unfavorable, unless the distribution is stabilized by negative charges on the walls of the pores. Phosphate and carboxyl groups are the most probable sources of such negative charges. Ion binding by these negatively charged groups is not limited to one specification (see Fig. 33). From a physicochemical viewpoint, therefore, the postulate of pores specific for sodium (or potassium) ions in the axon membrane is rather unrealistic.

The squid axon membrane is permeable to small neutral molecules, such as tritiated water (Nevis, 1958; Tasaki, Teorell and Spyropoulos, 1961) and C¹⁴-labelled urea or glycerol, but relatively impermeable to large molecules such as sucrose (Villegas, Blei and Villegas, 1965). Based on these observations, the *apparent pore diameter* has been calculated for the squid axon membrane. The concept of pore diameter appears to be a useful index of the compactness of the membrane, as long as it is realized that there are no geometrically well-defined, rigid pores in the membrane.

Chapter VII

TWO STABLE STATES OF AXON MEMBRANE

PROLONGED ACTION POTENTIALS

(7.1) The duration of the action potential discussed in preceding sections is generally of the order of one millisecond. However, there are excitable tissues which normally develop action potentials with much longer durations. Single cells of *Nitella*, an excitable alga, develop action potentials of about one second in duration. Individual cells of vertebrate heart muscle repetitively generate action potentials with a duration of the order of one second.

It is possible to convert the normal action potential of a squid giant axon into a prolonged action potential by various experimental procedures. Examples of such action potentials have already been encountered in axons treated internally with ficin and papain (see Figs. 19 and 29). Intracellular injection of tetraethylammonium (TEA) ions (Tasaki and Hagiwara, 1957) and internal perfusion with dilute solutions of various alkali metal ions (Tasaki and Shimamura, 1962; Adelman, Dyro and Senft, 1965) are well-known techniques for prolonging the action potential in the squid giant axon.

Prolonged action potentials have several interesting features which offer clues to the mechanism of nerve excitation.

A weak pulse of electric current applied to the axon membrane during the plateau of a prolonged action potential produces a potential variation with a roughly exponential time course superposed on the plateau (Fig. 37A, thick continuous line). After the end of the pulse, the membrane potential returns to the original level along an approximately exponential time course. Reversal of the polarity of the applied current produces similar potential variations of the opposite polarity (broken line in Fig. 37A). These potential variations reflect the resistance and the apparent capacity of the axon membrane in the excited state. Similar potential variations can be demonstrated in the resting state of the membrane (see Diagram A, left).

When the intensity of an outward-directed current pulse increases the potential variation across the membrane in the resting state increases. When the intensity reaches a certain critical level (threshold), the membrane potential does not fall exponentially after the end of the pulse. Following a brief current pulse of threshold intensity, the membrane potential may stay at a constant level for a variable period of time before either (a) the prolonged action potential is initiated (see diagram B) or (b) the potential returns to the resting level.

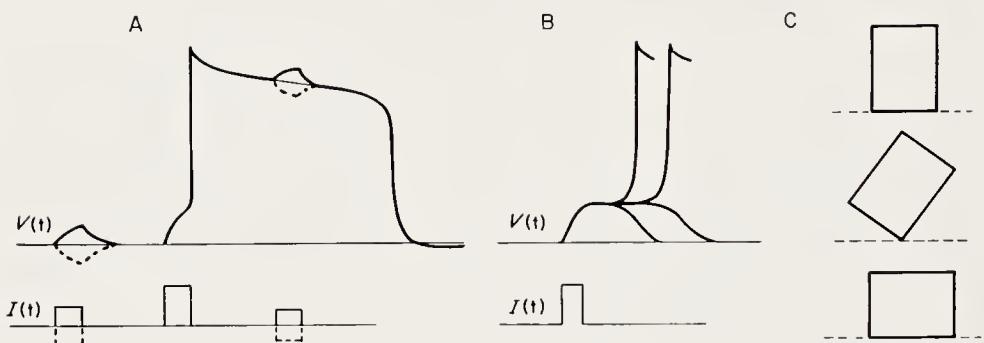


FIGURE 37. A: Diagram showing “stability” of the squid axon membrane in the resting state and during the plateau of a prolonged action potential. The lower trace, marked $I(t)$, shows the time courses of the rectangular current pulses applied to the axon. The upper trace, marked $V(t)$, shows the variation of the membrane potential produced by the applied rectangular current pulses.

B: Diagram showing variability in the potential-time curve in stimulation at threshold intensity; four traces represent the membrane potentials observed at the same stimulus intensity.

C: Diagram showing mechanical stability of a box on a table.

The electrical behavior of such an axon may be compared with the mechanical properties of the rectangular box shown in Figure 37C. When a small external force is applied, the box is tilted slightly; the box returns to its original position when the external force is withdrawn. If the force is strong enough, the box can fall into a new position. It exhibits *mechanical stability*. When the box is standing on one of its edges, it falls to one of the stable states (either the original position or the new position) after a variable period of time.

There is a formal similarity in properties between the box and the axon membrane. Hence, the term *stability* will be adopted to describe the tendency of the axon membrane to return to its initial state after the end of a small perturbation (a weak current pulse in this case).

Obviously, the similarity between the two systems is superficial; the stable positions of the box are given by the minima of the potential energy; the stability of a system consisting of a membrane and two solutions is described by the minimum of the entropy production of the system (see Prigogine, 1955).

The adequacy of the term *stability* can be illustrated by the potential time curves shown in Figure 38. These records were obtained from a single nerve cell in the dorsal root ganglion of the frog immersed in an isotonic barium chloride solution. The membrane potential of this nerve cell takes either of the two stable levels referred to as the "lower" and "upper" levels. When the membrane potential is at the lower stable level, a brief rectangular pulse of outward-directed current produces an abrupt potential rise in an all-or-none manner (see record A). The membrane potential can now stay at the upper stable level indefinitely. Under these conditions, another brief pulse of outward-directed current produces no shift in the membrane potential (see record B); but, a pulse of inward current does elicit an all-or-none potential change which returns the potential to the original lower level. This procedure of alternate stimulation by outward- and inward-directed currents produces a train of roughly rectangular potential variations (record C). Similar rectangular potential-time curves have been obtained from the squid giant axon under a variety of experimental conditions (see Fig. 29).

To summarize this section, some excitable cells develop action potentials of almost rectangular configuration under a variety of experimental conditions. This particular form of action potential can be regarded as representing transitions between two stable potential levels of an excitable system.

THE TEORELL NERVE MODEL

(7.2) Potential variations of a nearly rectangular configuration are encountered frequently in inanimate electrochemical systems. The nerve model proposed by Teorell (1956, 1959, 1960) is one of the best-understood systems in which the detailed mechanism of the production of potential variations of this type has been worked out (see also Kobatake *et al.*, 1964).

This model system consists of a sintered glass membrane separating two NaCl solutions of different concentrations. The nature of the

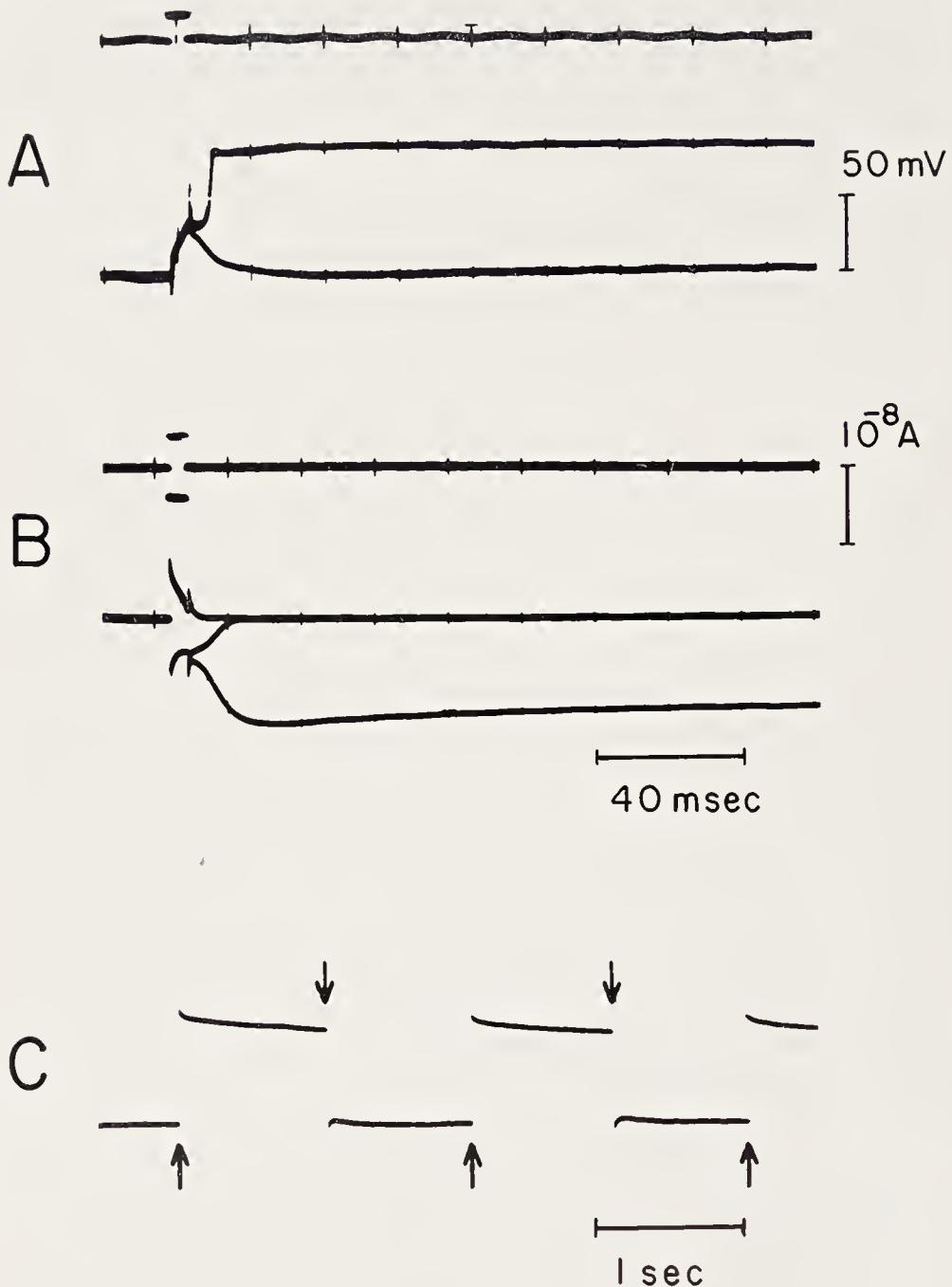


FIGURE 38. Approximately rectangular action potentials developed by a single nerve cell in the frog dorsal root ganglion immersed in a mixture of 80 mM BaCl_2 , 2 mM KHCO_3 and 1.5 mM CaCl_2 . The upper trace in Records A and B represents the current applied to the cell (through the microelectrode used for recording). The other trace shows the intracellular potential.

Record A shows the beginning of a rectangular action potential produced by

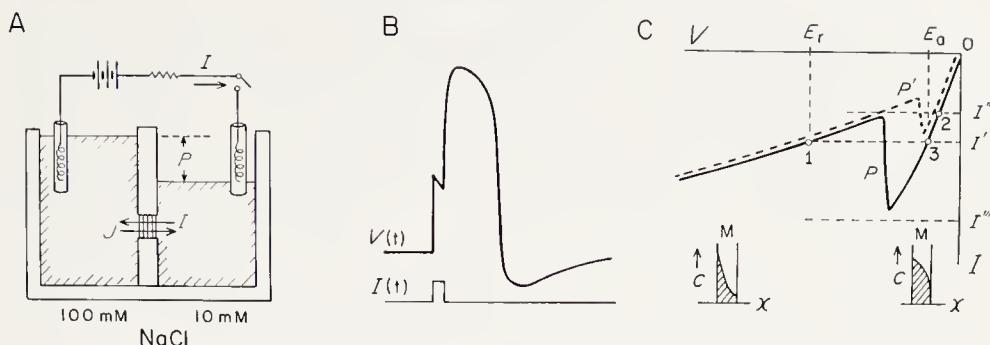


FIGURE 39. A: Simplified diagram of Teorell's model of nerve; a sintered glass membrane separates two solutions of sodium chloride; the arrows marked J and I indicate the direction of movement of the solution in the membrane caused by the hydrostatic pressure difference (P) and the electric current I , respectively.

B: Action potential produced in this model; the action potential amplitude is of the order of two volts and the duration is of the order of several minutes.

C: Current-voltage ($I-V$) relationship for Teorell's model determined at two different hydrostatic pressures, P and P' ($P' < P$); the resting potential, E_r , and the peak of the action potential, E_a , are indicated. The concentration profiles ($C-x$) in the membrane in the two stable states are shown below.

membrane and the conditions of the fluid media on both sides of the membrane in this system are very different from those in the living axon. The purpose of this discussion is to point out the importance of the concept of *two stable states* in systems capable of producing potential variations similar to those observed in living axons, rather than to analyze the detailed mechanism of production of potential variations in this inanimate system.

The experimental arrangement employed by Teorell is illustrated in Figure 39A. A sintered glass membrane with appropriate fixed charge

an outward-directed current of the threshold intensity, two oscillograph traces were superposed.

Record B shows the effects of an outward-directed current pulse and of an inward-directed current pulse applied during the "plateau" of a rectangular action potential; only an inward-directed current pulse produced an all-or-none response of the reversed polarity. Three oscillograph traces were superposed.

Record C shows the effect of short pulses of outward (indicated by arrows directed upward) and inward (downward-directed arrows) current applied alternately. (Adapted from Tasaki, *Nature*, 184:1574-1575, 1959.)

and pore size separates two compartments of a Lucite chamber. Two sets of electrodes are installed: one for delivering electric current and the other (not shown in the figure) for recording the potential difference across the membrane. The electrolyte solutions used were 100 mM and 10 mM NaCl. In this system, the hydrostatic pressure difference across the membrane plays an important role. The pressure difference can be fixed at a preselected level or allowed to change slowly when there is a flow of solution through the membrane.

The potential time curve obtained without a fixed pressure difference is shown in Figure 39B. The similarity between the potential-time curves in this system and the axon is clear.

In this model, the resting potential represents the potential drop maintained across the membrane by a constant current directed from the compartment filled with dilute NaCl solution to the other compartment. This current tends to create a flow of the pore fluid (electro-osmosis) in the direction of the current because of the negative fixed charge on the pore walls. However, there is a hydrostatic pressure difference between the two compartments which tends to oppose the effect of the electro-osmosis. One might say that the membrane is a battlefield between the two opposing forces: if the electric force dominates, the dilute solution becomes dominant in the membrane (resting state); if the hydrostatic force dominates, the concentrated NaCl fills the membrane (excited state).

The mechanism of production of a roughly rectangular action potential in this system will now be considered. The steady state current-voltage (I - V) relationships in this system are shown in Figure 39C for two different fixed pressure differences. These two curves were obtained by the voltage clamp technique. At pressure P , an N -shaped I - V curve is obtained (shown by the continuous line). A similar curve obtained at pressure P' ($P' < P$) is shown by the broken line. It is assumed that the system is initially in the stationary state marked by 1, with maintained current I' and pressure P . When the system is electrically "stimulated" by changing the current from I' to I'' , the system undergoes a transition to the state marked by 2 on the other (right-hand) limb of the I - V curve (because there is no stationary state on the left-hand limb at P and I''). At this moment, the current is changed from I'' back to I' (termina-

tion of stimulus). The system now reaches the state marked by 3. If the pressure is kept at this level, P , the system can stay at 3 for an indefinite period of time. If the pressure is not fixed, it falls gradually. (This fall is due to decreased electro-osmosis, which results from the fall in the membrane potential.) When the pressure reaches the level marked P' , the system can no longer stay in the state represented by the right limb of the N -shaped I - V curve, and undergoes a transition to the original state marked 1.

The left-hand limb of the N -shaped curve represents the resting state of the system, with a high membrane resistance. In this state the concentration profile in the membrane is concave upward, and the major portion of the membrane is occupied by the dilute NaCl solution (see Diagram C). The right-hand limb of the curve represents the excited state; in this region the concentration profile is concave downward, and the membrane is occupied predominantly by the concentrated NaCl solution.

In the region of the curve where the horizontal lines ($I = \text{constant}$) intersect the N -shaped I - V curves at three points, there are two different values of V that can be realized. These two values of V are reflections of two stable concentration-profiles in the membrane, each representing a state in which the salt solution on one side dominates the membrane. The intermediate limb of the N -shaped curve represents an unstable state: this state cannot be maintained.

The process of production of an action potential is based on the existence of two stable states. A pulse of stimulating current brings about a transition from the resting state to the excited state. While the system is in the excited state, there is a *gradual alteration* in the system; in Teorell's model this is a gradual fall in the hydrostatic pressure difference. As the result of this gradual alteration, it is not possible for the system to stay in the excited state indefinitely, and eventually the system undergoes a transition to the resting state.

In the following sections, the descriptive analysis presented in the last paragraph will be applied to the processes occurring in the axon membrane. As long as discussion is limited to formal expressions, such as *two stable states*, *gradual alteration*, etc., of the system, the process of excitation in the axon can be described in exactly the same manner as in Teorell's model.

INDISPENSABILITY OF DIVALENT CATIONS IN THE EXTERNAL MEDIUM

(7.3) Electrophysiologists are familiar with the fact that many excitable cells and tissues can maintain their excitability without any univalent cations in the external medium. Single cells of *Nitella* lose their excitability following prolonged immersion in distilled water, but they regain their ability to develop action potentials when CaCl_2 is added to the medium (Osterhout and Hill, 1933). Crustacean muscle fibers produce large, prolonged action potentials in media containing only salts of divalent cations (Fatt and Katz, 1953; Hagiwara, Chichibu and Naka, 1964). Nerve cells in frog dorsal root ganglia also maintain their excitability in media containing only divalent cation salts (Koketsu, Cerf and Nishi, 1959; Tasaki, 1959).

Squid giant axons under intracellular perfusion are not fundamentally different from the excitable tissues stated above; excitability can be maintained in media containing only CaCl_2 or certain other salts of alkaline earth metals (Tasaki, Watanabe and Singer, 1966). It is important to note that the excitability cannot be maintained in external media containing only salts of univalent cations (e.g., Na and/or K). (In the experiments with squid giant axons described in previous sections, the external media usually contained a mixture of salts of uni- and divalent cations.

An example of the experiments demonstrating action potentials in external media free of univalent cations is presented in Figure 40. Prior to initiation of intracellular perfusion, the external surface of the axon was exposed to a rapidly flowing solution of calcium salt prepared by mixing a 400 mM CaCl_2 solution with an equal amount of 12 (volumes) percent glycerol solution. The excitability of this unperfused axon was immediately suppressed by this procedure. Next, the axoplasm in the axon interior was removed by the technique of enzymatic digestion (see Fig. 9) and intracellular perfusion with 25 mM CsF (glycerol) solution was instituted. This treatment restored the excitability of the axon in a relatively short period of time (record A). The action potentials observed under these conditions were 60 to 100 mV in amplitude and 100 msec to 20 sec in duration. The excitability of such an axon could be maintained for more than two hours under continuous intra- and extracellular perfusion.

When the extracellular CaCl_2 solution was replaced with an isotonic choline chloride solution (Tasaki, Watanabe and Lerman 1967), the excitability of the axon was suppressed immediately (record B). Replacement of choline by sodium did not restore excitability (record C). However, when CaCl_2 was mixed with the Na-containing external medium, there was an immediate recovery of the ability of the axon to develop large action potentials (record D).

The results of these experiments can be summarized as follows:

1. Solutions of CaBr_2 , SrCl_2 , BaCl_2 , etc., can be used in place of the extracellular CaCl_2 solution.
2. Favorable intracellular perfusion media include dilute phosphate or fluoride salt solutions of Cs, Na, TEA or choline.
3. Although extracellular univalent cations are not required for the maintenance of excitability, addition of sodium, hydrazinium, or other favorable univalent cations to an extracellular calcium solution increases the action potential amplitude significantly (Sects. 8.4 and 8.5).
4. When an action potential is evoked by an outward-directed current, there is a concomitant fall in the membrane resistance (see Sects. 5.2 and 5.3). This fall can be seen in Figure 40A by comparing the magnitudes of the two potential variations at the end of the applied pulse.

All these characteristics of action potentials evoked in external media free of univalent cations indicate that this process of excitation is not fundamentally different from that in media containing both divalent and univalent cations. Ion fluxes in axons immersed in a pure calcium salt solution will be discussed in the Appendix.

The importance of divalent cations in various excitable tissues has been emphasized by a number of investigators, particularly by Loeb (1906), Höber (1926) and Heilbrunn (1952). More recently, Gordon and Welsh (1948), Brink (1954), Koketsu *et al.* (1959) and Liberman *et al.* (1961) have furnished more evidence in support of the view that divalent cations play an essential role in the process of excitation.

