

## THE CELLULAR RESTING AND ACTION POTENTIALS: INTERPRETATION BASED ON THE ASSOCIATION-INDUCTION HYPOTHESIS

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• *The Hodgkin, Huxley, and Katz theories of resting and action potentials are based on the membrane theory, which holds that cell  $K^+$  and water exist in the free state. Reviewed here are these theories of cellular potential along with the results of experimental testings. Reviewed also is Ling's association-induction (AI) hypothesis, which holds that all  $K^+$  is adsorbed selectively and singly on anionic protein sites and that cell water is adsorbed in multilayers on extended chains of "matrix proteins." In the development of the AI model, molecular mechanisms of cell permeation and electric potentials were presented according to which the potentials are surface-adsorption phenomena. Thus they resemble those suggested by Baur rather than the membrane potentials proposed by Ostwald and Bernstein. In the present review it is shown that the AI version of the surface adsorption model can account for evidence supporting the Hodgkin, Huxley, Katz approach as well as evidence against it – including extensive recent confirmation of the adsorbed state of  $K^+$  in muscle.*

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## I. INTRODUCTION

Electrical activities of living tissues have fascinated biologists since the days of **Galvani**<sup>1</sup> and **Matucci**.<sup>2</sup> With relatively crude tools, much information was obtained subsequently by pioneers like **DuBois-Reymond**,<sup>3</sup> **Hermann**,<sup>4</sup> and **Bernstein**.<sup>5,6</sup> But rapid strides were not made in quantitative and mechanistic appraisal of these major physiological phenomena until the development of techniques utilizing impalement of giant squid axons, voltage clamps, capillary microelectrodes, and the sucrose gap.

In the present overview, I first present a brief history of interpretation of resting and action potentials according to the membrane-pump theory of the living cell. I then present findings that led me to question the validity of that theory. Finally, I review evidence for a different interpretation of the resting potential and action potential based on an alternative model of the living cell – the association-induction (AI) hypothesis.

## II. CELLULAR ELECTRIC POTENTIALS ACCORDING TO THE MEMBRANE THEORY

### A. Cellular Resting Potential

1. THE BERNSTEIN CONCEPT. In 1841, some fifty-five years after **Galvani's** discovery of **bioelectricity**,<sup>1</sup> **Matucci** demonstrated that the intact surface of a muscle is electrically positive with respect to the cut end (the injury **potential**).<sup>2</sup> Another thirty-five years passed before **Pfeffer**<sup>7</sup> founded the membrane theory, based experimentally on the supposed near-perfect semipermeable properties of the copper ferrocyanide gel membrane of **Traube**.<sup>8</sup> **Traube's** suggestion that this membrane acts as a molecular sieve was not supported by later X-ray and electron diffraction studies revealing that the pores in copper-ferrocyanide gel were too large (ca. 150 Å) to bar passage of impermeant solutes like sucrose (diameter, 9.4 Å). (See **Glasstone**.<sup>9</sup>)

However, **Ostwald**<sup>10</sup> suggested that a similar semipermeable membrane could be responsible for the electrical potentials of living cells. **Bernstein**,<sup>5,6</sup> first proposed application of the membrane theory to the cellular electrical potential. His idea was that a difference of potential exists across the normal cell membrane, with the inside of the cell negative; the injury potential is a partial expression of this preexisting **resting potential**. In this "membrane theory" of electric potential, the cell membrane allows passage of **K<sup>+</sup>** but not **Na<sup>+</sup>**, the potential being described as a **K<sup>+</sup>** equilibrium potential:

$$\psi = \frac{RT}{F} \ln \frac{[K^+]_{in}}{[K^+]_{ex}} \quad (1)$$

where R, T, F have the usual meanings, and where  $[K^+]_{in}$  and  $[K^+]_{ex}$  are the inter- and extracellular **K<sup>+</sup>** concentrations. Three decades later this idea was reintroduced by **Boyle** and **Conway**,<sup>11</sup> who attempted to relate selective **K<sup>+</sup>** accumulation, cell swelling and shrinkage, and resting potential within a single framework. Their approach was in fact a direct application of **Donnan's theory**<sup>12</sup> of ionic equilibrium and membrane potential.

Contrary to the then widely held view, Boyle and **Conway** concluded that  $\text{Cl}^-$  is fully **permeant** and distributes between the muscle cell and its environment according to **Donnan's** theory:

$$\frac{[\text{K}^+]_{\text{in}}}{[\text{K}^+]_{\text{ex}}} = \frac{[\text{Cl}^-]_{\text{ex}}}{[\text{Cl}^-]_{\text{in}}} \quad (2)$$

where  $[\text{Cl}^-]_{\text{in}}$  and  $[\text{Cl}^-]_{\text{ex}}$  are the intracellular and extracellular  $\text{Cl}^-$  concentration respectively. Combining with Eq. 1 yields

$$\psi = \frac{RT}{F} \ln \frac{[\text{K}^+]_{\text{in}}}{[\text{K}^+]_{\text{ex}}} = \frac{RT}{F} \ln \frac{[\text{Cl}^-]_{\text{ex}}}{[\text{Cl}^-]_{\text{in}}} \quad (3)$$

It was ironic that Boyle and **Conway's** theory met with serious difficulty almost immediately before and after publication. Absolute membrane impermeability to  $\text{Na}^+$ , long considered established, and essential to the Boyle and **Conway** theory, had been disproven some months before by the isotopic  $\text{Na}^+$  studies of **Heppel**<sup>13</sup> and it was disproven again some months later by the studies of **Steinbach**.<sup>14</sup> Indeed, in 1948  $\text{Na}^+$  was shown to travel in and out of muscle cells with a half-time of only 30 minutes (Levi and **Ussing**<sup>15</sup>) whereas  $\text{K}^+$  takes many hours (Harris and **Burn**<sup>16</sup>).

The crisis created by Heppel's and Steinbach's findings (1940, 1941) set the stage for radical revamping of the membrane theory. Two major developments were

(I) Introduction of Na (and other) "pumps" functioning in lieu of an impermeable membrane — hence the membrane theory became referred to also as the **membrane-pump** theory. The quiescent resting cell now required a continuous supply of energy for various pumps.

(2) Introduction of the Hodgkin-Katz theory of the cell potential.

2. **HODGKIN-KATZ** THEORY OF CELLULAR RESTING AND ACTION POTENTIALS. In 1902 **Overton**<sup>17</sup> had shown that excitability of living cells depends on the presence of  $\text{Na}^+$  in the bathing medium. Some half a century later, Hodgkin and **Katz**<sup>18</sup> discovered that the magnitude of the action potential depends on the logarithm of the external  $\text{Na}^+$  concentration. This relation was represented in an equation of the cellular electrical potential known as the Hodgkin-Katz equation (often called the **Hodgkin-Katz-Goldman** equation because the model resembles the constant field construct of **Goldman**<sup>19</sup>):

$$\psi = \frac{RT}{F} \ln \frac{P_{\text{K}} [\text{K}^+]_{\text{in}} + P_{\text{Na}} [\text{Na}^+]_{\text{in}} + P_{\text{Cl}} [\text{Cl}^-]_{\text{ex}}}{P_{\text{K}} [\text{K}^+]_{\text{ex}} + P_{\text{Na}} [\text{Na}^+]_{\text{ex}} + P_{\text{Cl}} [\text{Cl}^-]_{\text{in}}} \quad (4)$$

where  $P_{\text{K}}$ ,  $P_{\text{Na}}$ , and  $P_{\text{Cl}}$  are the permeability for each of the ions indicated.