The emphasis on the importance of the extracellular divalent cations should not be allowed to overshadow the importance of the membrane macromolecules and the intracellular univalent cations in the process of excitation. In the following section (7.4) it will be

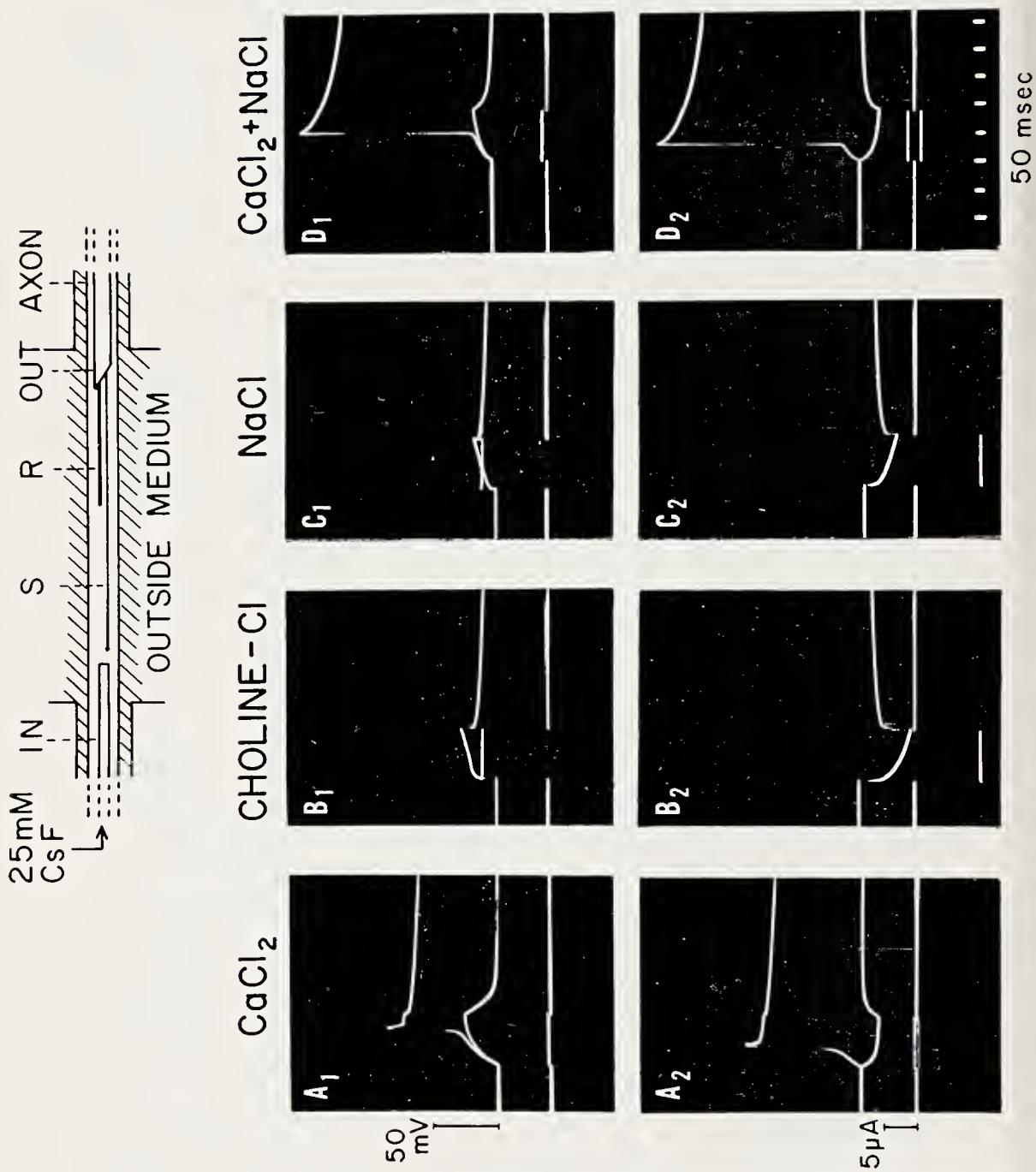


FIGURE 40. Oscillograph records demonstrating dispensability of univalent cations in the external medium for the maintenance of excitability of the squid giant axon. The bottom trace of each record represents the applied current, upward deflection signifying outward current. The top trace represents variation of the membrane potential. Deflections brought about by sub-threshold and suprathreshold stimuli are superimposed.

Records A_1 and A_2 show all-or-none action potentials produced in a medium containing 200 mM CaCl_2 and glycerol only; the intracellular perfusion fluid contained (25 mM) CsF and glycerol.

B_1 and B_2 show the effect of replacing the CaCl_2 -containing external medium with 600 mM choline chloride solution.

C_1 and C_2 show the effect of substitution of choline with NaCl (600 mM) mixed with 2mM EDTA; much stronger current pulses (not shown in the figure) failed to produce action potentials under these conditions.

D_1 and D_2 show restoration of excitability by replacement of half of NaCl with CaCl_2 ; note that the action potentials in the new mixture (containing 300 mM NaCl and 200 mM CaCl_2) are much larger than those shown in Records A . (Tasaki, Watanabe and Lerman, 1967.)

argued that alterations of the membrane macromolecules are normally caused by changes in the ratio of uni- and divalent cations, and that both of these cations play essential roles in the process of excitation.

“PHASE TRANSITION” IN THE AXON MEMBRANE

(7.4) Transition of the axon membrane from the resting state to the excited state is accompanied by a large change in the membrane resistance (Cole and Curtis, 1939). Under favorable experimental conditions, the membrane resistance falls to roughly 1/200 of the value in the resting state (Sects. 5.2 and 5.3).

In the field of physical chemistry, there are cases in which addition of a salt of divalent cation to the medium profoundly influences the resistivity. Clowes (1916) found that addition of CaCl_2 to a concentrated emulsion of Na oleate raises the resistance of the solution. This change in resistance is not a simple monotonic function of the amount of the added CaCl_2 ; a drastic increase in resistance takes place in a very narrow range of Ca ion concentration.

Figure 41 is adapted from a study by Waterman (1928), in which the resistance of a 1:1 mixture (emulsion) of 0.1 M NaOH and olive oil was measured with an impedance bridge and a pair of platinized platinum electrodes. When a CaCl_2 solution was gradually added to the mixture in small aliquots, there was a gradual decrease in the resistance, which reflected the increase in the amount of mobile ions in the system. When the amount of the added CaCl_2 solution reached a certain critical level, the resistance was found to *increase* suddenly and profoundly.

At the moment when the sudden rise in the resistance of the oleate emulsion takes place, there is a concomitant rise in the viscosity, and a visible change in the opacity of the mixture. These changes are attributed to a transition of the soap emulsion from an oil-in-water state to a water-in-oil state. With a sufficient amount of CaCl_2 in the medium, the fatty acid molecules arrange themselves around small water droplets and the nonaqueous material forms the continuous phase. On the other hand, at low CaCl_2 concentration, a continuous aqueous phase surrounds Na (or K) soap molecules. More recently, Parsegian (1966) discussed the nature of this type of phase transition from a statistical-mechanical point of view.

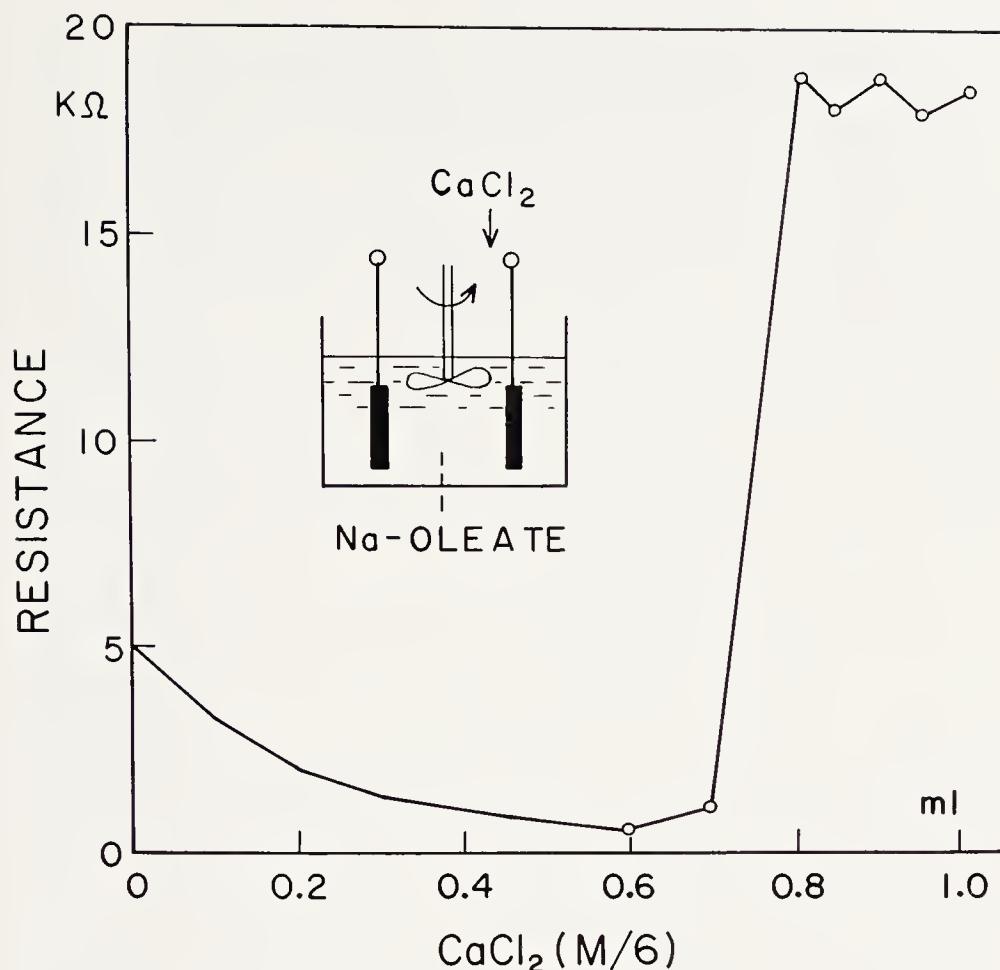


FIGURE 41. Changes in the electric resistances between two platinized platinum electrodes in Na-oleate emulsion caused by progressive addition of 1/6 M CaCl_2 solution. The original emulsion was prepared by mixing 60 ml of olive oil and 60 ml of 0.1 N NaOH. Note that a large increase in the resistance was observed between 0.7 ml and 0.75 ml of the added CaCl_2 solution (adapted from Waterman, in J. Alexander, *Colloid Chemistry*, Vol. II, Reinhold Publishing Corporation, New York, 1928).

Recent studies of the physical chemistry of synthetic and natural macromolecules have provided different examples of systems in which small variations in the uni- to divalent cation concentration ratio can produce a profound change in the state of macromolecules. Investigations of the effects of common neutral salts on collagen, RNase, DNase, etc., have shown that secondary and tertiary structures of these macromolecules are very strongly influenced by the neutral salts in the medium (Garret and Flory, 1956; Peller, 1959; Michaeli,

1960; Hamaguchi and Geiduschek, 1962; von Hippel and Wong, 1963, 1964). Also, the colloidal state of various polyelectrolyte emulsions can change abruptly within a very narrow range of Na and Ca ion concentration ratios in the medium. Overbeek and Vooran (1957) treated this phase-transition problem as a competition between the electric attraction which tends to assemble the charged groups in macromolecules and the entropy which tends to disperse them.

There is another type of phase-transition in regular and rigid ion exchangers. Barrer and Falconer (1956) have shown that the X-ray pattern of a Na-rich state of zeolite is different from that of the K-rich state. When the mole-fraction of K ion in the mixture of Na- and K-salts is increased continuously, a change from the Na-rich state to the K-rich state takes place at a certain mole fraction in the medium. In the reverse direction; i.e., when a K-rich form is converted to a Na-rich form, transition occurs at a different mole fraction in the medium (hysteresis). The transition mechanism proposed by Barrer and Falconer is based on the assumption that occupancy of neighboring sites by ions of the same kind is energetically more favorable than occupancy of adjacent sites by different ion species.

Tasaki (1963) suggested that the type of interaction between ions occupying two neighboring charged sites seen in zeolite could explain *phase transition* occurring in the axon membrane. When this type of interaction exists, a continuous change in the equivalent fraction of the univalent cation in the medium [$c_1/(c_1+2c_2)$], can produce a large, discontinuous change in the equivalent fraction in the membrane [$\bar{C}_1/(\bar{C}_1+2\bar{C}_2)$]. This situation is illustrated in Figure 42, where the three segments of the S-shaped isotherm represent the resting state (OABC), the excited state (DEF), and the unstable (unrealizable) state (CD). As the equivalent fraction of the univalent cation in the medium rises and falls, the corresponding fraction in the membrane varies along the hysteresis loop marked A-B-C-E-F-E-D-B-A. The discontinuous relationship between the membrane potential and the KCl concentration in the medium (Figs. 19 and 20) can be interpreted on the basis of this diagram. A discontinuous change in the equivalent fraction in the membrane is expected to produce a discontinuous change in the membrane potential.

More recently, Tasaki, Singer and Takenaka (1965) suggested that formation and dissolution of a "complex coacervate" by a

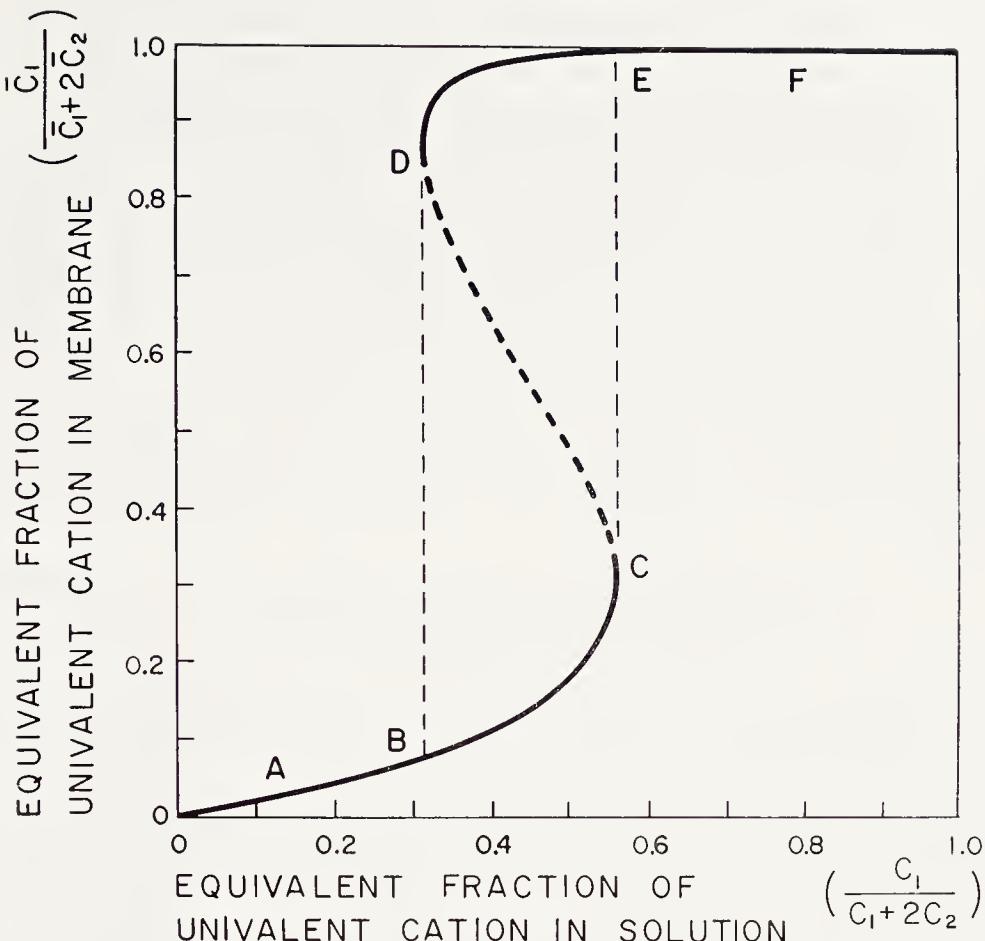


FIGURE 42. Theoretical ion-exchange isotherm calculated for a cation-exchanger membrane immersed in a salt solution containing univalent and divalent cations. In calculation, it was assumed that occupancy of two neighboring charge sites in the membrane by two cations of different valencies is energetically unfavorable. When the equivalent fraction of the univalent cation in the solution is increased continuously from zero to one, the corresponding fraction in the membrane increases along the course O , A , B , C , E , and F . When the equivalent fraction in the solution is decreased from one to zero, the corresponding fraction in the membrane changes along F , E , D , B , A , O . (For further details, see Tasaki, *J Gen Physiol*, 46:755-772, 1963.)

change in the fraction of the divalent cations in the external medium could also represent the phase transition which takes place in the axon membrane. Again, it is believed that this transition is associated with a large change in the fraction of the divalent cation in the membrane. Such a transition is expected to accompany a drastic change in the water content of the membrane.

One important factor to be considered in connection with the problem of phase transition in the axon membrane is the existence of electric interaction between two neighboring areas on the membrane. When a small area or spot in the axon membrane undergoes a transition from the resting state to the excited state, an electric current is generated between this spot and the neighboring resting area. This current is inward-directed through the excited area and outward-directed through the resting area. Therefore, the current tends to shift the resting area toward the excited state and the excited spots toward the resting state. In this sense, the process in the axon membrane is cooperative. The importance of electric interaction between different parts of the membrane will also be discussed in Chapter VIII.

ABOLITION OF ACTION POTENTIAL BY INWARD CURRENT PULSE

(7.5) The phenomenon of abolition of the action potential was first described in the study of the nodal membrane of the frog nerve fiber (Tasaki, 1956). The small size of a single node preparation requires a special technique for recording variations in the membrane potential (see Tasaki and Frank, 1955; Frankenhaeuser, 1957). The action potential of a single node of Ranvier is roughly triangular in configuration, and falls nearly linearly from its peak. There is a rather sudden fall in the membrane potential at the end of the action potential.

If a brief pulse of inward current is applied during the slowly descending period of the action potential (see Fig. 43A), the membrane potential falls during the period of current flow. Immediately after the end of the pulse, the membrane potential rises rapidly to the level which would have been reached had no current pulse been delivered (see Fig. 43A). As the intensity of the pulse is increased, the membrane potential falls further; but after the end of the pulse, the potential rises back to approximately the same level (see curve B). However, when the current exceeds a certain critical intensity, there is no potential rise back to the same level following termination of the pulse. Instead the membrane potential approaches the level of the resting potential (see curve D). Exactly at the critical intensity of the pulse, the membrane potential stays at an unstable level for

a variable period of time before it approaches either of the stable levels (see curve C).

Premature termination of an action potential by a pulse of inward current is called *abolition of the action potential*. The critical intensity of the current needed for abolition is high near the peak of the action potential, gradually decreases during the descending period, and approaches zero toward the end of the action potential.

The phenomenon of abolition has been demonstrated during pro-

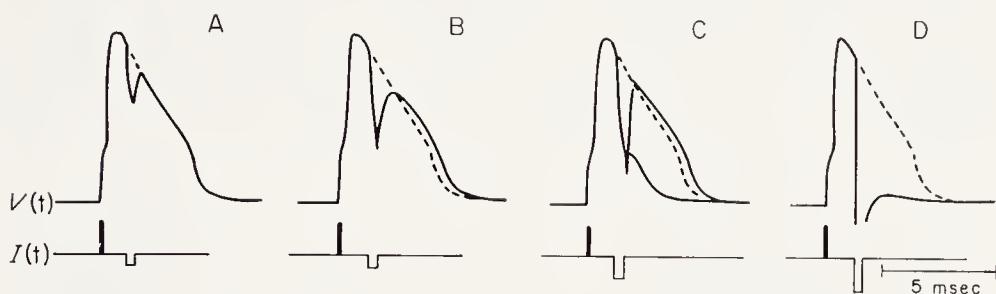


FIGURE 43. Abolition of the action potential of a single node of Ranvier by brief pulses of inward current. The tracing marked $I(t)$ shows the time course of the applied currents. (A downward deflection represents an inward-directed current.) The tracing marked $V(t)$ represents the potential variations across the nodal membrane. Abolishing current pulses were applied to the node during the descending period of the action potential. The current intensity was increased progressively from A to D. The broken lines indicate the potential levels that would be observed if the abolishing pulses were not delivered. (Tasaki, *J Gen Physiol*, 39:377-395, 1956.)

longed action potentials in the squid giant axon (Tasaki and Hagiwara, 1957). The general characteristics of this phenomenon are very similar to those found in the frog nerve fiber and in Teorell's nerve model. This phenomenon has also been demonstrated in squid giant axons immersed in media free of univalent cations (Tasaki, Watanabe and Lerman, unpublished).

Teorell (see Sect. 7.2) has demonstrated the phenomenon of abolition in his inanimate electrochemical system. In this model system, abolition of an action potential represents a transition from one stable concentration profile to another. As discussed in Section 7.2, there are two stable profiles at a given current I and pressure P (see points 3 and 1 in Fig. 39). When the system is in the excited state (represented by 3), a change in the current intensity from I' to I'''

for a brief period of time produces a transition to a resting state. (Note that there is only one stable state at this current and pressure.) Subsequently, when the current intensity is reduced to I' , the system returns to the original (resting) state 1.

The two stable states hypothesis and its macromolecular interpretation offer a satisfactory explanation for the phenomenon of abolition. An inward-directed membrane current tends to increase the equivalent fraction of divalent cations in the membrane. There is enhanced cation interdiffusion during excitation, and this process tends to oppose the effect of the applied current. When the current is strong enough, the equivalent fraction of the divalent cations is altered sufficiently and the membrane undergoes a transition from the excited state to the resting state. This explanation is strongly supported by the observation that action potentials produced by axons immersed in pure solutions of CaCl_2 (or SrCl_2) can be abolished by pulses of inward-directed current. The physicochemical nature of the process which gradually changes the threshold intensity for abolition during excitation will be discussed in Section 7.7.

ABOLITION BY CHEMICAL AND THERMAL MEANS

(7.6) The duration of the prolonged action potential observed in squid giant axons is sensitive to the divalent cation concentration in the medium; replacement of a portion of NaCl in the medium with CaCl_2 tends to decrease the duration. This result is expected from the analysis presented in the preceding section; an increase in the external Ca ion concentration accelerates the replacement of univalent cations in the membrane by Ca ion, and terminates the action potential prematurely.

In the single node preparation of the toad, the area of membrane involved in excitation is very small (Ch. II). Therefore, when a small amount of CaCl_2 solution is applied to the node, the Ca concentration rises nearly uniformly over the entire membrane surface. By using this preparation, Spyropoulos (1961) examined the effect of CaCl_2 application during the plateau period of a prolonged action potential. A small, electrically driven syringe was used to deliver an aliquot of isotonic CaCl_2 solution to the vicinity of the node of Ranvier. Since there was a continuous flow of normal Ringer's solution around the node, the Ca ion concentration around the nodal membrane was

raised only for a brief period of time. As shown in Figure 44A, an all-or-none, premature termination of the prolonged action potential was observed under these conditions. On the other hand, when isotonic KCl solution was used instead of CaCl₂, a prolongation of the action potential was observed. This observation is consistent

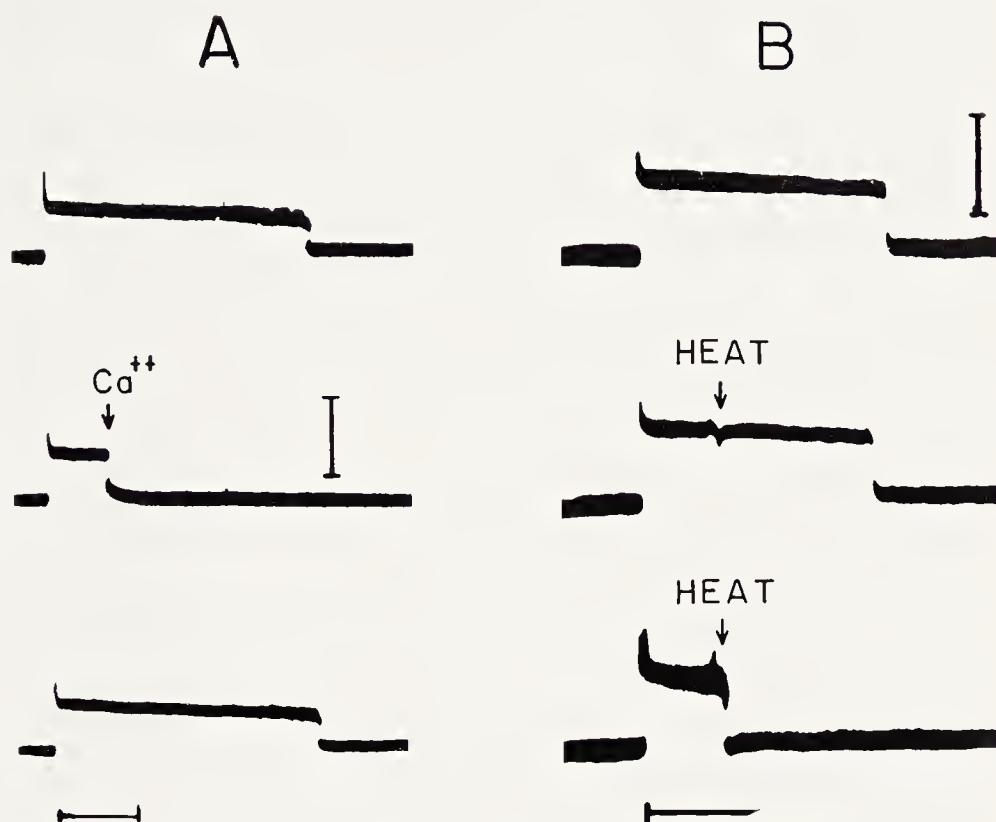


FIGURE 44. A: Abolition of the action potential of a Ni-treated node of Ranvier by exposing to a Ca-rich solution for a brief period of time.

B: Abolition of the action potential of a Ni-treated node by a heat pulse. (Adapted from Spyropoulos, *Amer J Physiol*, 200:203-208, 1961.)

with the view that divalent cations play a crucial role in the initiation and abolition of an action potential.

The replacement of Ca ion with K ions in sulfonic and phenolic cation-exchanger resins is exothermic. Coleman (1952) measured the change in enthalpy associated with this ion exchange by two different methods. In one series of experiments he used a direct calorimetric method; in the other series the enthalpy change was determined from the temperature dependence of the selectivity coefficient. He esti-

mated that the heat evolved when K ions replace Ca ions is approximately 2.7 kcal/mole. The enthalpy change associated with substitution of K ions for Na ions is on the order of 0.5 kcal/mole. Since the temperature dependence of the selectivity of carboxyl and phosphate resins seems similar to that of sulfonic resin, the enthalpy change in these ion-exchangers may well be qualitatively the same as in the sulfonic resins.

On this basis, the onset of the action potential should be associated with heat production. Although there is no direct measurement of heat production associated with a prolonged action potential, experiments by Abbott, Hill and Howarth (1958) indicate that there is actually a rise in temperature at the onset and a fall in temperature at the end of the action potential. The theory of action potential production involving uni- and divalent cation exchange is consistent with this experiment; uni- univalent cation exchange (e.g., Na-K) cannot account for these results.

According to the present theory, the terminal potential jump (to the resting level) at the end of a prolonged action potential should be associated with a sudden cooling of the axon. Since an endothermic reaction is encouraged by heating, a rise in temperature is expected to decrease the duration of the action potential. Spyropoulos (1961) examined the effect of a brief heat pulse applied during the plateau period of the nodal action potential. He found that the action potential could be terminated by a brief heat pulse in an all-or-none manner (see Fig. 44B). This observation strongly supports the view that the termination of the action potential is an endothermic ion-exchange reaction.

The process of initiation of an action potential is exothermic; therefore, it is expected that the process be encouraged by cooling. In fact, an action potential can be evoked by sudden cooling in various excitable tissues (Hill, 1934-35; Spyropoulos, personal communication), and cooling lowers the threshold for excitation (see Tasaki and Spyropoulos, 1957).

FALLING LIMB OF THE ACTION POTENTIAL AND REFRACTORY PERIOD

(7.7) It is well known that the axon membrane is absolutely refractory to a second pulse of outward-directed current applied

during the descending limb of an action potential (see Ch. II). There is a gradual recovery in the ability of the axon membrane to respond to the second stimulus after the end of the action potential. The time course of this recovery process is shown in Figure 45A for the single

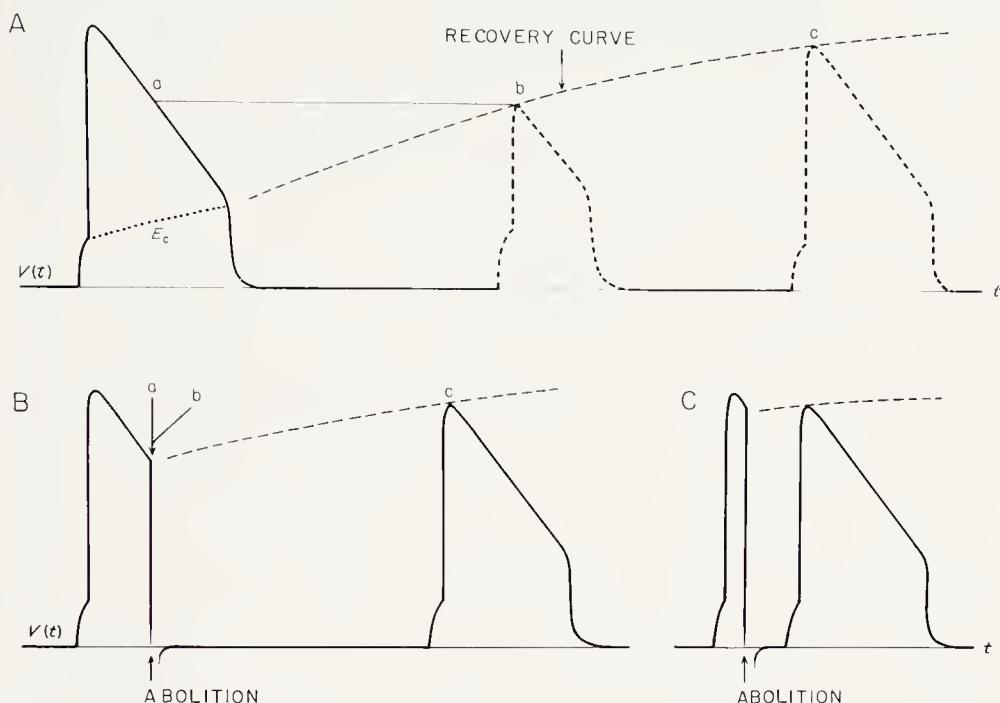


FIGURE 45. A: Recovery of a node of Ranvier following production of an action potential; recovery curve represents the curve connecting the peaks of the second action potentials elicited at various intervals after the first action potential; E_c represents the critical level of the membrane potential required for abolition. When the membrane potential (continuous line) reaches E_c "spontaneously" or by application of an inward current pulse, the action potential is terminated.

B and C: Recovery of a node following an action potential abolished in the middle of its descending limb; note that the period between a and b in diagram A has been eliminated by abolition.

C: Recovery of a node following abolition near the peak of an action potential. (Adapted from Tasaki, *J Gen Physiol*, 39:377-395, 1956.)

node preparation of the toad (Tasaki, 1956). The broken line represents an envelope of the peaks of the action potentials which could be elicited at various intervals after the first conditioning action potential. The time course of this recovery is very similar to the recovery curve for the threshold.

When the conditioning action potential is abolished by a pulse of strong inward current, an entirely different recovery curve is observed (Tasaki, 1956). After an action potential is abolished at one-half of the peak value, the amplitude of the response that can be elicited immediately after abolition is about one-half of the normal amplitude. Since recovery starts at this level, the after-effect of abolition is equivalent to elimination of the portion of the normal recovery curve indicated by the line a-b in the figure (compare diagram A with B). When the action potential is abolished at its peak, there is no refractoriness at all (diagram C).

These properties of the axon membrane can be compared with corresponding processes in Teorell's model of nerve (Fig. 39). In Teorell's system, both the fall in the action potential amplitude and the refractoriness are consequences of the enhanced transport process during excitation. Axonologists are familiar with the fact that interdiffusion of cations across the axon membrane is enormously enhanced during excitation. This enhanced interdiffusion tends to raise the concentration of internal cations near the external membrane surface and the concentration of external cations near the internal surface. Both a rise in the *external* cation concentrations in the axon *interior* and a rise in the *internal* cation concentration *outside* the axon lower the action potential amplitude; therefore, *the fall of the membrane potential from the peak of the action potential may be considered as the result of accumulation of the interdiffusing cations in and near the axon membrane.*

The electric resistance of the axon membrane is relatively high in the resting state (see Sect. 5.3). Therefore, the effects of "unstirred layers" (see Sect. 3.6) on the concentration profiles of individual ions are relatively small. But, when the membrane resistance is drastically reduced at the onset of excitation, it is expected that the system would begin to deviate from the original, "membrane diffusion-controlled" situation and approach the "film diffusion-controlled" state. (For the concept of film diffusion-controlled state, see Helfferich, 1962, p. 346 and 362). This inference is supported by the fact that the layer of axoplasm or perfusion fluid in the axon interior makes a significant contribution to the overall membrane resistance in the excited state (see Sect. 5.3). Even extremely violent stirring of the solutions does not eliminate the unstirred layers under these

conditions. Thus, a transition of the axon membrane from the resting state to the excited state is expected to create a gradual alteration of the ionic concentration profiles in and near the axon membrane.

When the action potential is terminated, the membrane resistance returns to the original, high level. The ionic concentration profiles are then expected to return gradually to the original, membrane diffusion-controlled state. According to this interpretation, the rate of this recovery process should be determined by diffusion of the accumulated cations near the membrane into the surrounding fluid media. The problem of diffusion of K ions into the surrounding medium following repetitive stimulation has been discussed previously by Frankenhaeuser and Hodgkin (1956).

[The diffusion of cations into the surrounding medium should result in an approximately exponential fall in concentration, with a time constant of $(2l)^2/(\pi^2 D)$; D represents the average value of the diffusion coefficients of the cations, and l is the thickness of the surrounding diffusion layer (or film). The observed recovery curve for the toad nerve fiber has a time constant of the order of 10 msec at room temperature. Based on the assumption that the diffusion coefficient is of the order of 10^{-5} cm²/sec, the calculated effective thickness of the film outside the axon membrane is of the order of 5 μ . This figure is a reasonable value for the thickness of the Schwann cell layer outside the nodal membrane. The effective thickness of the film on the inner side of the membrane is not well defined.]

Under ordinary experimental conditions, enhanced interdiffusion continues during the entire period of the action potential of a node of Ranvier. Hence, interdiffusing cations continue to accumulate in and near the membrane while the membrane potential falls from the peak of the action potential. When the action potential is terminated prematurely by a brief pulse of inward-directed current (abolition), the rate of interdiffusion is immediately reduced to the resting level, and the accumulated cations start to diffuse away from the membrane surface. Since the ionic composition near the membrane has not been altered in this case as much as at the end of a normal (unabolished) action potential, recovery should be much faster (Fig. 45). In actual abolition experiments using current pulses of a relatively long duration, the ionic composition near the membrane can be significantly altered by the applied current.

The intensity of an inward-directed current required to abolish an action potential gradually decreases during excitation (Sect. 7.5). This gradual fall in the abolition threshold can now be interpreted as a result of cation accumulation in and near the membrane. The abrupt potential jump at the end of the nodal action potential (see Fig. 45) can then be taken as a sign of a spontaneous transition (i.e., without applied current) from the excited state to the resting state, caused by a gradual alteration of the ionic environment of the axon membrane.

According to the present theory, reduction of the interdiffusion during excitation is one factor which tends to prolong the action potential. Now it is possible to understand why dilution of the internal salt solution, which is expected to reduce the interdiffusion, prolongs the action potential (see Tasaki and Shimamura, 1962; Adelman *et al.*, 1965). The use of large polyatomic cations such as guanidinium as replacements for external sodium reduces the interdiffusion during excitation; the duration of the action potential observed under these conditions is definitely longer than that of a normal action potential (Tasaki, Singer and Watanabe, 1965, 1966). However, it should be noted that the intensity of interdiffusion is only one of the many factors which determine the duration of the action potential. Other factors (such as a low divalent cation concentration in the external medium) may strongly influence the action potential duration under various experimental conditions.

In unperfused squid axons immersed in natural sea water, interdiffusion of cations during excitation is much greater than in frog nodes or TEA-treated squid giant axons. (Note that the membrane conductance increases by a factor of about two hundred in normal squid axons, while the increase is only about ten fold in frog nodes and in TEA-treated squid axons.) The high intensity of interdiffusion accounts for the rapidity of the falling limb of the action potential observed in the "normal" squid giant axon, and for the difficulty in examining the stability of the membrane in the excited state.

DISSIPATION OF FREE ENERGY IN AXONS

(7.8) In this section, a thermodynamic problem related to the transition process in the axon membrane between the resting and excited states will be described briefly. In the preceding section, it was shown that termination of an action potential is a spontaneous

process, which results from a gradual alteration of the ionic environment of the membrane. When the membrane undergoes such a spontaneous transition in the critical ionic environment, the free energy of the system (consisting of the membrane and its environment) should decrease:

$$(7.8.1) \quad \Delta G = (\Delta H - T\Delta S) < 0$$

where ΔG is the change in the free energy of the system, ΔH is the change in the enthalpy, and ΔS is the change in the entropy. Since this process is spontaneous and endothermic, ΔG is negative and ΔH is positive; therefore, $T\Delta S$ is positive, and larger than ΔH . In other words, there is a large increase in the entropy of the system at the end of the action potential.

According to the macromolecular interpretation of excitation, transition from the excited state to the resting state is associated with formation of linkages between membrane macromolecules. This transition tends to restrict possible movement of the macromolecules, and therefore tends to *decrease* the entropy of the system. However, formation of salt-linkages is known to be associated with a large *increase* in the entropy of the system, which is attributed to a decrease in the amount of highly structured water around the macromolecules (Kauzmann, 1959). Therefore, the entropy increase at the termination of the action potential can be attributed to the rearrangement of water molecules around the membrane macromolecules.

Following stimulation of an axon by a brief pulse of electric current, the system undergoes transition from the resting state to the excited state. This process is exothermic (ΔH is negative), and the water around the membrane macromolecules becomes more ordered (ΔS is negative). Since the process of phase transition in the axon can be readily reversed, the change in free energy associated with this transition must be small.

In the resting state of the axon, there is continuous dissipation of the free energy of the system due to cation interdiffusion across the membrane. The rate of free energy dissipation increases enormously during excitation because the rate of interdiffusion increases. The changes in the enthalpy and entropy associated with phase transition are superposed on the dissipation of free energy by interdiffusion.

Chapter VIII

THE AXON MEMBRANE UNDER VOLTAGE CLAMP

THRESHOLD STIMULATION

(8.1) According to the two stable states hypothesis of nerve excitation described in the preceding chapters, every surface element of the membrane may take either of two stable macromolecular conformations, called the *resting* and *excited* states. The macromolecular conformations intermediate between these two stable states are assumed to be short-lived, and to change spontaneously to one of the two stable conformations.

When a brief pulse of (stimulating) electric current directed outward through the resting axon membrane converts a limited portion of the membrane into the excited state, an electric current begins to flow between the resting and excited portions. This eddy, or local current, acts as a stimulus for the resting area of the membrane and continues to act after termination of the brief, applied current pulse. The local current is inward-directed in the excited portion of the membrane and outward-directed in the resting portion. If only a small portion of the membrane is brought to the excited state at the end of the applied current pulse, the inward-directed current through the excited portion has a relatively high density and tends to terminate the excitation process prematurely. If a sufficiently large area of the axon membrane is excited by the applied current pulse, the density of the outward current will be enough to bring the remaining resting area into the excited state.

It is reasonable to assume that a brief pulse of stimulating current produces spots and patches in the membrane which are in the excited state, because the axon membrane has various morphological irregularities which tend to make the effect of a stimulating current non-uniform. Even in chemically pure uniform systems, the effect of an electric current is frequently nonuniform. For example, Stephenson

and Bartlett (1954) showed that when currents are passed through the apparently smooth surface of a pure copper sheet immersed in hydrochloric acid, microscopically visible spots appear on the surface of the copper. It is of interest that Matumoto and his associates (1958) found that stimulation of the Ostwald-Lillie iron wire model for nerve is characterized by the appearance of active (excited) spots on the surface of the iron.

The interaction between the resting and excited portions of the axon membrane has been analyzed on the basis of the N -shaped current-voltage (I - V) relationship determined by the voltage clamp technique (Sect. 5.3). In principle, the procedure of voltage clamping is equivalent to placing the axon membrane between two metal electrodes across which a rectangular voltage pulse of the preselected amplitude V is applied (see Fig. 46A). Since there is some space between the axon membrane and the metal electrodes, it is not possible to eliminate flow of electricity between the resting and excited areas completely. However, the major portion of the current passes from one metal plate to the other plate through the membrane.

The ratio of the membrane area in the excited state to the total area under study will be designated by α , the *active fraction* of the membrane. The fraction of the membrane in the resting state is then $(1 - \alpha)$. The total current, I , passing through a unit area of membrane is given by the sum of the current through the resting area and through the excited area:

$$(8.1.1) \quad I = (1 - \alpha)g_r(V - E_r) + \alpha g_a(V - E_a)$$

where V is the voltage at which the membrane is clamped, g_r and E_r are the conductance and *emf* of the membrane in the resting state respectively; g_a and E_a are the corresponding quantities in the excited state (see Fig. 46B). This equation (8.1.1) is approximate because there are uncontrollable local currents in the membrane.