In the derivation of Eq. 4, it was assumed that the cell membrane is a homogeneous and isotropic medium containing no fixed ionic sites. Therefore diffusion of ions in the membrane would be independent of the presence of other ions within it. Thus adjustment of the different permeability constants for  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  would be sufficient for the equation to yield the correct magnitudes of both the resting potential and the action potential.

The relation between  $\psi$  and  $T$  and between  $\psi$  and  $\ln[K]_{\text{ex}}$  (Eq. 5 below) had already been verified, within limits, by Ling and Woodbury,<sup>20</sup> Hodgkin and Katz,<sup>21</sup> Corabouef and Weidemann,<sup>22</sup> Curtis and Cole,<sup>23</sup> Ling and Gerard,<sup>24</sup> and Huxley and Stämpfli.<sup>25</sup> The dependence of  $\psi$  at the height of the action potential on  $\ln[Na]_{\text{ex}}$  had also been verified in a variety of living cells by Huxley and Stämpfli,<sup>25</sup> Hodgkin and Katz,<sup>26</sup> and Nastuk and Hodgkin.<sup>27</sup>

However, the expected dependence of the steady potential  $\psi$  on external  $Cl^-$  concentration predicted by the Hodgkin-Katz-Goldman equation was not observed (Hodgkin and Horowicz<sup>28</sup>). This independence of  $\psi$  led to revision of Eq. 4 into the following form (Katz<sup>29</sup>):

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{\text{in}} + P_{Na} [Na^+]_{\text{in}}}{P_K [K^+]_{\text{ex}} + P_{Na} [Na^+]_{\text{ex}}} \quad (5)$$

or

$$\psi = \frac{RT}{F} \ln \frac{[K^+]_{\text{in}} + b [Na^+]_{\text{in}}}{[K^+]_{\text{ex}} + b [Na^+]_{\text{ex}}} \quad (6)$$

where

$$b = \frac{P_{Na}}{P_K}. \quad (7)$$

## B. The Action Potential According to the Membrane Theory

1. **BERNSTEIN'S THEORY.** After Matucci's discovery of the injury potential, DuBois-Reymond,<sup>3</sup> observed that the "Muskelstrom" (electric current measured between the surface of the muscle and its tendon) decreased during tetanic contraction of the muscle. DuBois-Reymond named this "die negative Schwankung" or negative variation. Later the transient negative variation that traveled along the length of nerve and muscle cell during excitation was called the action potential. In Bernstein's original membrane concept,<sup>5,6</sup> the action potential was seen as an annulment of the preexisting membrane potential due to a sudden increase of permeability of the cell membrane. That there is indeed a transient large increase of ionic permeability concomitant with the action potential was confirmed in both the giant plant cell *Nitella* (Cole and Curtis<sup>30</sup>) and the giant squid axon (Cole and Curtis<sup>31</sup>).

On the basis of Bernstein's theory, the action potential would be expected never to exceed the magnitude of the resting potential. Later work did not confirm this specific prediction. Instead, the action potential (90 mV) was found to be considerably larger than the resting potential (50 mV) in squid axon (Hodgkin and Huxley<sup>32</sup>) and other excitable tissues studied. In other words, the action potential does not represent merely an annulment of the resting potential; it actually involves a reversal of the polarity of the resting potential.

2. **THE SODIUM HYPOTHESIS OF HODGKIN AND KATZ.** In 1949 Hodgkin and Katz<sup>26</sup> proposed that the transient increase of permeability postulated by Bernstein and confirmed by Cole and Curtis,<sup>30,31</sup> is limited to Na'. That is, the permeability shifts

from one favoring  $\mathbf{K}^+$  to one favoring  $\mathbf{Na}^+$ . Thus while the cell surface at rest behaves like a  $\mathbf{K}^+$  electrode as shown in Eq. 1, during the action potential the active section of the cell surface transiently behaves like a  $\mathbf{Na}^+$  electrode:

$$\psi_{\text{act}} = \frac{RT}{F} \ln \frac{[\mathbf{Na}^+]_{\text{in}}}{[\mathbf{Na}^+]_{\text{ex}}} . \quad (8)$$

In support of this hypothesis, the action potential was indeed found to vary with the logarithm of the external  $\mathbf{Na}^+$  concentration just as the resting potential varies with the logarithm of the external  $\mathbf{K}^+$  concentration. Both Eq. 1 and Eq. 8 are variations of Eq. 6 with different values of  $b$ , which is the ratio of the permeability coefficient of  $\mathbf{Na}^+$  to that of  $\mathbf{K}^+$ . For resting cells,  $b$  is small; it becomes larger transiently during activity.

**3. THE HODGKIN-HUXLEY THEORY OF THE ACTION POTENTIAL.**<sup>32-37</sup> Adopting the voltage clamp method introduced by Cole<sup>38</sup> and Marmont,<sup>39</sup> Hodgkin and Huxley presented their classic studies of the action potential of squid giant axon in 1952. The method allows the investigator to set the electrical potential difference between the inside and the outside of an axon at any level chosen and to hold it there by feedback circuitry. Since the electrical potential is altered uniformly throughout the whole axon at any one time, no propagation occurs along the length of the axon so that analysis of the current is simpler. The electric current across the cell surface can be divided into a *capacity current* involving charge distribution within the cell surface and an *ionic current* involving current passing through the membrane. By holding the voltage,  $V$ , constant, the capacity current is made to vanish. Hence the voltage clamp method allows study of the ionic current uncomplicated by capacity current effects.

The ionic current,  $I_i$ , in turn can be separated into three components: the *sodium current* ( $I_N$ ), the *potassium current* ( $I_K$ ), and the remainder, called the *leakage current* ( $I_L$ ).  $I_N$ , and  $I_L$ , but not  $I_K$ , vary in the course of an action potential.

Capacity current is extremely rapid (completed within 50  $\mu\text{sec}$  at 8°C in squid axon) and not easily discernible unless observed at a very high time-base and low amplification. It is readily recognized by its symmetrical current records with equivalent anodal and cathodal currents. Ionic currents are much slower (on a msec scale). Since  $I_K$  is constant during the action potential, the main events occurring in an action potential concern  $I_{\mathbf{Na}}$  and  $I_L$ .

a. *Some major experimental observations.* At the region of excitation, the electric potential reverses its sign so that the outside becomes negative with respect to the inside of the nerve. The flow of local current causes depolarization at the neighboring region and propagation of the action potential. With a voltage clamp, a similar depolarization is artificially applied as a short pulse across the resting membrane. If it exceeds 12 to 15 mV, it produces a nonpropagated action potential of about 100 mV. There is a surge of inward current that reaches a maximum a fraction of a millisecond after application of the shock; this inward current decreases and at about 2 milliseconds an outward current occurs that then slowly returns to the resting level.