If the voltage V at which the current I vanishes is denoted by E_c and the fraction of the membrane in the excited state at this voltage by α_c , then

$$(8.1.2) \quad 0 = (1 - \alpha_c)g_r(E_c - E_r) - \alpha_c g_a(E_a - E_c).$$

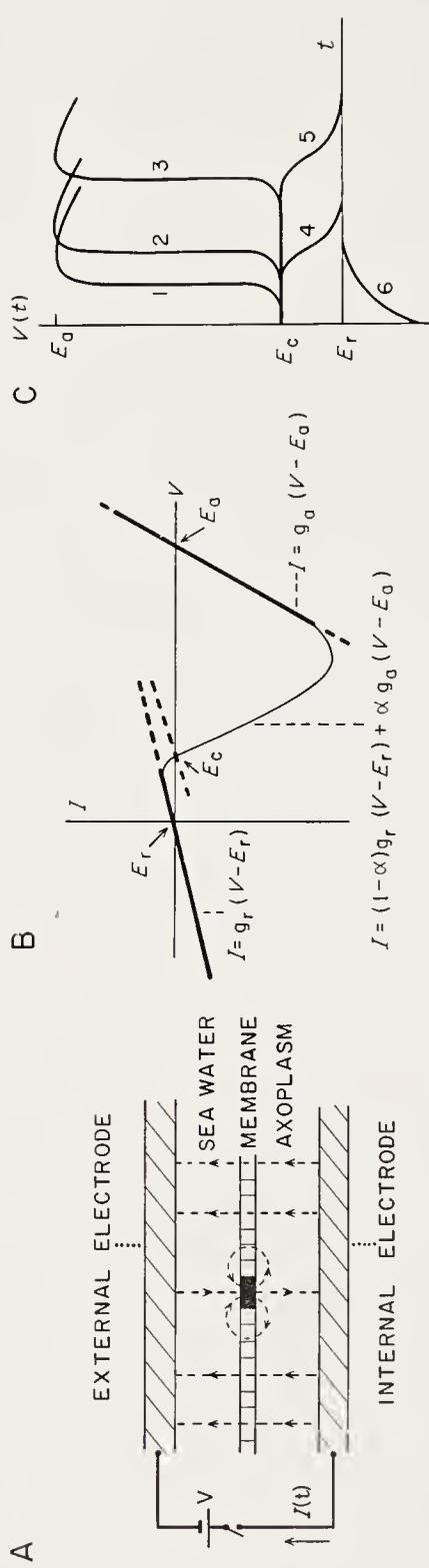


FIGURE 46. A: Schematic diagram showing the state of the squid axon membrane under voltage clamp; the potential difference between the internal and external metal electrodes is maintained at a preselected level, V , and the membrane current, $I(t)$, is determined; the black area represents the excited portion of the membrane; the broken lines and the arrows indicate the lines of current flow.

B: The $I-V$ relationship in the resting state (shown by the straight line on the left), in the excited state (straight line on the right), and in the mixed state (intermediate thin line).

C: Potential-times curves of an axon stimulated by a current pulse of the threshold strength.

From this equation it follows that

$$(8.1.3) \quad \alpha_e = \frac{1}{\frac{g_a(E_a - E_e)}{g_r(E_e - E_r)} + 1}$$

This equation determines the critical active fraction, α_e , of the axon membrane across which there is no (net) electric current (see E_e in Fig. 46). Under these conditions, the inward current through the excited spots is equal to the outward current through the resting area (see Fig. 46A).

When the axon membrane is stimulated by a brief pulse of outward current at the threshold intensity (without voltage clamping), a quantity analogous to α_e can be defined. In this case, the membrane potential remains at a constant level for a considerable period of time before it starts to deviate either upward or downward (see Figs. 37B, 46C). Since there is no (net) membrane current during the period of constant membrane potential, this constant potential level should coincide with E_e in equation 8.1.2 or 8.1.3. Therefore, the fraction of the membrane in the excited state in this case is also given by equation 8.1.3.

In single node preparations of the toad, the conductance ratio, g_a/g_r is of the order of ten (Tasaki and Freygang, 1955; Dodge and Frankenhaeuser, 1959). The critical membrane potential E_e is approximately 25 mV above the resting potential E_r , and the action potential amplitude is about 105 mV. Introduction of these values into equation 8.1.3 leads to a value of approximately 0.03 for the critical fraction α_e . This finding indicates that excitation of only 3 percent of the entire membrane surface is sufficient to throw the remaining 97 percent of the membrane into the excited state.

A similar calculation for the squid axon membrane under favorable conditions shows that the critical fraction is far smaller than that for the nodal membrane. The critical fraction increases as the condition of the axon becomes unfavorable.

It is known that there is a small reduction in the membrane impedance during the period in which the membrane potential is maintained at the critical level E_e (see Tasaki, 1958). The presence of this small impedance loss is consistent with the existence of only a small fraction of the membrane in the excited state. According to the

present interpretation, the all-or-none property of the action potential derives from the condition that the critical fraction α_c is far smaller than unity. The axon membrane loses its ability to develop all-or-none action potentials when the critical fraction defined by equation 8.1.3 approaches unity.

The critical potential level (E_c) is known to rise when the divalent cation concentration in the external medium is increased; this rise is expected from the argument stated in Section 7.4. However, it is seldom that E_c rises to a level $2RT/F$ (50.4 mV) above the resting potential. The distribution of cations within the membrane is significantly altered when the potential drop caused by an outward current reaches $2RT/F$ (see Appendix). Therefore, the critical level for initiation of an all-or-none action potential is not expected to exceed about 50 mV.

The instability of the membrane potential at and near the critical level, E_c (see Fig. 46C) can be expressed formally by

$$(8.1.4) \quad \frac{dV}{dt} = + k(V - E_c)$$

where k is a positive coefficient of proportionality. This expression indicates that when $V = E_c$, the membrane potential can stay at E_c indefinitely, but the slightest upward or downward deviation in V leads to an accelerated deviation from the initial state. This instability arises from the strong dependence of the fraction α on the membrane potential V . In physiological terms, the delayed decline in V at the level of E_c is called a *subthreshold response* (Hodgkin, 1938).

In the iron wire model of nerve (see Sect. 1.3), dilution of the surrounding acid solution often leads to formation of active spots in the absence of stimulating currents. The number of such spots in a given area may vary with time; in such cases, the potential of the wire (referred to a Pt-electrode in the surrounding medium) exhibits an oscillatory change. The phenomenon of subthreshold oscillation studied by Arvanitaki (1939) can be considered analogous to that in the iron wire model, and to represent a temporal variation in fraction α . A decrease in the divalent cation concentration in the external medium is the best-known method of producing a subthreshold oscillation in various nerves.

INTERMEDIATE PORTION OF CURRENT-VOLTAGE CURVE

(8.2) Based on the concept of the “active fraction,” α , a reasonable interpretation of the intermediate portion of the current-voltage (I - V) curve may be offered (see Fig. 47). As has been stated previously (Sect. 5.3), the approximately straight portion of the I - V curve passing through the origin ($I=0$, $V=E_r$) represents the ohmic character of the axon membrane in the resting state. Unless the axon is on the verge of firing action potentials spontaneously, the membrane may be considered macroscopically uniform. In this state the active fraction α is approximately zero.

The approximately linear portion of the curve passing through the peak value of the action potential ($I=0$, $V=E_a$) represents the ohmic property of the axon membrane in the excited state; the membrane can be regarded as macroscopically uniform at the peak of excitation ($\alpha \approx 1$).

With a clamping voltage (V) sufficient to bring some finite fraction of the membrane into the excited state ($0 < \alpha < 1$), the situation is somewhat complicated. However, as an approximation it is assumed that the passage of electric currents between the resting and excited areas of the membrane is negligible under voltage clamp. Under this assumption, different portions of the axon membrane respond to the clamping voltage independently, and proceed with their own recovery processes without being affected by other portions of the membrane.

As discussed in the preceding section, the current-voltage (I - V) relationship for a nonuniformly excited membrane can be formally expressed by

$$(8.2.1) \quad I \approx (1 - \alpha)g_r(V - E_r) + \alpha g_a(V - E_a)$$

Since the fraction α varies with the membrane potential V , this expression does *not* represent a linear relationship between the membrane current, I , and the clamping voltage V . The active fraction, α , can be determined as a function of V by applying this equation to the observed I - V curve. For a given value of α , the relation between I and V is linear; therefore, the voltage dependence of α can be determined graphically by the procedure shown in Figure 47A, and diagram B shows an example of this determination.

There is an independent method of determining the voltage dependence of the active fraction α . When a high-frequency alternating voltage of a low amplitude is superposed on the rectangular clamping voltage, the observed membrane current is composed of the original smooth component and the additional sinusoidal component. Since the smooth component has the same amplitude and time course as the membrane current observed in the absence of the sinusoidal component, it may be safely assumed that the value of α is not affected by the added a.c. Let the amplitudes of the sinusoidal voltage and

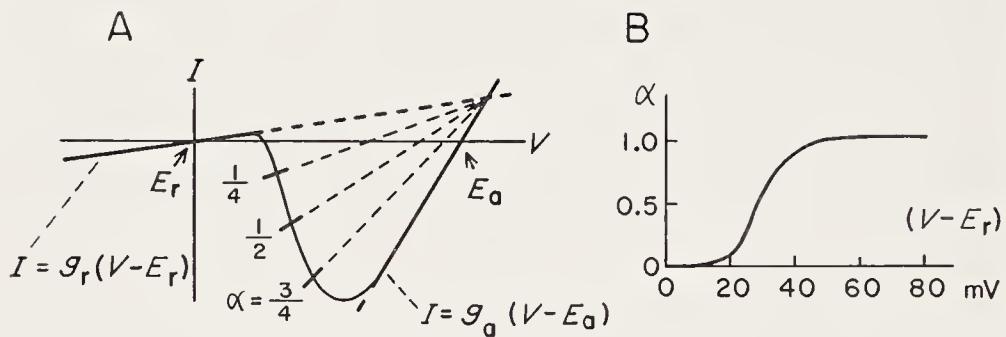


FIGURE 47. A: Diagram showing that the I - V relationship given by equation 8.2.1 for a fractional value of α passes through the intersection of the straight lines $I = g_r(V - E_r)$ and $I = g_a(V - E_a)$.

B: The dependence of the active fraction α on the clamping voltage V , as determined by the method of diagram A for a node of Ranvier.

current be denoted by ΔV and ΔI . Under these conditions, the relationship between the membrane current and potential can be written as

$$(8.2.2) \quad I + \Delta I \cdot e^{j(\omega t+\phi)} \approx (1 - \alpha)g_r[(V + \Delta V \cdot e^{j\omega t}) - E_r] \\ + \alpha g_a[(V + \Delta V e^{j\omega t}) - E_a]$$

where conventional complex number notations are used to describe the a.c. components. (The angular frequency and the phase shift are denoted by ω and ϕ , respectively.) The relationship between the sinusoidal voltage and current can be obtained by subtracting 8.2.1 from 8.2.2:

$$(8.2.3) \quad \Delta I \cdot e^{j\phi} \approx [g_r + (g_a - g_r)\alpha]\Delta V$$

Under favorable experimental conditions, the membrane conductance at the peak of excitation (g_a) is far larger than that at rest (g_r). Equa-

tion 8.2.3 can be simplified by introducing the condition that $g_a \gg g_r$, and assuming that the phase shift produced in the excited area is negligible. Equation 8.2.3 then becomes

$$(8.2.4) \quad \alpha \approx \frac{\Delta I}{g_a \Delta V}$$

Thus, for a given value of ΔV , the a.c. amplitude (ΔI) is expected to be proportional to the active fraction α .

A direct experimental test to see if the active fraction (α) determined by a.c. and d.c. methods agree was made by Tasaki and Spyropoulos (1958). The results obtained at about 18 kc a.c. with unperfused axons immersed in sea water were found to agree with that obtained by the d.c. method. Although this agreement does not preclude other explanations, it indicates that the present theory is self-consistent.

The active fraction α increases rather abruptly within a narrow range of voltage and approaches unity (Fig. 47B). An abrupt rise in α occurs when the membrane potential reaches about 25 mV above the resting level. Interdiffusion of cations across the membrane is altered significantly when the potential drop caused by the current exceeds about RT/F (25.2 mV); since the transition to the excited state can take place only when the cation distribution in the membrane is significantly altered by the current, the abrupt rise is expected to begin at about this voltage.

The voltage-dependence of α corresponds to the dependence of the sodium-conductance on the membrane potential in the theory proposed by Hodgkin and Huxley (1952). However, since determination of α can be made in axons immersed in sodium-free media (see Fig. 25), α is not specifically related to sodium. Furthermore, the inward current under voltage clamp is not carried purely by sodium ions even when sodium is the major cation in the external medium (Sect. 5.3; Appendix).

TIME DEPENDENT MEMBRANE POTENTIAL

(8.3) As an approximation, an axon developing an action potential can be treated as a system in a quasi-stationary state (see Sect. 5.3). When compared with the relaxation time (or time constant), the membrane potential changes very slowly during excitation; therefore, the system can be regarded as shifting very gradually from one sta-

tionary state to another. Based on this assumption, the time-dependence of the *emf* of the axon membrane during voltage clamping can be determined. The method of superposing a weak sinusoidal voltage on the clamping voltage was used for this determination.

The time-dependent membrane conductance and *emf* are denoted by $g(t)$ and $E(t)$, respectively; these quantities are assumed to vary slowly compared with the time required to measure them. The membrane current $I(t)$ during the period of clamping with a rectangular pulse, V , is given by

$$(8.3.1) \quad I(t) = g(t)[V - E(t)]$$

At the peak of excitation, $g(t)$ is equal to g_a and $E(t)$ is equal to E_a (see Sect. 5.3). In this case, a large value of V is chosen so that the entire membrane is thrown uniformly into the excited state.

When a weak sinusoidal voltage $\Delta V \cdot e^{j\omega t}$ is superposed on V , the total current is given by

$$(8.3.2) \quad I(t) + \Delta I(t) \cdot e^{j\omega t} \approx g(t)[(V + \Delta V \cdot e^{j\omega t}) - E(t)]$$

where the phase shift is omitted by adopting the approximation that the relaxation time (time constant) is far shorter than the period of the measuring a.c. When ΔV is small, $I(t)$ in the record with superposed a.c. is practically identical with $I(t)$ in the record without a.c. Subtraction of 8.3.1 from 8.3.2 yields

$$(8.3.3) \quad g(t) \approx \Delta I(t) / \Delta V.$$

This relation makes it possible to determine the membrane conductance, $g(t)$, during the entire period of voltage clamping. Next, $E(t)$ can be calculated by introducing this function, $g(t)$, into the relation of 8.3.1. The major advantage of this a.c. superposition technique is that the entire time course of $g(t)$ and $E(t)$ can be determined from a single oscilloscope record displaying the time course of a superposed current

$$[I(t) + \Delta I(t) \cdot e^{j\omega t}].$$

An example of the determination of $E(t)$ and $g(t)$ by this method for unperfused axons is shown in Figure 48. The initial portion of the *emf* time curve, $E(t)$ is very similar to the time course of the action potential (recorded with no membrane current). This result is expected from the fact that the cation interdiffusion fluxes during ex-

citation are usually much larger than the cation fluxes associated with the membrane current, $I(t)$, observed during the voltage clamp (see Tasaki, Singer and Watanabe, 1967). In the present theory, the decrease in emf during excitation is the result of increased cation interdiffusion (see Sect. 7.7). Since the passage of the membrane current

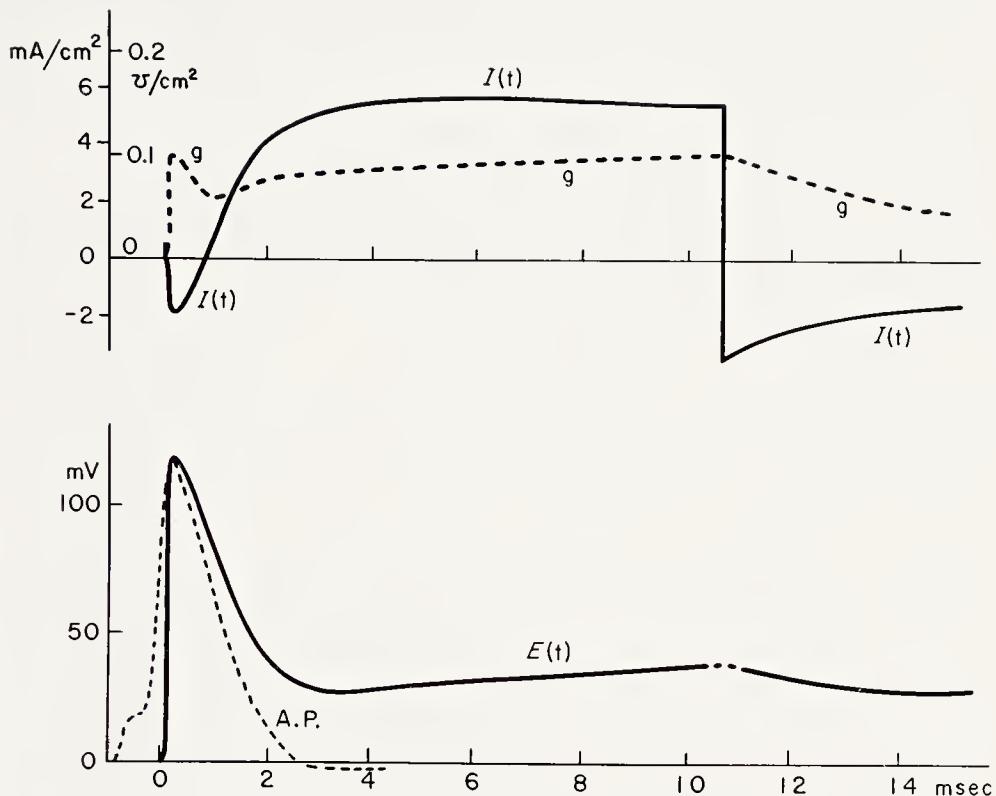


FIGURE 48. An example of the results of determination of $g(t)$ and $E(t)$ in equation 8.3.1 by the a.c. method. The broken line shows the action potential recorded from the same giant axon. (Tasaki and Spyropoulos, *Amer J Physiol*, 193:309-317, 1958.)

does not alter the interdiffusion fluxes significantly, little difference is expected between the time course of $E(t)$ for $I = 0$ and that for a finite (but weak) current.

The late portion of the emf -time curve is different from that of the normal action potential. This difference can be attributed to the maintained outward current under voltage clamp. The outward current is expected to raise the K ion concentration in both the membrane and the external fluid medium adjacent to the membrane (see

Sect. 5.1). The effect of this K ion accumulation is equivalent to raising the K ion concentration in the external fluid medium; therefore, the *emf* is expected to remain at a low level (see Sect. 4.5).

It is interesting to note that neither $E(t)$ nor $g(t)$ shows any discontinuity at the termination of the clamping pulse; but the membrane current, $I(t)$, changes discontinuously at this moment. The slow approach of $E(t)$ and $g(t)$ toward their resting levels during voltage clamping is attributed to slow diffusion of the accumulated K salt away from the membrane (Fig. 48, 11 to 15 msec).

SUBSTITUTION OF EXTERNAL CATIONS

(8.4) The intensity of the inward membrane current observed in voltage clamp experiments varies with the chemical nature of the ions present in the external medium. In fact, the foundation of the nerve excitation theory of Hodgkin and Huxley (1952) is built on observations and interpretations of the effects of substituting choline ions for Na ions in the external fluid medium. When such a substitution is performed, the process of action potential production is strongly suppressed; practically no inward current can be observed under voltage clamp.

In the original analysis of these results by Hodgkin and Huxley, it was tacitly assumed that the axon membrane is practically impermeable to choline ions. Based on this assumption, the axon membrane was treated as a Na-sensitive glass membrane. Hodgkin (1951) proposed that the membrane potential at the peak of excitation is given approximately by the Nernst equation:

$$(8.4.1) \quad E_a = \frac{RT}{F} \ln \frac{C_{\text{Na}}'}{C_{\text{Na}}''}$$

where C_{Na}' and C_{Na}'' are the external and internal Na ion concentrations (or activities), respectively. At that time it was believed that only Li ion could replace Na ion without a loss of excitability.

When the permeability of the axon membrane to external choline ion was examined with C^{14} -labelled choline (Tasaki and Spyropoulos, 1961), it was found that choline accumulates within the axon. In addition, when C^{14} -labelled choline was injected into the axoplasm, there was an appreciable efflux of radioactive material through the axon membrane. Therefore, the axon membrane does not behave as

a membrane specifically permeable to K or Na ion. [The assumption that the membrane is permeable to only one cation at the peak of excitation is also inconsistent with the existence of increased cation interdiffusion and increased membrane conductance (see Sect. 5.5).]

More recently, it was found that a large number of cations can replace external Na ion without eliminating the excitability of the axon

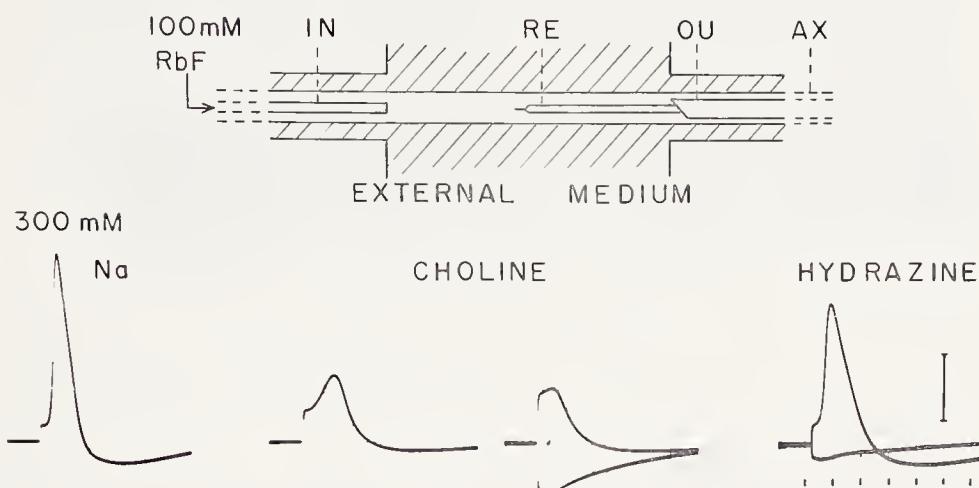


FIGURE 49. *Top:* Experimental arrangement used for demonstration of excitability of squid giant axon immersed in Na-free medium (not to scale). *AX*, giant axon; *RE*, recording electrode (wire enclosed in glass capillary); *OU*, outlet cannula, *IN*, inlet cannula.

Bottom: An example of the action potential records obtained with the arrangement above. The composition of the external fluid medium was 300 mM NaCl and 200 mM CaCl₂ (left record), 300 mM choline chloride and 200 mM CaCl₂ (center records), and 300 mM hydrazine chloride and 200 mM CaCl₂ (right record). The latter two media were Na-free. The perfusion zone was 12 mm in length, and the axon diameter was 620 μ . (Tasaki, Singer and Watanabe, *Proc Natl Acad Sci USA*, 54:763-769, 1965.)

(Tasaki, Singer and Watanabe, 1965, 1966 and 1967). Many of the favorable external cations used for maintaining excitability in squid axons are the same as those found for frog nerve trunks by Lorente de Nô *et al.* (1957) and Larramendi *et al.*, (1956).

An example of the experiments demonstrating a large action potential in a Na-free external and K-free internal environment is shown in Figure 49. In this case, an axon was internally perfused with 0.1 M RbF solution. After an action potential was recorded in a Na-containing (and CaCl₂-rich) medium,

was completely replaced with choline ion. The excitability of the axon was rapidly suppressed by this procedure. When choline ion was replaced with hydrazinium ion the ability of the axon to produce action potentials was immediately restored. The external CaCl_2 concentration was maintained at a constant level of 200 mM during this observation.

Under favorable intracellular conditions, ammonium ion is also an effective Na substitute. Sizable action potentials (about 80 mV in amplitude) can be obtained at relatively low NH_4 concentrations (approximately 100 mM NH_4Cl used in combination with CaCl_2 and tetraethylammonium-chloride). However, at slightly higher concentrations, there is a sudden rise in the internal potential and a loss of excitability (depolarization). This effect of ammonium ion is similar to that of K ion discussed previously (see Sect. 4.6).

The effectiveness of various univalent nitrogenous cations as Na substitutes can be compared in terms of relative favorability. The term *favorable* is applied to an external cation when the action potentials elicited are large in amplitude and exhibit a sharp ascending limb. When the axon produces action potentials with only small, variable (or graded) amplitudes, the cation substituted for sodium is called *unfavorable*. There are a number of homologous series which have been tested by this physiological technique. The orders of favorability found are:

1. $(\text{CH}_3)_4\text{N} < (\text{CH}_3)_3\text{NH} < (\text{CH}_3)_2\text{NH}_2 < (\text{CH}_3)\text{NH}_3 < \text{NH}_4$
2. $(\text{C}_2\text{H}_5)_4\text{N} < (\text{C}_2\text{H}_5)_3\text{NH} < (\text{C}_2\text{H}_5)_2\text{NH}_2 < (\text{C}_2\text{H}_5)\text{NH}_3 < \text{NH}_4$
3. $(\text{C}_3\text{H}_7)\text{NH}_3 < (\text{C}_2\text{H}_5)\text{NH}_3 < (\text{CH}_3)\text{NH}_3 < \text{NH}_4$
4. $(\text{C}_4\text{H}_9)_4\text{N} < (\text{C}_3\text{H}_7)_4\text{N} < (\text{C}_2\text{H}_5)_4\text{N} < (\text{CH}_3)_4\text{N} < \text{NH}_4$
5. $(\text{C}_2\text{H}_5)_2\text{NH}_2 < (\text{CH}_3)_2\text{NH}_2 < \text{NH}_4$.
6. $\text{CH}_3\text{NH}_3 < \text{HNH}_3 < \text{HONH}_3 \sim \text{H}_2\text{NNH}_3$
7. $\text{HO}(\text{CH}_2\text{CH}_2)\text{N}(\text{CH}_3)_3 < \text{HO}(\text{CH}_2\text{CH}_2)\text{NH}_3 < \text{HONH}_3$
8. $(\text{HOCH}_2\text{CH}_2)_2\text{NH}_2 < (\text{HOCH}_2\text{CH}_2)\text{NH}_3$
9. $\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_3 < \text{HOCH}_2\text{CH}_2\text{NH}_3$
10. $(\text{CH}_3)_2\text{CHNH}_3 \sim \text{CH}_3\text{CH}_2\text{CH}_2\text{NH}_3 < \text{CH}_3\text{CH}_2\text{NH}_3$
 $< \text{HOCH}_2\text{CH}_2\text{NH}_3$
11. $\text{H}_2\text{NC}_6\text{H}_4\text{NH}_3 < \text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_3 < \text{H}_2\text{NNH}_3$
12. $\text{H}_2\text{NHN}(\text{H}_2\text{N})\text{C:NH}_2 \sim (\text{H}_2\text{N})_2\text{C:NH}_2 < \text{H}_2\text{NNH}_3$.

OUTSIDE : KCl, TMA - Cl, CaCl₂
 INSIDE : CsF, Cs - PHOSPHATE

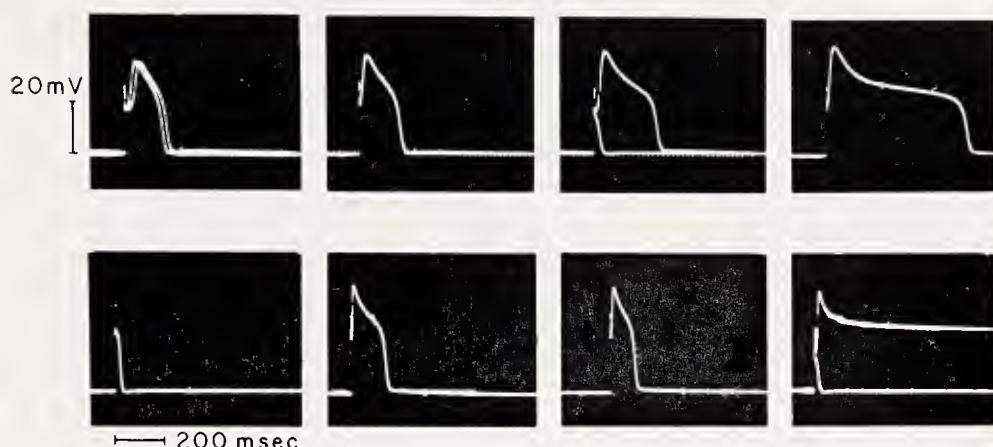
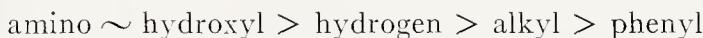


FIGURE 50. Effects of replacement of external sodium ion with potassium ion. The external medium was a mixture of KCl, TMA-Cl and CaCl₂; the potassium ion concentration was approximately 30 to 90 mM (see text). The internal solution was a mixture of 90 mM CsF and 10 mM Cs-phosphate (pH 7.2). The upper and lower sets of records were taken from two different axons. Subthreshold responses are shown in the third, fifth, and eighth records. The third and eighth records also show superposed suprathreshold responses. All other records show suprathreshold responses. The eighth record shows a marked plateau period; such plateaus may last more than one second. These responses can also be maintained under internal perfusion with RbF and are more resistant to tetrodotoxin than the responses of axons immersed in pure CaCl₂ solution (see Fig. 40). (Tasaki, Singer and Watanabe, *Amer J Physiol*, 211:746-754, 1966.)

From these results, it is found that (a) the Na-substituting ability of polyatomic univalent cations decreases with the number and length of the hydrocarbon chains attached to the nitrogen atom, and (b) the Na-substituting ability of univalent cations containing various side groups decreases in the following order:



It was reported that both KCl and RbCl could be used to replace NaCl in the external fluid medium during internal perfusion with 100 mM CsF solution. As in the case of ammonium ion, the concentration of these salts had to be limited to relatively low levels because higher concentrations tend to depolarize the axon (i.e., to lower the membrane potential and resistance and to suppress excitability). Two examples of the action potentials observed with exter-

nal K ion in place of Na ion are shown in Figure 50. In these observations, the axon was initially rendered inexcitable by immersion in a medium containing a mixture of tetramethylammonium and calcium chloride. After initiating internal perfusion with the CsF solution, an isotonic solution of KCl was added to the medium in small aliquots until all-or-none action potentials could be elicited from the axon.

It is worth mentioning that the ability of the squid axon to produce all-or-none action potentials can be maintained for a considerable period of time with an internal medium containing only sodium salts (Tasaki and Shimamura, 1962). In axons perfused with a dilute solution of NaF, excitability can be maintained when the Na ion in the external medium is replaced with hydrazinium ion or even when a simple CaCl₂ solution is used externally (Sect. 7.3).

The current-voltage (*I-V*) relationship of axons immersed in Na-free media has been examined by the voltage clamp technique (Tasaki, Singer and Watanabe, 1967). An example of the results obtained has been presented in Section 5.3 (see Fig. 25). Whenever an axon is capable of producing all-or-none action potentials, the *I-V* curve obtained shows a typical *N*-shaped configuration. The maximum value of the inward current varies with the compositions of the internal and external fluid media.

EXPLANATION OF THE RESULTS OF SUBSTITUTION EXPERIMENTS

(8.5) The results of the substitution experiments described in the preceding section are very complex. However, they can be at least qualitatively interpreted on the basis of the macromolecular hypothesis proposed in recent articles from this laboratory (Tasaki and Singer, 1965, 1966).

The macromolecules in the critical layer of the membrane are assumed to be bound together by the divalent cations from the external medium and form *complex coacervates* (see Bungenberg de Jong and Saubert, 1936; Bungenberg de Jong, 1949, p. 335, 337 and 289). Such complexes can be disrupted (reversibly) by the addition of univalent cations which have a strong affinity for the negatively charged sites contributing to the complex formation. (It is assumed that the membrane has other negative sites which confer the cation exchanger properties on the membrane.)

The cation sequence determined by the ability of a cation to suppress complex formation and to depolarize the axon membrane varies with the chemical nature of the negative sites. The sequence of externally applied cations for production of depolarization is:

$$\text{Na} < \text{Cs} < \text{Rb} < \text{K}$$

(Höber, 1926; Tasaki, Takenaka and Yamagishi, unpublished).

The favorable internal cations (K, Rb or Cs) have a stronger tendency to depolarize the membrane than the usual favorable external univalent cations (Li, Na, hydrazinium, etc.). Consequently, transport of the internal, more depolarizing cations into the critical layer of the axon membrane by a stimulating current pulse is very effective in rapidly disrupting the macromolecular complex in the membrane. This reversible disruption is thought to convert the membrane macromolecules from their resting conformation to their excited conformation.

External polyatomic univalent cations with weak depolarizing ability are expected to replace the relatively weakly bound Na in the membrane without displacing the divalent cations. The tendency of the membrane to accumulate polyatomic cations would increase if a hydrophobic group was added to the cation. (Note that complex coacervates have a low water content; therefore, formation of hydrophobic bonds within the complex is highly probable.) When a pulse of stimulating (outward) current is delivered to the membrane under these conditions, transition to the excited conformation would be delayed by the presence of the additional hydrophobic bonds between the membrane macromolecules and the added polyatomic cations. Thus, the suppressive effect of substituting hydrophobic group-containing cations (tetraethylammonium, choline, etc.) for the external Na ion could result from a decrease in the *rate of conformational change*. (This would account for the absence of an initial inward current under voltage clamp.) When a strong outward current is maintained through the membrane, the membrane macromolecules eventually undergo transition to the excited state. (Therefore, the delayed outward membrane current under voltage clamp remains unaffected by substitution of choline for Na ion.) Polyatomic cations with hydrophylic groups interfere with this transition to a lesser extent because they do not form hydrophobic bonds with the membrane macromolecules. The effect of large polyatomic cations, such as

tetrodotoxin, may be interpreted in a similar manner (Watanabe *et al.*, 1967). In such cases, Van der Waals forces and other short-range forces may contribute to decrease the rate of the conformational change suggested above.

When only part of the external Na ion is replaced with choline ion, the axon is still capable of developing action potentials. In this case, some of the negatively charged sites in the membrane are still occupied by Na and Ca ions; the macromolecules at these sites should undergo a normal transition when stimulated. However, the portion of the membrane occupied by choline is expected to remain relatively unchanged at the peak of excitation because of the slow rate of the ion exchange process. Thus, the cation composition in the excited portions of the membrane is expected to remain unaffected by addition of choline ion to the external medium. The phase-boundary potential, E , between the external medium and the membrane, should then vary with the logarithm of the Na ion activity in the external medium a_{Na} :

$$(8.5.1) \quad E = \frac{RT}{F} \ln a_{\text{Na}} + \text{constant}$$

(Note that this equation simply describes the continuity of the electrochemical potential of Na ion at the phase boundary. At the peak of excitation, there is no continuity for the electrochemical potential of choline ion because equilibrium has not yet been reached in the choline-occupied portion of the membrane.)

To obtain the total membrane potential, the intramembrane diffusion potential and the phase-boundary potential at the other interface have to be added. In the excited portion of the membrane, neither the intramembrane diffusion potential nor the phase-boundary potential at the inner interface is affected significantly by the presence of choline ion in the medium. Therefore, equation 8.5.1 describes the dependence of the peak value of the entire action potential on the external Na ion activity. The membrane conductance at the peak of excitation decreases as the choline concentration in the medium increases, because the active fraction of the membrane decreases.

One of the necessary conditions for the system consisting of the axon membrane and the two contiguous fluid media to exhibit ex-

citability is that the system can be brought readily to a critical, unstable state in which spontaneous transition to one of two stable states can take place. An unperfused squid giant axon immersed in a pure, isotonic solution of CaCl_2 or MgCl_2 is well stabilized in the resting state. One way to make this system excitable is to introduce salts of various univalent cations externally and thus into the critical layer of the membrane, bringing the system close to the unstable state. [Another way of making the system excitable is to perfuse the axon interior with a more favorable, more strongly depolarizing cation (see Fig. 40).] In axons immersed in a salt solution of pure univalent cation, the system is stabilized in the excited (depolarized) state; in order to restore excitability, it is necessary to introduce divalent cations into the critical layer of the membrane. The ion antagonism in excitable tissues (see Loeb, 1906; Höber, 1926; Heilbrunn, 1952) essentially deals with the problem of maintaining membranes in a stable, "but-not-too-far-from-unstable," state.

EQUIVALENT CIRCUIT THEORY

(8.6) The interpretation of the mechanism of excitation described in this monograph is very different from the widely accepted interpretation based on the theory proposed by Hodgkin and Huxley (1952). In their theory, the axon membrane is represented by the equivalent electrical circuit shown in Figure 51. The apparent membrane capacity, C , is approximately $1 \mu\text{F}/\text{cm}^2$; it is considered to be purely dielectric in nature. The battery marked E_K has an *emf* given by

$$(8.6.1) \quad E_K = \frac{RT}{F} \ln \frac{C_K'}{C_K''}$$

and E_{Na} by equation 8.4.1; these *emf*'s are determined solely by the potassium and sodium ion concentrations (or activities) on both sides of the membrane. Two conductors marked g_K and g_{Na} are considered as functions of the membrane potential V and time t . It was possible to fit the observed membrane currents under voltage clamp to their set of equations by proper choice of the functions $g_{Na}=g_{Na}(V, t)$ and $g_K=g_K(V, t)$.

This process of curve fitting was carried out by adopting the following form of function for g_K :

$$(8.6.2) \quad g_K = \bar{g}_K n^4$$

where \bar{g}_K is the maximum value of g_K and n is described

$$(8.6.3) \quad \frac{dn}{dt} = a_n(1 - n) - \beta_n n$$

where a_n and β_n are factors which depend on the membrane poten-

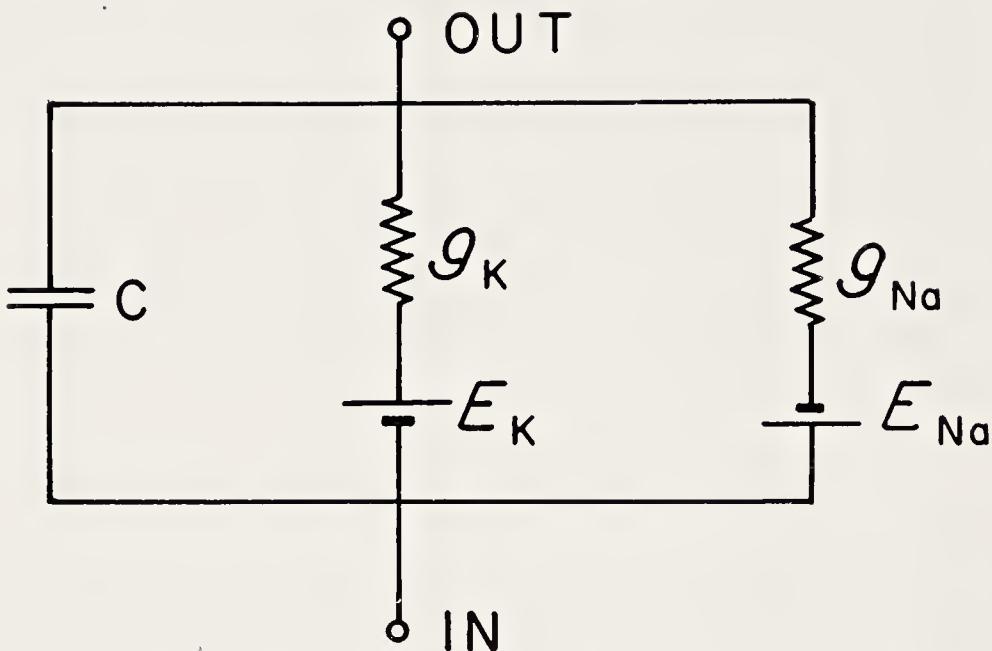


FIGURE 51. The equivalent circuit of the squid axon membrane proposed by Hodgkin and Huxley.

tial V (but not on t). Similarly, g_{Na} is described by

$$(8.6.4) \quad g_{Na} = \bar{g}_{Na} m^3 h$$

where both m and h are given by expressions of the same form as for n .

Under voltage clamp, g_K increases with time t monotonically; g_{Na} rises initially, and then falls as the factor h starts to dominate. The factor h is called the "Na-inactivation" process. In these equations, there are many constants which can be adjusted to fit the observed current-time curves at different clamping voltage V . By using these functions, Hodgkin and Huxley calculated the time course of the action potential (observed without voltage clamp), and obtained excellent agreement with the observed action potential.

A system consisting of two batteries of constant *emf* and two variable resistors (Fig. 51) is equivalent to a system with one battery of variable *emf* and one variable resistor in series. The circuit shown in Figure 51 has the same electrical properties as a circuit consisting of one battery of *emf* $(g_{\text{Na}}E_{\text{Na}} + g_{\text{K}}E_{\text{K}})/(g_{\text{Na}} + g_{\text{K}})$ and one series resistance $1/(g_{\text{K}} + g_{\text{Na}})$. Therefore, it is not possible to decide by purely electrical measurements which one of the two alternatives (two batteries with fixed *emf*'s or one battery with a variable *emf*) represents the real axon membrane.

As discussed in Section 5.4, there are large cation fluxes across the axon membrane in the excited state (carrying no net membrane current). The inward-directed membrane current observed under voltage clamp does not significantly alter the rate of transfer of cations associated with this interdiffusion. Consequently, neither the time course of the *emf* nor that of the membrane conductance under voltage clamp is very different from that observed with no membrane current (see Fig. 48). The process of calculating the action potential by the use of equations 8.6.2 and 8.6.4 is not fundamentally different from the process of determining the *emf* by the a.c. method in Section 8.3, because the conductances in these equations are determined by changing the membrane potential *V* in small steps. Therefore, it is expected that computation of the action potential by the use of equations 8.6.2 and 8.6.4 yields a good approximation of the observed action potential. (Since the discrepancy between $E(t)$ and the action potential in Figure 48 is due to a strong outward current during voltage clamp, the agreement between the computed and observed action potentials is greatly improved when the effect of reducing the membrane current is taken into consideration.)

In recent years the equivalent circuit theory has greatly stimulated the interest of biophysicists. However, from a physicochemical point of view, there are a number of difficulties in this theory:

1. In this theory the membrane potential is described in terms of single ion activities in mixed salt solutions which cannot be defined without arbitrariness (see Guggenheim, 1957, p. 379; Robinson and Stokes, 1959, p. 26). In the experimental procedure of determining the membrane potential, there is always ambiguity arising from the liquid junctions (see MacInnes, 1939; Bates, 1964). Therefore, the theory lacks a rigorous thermodynamic foundation.

2. The equivalent circuit model (Fig. 51) may show the relative importance of the major univalent cations determining the membrane potential, but the membrane resistance, the interdiffusion fluxes, and the indispensable role of divalent cations cannot be treated properly by this circuit (see Sect. 5.4 and 7.3).

3. Since there is a strong interdiffusion of cations across the membrane both in the resting and excited states (see Sect. 4.2 and 5.4), it is not possible to unambiguously determine the component of the membrane current carried by Na ion with the voltage clamp technique.