Under the voltage clamp, the initial part of the current flow is quite similar to that of a normal action potential. The later phase of the action potential, however, is exaggerated by the creation of a sustained delayed outward current. This delayed outward current then returns to the normal resting level if the voltage clamp is turned off.

The above observations come from Hodgkin and Huxley. But from the earlier work mentioned above it was known that the action potential involves a local exchange of  $K^+$  and  $Na^+$  between the cell and its surrounding medium. This gave rise to the next question: What is the precise contribution of each of these ions to generation of the action potential? To arrive at answers, the voltage clamp technique again offers unique advantage. In the laboratory, if all the  $NaCl$  in sea water medium is replaced by choline chloride, the nerve becomes inexcitable; the natural action potential does not occur and the early inward current vanishes. But in the voltage clamp method, the delayed outward current remains largely unchanged. A more careful look at the record shows that the normal large inward current is replaced by a very small current in the opposite direction.

On the strength of the above findings, Hodgkin and Huxley then argued that the cathodic depolarization opens the cell membrane to the passage of  $Na^+$ . They viewed the small *outward* hump in choline sea water as well as the normal initial *inward* current in normal sea water as quantitatively determined by the balance of the applied electrical potential difference and the sodium potential,  $V_{Na}$ , where

$$V_{Na} = \frac{RT}{F} \ln \frac{[Na^+]_{in}}{[Na^+]_{ex}}. \quad (9)$$

When there is much more  $Na^+$  in the external solution, as in the case of normal sea water,  $V_{Na}$  provides the driving force for the surge of inward movement of  $Na^+$ . When there is more  $Na^+$  in the cell water than in the external medium, as in the case of choline sea water,  $V_{Na}$  drives  $Na^+$  to move outward. In normal sea water, when the inside of the axon is made electrically negative to the outside by about 108 mV, neither the initial nor the inward current occurs. In sea water containing only 30% of its normal  $Na^+$ , a negative potential of merely 79 mV induces the same state of balance.

All these observations of Hodgkin and Huxley are in harmony with the notion that the early ionic current is carried by  $Na^+$  moving in response to both joint electrical and concentration gradients. The driving force for the  $Na^+$  movement is the difference between the membrane potential ( $V$ ) and the  $Na^+$  equilibrium potential and is represented by  $(V - V_{Na})$ . The  $Na^+$  permeability is then represented as a  $Na^+$  conductance,  $g_{Na}$ :

$$g_{Na} = \frac{I_{Na}}{V - V_{Na}}. \quad (10)$$

By making certain simplifying assumptions, Hodgkin and Huxley found it possible to separate the experimentally observed ionic current into its sodium and potassium components and to show that during the action potential the sodium conductance rises rapidly to a maximum and then declines along an exponential



curve. The potassium conductance, on the other hand, rises more slowly along an S-shaped curve and in the voltage clamp is maintained at a high level indefinitely. The  $K^+$  conductance,  $g_k$ , may be represented as

$$g_k = \frac{I_k}{V - V_k} \quad (11)$$

Hodgkin and Huxley's sodium potential seems explained by the concentration gradient calculated from the  $Na^+$  concentrations in the cell water and the sea water. But the effect on the  $K^+$  potential of changing external  $K^+$  concentration, though in the right direction, is far from being quantitatively precise. Indeed the observed potassium potential change as determined from the potential needed to annul  $I_k$  is only half that calculated on the basis of the known intracellular  $K^+$  concentration.

Finally, consideration must be given the phenomenon called "inactivation," produced by a steady small depolarization. Inactivation means a loss of the ability to undergo the usual large increase of  $Na^+$  conductance with further *depolarization*. It can be overcome by a small *hyperpolarization* (making the outside more positive). Inactivation is quantitatively related to the magnitude of the membrane potential and is virtually complete at  $-30$  mV or lower but vanishes at  $+30$  mV or higher.

b. *Hodgkin-Huxley theory of permeability changes during the action potential.* At least three alternative mechanisms underlying the action potential were considered by Hodgkin and Huxley. Of these, a carrier model and a model of one channel for both  $K^+$  and  $Na^+$  were either rejected or not pursued. A brief description of the third model, as developed by those authors, follows:

The advancing front of an action potential causes depolarization of the neighboring quiescent region. This electrical depolarization causes a certain number of charged sodium particles ( $CP_{Na}$ ) to migrate from one position (A) to another (B).  $CP_{Na}$  are not carriers but they allow  $Na^+$  to pass through the membrane when they occupy position B although not when they occupy position A. The observed decline of the  $Na^+$  conductance is attributed to either a chemical change of  $CP_{Na}$  after leaving position A or to another charged slow-migrating particle ( $CP'_{Na}$ ) that blocks  $Na^+$  movement. The chemically changed  $CP_{Na}$  blocking the channel would of course have to be subsequently removed to allow the next impulse to go through.

Similarly, the electrical depolarization brought on by the front of the advancing action potential moves a set of charged potassium particles ( $CP_k$ ) from a position C to a position D, thereby opening a separate channel to allow passage of  $K^+$ .  $CP_k$  would then have to return to position C for the initiation of the next impulse.

Hodgkin and Huxley postulated that a K channel is formed when four  $CP_k$  move to position D. Using  $n$  to represent the probability that a single  $CP_k$  moves to position D, and representing K conductance by  $g_k$ , they introduced the equation

$$g_k = \bar{g}_k n^4 \quad (12)$$

where  $\bar{g}_k$ , a constant, is the maximum  $K^+$  conductance. For the Na channel,

$$g_{Na} = \bar{g}_{Na} m^3 h \quad (13)$$

where  $\bar{g}_{Na}$  is a constant equal to the maximum  $Na^+$  conductance;  $m$  is the probability

that a single  $CP_{Na}$  occupies position B, and it takes the movement of three  $CP_{Na}$  from position A to position B to open the Na channel;  $h$  is the probability of a single  $CP'_{Na}$  moving to the blocking position and being adequate to block the Na channel. The rate of change of the probabilities  $n$ ,  $m$ , and  $h$  with time are described by

$$\frac{dn}{dt} = \alpha_n(1 - n) - \beta_n n \quad (14)$$

$$\frac{dm}{dt} = \alpha_m(1 - m) - \beta_m m \quad (15)$$

$$\frac{dh}{dt} = \alpha_h(1 - h) - \beta_h h \quad (16)$$

where  $\alpha$  and  $\beta$  are rate constants. It should be emphasized, however, that these are not true constants but vary with the membrane potential. Depolarization (i.e., the cell interior becoming more positive) increases  $\alpha_n$  and  $\alpha_m$ , decreases  $\beta_n$  and  $\beta_m$ , increases  $\beta_h$ , and decreases  $\alpha_h$ .

The complete Hodgkin-Huxley equation for the membrane current density,  $I$ , is

$$I = C \frac{dV}{dt} + (V - V_K) \bar{g}_K n^4 + (V - V_{Na}) \bar{g}_{Na} m^3 h + (V - V_l) \bar{g}_l \quad (17)$$

where  $C$  is the membrane capacity, and the four terms on the right are the capacity current, the K current, the Na current, and the leakage current respectively.

### C. Experimental Testing of the Membrane Theory of the Action and Resting Potentials

The Hodgkin-Huxley theory of the potentials, as summarized above, instigated extensive research on the action potential of a variety of excitable cells. In a general way, certain basic concepts introduced were widely confirmed and extended. However, some contradictions were observed.

1. CONFIRMATIONS. The discovery of more or less specific blockers of the Na channels (e.g., tetrodotoxin, saxitoxin) and of the K channels (e.g., tetraethylammonium, 4-aminopyridine) was in accord with the two-channel concept. Concepts of these channels include Hille's Na channel model comprised of a rigid pore of  $3 \times 5 \text{ \AA}$  surrounded by eight oxygen atoms,<sup>40,41</sup> and Armstrong's  $K^+$  channel model with a narrow mouth facing the outside and a wide mouth facing the cell interior.<sup>42</sup> Armstrong's idea was supported by research of Hucho and Schiebler<sup>43</sup> using a photo-labeling technique. Similarly, the discovery of the gating current (1975) offered support for the Hodgkin-Huxley theory (Lansdowne et al.<sup>44</sup>).

2. CONFLICTING FINDINGS. Contradictions gradually accumulated as research continued. Of these, perhaps the most striking pertained to two aspects of the Hodgkin-Huxley work. First, the "independence principle." Second, predicted relations between the resting or action potentials and various ionic conditions.

a. *The independence principle.* This major premise of the Hodgkin-Huxley action potential theory holds that the cell membrane acts as a homogeneous isotropic medium through which the diffusion of  $\text{Na}^+$  and  $\text{K}^+$  is independent of the diffusion of other ions of the same or different species. The principle was implicitly assumed in the development of the Hodgkin-Katz-Goldman equation; earlier testing had suggested independence in the relation between  $\text{Na}^+$  concentration and sodium current during excitation (Hodgkin and Huxley<sup>37</sup>).

Since 1952, however, many studies raised questions about the validity of the principle. One example is Hodgkin and Huxley's<sup>45</sup> own discovery that during an impulse the influx of labeled  $\text{K}^+$  observed is only about 1/6 of that calculated on the basis of the independence principle. (It was this discovery that led to postulation of the "long-pore model" by Hodgkin and Keynes.<sup>46</sup>) Chandler and Meves<sup>47</sup> showed that in perfused squid axons, alkali metal ions in the internal perfusing solution reduce delayed current in decreasing effectiveness:  $\text{Cs}^+ > \text{Rb}^+ > \text{Na}^+$ . That finding was confirmed by Adelman and Senft<sup>48</sup> and by Bezanilla and Armstrong.<sup>49</sup> Similarly Hagiwara et al.<sup>50</sup> found that in giant barnacle muscle fibers the inward  $\text{Ca}^{2+}$ -current, which plays the same role as the Na current in squid axons, saturates at high  $\text{Ca}^{2+}$  concentration. Both K conductance and Na conductance were shown to be reversibly depressed by low pH (Hille,<sup>51,52</sup> Drouin and The,<sup>53</sup> Stillman et al.,<sup>54</sup> Woodhull,<sup>55</sup> Schauf and Davis<sup>56</sup>). Although some investigators thought that the  $\text{H}^+$  effect is indirect and via the gating process (Fishman et al.<sup>57</sup>), others regarded the  $\text{H}^+$  effect as directly blocking  $\text{Na}^+$  migration and thus contradicting the independence principle.

Finally, Chang's finding that the null potential induced by the voltage clamp is not equal to the  $\text{Na}^+$  potential in squid axons<sup>5a</sup> raised doubts about what had long been regarded as the best evidence in support of the independence principle.

b. *Opening and closing of  $\text{K}^+$  and  $\text{Na}^+$  channels in the cell membrane.* This concept is in conflict with the knowledge that the resting cell membrane is already quite permeable to both  $\text{K}^+$  and  $\text{Na}^+$ . After the advent of radioactive tracer technique, the old idea that the membrane is impermeable to  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  became untenable. For example, the halftime of exchange of labeled  $\text{Na}^+$  has been shown to be seconds in *Spiroga* (Brooks<sup>58</sup>) and (a few) minutes in frog muscle cells (Ling<sup>5ab</sup>). With such rapid traffic of Na at all times it is awkward, to say the least, to postulate specific all-or-none opening and closing of  $\text{Na}^+$  (and K) gates during an action potential.

c. *Predicted relation between the action potential and intracellular  $\text{K}^+$  and  $\text{Na}^+$ .* Baker, Hodgkin, and Shaw<sup>59</sup> showed that in agreement with prediction of the Hodgkin-Huxley theory, replacement of 1/4 or 1/2 of potassium sulfate in the internal perfusing solution in squid axon by sodium sulfate decreases the height of the action potential. Using Chilean squid axon, Tasaki et al.<sup>60</sup> found a similar fall of the action potential with a decrease of the K concentration from 0 to 350 mM in the internal perfusing solution. However, in contradiction to the Hodgkin-Huxley theory, there remained an *overshoot* when both external and internal solutions contained the same concentrations of  $\text{Na}^+$  (350 mM) (see also Tasaki and Takenaka<sup>61</sup>).

Chandler and Meves,<sup>47</sup> studying perfused squid axons with axoplasm removed by the method of Baker, Hodgkin, and Shaw,<sup>59</sup> observed an apparent equilibrium Na

potential (see Eq. 9) of 69 mV even though the internal perfusing fluid contained no Na and hence there could not be an equilibrium Na potential. Similar questions of the origin of the observed  $\text{Ca}^{2+}$  equilibrium potential in barnacle muscle fiber injected with the  $\text{Ca}^{2+}$ -chelating agent EDTA, which reduces internal free  $\text{Ca}^{2+}$  to near zero, were raised by Hagiwara et al.<sup>62</sup>

d. Predicted relation between resting potential and the external  $\text{Cl}^-$  concentration. Substitution of external  $\text{Cl}^-$  by the much less permeable  $\text{SO}_4^{2-}$  was found to produce no significant change in the resting potential of frog muscle cells. The  $\text{Cl}^-$  terms were then deleted from the original Hodgkin-Katz-Goldman equation to yield Eqs. 5 and 6. The reasons given for the deletion were that the  $\text{Cl}^-$  ions redistribute themselves between the extracellular and intracellular fluid (Adrian<sup>63</sup>) and that they are in equilibrium in resting muscle tissue (Katz, ref. 29, p. 62). I do not believe that these or any other reasons justify deleting parts of the key theoretical equation in order to fit the data. Indeed, in neither the Nernst equation (Eq. 1) nor the Donnan (equilibrium) equation (Eq. 2), do electrical potentials disappear with the attainment of equilibrium of either  $\text{K}^+$  or  $\text{Cl}^-$ .

e. Predicted relation between intracellular  $\text{K}^+$  and the resting potential. Regarding the predictive value of the membrane theory of the resting potentials, important data came to light in 1954 and the years following. These concerned (1) axon, (2) muscle, and (3) egg:

(1) Grundfest et al.<sup>64</sup> injected 1.3 M K-aspartate into squid giant axon (*Loligo*) but could not find significant change of the resting potential. Hodgkin and Keynes,<sup>65</sup> however, found that injection of 1.0 M NaCl and 1.0 M KCl into squid giant axon (*Loligo*) did cause the small changes of resting potential theoretically expected.