4. The equivalent circuit may be regarded as a graphic representation of the discontinuous treatment of membrane phenomena described in Section 3.4. It has been pointed out that such a treatment involves serious difficulties when applied to internally perfused axons in which there are no common ions on two sides of the membrane; e.g., g_{Na} is expected to vary with the concentrations of other ions (potassium, calcium, etc.) of the system. The constant field equation (3.7.9) has often been used instead of equation 8.6.1 to describe the *emf*'s of the batteries in the equivalent circuit. But, the assumptions made to derive this equation (e.g., continuity of φ , a_i , $\mu_i^{(o)}$, etc., at the surfaces of the membrane) seem too restrictive to be applicable to biological membranes (see Sect. 3.7).

5. The equivalent circuit theory is based on the assumption that the mobility ratio, $\bar{u}_{\text{Na}}/\bar{u}_{\text{K}}$, changes drastically during excitation. This ratio varies only slightly under different conditions in most inanimate membranes studied (see Helfferich, 1962, p. 302). Even if this ratio is assumed to change drastically, it is difficult to explain the observed increase in the membrane conductance and the interdiffusion flux (see Sect. 5.5).

6. The effect of dilution of the external and internal fluid media with an isotonic solution of nonelectrolyte (see Sect. 4.5) shows that both the equivalent circuit and equation 3.7.9 are inadequate for description of the membrane potential in the squid axon.

7. In general, the ion selectivities and mobilities vary with the composition of the electrolytes in the surrounding medium (see Sect. 7.4 and Appendix). In formulation of the equivalent circuit theory, little consideration was given to this problem.

In short, the equivalent circuit model is designed to explain mainly

electrical events associated with production of action potentials and does not cover many important physicochemical features of nerve excitation. On the other hand, the macromolecular hypothesis described in this monograph offers reasonable explanations for the observed anion and cation sequences (Sect. 6.2 and 8.4), the phenomenon of abolition of action potentials by chemical and thermal means (Sect. 7.6), abrupt depolarization (Sect. 4.6), excitation in univalent cation-free media (Sect. 7.3), and other phenomena for which the equivalent circuit model offers no interpretation.

SUMMARY

THE historical background of electrophysiology and basic physicochemical concepts presented in the first three chapters of this monograph are not new to students and investigators interested in biological and nonbiological membranes. These chapters were written in order to familiarize the reader with the facts and concepts needed for the arguments developed in the subsequent chapters. The two stable states theory of excitable membranes and the macromolecular interpretation developed in the remainder of this book may be unfamiliar to many students of physiology, particularly for those who are accustomed to regarding the membrane as a boundary without thickness between two aqueous phases. Therefore, it seems worthwhile to briefly summarize the major arguments that have been presented in the last five chapters.

The excitable membrane of the squid giant axon is visualized as a macromolecular complex of proteins and phospholipids. A relative excess of fixed negative charge at the external layer of the membrane confers cation-exchanger properties on the system. Interactions between this macromolecular system and its ionic environment may be thought of in terms of direct effects within the cation-exchange process, as well as indirect effects on the underlying macromolecular structure.

The process of excitation involves a rapid, reversible cation-exchange process involving transitions between "two stable (conformational) states" of the membrane macromolecules. In the resting stable state, the anionic sites in the membrane are occupied primarily by divalent cations derived from the external medium; in the excited stable state these sites are occupied predominantly by univalent cations. The conformation of the membrane macromolecules and the properties of the cation exchanger are determined primarily by the univalent/divalent cation ratio within the membrane.

When an outward electric current is delivered to the axon membrane, the excitation process is triggered by the transport of internal univalent cations into the membrane. These univalent cations are capable of competitive displacement of divalent cations from the anionic sites of the membrane macromolecules. The continued displacement of the divalent cations by cooperative processes results in an increase in the univalent/divalent cation ratio, and produces a conformational change of the membrane macromolecules.

The difference in macromolecular conformation in the excited and resting states is responsible for the spread of the excitation process along the axon membrane. When a limited area of the membrane is transformed into its excited conformation, a local circuit is effected: current is directed inward through the excited area and outward through the resting regions. The internal cations which are driven into resting regions of the membrane by the outward-directed currents convert these regions to new excited patches; eventually, if the initial excited area is large enough, the entire membrane is converted from the resting stable conformatio to the excited stable conformation. This conformational change is exothermic and is accompanied by a large increase in membrane water content and in the density of the charged sites available for transmembrane ion transfer; consequently, there is a large increase in membrane conductance and a large change in membrane potential during excitation.

The increase in membrane conductance during excitation is associated with an increased interdiffusion of cations across the membrane. In the absence of a (net) membrane current, the external cations are transported into the axon interior, and simultaneously, the internal cations are transported through the membrane into the external medium. This increased interdiffusion gradually alters the ionic milieu within and near the membrane. The change in the ionic milieu is responsible for the gradual alteration in the physicochemical properties of the membrane during excitation.

Excitation is terminated when divalent cations derived from the external medium once again form stable complexes with the fixed anionic sites. This is associated with a reduction in the univalent/divalent cation ratio in the membrane macromolecules and a return of macromolecular conformation to that of the resting state. The pro-

cess of transition from the excited to the resting state is endothermic.

The two stable states theory for the excitation process, and the macromolecular model for the squid axon membrane provide a qualitative and semiquantitative interpretation of the mechanism of generation and propagation of the nerve impulse. It is hoped that future investigations based on this physicochemical approach to the excitation process will yield a more quantitative interpretation.

ADDENDUM

Quite recently, excitation of squid giant axons in external media free of univalent cations (see Sect. 7.3) was studied in greater detail (Watanabe, Tasaki and Lerman; Biol. Bulletin, Oct. 1967). Action potentials obtained from axons perfused internally with dilute sodium phosphate solutions and externally with calcium chloride solutions were found to be qualitatively similar to those shown in Figure 40 (p. 114). This finding strongly supports the theory of excitation summarized above, and is inconsistent with the conventional assumption that initiation of an action potential is the consequence of a membrane permeability increase specific to sodium ions.

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Appendix I

A THEORY OF MEMBRANE POTENTIAL

I. TASAKI AND Y. KOBATAKE

MODERN quantitative theories of membrane potentials across charged membranes can be said to have started in 1935 with the work of Teorell¹ and Meyer and Sievers.² These authors obtained a mathematical expression for the potential difference across an ion-exchanger membrane by integrating the Nernst-Planck equation for diffusion of ions within the membrane, and subsequently adding the two phase boundary (Donnan) potentials to the intramembrane diffusion potential. This method of calculating the membrane potential was expanded by Schlägl³ to include systems containing a number of uni- and multivalent ions. Helfferich and Ocker⁴ extended this method to take the effect of the ion selectivity of the membrane into consideration. Eisenman and his coworkers^{5,6} applied this improved and extended method to glass membranes.

It is difficult, however, to apply this method of calculating the membrane potential to nonaqueous or dense ion-exchanger membranes, mainly because of the mathematical complexity involved in the treatment of the ion activities within the membrane. In order to calculate the intramembrane diffusion potential, it is necessary to integrate mathematical expressions involving single ion activities in the membrane interior (see Helfferich and Ocker⁴). Since the counter-ions in an ion-exchanger membrane are far from being dilute, the electric potential within the membrane is an unmeasurable quantity in Guggenheim's sense.^{7,8}

This portion of the Appendix is designed to demonstrate that the membrane potential can be calculated without treating the unmeasurable intramembrane diffusion potential directly. The new method of calculation has been developed from the theory of Kobatake,⁹ which deals with a capillary model of the charged membrane. This method involves consideration of the equilibrium between an imag-

inary droplet of aqueous solution and a layer of the membrane of infinitesimal thickness with which the droplet is in contact. Consideration of such an equilibrium is consistent with the concept of local equilibrium adopted in the basic formulation of the thermodynamics of irreversible processes.¹⁰ (The consideration of the electrochemical potential of such an imaginary aqueous solution is analogous to the consideration of the local temperature in nonisothermal systems.) The advantage gained by such consideration has been stressed in a recent article by Kobatake *et al.*¹² dealing with membrane potentials in a system involving diffusion of single uni-univalent electrolytes. The method introduced in this article is consistent with Sollner's view that the concentration of the critical ions and their mobilities in the membrane play decisive roles in determining the bi-ionic and multi-ionic potentials.¹³ The method is very general and can be applied to nonaqueous membranes, as well as to ordinary ion-exchanger membranes. This method can also be applied to systems involving a mixture of uni- and divalent cations. In the present discussion, application of the method is confined to diffusion of a mixture of uni-univalent electrolytes or di-univalent electrolytes across a dense ion-exchanger membrane. The method can also be applied to calculation of interdiffusion fluxes and membrane resistance.

BASIC DIFFERENTIAL EQUATION FOR MEMBRANE POTENTIAL

We consider two phases of aqueous solutions, separated by a membrane bounded by two planes normal to the x -axis at $x=0$ and $x=\delta$. We assume that the fluxes of all mobile species under consideration are only in the direction of the x -axis. The existence of nonhomogeneity in the direction of the y - and z -axes is permissible insofar as the direction of fluxes is not affected by the nonhomogeneity. However, it is important to note that the present treatment is limited to membranes in which the electrochemical potentials for all the mobile species do not vary in the direction of the y - and z -axes. In general, the electrochemical properties of the membrane may vary in the x -direction. In the present article, we deal only with the cases where there are no differences in temperature or hydrostatic pressure between the two aqueous solutions.

We take the membrane as the reference component for the fluxes under consideration. There are n mobile components in the system. As long as we ignore the mass movement across the membrane, at any point in the membrane, we have the following relation between fluxes J_i ($i=1, 2, \dots, n$) and the gradient of the electrochemical potentials $\bar{\mu}_k$ of mobile species, k ;

$$(A.1) \quad J_i = \sum_{k=1}^n \Omega_{ik} \frac{d\bar{\mu}_k}{dx} \quad (k = 1, 2, \dots, i, \dots, n)$$

where Ω_{ik} are mass-fixed phenomenological coefficients averaged over the y - z plane¹² and obey the Onsager reciprocal relation. [For further discussion of equation A.1 cf. Kirkwood¹⁴ or Fitts¹⁰(see Ch. 4).] The condition of no electric current across the membrane may be expressed by

$$(A.2) \quad \sum_{i=1}^n z_i J_i = 0,$$

where z_i is the valence of ion species i . When equation A.1 is introduced into equation A.2, we have

$$(A.3) \quad \sum_i \sum_k z_i \Omega_{ik} \frac{d\bar{\mu}_k}{dx} = 0$$

where the summation tends over all mobile species. We now reverse the order of summation in this equation and rewrite it in the form

$$(A.4) \quad \sum_k \left(\sum_i z_i \Omega_{ik} \right) \frac{d\bar{\mu}_k}{dx} = 0.$$

We next introduce the new average quantity \bar{u}_k defined by the following equation:

$$(A.5) \quad z_k \bar{u}_k \bar{C}_k = \sum_i z_i \Omega_{ik}$$

where \bar{C}_k stands for the average concentration of ion species k in a membrane layer of infinitesimal thickness at position x . In general, \bar{u}_k may vary with x . By the use of the Onsager reciprocal relation, $\Omega_{ik} = \Omega_{ki}$ ($i \neq k$), it can be shown that $z_k \bar{u}_k$ is the proportionality

constant between the increment in the average velocity of ion species k and a small variation in the local electric field $d\bar{\varphi}/dx$. Therefore, \bar{u}_k is the average value of the mobility relative to the local center of mass of species k in plane x . From equations A.4 and A.5, it follows that

$$(A.6) \quad \sum_k z_k \bar{u}_k \bar{C}_k \frac{d\bar{\mu}_k}{dx} = 0$$

The next step in our argument is to consider an imaginary droplet of aqueous solution which is in equilibrium with the infinitesimally thick layer of the membrane at x . Except in the case where co-ions are completely excluded from the membrane interior, the chemical composition of the droplet is uniquely defined by the conditions of the postulated equilibrium. (In the case of complete co-ion exclusion, the composition of electrolytes in the droplet can not be defined by the equilibrium conditions only; but, as we shall see later, this ambiguity does not create any problem in the determination of the membrane potential.) It is obvious that the composition of the electrolytes in the droplet varies with x . At $x=0$ and $x=\delta$, the composition should coincide with that of the bulk solutions on either side of the membrane.

We denote the electrochemical potential of species k in the hypothetical droplet by $\bar{\mu}_k^{(h)}$; the condition of equilibrium between the droplet and the layer at x is described by

$$(A.7) \quad \bar{\mu}_k^{(h)} = \bar{\mu}_k$$

and

$$(A.8) \quad \bar{\mu}_k^{(h)} = \mu_k^{0(h)} + RT \ln a_k^{(h)} + z_k F \varphi^{(h)}$$

where $a_k^{(h)}$ is the activity of the mobile ion species k in the droplet located at x , $\varphi^{(h)}$ is the electric potential of the droplet, and R , T , and F have their usual thermodynamic meanings. The reference value of the chemical potential of species k in the aqueous solution, $\mu_k^{0(h)}$, is a function of pressure and temperature and is constant in the present treatment.

By combining equations A.6, A.7 and A.8 and rearranging the terms, we obtain the following equation for $\varphi^{(h)}$:

$$(A.9) \quad \frac{d\varphi^{(h)}}{dx} = \frac{-RT}{F} \frac{\sum_k z_k \bar{n}_k \bar{C}_k (d \ln a_k^{(h)} / dx)}{\sum_k z_k^2 \bar{n}_k \bar{C}_k}.$$

It is important to realize that $\varphi^{(h)}$, the electric potential of the droplet at x , is *not* the potential in the interior of the membrane.

The final step in our method of calculating the membrane potential is to express the average concentration in the membrane, \bar{C}_k , in terms of the activities, $a_k^{(h)}$, in the aqueous solutions in contact with the membrane, and to integrate the right-hand member of equation A.9 with respect to x . When this integration is accomplished, we have the desired expression for the membrane potential, $\Delta\varphi$:

$$(A.10) \quad \Delta\varphi = \Delta\varphi^{(h)} = \int_0^\delta \frac{d\varphi^{(h)}}{dx} dx.$$

As a rule, we are not interested in the unmeasurable potential profile within the membrane. Instead, we are interested in the measurable potential difference across the whole membrane, which is uniquely determined by the activities of all the mobile species on both sides of the membrane. For this reason, we express equation A.9 in the following form:

$$(A.11) \quad d\varphi^{(h)} = \frac{-RT}{F} \frac{\sum_{k=1}^n z_k \bar{n}_k \bar{C}_k d \ln a_k^{(h)}}{\sum_{k=1}^n z_k^2 \bar{n}_k \bar{C}_k}$$

and regard $\varphi^{(h)}$ as a function of variables $a_1^{(h)}, a_2^{(h)}, \dots, a_n^{(h)}$ or of combinations of these variables (e.g., $a_1^{(h)}/a_n^{(h)}, a_2^{(h)}/a_n^{(h)}, \dots$). If $d\varphi^{(h)}$ is expressed in a form of an exact differential of these variables, $\varphi^{(h)}$, hence φ , is determined by the limits of integration of these variables, irrespective of their paths. In this manner, we can arrive at an expression relating the transmembrane potential to the activities of the mobile species in the bulk solutions on both sides of the membrane.

RELATIONSHIP BETWEEN $\varphi^{(h)}$ AND INTRAMEMBRANE DIFFUSION POTENTIAL

We now show that $d\varphi^{(h)}$ in equation A.11 is directly related to the intramembrane diffusion potential in previous treatments of the membrane potential.¹⁻³ It has been customary to make a nonoperational split of electrochemical potential $\bar{\mu}_k$ in the membrane into a chemical part and an electric part:

$$(A.12) \quad \bar{\mu}_k = \mu_k^0 + RT \ln \bar{a}_k + z_k F \bar{\varphi}$$

where \bar{a}_k is the activity of species k , $\bar{\varphi}$ the electric potential at x , and μ_k^0 is the reference value of the chemical potential of species k within the membrane.

The Nernst-Flanck flux equation,

$$(A.13) \quad J_k = -\bar{u}_k \bar{C}_k \frac{d(RT \ln \bar{a}_k + z_k F \bar{\varphi})}{dx}$$

is the starting point in all the previous treatments.¹⁻⁵ By combining this equation with

$$\sum_{i=1}^n z_i J_i = 0$$

and by using a procedure entirely analogous to that used in the preceding section, we arrive at

$$(A.14) \quad d\bar{\varphi} = \frac{-RT}{F} \left[\frac{\sum_k z_k \bar{u}_k \bar{C}_k (d \ln \bar{a}_k)}{\sum_k z_k^2 \bar{u}_k \bar{C}_k} \right].$$

This equation has a form similar to that of equation A.11. There is, however, an important distinction between the two: the electric potential and activities in the last equation are quantities within a charged membrane, whereas the corresponding symbols, $\varphi^{(h)}$ and $a_k^{(h)}$, represent quantities in an aqueous solution.

A phase boundary potential, ψ , between the membrane and the aqueous solution in contact with the membrane is defined formally as the difference between $\varphi^{(h)}$ in equation A.8 and $\bar{\varphi}$ in equation A.12; namely,

$$(A.15) \quad \psi = \frac{1}{z_k F} [(\mu_k^0 - \mu_k^{0(h)}) + RT (\ln \bar{a}_k - \ln a_k^{(h)})].$$

Hence, the difference in phase boundary potential at two points at a distance of dx is given by

$$(A.16) \quad d\psi = \frac{RT}{z_k F} [d \ln \bar{a}_k - d \ln a_k^{(h)}].$$

Since all the mobile species are assumed to have achieved equilibrium distribution across the phase boundary, equation A.16 applies to all ionic species in the system. Elimination of \bar{a}_k in equation A.14 by the use of the relationship of equation A.16 leads to

$$(A.17) \quad d\bar{\varphi} + d\psi = \frac{-RT}{F} \left[\frac{\sum_k z_k \bar{u}_k \bar{C}_k (d \ln a_k^{(h)})}{\sum_k z_k^2 \bar{u}_k \bar{C}_k} \right] = d\varphi^{(h)}$$

which is identical to equation A.11. This alternative derivation of our basic equation indicates that $d\varphi^{(h)}$ is nothing but the sum of the difference in the intramembrane diffusion potential, $d\bar{\varphi}$, and the difference in phase boundary potentials, $d\psi$, at two points a distance of dx apart. Therefore, integration of the right-hand member of the last equation gives the sum of the intramembrane diffusion potential from $x=0$ to $x=\delta$, and the difference in phase boundary potentials at the two boundaries, as is required by previous theories of membrane potential.

APPLICATION OF THE THEORY

Diffusion of Single Uni-univalent Salt Across Uniform Membrane

The potential difference across a uniform membrane separating two solutions of the same uni-univalent electrolyte at different concentrations has been discussed extensively by previous investigators.^{1-3, 9, 11, 15, 16} We will now demonstrate that the method of calculating the membrane potential described in the preceding pages yields an equation identical to that obtained previously.

Equation A.11 applied to a single electrolyte of the uni-univalent type ($z_1=1$ and $z_2=-1$) reduces to

$$(A.18) \quad d\varphi^{(h)} = \frac{-RT}{F} \left[\frac{(\bar{u}_1 \bar{C}_1 - \bar{u}_2 \bar{C}_2) d \ln a^{(h)}}{\bar{u}_1 \bar{C}_1 + \bar{u}_2 \bar{C}_2} \right]$$

where the convention suggested by Guggenheim,¹⁷ namely, $a_1^{(h)} = a_2^{(h)} = a^{(h)}$, has been used. We assume that both the mobility ratio, \bar{u}_2/\bar{u}_1 , and the fixed charge density, \bar{X} , are independent of x .

In order to integrate equation A.18, it is necessary to introduce an explicit relationship between the average ion concentrations in the membrane, \bar{C}_1 (counter-ion) and \bar{C}_2 (co-ion), and the mean activity a of the electrolyte component in the aqueous solution which is in equilibrium with the membrane at position x . In the Teorell-Meyer-Sievers theory, the so-called Donnan distribution has been adopted. The Donnan distribution can be written in the following form:¹⁸

$$(A.19) \quad \bar{C}_1 \bar{C}_2 = k a^2$$

where k is a constant representing the reciprocal of the square of the mean activity coefficient of the electrolyte in the membrane. Equation A.19 combined with the electroneutrality condition,

$$(A.20) \quad \bar{C}_1 = \bar{X} + \bar{C}_2,$$

leads to the following relationships:

$$(A.21) \quad \begin{aligned} \bar{C}_1 &= \frac{1}{2}(\beta + \bar{X}), \quad \text{and} \\ \bar{C}_2 &= \frac{1}{2}(\beta - \bar{X}), \end{aligned}$$

where β stands for $(4ka^2 + \bar{X}^2)^{\frac{1}{2}}$. Substitution of β for \bar{C}_1 , \bar{C}_2 and $a^{(h)}$ (in place of a) changes equation A.18 into a form that can be integrated immediately. This leads to the well-known formula of Teorell.^{1,2}

$$(A.22) \quad \frac{F}{RT} \Delta\varphi = U \ln \left(\frac{\beta' + U\bar{X}}{\beta'' + U\bar{X}} \right) + \frac{1}{2} \ln \frac{(\beta' - \bar{X})(\beta'' + \bar{X})}{(\beta'' - \bar{X})(\beta' + \bar{X})}$$

where $U = (\bar{u}_1 - \bar{u}_2)/(\bar{u}_1 + \bar{u}_2)$, $\Delta\varphi$ represents the value $(\varphi'' - \varphi')$, β' and β'' are the values of $(4ka^2 + \bar{X}^2)^{\frac{1}{2}}$ in which the activities of the electrolyte component in the two bulk solutions, a' and a'' , are introduced. It should be noted that there was no splitting of the total membrane potential into the diffusion and phase boundary potentials in the present derivation.