In general agreement with the Hodgkin-Katz-Goldman equation, Baker, Hodgkin, and Shaw<sup>59</sup> demonstrated that by internally perfusing isolated squid axon with a sodium medium containing varying amounts of KCl (in mixtures of KCl and NaCl) while the axon was bathed externally in a solution containing 10 mM K, the resting potential varied with the  $[\text{K}]_{\text{in}}$ . On the other hand, when Tasaki et al.<sup>60</sup> bathed Chilean squid giant axon in an external solution containing 350 mM  $\text{Na}^+$ , the resting potential remained entirely unchanged when the  $\text{K}^+$  perfusing solutions contained either 350 mM Na and 150 mM K or 500 mM K and no Na.

(2) Adrian<sup>63</sup> altered intracellular  $\text{K}^+$  concentration in muscle through exposure to hypo- and hypertonic solutions; he concluded that  $\psi$  varies with the intracellular  $\text{K}^+$  concentration. A similar but more qualified conclusion was reached by Hagiwara et al.,<sup>62</sup> who perfused giant barnacle muscle fiber with solution containing varying  $\text{K}^+$  concentrations up to 200 mM. (Beyond 200 mM, the observed  $\psi$  change was in the wrong direction relative to the expected direction.)

In contrast to these observations, leaching  $\text{K}^+$  from muscle cells in distilled water (Tobias<sup>66</sup>) or in half-isotonic sucrose solution (Koketsu and Kimura<sup>67</sup>) did not produce the drastic reduction of the resting potential predicted from the measured low levels of  $\text{K}^+$  in the cells. Nor did injection of 3 M KCl or 3 M NaCl into frog muscle fiber cause the predicted rise of the resting potential (Falk and Gerard<sup>68</sup>). A similar failure to demonstrate the expected relation between the resting potential of frog muscle and intracellular  $\text{K}^+$  and  $\text{Na}^+$  concentration was reported by Shaw and coworkers.<sup>69,70</sup>

As time went on, still more experimental observations were reported showing that the resting potential of various types of cells behaved differently than predicted by the Hodgkin-Katz-Goldman equation (Eq. 4) or its modified version (Eq. 6). As a rule, the resting potential measured was substantially higher than predicted on the basis of Eqs. 4 and 6 and the measured intracellular  $K^+$  concentrations. The most often reported observation dealt with amphibian voluntary muscle deprived of some of its normal  $K^+$  content and then allowed to regain this ion by restoring  $K^+$  to the bathing solution (Kernan,<sup>71</sup> Hashimoto,<sup>72</sup> Sato et al.<sup>73</sup>). A similar discrepancy between resting potential measured and that predicted from intracellular  $K^+$  concentration was reported following exposure of mammalian smooth muscle to adrenaline (Burnstock<sup>74</sup>), on removal of ouabain from the bathing medium (Casteels<sup>75</sup>), or on rewarming chilled mammalian heart muscle (Page and Strom,<sup>76</sup> Tamai and Kagiya<sup>77</sup>).

(3) While in most of the above observations  $\psi$  is higher than predicted from the measured concentrations of intracellular  $K^+$ , discrepancy of a different kind was reported for *Fundulus* eggs. These exhibit no measurable resting potential despite the presence of the usual normal intracellular  $K^+$  and  $Na^+$  concentrations (Kao<sup>78</sup>).

Discrepancies of resting potential change in response to variation of internal  $K^+$  ion concentration have led some to suggest changes of  $K^+$  ion permeability ( $P_K$ ) with changing  $K^+$  ion concentration. But these and other similar *ad hoc* postulations are unacceptable on at least two grounds. First, the original Hodgkin-Katz-Goldman equation was derived on the basis that  $P_K$ , etc. are constant. Second, variation of the potential with external  $K^+$  has been shown to conform to the original equation incorporating the assumption of constancy of  $P_K$ . If  $K^+$  ion can traverse the cell surface it is unreasonable to assume that changes of internal  $K^+$  ion concentration would affect the potential while changes in the external  $K^+$  would not (Baker et al.,<sup>79</sup> Stämpfli<sup>80</sup>).

f. **Conclusion.** We see from all the above that the membrane theory of the cell potentials can be only partially correct because it does not account for many observed contradictions. These have to do with the independence principle, the role of  $Cl^-$  (and other anions), the variation of cell  $K^+$  and the action potential, and the variation of cell  $K^+$  and the resting potential.

It is necessary, therefore, to reassess the massive accumulation of data on the electrical potentials of the living cell—data thus far interpreted predominantly from one viewpoint, that of the membrane theory. For this essential reassessment, we may draw upon the history as well as substance of the major alternative view of the living cell, the association-induction (AI) hypothesis. Over the years, various experimental findings, supportive observations and predictive successes, as well as tested refinements and extensions, have given to the hypothesis the full force of a formal theory. It is the subject of the following sections.

### III. LING'S FIXED CHARGE (LFC) HYPOTHESIS AND THE MOLECULAR MECHANISM OF ION SELECTIVITY

Between 1951 and 1955 I made several theoretical suggestions concerning cellular selective ionic accumulation, permeability, and the resting potentials. These proposals were based on the then new concept of selective adsorption of  $K^+$  on fixed

anionic sites in the living cell. This concept and the related proposals were referred to as Ling's fixed charge (LFC) hypothesis.

### A. A Molecular Mechanism for $K^+$ Selectivity Over $Na^+$

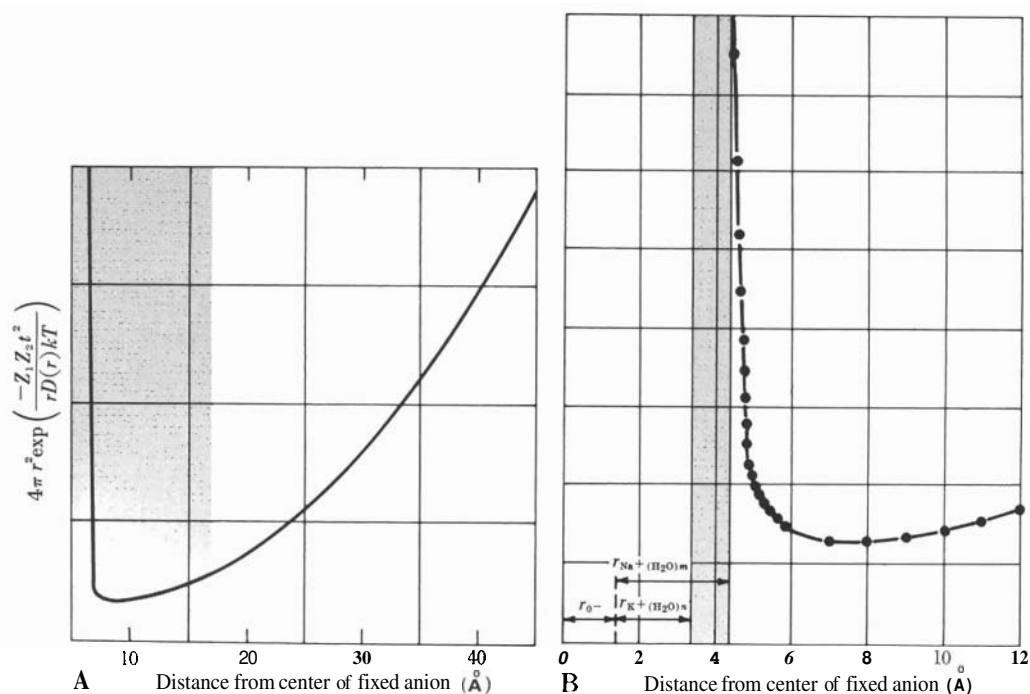
Arrhenius<sup>80a</sup> successful ionic dissociation theory had led to wide acceptance of near total ionic dissociation in electrolyte solutions of moderate strength. Counterion-fixed ion interaction was traditionally regarded as being negligible. It was in this atmosphere that the role of fixed charges was reconsidered in the LFC hypothesis.

The existence of fixed charges at the cell surface (but not in the cell interior) had been discussed long before by **Bethe** and **Toropoff**,<sup>80b</sup> **Michaelis**,<sup>81</sup> **Teorell**,<sup>82</sup> **Meyer** and **Sievers**,<sup>83</sup> **Sollner**.<sup>84</sup> None of those workers regarded the degree of counterion association as significant (Ling, ref. 85, p. 214, footnote). *But the new hypothesis suggested a basic molecular mechanism for specific selectivity of one alkali-metal ion ( $K^+$ ) over another ( $Na^+$ )* and included two proposals:

(1) A high degree of association of free counterions with the fixed ionic sites of opposite charge results from the charge fixation.

(2) Preference of adsorption of  $K^+$  over  $Na^+$  results from a more favorable (electrostatic) adsorption energy of the small hydrated  $K^+$  over that of the larger hydrated  $Na^+$ .

Figure 1 portrays the theoretical model presented in 1952.<sup>87</sup> Each fixed anionic site, represented by a singly charged anionic oxygen atom, creates an electrical field



**FIGURE 1.** Probability of finding a counteranion at various distances from the center of a microcell. Shaded area in A represents volume available to the smaller hydrated  $K^+$  ion (radius =  $r_{K^+(H_2O)_n}$ ) but not to the larger hydrated  $Na^+$  ion (radius =  $r_{Na^+(H_2O)_n}$ );  $r_o$  stands for the radius of the singly charged oxygen. In B, taken from Bjerrum,<sup>86</sup> shaded area corresponds to volume of a microcell of the size shown in A.

that decreases exponentially with the square of the distance between the center of this anionic charge and the center of a counterion. That is to say, the field is much more intense at close distance between the counterion and the fixed anion than further away. The steepness of the rising field strength at close proximity is further enhanced by the dielectric saturation phenomenon; *i.e.*, the effective dielectric constant of water in the immediate neighborhood of the fixed anion is close to unity while at far distance it is characteristic of that of normal water.<sup>83</sup> If the field strength distribution is known, it then is easy to calculate on the basis of Boltzmann's law that the probability of  $K^+$  occupying an anionic site is many times greater than the probability of  $Na^+$  occupying that site. Being smaller, the  $K^+$  can come closer to the fixed anion than the larger hydrated  $Na^+$  and as a result  $K^+$  can occupy a shell of space of high probability denied to  $Na^+$ .

#### B. Selective $K^+$ Accumulation in Living Cells, Ion-Exchange Resins, and Other Fixed Charge Systems

In presenting the LFC model, evidence was given that the fixed anionic sites in living cells on which  $K^+$  is normally adsorbed are the  $\beta$ - and  $\gamma$ -carboxyl groups belonging respectively to the **aspartic** and glutamic acid residues of cell proteins. Calculation from data available indicated that myosin, found only in the A-band of striated muscle cells, carries a major share of these anionic sites (Ling<sup>87</sup>) Recent confirmation of this concept is discussed below (p. 71).

In support of the fundamental LFC premise that selective adsorption underlies the accumulation of  $K^+$  over  $Na^+$  in living cells, I cited and discussed a number of artificial fixed charge systems including permutites, soils, and synthetic sulfonate ion-exchange resins. All are known to selectively accumulate  $K^+$  over  $Na^+$ . Thus the LFC hypothesis as presented is actually a general theory not only for selective ionic accumulation in all living cells including bacteria but for nonliving systems as well. As a theory for the selective accumulation of  $K^+$  over  $Na^+$  in synthetic ion-exchange resin, it stands apart from that of **Gregor**,<sup>88,89</sup> whose swelling pressure theory of selective  $K^+$  accumulation over  $Na^+$  was based on the assumption of total counterion dissociation. In 1956, however, Harris and Rice<sup>90</sup> did present a theory similar to mine for selective  $K^+$  accumulation in ion-exchange resins.

#### C. Selective Ionic Permeability of Living Cells

From the first, it was set forth as intrinsic to Ling's fixed charge hypothesis that the cell surface is in essence a two-dimensional version of the bulk phase three-dimensional fixed charge system (**Ling<sup>87</sup>**). The selectivity for  $K^+$  seen in its bulk phase accumulation offers the basis for selective permeability (**Ling<sup>91</sup>**). Here the major route of  $K^+$  entry is by adsorption onto fixed anionic sites, followed by libration around those fixed ionic sites and desorption and entry (the adsorption-desorption route) as depicted in Fig. 2. Another route is the "saltatory" route through the interstices, also illustrated in Fig. 2. Since the number of surface anionic sites is limited, and since a counterion like  $K^+$  or  $Rb^+$  must associate with these **cationic** sites first before entry, the rate of  $K^+$  or  $Rb^+$  permeation can reasonably be assumed to depend on the density of surface anionic sites associated with an external  $K^+$  or

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$Rb^+$ . The rate of entry would then follow an equation similar to that which Michaelis and **Menton** derived for enzyme activity rate (Ling<sup>90</sup>). Plotted reciprocally, that equation takes the form

$$\frac{1}{V_K} = \frac{1}{V_{max}} \left( K_K + \frac{K_K [Rb]_{ex}}{K_{Rb}} \right) \frac{1}{[K]_{ex}} + \frac{1}{V_{max}} \quad (18)$$

where  $V_K$  is the rate of entry of radioactive  $K^+$ ,  $V_{max}$  is the maximum rate of  $K^+$  entry.  $K_K$  and  $K_{Rb}$  are the adsorption constants of  $K^+$  and  $Rb^+$  on the cell surface anionic sites,  $[K]_{ex}$  and  $[Rb]_{ex}$  are the external ion concentrations. A plot of  $1/V_K$  against  $1/[K]_{ex}$  at different values of  $[Rb]_{ex}$ , yields a family of straight lines converging on the same locus on the ordinate and equal in value to  $1/V_{max}$ . Equation 18 embodies what is often referred to as *saturability* and *competition* (Christensen<sup>92</sup>). It should be clear that permeation possessing these characteristics does not require postulation of a carrier mechanism.