In a more recent theory proposed by Kobatake *et al.*,¹¹ the Don-

nan distribution, equation A.19, was replaced with

$$(A.23) \quad \bar{C}_1 \bar{C}_2 = k(\bar{X} + c)c$$

where c is the concentration of the electrolyte component in the droplet. This replacement yields the following expression for the membrane potential:

$$(A.24) \quad -\frac{F}{RT} \Delta\varphi = \ln \frac{c''}{c'} - 2(1 - \alpha) \ln \left(\frac{c'' + \alpha \bar{X}}{c' + \alpha \bar{X}} \right),$$

where c' and c'' are the concentrations in the two external solutions, and α stands for $\bar{u}_1/(\bar{u}_1 + \bar{u}_2)$. This equation was shown to agree satisfactorily with the available experimental data.

Uniform Membrane with Interdiffusing Counter-ions of Equal Valence

The problem to be considered here is calculation of the membrane potential in a system where interdiffusion of counter-ions of the same valence takes place across the membrane. In the present treatment, the fixed charge density need not be constant throughout the membrane. If there is one layer in the membrane where exclusion of co-ions is practically perfect, the co-ion fluxes across the whole membrane should be negligible and only interdiffusion of counter-ions should take place in the system. In this case equation A.11 takes the form:

$$(A.25) \quad d\varphi = \frac{-RT}{zF} \left[\frac{\sum_k \bar{u}_k \bar{C}_k (d \ln a_k^{(h)})}{\sum_k \bar{u}_k \bar{C}_k} \right]$$

where z is the valence of the interdiffusing counter-ions. After omission of the superscript h , we rewrite this equation as follows.

$$(A.26) \quad d\varphi = \frac{-RT}{zF} \left(\frac{Q_1 da_1 + Q_2 da_2 + \dots + Q_n da_n}{Q_1 a_1 + Q_2 a_2 + \dots + Q_n a_n} \right)$$

where Q_i ($i = 1, 2, \dots, n$) is defined by

$$(A.27) \quad Q_i = \frac{\bar{u}_i \bar{C}_i a_r}{\bar{u}_r \bar{C}_r a_i}; \quad Q_r = 1.$$

The subscript r refers to an arbitrary ion species chosen as reference.

The quotient $(\bar{C}_i a_r) / (\bar{C}_r a_i)$ in equation A.27 is closely related to, and is sometimes designated as, the selectivity coefficient of species i over species r at equilibrium. When the salt concentrations in the aqueous solution are low enough to use the Debye-Hückel approximation for activity coefficients, the activity ratio a_r/a_i is equal to the concentration ratio; therefore, $(\bar{C}_i a_r) / (\bar{C}_r a_i)$ is identical to the equilibrium selectivity coefficients defined by many investigators.^{4,5} Ordinarily, the mobility ratio \bar{u}_r/\bar{u}_i is not very different from unity. Hence, \bar{Q}_i is not very different from the static or equilibrium selectivity. For simplicity's sake, we call Q 's "kinetic selectivity coefficients" in this Appendix.

We now assume that these coefficients do not vary with x ; a case in which the coefficient is variable will be discussed in the next section. When Q 's are constant, equation A.26 can be integrated immediately, yielding

$$(A.28) \quad \Delta\varphi = \frac{RT}{zF} \ln \left(\frac{Q_1 a_1' + Q_2 a_2' + \cdots + Q_n a_n'}{Q_1 a_1'' + Q_2 a_2'' + \cdots + Q_n a_n''} \right),$$

which relates the membrane potential to the ion activities in the bulk solution on each side of the membrane (prime and double prime). Equation A.28 has been derived previously by Helfferich and Schlögl,¹⁹ Wyllie²⁰ and more recently by Conti and Eisenman.⁶ As mentioned earlier, this equation is valid even when the fixed charge density varies within the membrane.

Membrane with Variable Q

We shall now consider a membrane whose kinetic selectivity, as defined by equation A.27, varies within the membrane. Here we limit our argument to a system containing two counter-ions of the same valence interdiffusing across the membrane. Equation A.26 applied to this case has the following form:

$$(A.29) \quad d\varphi = \frac{-RT}{zF} \left(\frac{Q da_1 + da_2}{Q a_1 + a_2} \right)$$

where Q is the kinetic selectivity coefficient of ion species 1 over species 2, namely,

$$(A.30) \quad Q = \frac{u_1 \bar{C}_1 a_2}{\bar{u}_2 \bar{C}_2 a_1}.$$

We now introduce a new set of variables ξ and λ defined by

$$(A.31) \quad a_1 + a_2 \equiv \xi; \quad \frac{a_1}{a_1 + a_2} \equiv \lambda.$$

We introduce $a_1 = \lambda \xi$ and $a_2 = (1 - \lambda) \xi$ into equation A.29 and obtain

$$(A.32) \quad -\frac{zF}{RT} d\varphi = \frac{d\xi}{\xi} + \frac{(Q - 1)d\lambda}{(Q - 1)\lambda + 1}.$$

When Q is known as a function of λ , this equation can be integrated immediately. For example, if the λ -dependence of Q is given by

$$(A.33) \quad Q - 1 = p + q\lambda$$

where p and q are constant, an explicit expression for the membrane potential can be obtained. (Note that the case where $q=0$ corresponds to a membrane with a constant Q ; and the case in which $p=q=0$ represents a membrane with unit kinetic selectivity.) By introducing equation A.33 into equation A.32, we have

$$-\frac{zF}{RT} d\varphi = \frac{d\xi}{\xi} + \frac{(q\lambda + p)d\lambda}{q\lambda^2 + p\lambda + 1}$$

which on integration by the method of partial fractions leads to

$$(A.34) \quad -\frac{zF}{RT} \Delta\varphi = \ln \frac{\xi''}{\xi'} + \frac{1}{2} \ln \left(\frac{q\lambda''^2 + p\lambda'' + 1}{q\lambda'^2 + p\lambda' + 1} \right) \\ + \frac{p}{2\sqrt{p^2 - 4q}} \ln \frac{(2q\lambda'' + p - \sqrt{p^2 - 4q})(2q\lambda' + p + \sqrt{p^2 - 4q})}{(2q\lambda' + p - \sqrt{p^2 - 4q})(2q\lambda'' + p + \sqrt{p^2 - 4q})}$$

It can be shown that equation A.34 reduces to equation A.28 with $n=2$, when q approaches zero and p is replaced with $(Q-1)$.

The variation of the experimentally determined selectivity coefficients (see Gregor *et al.*²¹ and Faucher and Thomas²²) appears to follow the linear relation of equation A.33 only in limited ranges of the mole fraction. Equation A.34 is expected to describe the effect of the variation of selectivity on the membrane potential in the experimentally observed linear range of the mole fraction.

Eisenman *et al.*^{5,6} published a set of experimental data indicating that Q varies with the concentration ratio, \bar{C}_2/\bar{C}_1 , in the membrane, rather than with the mole fraction λ in the aqueous phase. In their systems, the ion distribution between the membrane and the aqueous phase is described by

$$\frac{\bar{C}_1}{\bar{C}_2} \left(\frac{a_2}{a_1} \right)^{1/n} = k$$

where k is a constant. In this case, we choose a_1 and a_2/a_1 as a set of independent variables and rewrite equation A.29.

$$\frac{zF}{RT} d\varphi = d \ln a_1 + \frac{d \ln (a_2/a_1)}{[(\bar{u}_1 \bar{C}_1 / \bar{u}_2 \bar{C}_2) + 1]}.$$

Integration can be carried out by substituting $a_2/a_1 = \beta^n$, yielding

$$(A.35) \quad - \frac{zF}{RT} \Delta\varphi = n \ln \left(\frac{a_2'^{1/n} + (\bar{u}_1 k / \bar{u}_2) a_1'^{1/n}}{a_2'^{1/n} + (\bar{u}_1 k / \bar{u}_2) a_1'^{1/n}} \right)$$

which is known to fit the experimental data for glass electrodes obtained by Eisenman.⁵

Uniform Membrane with Two Counter-ions of Different Valences

When applied to a system consisting of a uniform membrane and two counter-ions of different valences ($z_1=1$ and $z_2=2$) interdiffusing across the membrane, equation A.11 is of the following form.

$$(A.36) \quad d\varphi^{(h)} = \frac{-RT}{F} \frac{\bar{u}_1 \bar{C}_1 d \ln a_1^{(h)} + 2\bar{u}_2 \bar{C}_2 d \ln a_2^{(h)}}{\bar{u}_1 \bar{C}_1 + 4\bar{u}_2 \bar{C}_2}.$$

We rewrite this equation

$$(A.37) \quad - \frac{2F}{RT} d\varphi = d \ln a_2^{(h)} - \left(\frac{\bar{u}_1 \bar{C}_1 d \ln \nu}{\bar{u}_1 \bar{C}_1 + 4\bar{u}_2 \bar{C}_2} \right)$$

where ν is defined by

$$\nu \equiv \frac{a_2^{(h)}}{[a_1^{(h)}]^2}.$$

Generally, the equilibrium selectivity coefficient, K , is defined by

$$(A.38) \quad K \equiv \frac{\bar{C}_2}{\bar{C}_1^2 \nu} = \frac{\bar{C}_2}{\bar{C}_1^2} \frac{[a_1^{(h)}]^2}{a_2^{(h)}}.$$

By combining this expression with the condition of electroneutrality, $\bar{C}_1 + 2\bar{C}_2 = \bar{X}$, the concentrations in the last term in equation A.37 can be expressed in terms of ν only, because we find that

$$\frac{\bar{C}_2}{\bar{C}_1} = \frac{1}{4} [(1 + 8\bar{X}K\nu)^{1/2} - 1].$$

We have

$$(A.39) \quad -\frac{2F}{RT} d\varphi = d \ln a_2^{(h)} - \frac{\bar{u}_1(d \ln \nu)}{\bar{u}_1 + \bar{u}_2(\sqrt{1 + 8KX\nu} - 1)}.$$

When both the mobility ratio, \bar{u}_2/\bar{u}_1 , and the product of the selectivity coefficient and the fixed charge density, $K\bar{X}$, are known as functions of ν , it is possible to calculate the last term of equation A.39.

In the particular case where \bar{u}_2/\bar{u}_1 and $K\bar{X}$ are independent of ν , an explicit form of the membrane potential can be obtained. We make the following substitution.

$$(A.40) \quad 1 + 8K\bar{X}\nu = \gamma^2$$

After substituting for ν in terms of γ , the last term in equation A.39 can be integrated by the method of partial fractions, yielding

$$(A.41) \quad -\frac{2F}{RT} \Delta\varphi = \ln \frac{a_2''}{a_2'} - \ln \frac{\gamma'' - 1}{\gamma' - 1} + \frac{\bar{u}_1}{2\bar{u}_2 - \bar{u}_1} \ln \left(\frac{\gamma'' + 1}{\gamma' + 1} \right) + \frac{2\bar{u}_2 - 2\bar{u}_1}{2\bar{u}_2 - \bar{u}_1} \ln \left(\frac{\bar{u}_2\gamma'' - \bar{u}_2 + \bar{u}_1}{\bar{u}_2\gamma' - \bar{u}_2 + \bar{u}_1} \right).$$

This equation can be converted readily, by the use of equations A.40 and A.38, into an alternative form

$$(A.42) \quad -\frac{F}{RT} \Delta\varphi = \ln \frac{a_1''}{a_1'} + \frac{\bar{u}_2}{2\bar{u}_2 - \bar{u}_1} \ln \frac{\gamma'' + 1}{\gamma' + 1} + \frac{\bar{u}_2 - \bar{u}_1}{2\bar{u}_2 - \bar{u}_1} \ln \left(\frac{\bar{u}_2\gamma'' - \bar{u}_2 + \bar{u}_1}{\bar{u}_2\gamma' - \bar{u}_2 + \bar{u}_1} \right),$$

where γ'' and γ' denote the value of $(1 + 8KXa_2/a_1^2)^{1/2}$, in which a_1 and a_2 represent the activities in the bulk solutions on each side of the membrane.

It is easy to show that when either K or \bar{u}_2/\bar{u}_1 approaches ∞ , $\Delta\varphi = RT/2F \ln a_2'/a_2''$. In either case the membrane potential is

determined by the activity ratio of the divalent cation only. Conversely, when K or \bar{u}_2/\bar{u}_1 approaches zero, $\Delta\varphi = RT/F \ln a_1'/a_1''$. In this case the ratio of the univalent cation in the two bulk solutions determined the membrane potential. For a given set of concentrations in the bulk solutions, the membrane potential varies continuously with the mobility ratio \bar{u}_2/\bar{u}_1 . When we introduce the conditions $K=1$, equation A.41 is identical to the solution of the Nernst-Planck flux equation obtained by Schlögl.³

Under the conditions that $a_1'=a_2''=0$, equation A.41 reduces to equation 4.3.2, described in the text.

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Appendix II

COUNTER-ION INTERDIFFUSION ACROSS CHARGED MEMBRANE

Y. KOBATAKE AND I. TASAKI

EQUATION OF RESISTANCE-FLUX PRODUCT

THE system considered is composed of a cation-exchanger membrane of uniform thickness and two aqueous solutions of salts of different univalent cations separated by the membrane. Fluxes of the two mobile cation species occur in the direction of the space coordinate x normal to the surface of the membrane. No gradient of the electrochemical potential exists in the direction perpendicular to the x -axis. Fluxes of co-ions are assumed to be small and are neglected in the present discussion. Only stationary (time-independent) states are considered. In general, the density of the negative fixed charge of the membrane is assumed to vary with the space coordinate x .

The Nernst-Planck equations describing cation fluxes through the membrane are

$$J_1 = -\bar{u}_1 \bar{C}_1 \left(RT \frac{d \ln \bar{a}_1}{dx} + F \frac{d\bar{\varphi}}{dx} \right)$$

(A.43) and

$$J_2 = -\bar{u}_2 \bar{C}_2 \left(RT \frac{d \ln \bar{a}_2}{dx} + F \frac{d\bar{\varphi}}{dx} \right)$$

where subscripts 1 and 2 refer to the two cation species under study. The mobilities (\bar{u}_1 and \bar{u}_2), the cation concentrations (\bar{C}_1 and \bar{C}_2), the cation activities (\bar{a}_1 and \bar{a}_2) and the potential $\bar{\varphi}$ represent the quantities within the membrane. The mass movement within the membrane is neglected. The condition of electroneutrality is given by

$$(A.44) \quad \bar{C}_1 + \bar{C}_2 = \bar{X}$$

where the density of negative fixed charges, \bar{X} , is a function of the space coordinate x . The electric current, I , across the membrane is related to the cation fluxes by the equation

$$(A.45) \quad I = F(J_1 + J_2).$$

When there is no electric current across the membrane, $J_1 = -J_2$. Combination of equation A.43 under these zero current conditions leads to

$$(A.46) \quad J_1 \left(\frac{1}{\bar{u}_1 \bar{C}_1} + \frac{1}{\bar{u}_2 \bar{C}_2} \right) = RT \frac{d \ln (\bar{a}_2 / \bar{a}_1)}{dx}.$$

By introducing the (static) selectivity, κ , defined by

$$(A.47) \quad \kappa = \frac{\bar{C}_2 \bar{a}_1}{\bar{C}_1 \bar{a}_2}$$

and also using equations A.44 and A.45, equation A.46 can be re-written in the following form:

$$(A.48) \quad J_1 = -J_2 = \frac{RT \bar{u}_2 \kappa \bar{X} (d\beta/dx)}{(1 + Q\beta)(1 + \kappa\beta)}$$

where β is defined by $\beta = \bar{a}_2 / \bar{a}_1$ and Q by $Q = \kappa \bar{u}_2 / \bar{u}_1$. Equation A.48 can be integrated when \bar{u}_1 , \bar{u}_2 , κ and \bar{X} are known as functions of β . In a special case where these quantities are constant, integration of equation A.48 from $\beta = 0$ to ∞ leads to the second half of equation 5.5.1.

When a current, I , passes through the membrane, the potential difference $\Delta\bar{\varphi}$ across the membrane (excluding the phase boundary potentials) can be obtained by combining equations A.43 and A.45:

$$(A.49) \quad -\Delta\bar{\varphi} = \frac{1}{F^2} \int'' \frac{Idx}{\bar{u}_1 \bar{C}_1 + \bar{u}_2 \bar{C}_2} + \frac{RT}{F} \int'' \frac{\bar{u}_1 \bar{C}_1 d \ln \bar{a}_1 + \bar{u}_2 \bar{C}_2 d \ln \bar{a}_2}{\bar{u}_1 \bar{C}_1 + \bar{u}_2 \bar{C}_2}.$$

The second term in the right-hand side of equation A.49 represents the membrane potential for $I = 0$; this potential difference is denoted by $\Delta\bar{\varphi}_m$. The membrane resistance, r , is defined as the limiting value of $(\Delta\bar{\varphi} - \Delta\bar{\varphi}_m)/I$ when I approaches zero. Hence,

$$(A.50) \quad r = \frac{1}{F^2} \int_{\epsilon}'' \frac{dx}{\bar{u}_1 \bar{C}_1 + \bar{u}_2 \bar{C}_2}$$

where \bar{C}_1 and \bar{C}_2 represent the concentration profiles for $I=0$. By the use of the definition $\beta=a_2/a_1$ and $Q=\kappa\bar{u}_2/\bar{u}_1$, equation A.50 can be rewritten as

$$(A.51) \quad r = \frac{1}{F^2} \int_{\epsilon}'' \frac{(1 + \kappa\beta)d\beta}{\bar{u}_1 \bar{X}(1 + Q\beta)(d\beta/dx)}.$$

By substituting $(d\beta/dx)/(1+\kappa\beta)$ from equation A.48 into equation A.49, we obtain

$$(A.52) \quad rJ_1 = \frac{RT}{F^2} \int_{\epsilon}'' \frac{Qd\beta}{(1 + Q\beta)^2}.$$

When Q is known as a function of β , this equation can be integrated immediately. In case where Q is independent of β , integration from $\beta=0$ to ∞ yields

$$(A.53) \quad rJ_1 = \frac{RT}{F^2}.$$

Equation A.53 is valid even when the density of the fixed charge varies within the membrane. Some deviation is expected when Q varies within the membrane or when the valences of the two inter-diffusing cations are different.

EXPERIMENTAL DETERMINATION OF THE rJ -PRODUCT *Membrane and Cells*

Commercially available cation-exchanger membrane (Ionac, MC=3235) and Sollner's oxidized collodion membrane¹ were employed. Solutions were prepared by dissolving three different univalent salts, RbCl NaCl and LiCl of the analytical grade, in glass-distilled water.

The cell used for measurements of membrane resistances and ion fluxes are illustrated schematically in Figure 52. The volume of the solution introduced in each compartment of the cell was 50 ml. The area of the membrane was 0.78 cm² (circular). Two pairs of platinized platinum electrodes were installed, one pair for delivering weak alternating current and the other for detecting potential variations

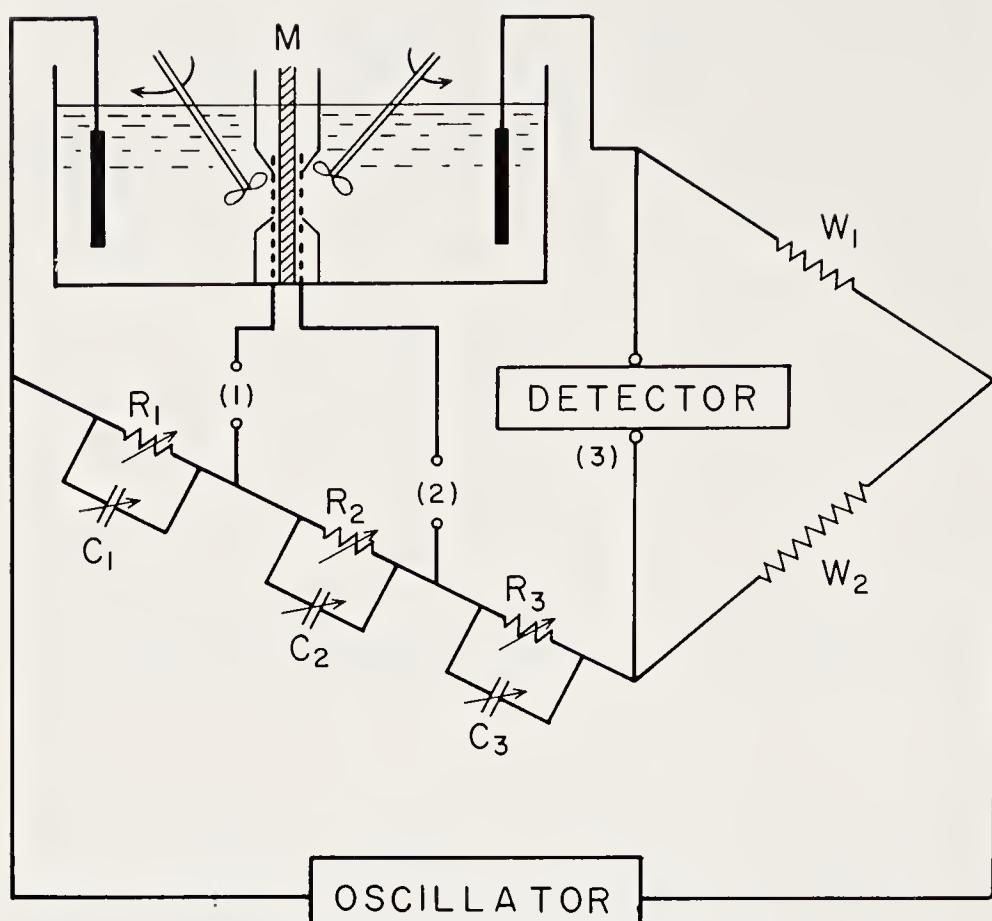


FIGURE 52. Schematic diagram showing the experimental setup used to determine the resistance-flux product in bi-ionic systems. Each compartment of the Lucite chamber has a capacity of 50 ml. The ac for measuring the impedance was applied through a pair of platinized platinum plates (3 cm^2 each). Two platinized platinum wires, 50μ in diameter, were placed directly on the surface of the membrane and were connected to the terminals indicated by (1) and (2). The output of the oscillator was attenuated to give one to five volts (peak-to-peak). The detector (Tektronix® oscilloscope, type 502) was connected successively to terminals marked (1), (2) and (3), and the bridge was balanced at all three positions. For determination of ion fluxes, the radio-tracer technique was used.

across the membrane. The current electrodes were approximately 3 cm^2 each in size. The potential electrodes were 50μ in diameter and were placed directly on the surfaces of the membrane. A small motor-driven propeller placed near each surface of the membrane was used to stir the solution.