1. **CONFIRMATION.** Figure 3A shows that radioactively labeled  $K^+$  entry into frog muscle follows the expectation of Eq. 18, as had already been shown in 1952 by Epstein and **Hagen**<sup>93</sup> for  $Rb^+$  entry into barley roots. Although Epstein and **Hagen** as well as a large number of later investigators interpreted their data on the basis of the carrier concept, serious arguments against the carriers were mentioned by Hodgkin and **Huxley**<sup>37</sup> (see also **Armstrong**<sup>42</sup>).

On the other hand, the successes of our laboratory in demonstrating the obedience

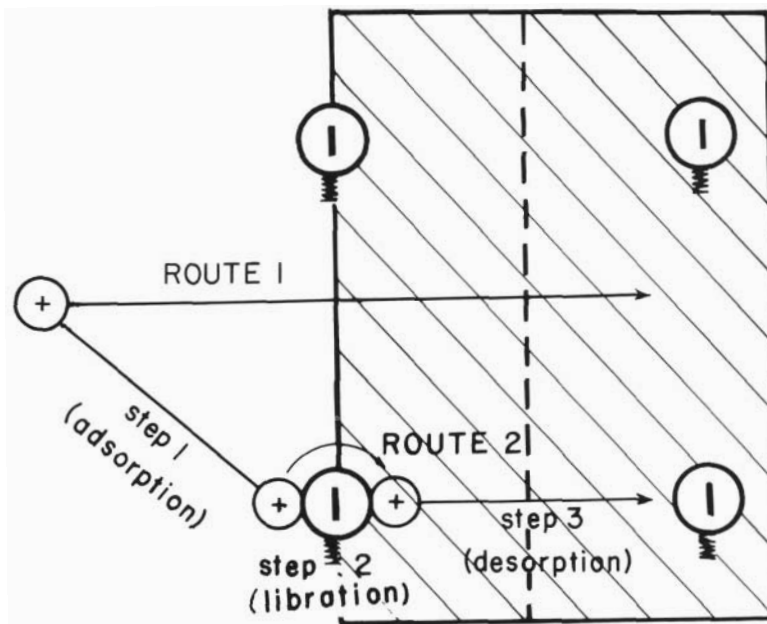


FIGURE 2. Representation of two routes of ion entry into a fixed-charge system. Shaded area represents a microscopic portion of the surface of a fixed-charge system in which four fixed anions are shown. Route 1 is the saltatory route. Route 2, the adsorption-desorption route, involves a sequence of three steps; adsorption, libration around the fixed anion, and desorption. This adsorption-desorption route corresponds to the doublet type, since two ions are involved (the free cation and the fixed anion).



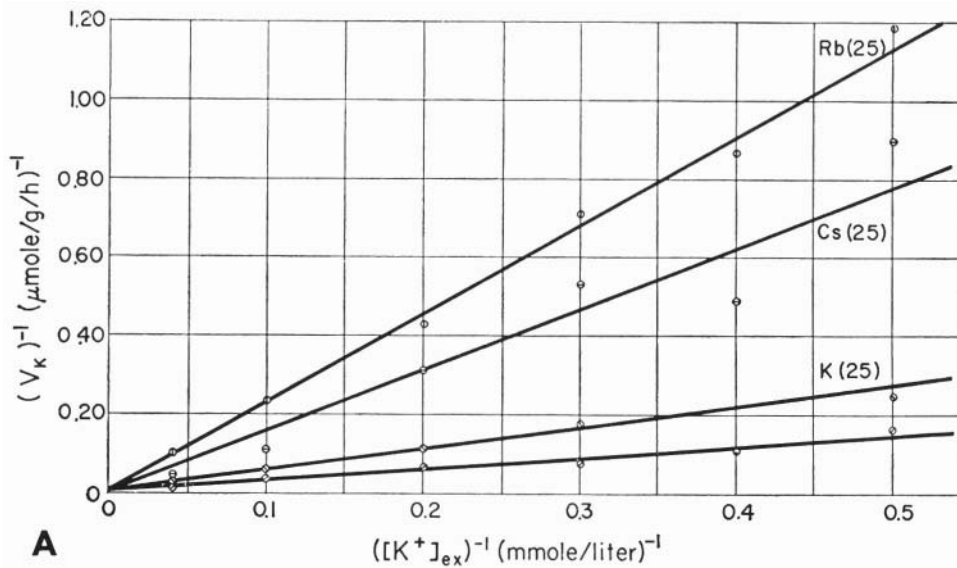


FIGURE 3A. Inhibitory effect of 25 mmole/liter of Rb<sup>+</sup>, Cs<sup>+</sup>, and nonlabeled K<sup>+</sup> ion on initial rate of entry of labeled K<sup>+</sup> ion into frog sartorius muscles. Lowest (unlabeled) curve represents rate of K<sup>+</sup> ion entry with no added competing ion. Muscles were soaked 30 min at 24°C, then washed 10 min at 0°C. Each point represents a single determination on two sartorius muscles.

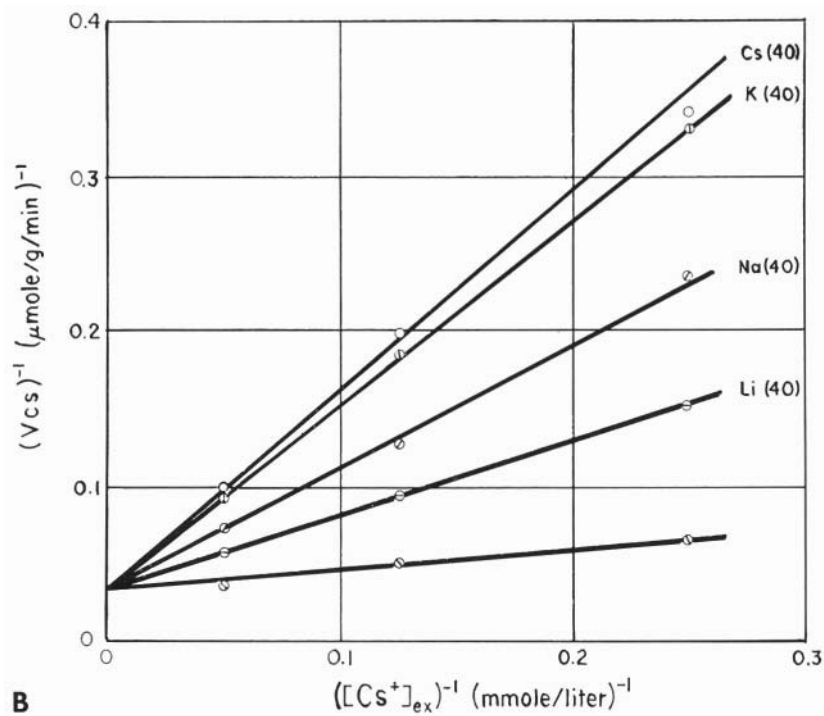


FIGURE 3B. Effects of CsCl, KCl, NaCl, and LiCl on the initial rate of entry of labeled Cs<sup>+</sup> ion into exchange resin sheets. Nalfilm-1 strips were soaked 2 min at 5°C in an experimental solution containing (approximately) 2.5 mmole tris buffer at pH 7.0, the labeled entrant ions and nonlabeled ion (40 mmole/liter) as indicated in the figure. Strips were washed for 10 sec in cold distilled water (0°C) before counting.

to Eq. 18 of ion entry into a variety of *nonliving* systems including anionic ion-exchange resin sheets, sheep's wool, and oxidized collodion membrane, all known to bear fixed anionic sites, lends additional support to the LFC model (Fig. 3b). These findings also establish clearly that "facilitated diffusion" cannot be equated with carrier mechanisms.