Impedance Bridge

The resistance of the membrane under bi-ionic conditions was measured with a modified Kohlrausch bridge, diagrammatically shown in Figure 52. The procedure of measurement was as follows: First, the differential input of an oscilloscope (detector) was connected to the terminals marked (1) in the figure and the bridge was balanced by adjusting capacitor C_1 and resistor R_1 . Since resistances W_1 and W_2 ($50 \text{ K}\Omega$ each) were much larger than the resistances between the two current electrodes, this balancing was insensitive to the values of R_2 and R_3 . Next, the detector was connected to the terminals (2) and the bridge was balanced by varying R_2 and C_2 . Then, the detector was switched to the position (3) and the bridge was balanced with R_3 and C_3 . By repeating this procedure several times, complete balance of the bridge at three positions was accomplished. The frequency of the measuring a.c. was varied between 400 and 50 cps and the output of the oscillator was varied between one and five volts (peak-to-peak). The value of R_2 extrapolated to zero frequency and zero intensity of a.c. was taken as the measure of the membrane resistance. The accuracy of these measurements was approximately 4 percent.

Radiotracer Technique

Fluxes of Na, Rb and Cl ions were measured by the use of Na^{22} , Rb^{86} and Cl^{36} . One of these radiotracers was added to the solution in the compartment containing the nonradioactive analog of the tracer, and the radioactivity appearing in the other ("cold") compartment was determined at intervals between ten and thirty minutes. (The volume of the radioactive samples taken from the "cold" compartment was between 0.03 and 0.1 ml and the radioactivity of an individual sample was between 100 and 5000 counts /min.) After a hold-up time, which was less than sixty minutes in most of the present measurements, the radioactivity in the "cold" compartment rose linearly with time. The flux of the radioisotope of ion species i , J_i^* , was determined from the slope of the linear rise in the radioactivity in the cold compartment. The flux of the nonradioactive species, J_i , expressed in $\text{mole} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$, was determined by the equation

$$J_i = J_i^* c_i / n_i^*$$

$$(i = 1, 2)$$

where c_i is the concentration ($\text{mole} \cdot \text{cm}^{-3}$) of the nonradioactive ion in the hot compartment, J_i^* is the flux of the radioactive species ($\text{CPM} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$). The level of n_i^* is the radioactivity in the hot compartment ($\text{CPM} \cdot \text{cm}^{-3}$). The level of n_i^* did not change appreciably during one series of measurements which lasted from two to

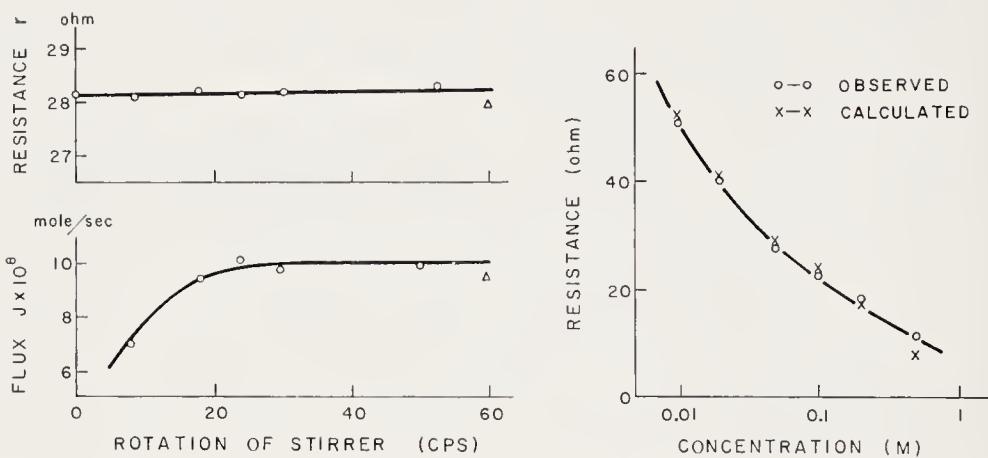


FIGURE 53. *Left:* Effect of stirring of the solutions on the membrane resistance and the interdiffusion-flux of cations.

Right: Dependence of the membrane resistance on the concentration of the salt solutions. The electrolytes used were NaCl on one side and RbCl (of the same concentration) on the other side of the membrane. Ionac, MC-3235 membrane was used. The calculated values of the membrane resistance were obtained by dividing the measured interdiffusion-flux by RT/F^2 . 24° C

five hours. No electric current was applied to the membrane during flux measurements.

Experimental Results

An example of the results obtained from one particular sheet of Ionac membrane is shown in Figure 53. The salts used were NaCl on one side of the membrane and RbCl on the other side. The effects of stirring on the membrane resistance and ion flux are shown by the left diagram. It is seen that the ion flux is very sensitive to the thickness of the stagnant layer on the surface of the membrane associated with changes in the speed of stirrer rotation. The effect of varying

the salt concentration on the membrane resistance and the ion flux is shown by the right-hand diagram in the figure. The salt concentrations in the two compartments were varied simultaneously, so that there was no detectable water flux across the membrane in these experiments. Ion fluxes measured with Na^{22} agreed well with the values obtained with Rb^{86} . Except at concentrations higher than

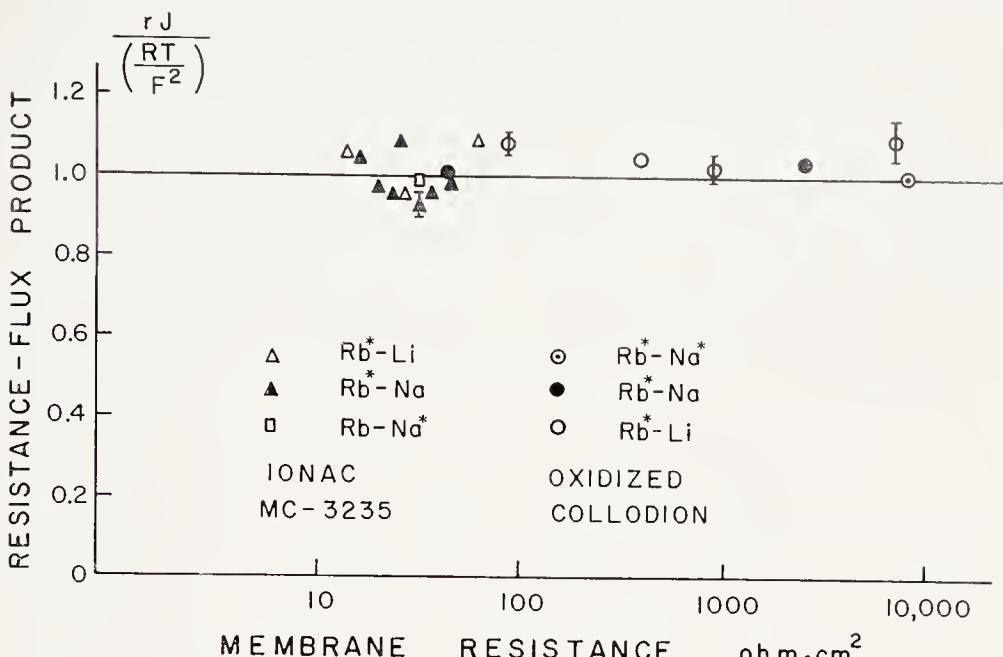


FIGURE 54. Resistance-flux product (normalized) plotted against the membrane resistance. Measurements of the membrane resistances and the interdiffusion-fluxes were made with the arrangement shown in Figure 52. The type of membrane and the cations used are indicated. The asterisk indicates the cation species traced by its radioisotope.

about 0.5 mole/l, the observed flux and resistance under a given experimental condition satisfied the expected relationship, $rJ = RT/F^2$. The deviation from this relationship at high salt concentration is due to imperfect co-ion exclusion from the membrane; this conclusion was supported by the tracer measurement using Cl^{36} .

The results of eighteen measurements of rJ -products under various experimental conditions are summarized in Figure 54. Over the entire range of the membrane resistance between 10 and 10,000 $\text{ohm}\cdot\text{cm}^2$, the observed values of rJ -product agreed with the theoretical value, RT/F^2 ($= 2.6166 \times 10^{-7} \text{ ohm}\cdot\text{equiv}\cdot\text{sec}^{-1}$ at 20°C), within approxi-

mately 10 percent. Gotlieb and Sollner (personal communication) made similar observations and arrived at the same conclusion.

From these observations, it may be concluded that the rJ -product of a uniform membrane with a high fixed-charge density is equal to RT/F^2 , irrespective of the nature of the membrane or of the chemical nature of the interdiffusing (univalent) cations.

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Appendix III

RELATIONSHIP BETWEEN CATION FLUXES AND MEMBRANE POTENTIAL IN IONIC MEMBRANES

SOLID giant axons are capable of developing action potentials in an external medium containing only a salt of a divalent cation if the axon interior is perfused with a dilute Cs or Na salt solution (see Sect. 7.3). The ionic environment of the axon membrane is simple and well defined under these conditions. The fluxes of cations across such a membrane can be described by the following Nernst-Planck equations.

$$(A.54) \quad J_1 = -\bar{u}_1 \bar{C}_1 \left\{ RT \frac{d \ln \bar{a}_1}{dx} + F \frac{d\bar{\varphi}}{dx} \right\}$$

$$(A.55) \quad J_2 = -\bar{u}_2 \bar{C}_2 \left\{ RT \frac{d \ln \bar{a}_2}{dx} + 2F \frac{d\bar{\varphi}}{dx} \right\}.$$

Some properties of these cation fluxes in relation to the membrane potential will be discussed briefly in this section (see Fig. 55).

The fluxes (J_1 and J_2), the concentrations (\bar{C}_1 and \bar{C}_2) and the ion activities (\bar{a}_1 and \bar{a}_2) of the univalent and the divalent cation (respectively) satisfy the following conditions at every point x in the membrane:

$$(A.56) \quad J_1 + 2J_2 = \frac{I}{F}$$

$$(A.57) \quad \bar{C}_1 + 2\bar{C}_2 = \bar{X}$$

$$(A.58) \quad \frac{\bar{C}_2}{\bar{C}_1^2} = \kappa \frac{\bar{a}_2}{\bar{a}_1^2}$$

where I is the (net) current through the membrane, \bar{X} the density of the fixed charge, and κ the activity coefficient ratio. Both \bar{X} and κ should be treated as functions of the space coordinate x normal to

the surface, and of the ratio \bar{a}_2/\bar{a}_1^2 . However, since analysis of such general cases is very complex, the present discussion is limited to a special case where \bar{X} and κ are treated as constants. The *qualitative* conclusions of the following discussion are expected to apply to the actual axon membrane.

The electric potential across a uniform cation-exchanger membrane separating a species of divalent cation from a species of univalent cation has been discussed previously (equations 4.3.2, A.41 and A.42.) In given ionic media, the membrane potential (for $I=0$) is a function of the mobility ratio (\bar{u}_2/\bar{u}_1), the fixed-charge density \bar{X} and the ion selectivity (K or κ). A rise in the internal potential can be brought about by an increase in one or more of these variables.

The interdiffusion flux under these conditions has been discussed by Helfferich and Ocker.¹ When $I=0$, the cation fluxes are given by

$$(A.59) \quad J_1 = -2J_2 = \frac{RT\bar{u}_1\bar{u}_2\bar{X}}{(\bar{u}_1 - 2\bar{u}_2)\delta} \left\{ 1 + \frac{2(\bar{u}_1 - \bar{u}_2)}{(\bar{u}_1 - 2\bar{u}_2)} \ln \frac{2\bar{u}_2}{\bar{u}_1} \right\}.$$

It should be noted that the interdiffusion flux is governed predominantly by the *slower* cation species. When $\bar{u}_2 \ll \bar{u}_1$, equation A.59 becomes

$$(A.60) \quad J_1 = -2J_2 = \frac{RT\bar{u}_2\bar{X}}{\delta} \left\{ 1 + 2 \ln \frac{2\bar{u}_2}{\bar{u}_1} \right\}.$$

The domination of the slower species is evident also in interdiffusion involving two univalent cations (see Sect. 3.3). This effect is due to accumulation of the slower cation species in the membrane.

The relationship between the fluxes, J_1 and J_2 , and the potential difference $\Delta\bar{\varphi}$ for a nonzero membrane current has been discussed by Schlögl.² The relationship in this case can be described by

$$(A.61) \quad -\frac{F\Delta\bar{\varphi}}{RT} = \frac{\frac{1}{2}J_1/\bar{u}_1 + J_2/\bar{u}_2}{J_1/\bar{u}_1 + J_2/\bar{u}_2} \ln \frac{-J_2/\bar{u}_2}{J_1/\bar{u}_1} = \frac{1}{2} + \frac{J_1/\bar{u}_1 + J_2/\bar{u}_2}{RT\bar{X}/\delta}.$$

(Note that the phase boundary potentials have to be added to $\Delta\bar{\varphi}$ above to obtain the membrane potential.)

Equation A.61 can be used to illustrate one important feature of the process of interdiffusion across a charged membrane. In Figure 55, the univalent cation species of the system is Cs and the divalent cation species is Ca. When the (net) membrane current is zero (see

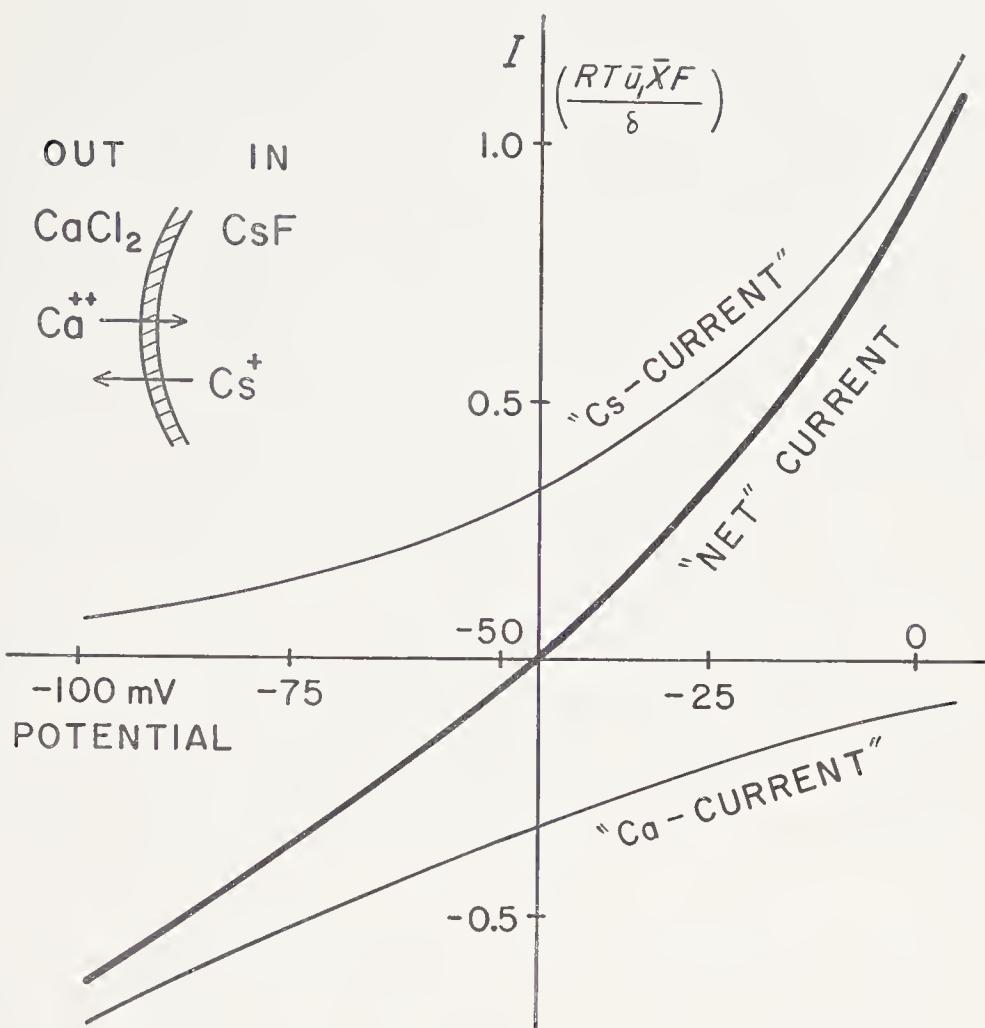


FIGURE 55. Theoretical membrane current (thick line), "cesium current" and "calcium current" (thin lines) plotted against the intramembrane diffusion potential. Equation A.61 was used for calculation with the mobility ratio (\bar{u}_2/\bar{u}_1) taken as 0.1. The abscissa is in mV and the ordinate is expressed in the unit of $RT\bar{u}_1\bar{X}F/\delta$.

$\Delta\bar{\varphi} = -46$ mV), $J_1 = -2J_2$; in this case, the "Ca current," $2J_2F$, is equal in intensity and opposite in sign to the "Cs current," J_1F . When the potential is raised above this particular level, the Cs current increases and Ca current decreases in intensity; both cation species contribute to the net current.

There is a roughly linear relation between the net current and the deviation of the membrane potential from the level for $I=0$; in other words, Ohm's law is valid within a limited range of potential.

The potential range in which this simple law is applicable is usually of the order of $\pm RT/F$ (i.e., ± 25 mV), which is the potential needed to accelerate ions (*in vacuo*) roughly to the average velocity of their thermal motion. In this range of potential, a net inward current consists of a Cs current and Ca current. Similarly, a net outward current is carried by both Cs and Ca ions.

When the membrane potential deviates from the level for $I=0$ by more than two or three times RT/F (i.e., 50–75 mV), the situation becomes very different. In these cases, an inward membrane current is carried predominantly by the external cations (Ca), and an outward current is carried mainly by the internal cations (Cs).

The importance of the concept of interdiffusion in interpretation of the current-voltage relationship in the squid giant axons has been stressed in recent articles from this laboratory.^{3,4}

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GLOSSARY OF SYMBOLS

a_{\pm}' and a_{\pm}''	Mean activity of salt in aqueous solution on left and right side of membrane, respectively.
a_i' and a_i''	Activity of ion species i in aqueous solution on left (out-) and right (in-) side of membrane (axon), respectively.
\bar{a}_i	Activity of ion species i <i>within</i> membrane.
c_i' and c_i''	Concentration of ion species i in aqueous solution on left and right side of membrane, respectively.
\bar{C}_i	Concentration of ion species i <i>within</i> membrane.
E_r and E_a	Membrane potential for $I=0$ in resting and excited state, respectively.
F	Faraday constant (96,500 coulomb/equivalent).
g_r and g_a	Membrane conductance in resting and excited state, respectively.
I	Electric current through unit membrane area.
J_i	Flux of ion species i (in $\text{mole}\cdot\text{cm}^{-2}\cdot\text{sec}^{-1}$).
K_j^i	Selectivity coefficient for exchange of species i for j .
Q_j^i	Kinetic selectivity coefficient ($= K_j^i \bar{u}_i / \bar{u}_j$).
r and r_a	Membrane resistance in resting and excited state, respectively.
R	Gas constant.
t	Time.
T	Absolute temperature.
\bar{u}_i	Mobility of ion species i .
$V(t)$ or V	Change in membrane potential produced by (applied) electric current.
x	Space coordinate normal to membrane surface.
\bar{X}	Concentration of fixed negative charge in membrane.
z_i	Valence of ion species i (negative for anions).
δ	Thickness of membrane.
$\Delta\varphi$	Electric potential of right-hand (internal) electrode referred to left-hand (external) electrode.
$\bar{\mu}_i$	Electrochemical potential of ion species i .
φ' and φ''	Electric potential of aqueous solution on left (out-) and right (in-) side of membrane (axon), respectively.
$\bar{\varphi}$	Electric potential at point x in membrane.
τ_m	Membrane time constant.

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