Continued study of the effect of one alkali-metal ion on the permeation rate of another had led me to the conclusion in 1952 that the permeability of these ions follows the rank order  $K^+ > Rb^+ > Cs^+$ . The adsorption constants derived from studies of competition in ion entry  $K_{Rb}$ ,  $K_{Cs}$ , and  $K_K$ , on the other hand had been found to follow a different order:  $Rb^+ > Cs^+ > K^+$  (see Ling, ref. 85, p. 309). That  $Cs^+$  and  $Rb^+$  reduce  $K^+$  permeability or conductance was repeatedly confirmed by later studies utilizing different techniques in different cells (Chandler and Meves,<sup>47</sup> Bezanilla and Armstrong,<sup>49</sup> French and Adelman<sup>94</sup>).

Equally supportive was the demonstration in 1965 (Ling and Ochsenfeld<sup>95</sup>) that the entry of labeled  $K^+$  into frog sartorius muscle is influenced by low pH (Fig. 4). Indeed, these data agree well with the assumption<sup>91</sup> that it is the  $\beta$ - and  $\gamma$ -carboxyl groups (with a  $pK_a$  of 4.6) carried by surface proteins that mediate the adsorption-desorption route as the major route for  $K^+$  entry. (Findings later achieved by the somewhat different approaches of Hille,<sup>51,52</sup> Drouin and The,<sup>53</sup> Woodhull,<sup>55</sup> Schaaf and Davis<sup>56</sup> are consistent with the LFC model I first proposed in 1953<sup>91</sup>; see p. 89 for further discussion).

2. EXPERIMENTAL OBSERVATIONS NOT IMMEDIATELY PREDICTED BY THE SIMPLE LFC MODEL. Figure 5 shows that the influx of labeled  $Na^+$  is inhibited by  $K^+$  as

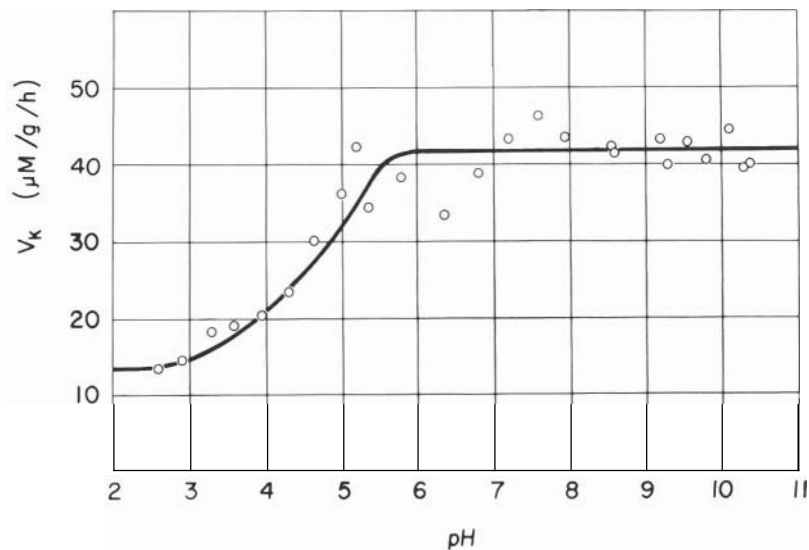


FIGURE 4. Effect of pH on the initial rate of entry of labeled  $K^+$  ion into frog sartorius muscles. Labeled  $K^+$  ion concentration was 20 mmole/liter; concentration of phosphate buffer was 10.8 mmole/liter; concentration of the glycine, succinate, and veronal buffers was 5.4 mmole/liter. Muscles had been isolated on the previous day and kept overnight at 2°C in Ringer's solution. Fifteen min of soaking at 25°C were followed by 10 min of washing at 0°C. pH of experimental solution measured after the experiment. Each point represents the average of three determinations.

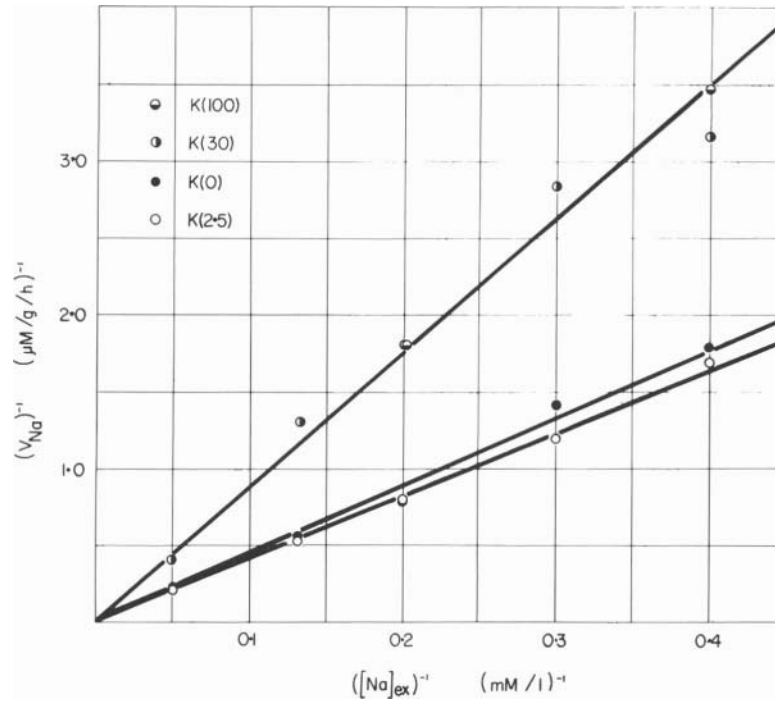
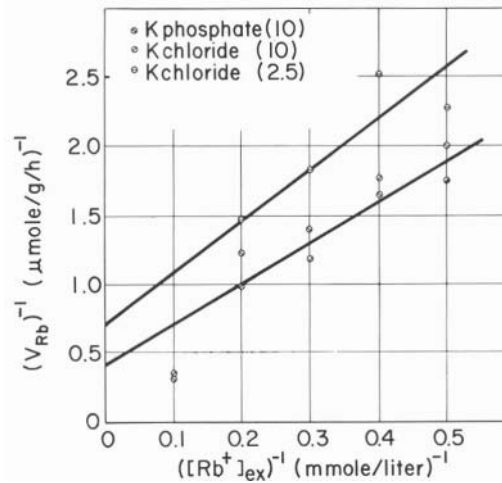


FIGURE 5. Effect of various concentrations of  $K^+$  ion on the initial rate of entry of  $Na^+$  ion into frog sartorius muscles. Increasing  $K^+$  ion concentration from 30 mmole/liter to 100 mmole/liter shows no apparent effect on rate of  $Na^+$  ion entry, whereas reduction from 30 mmole/liter to 2.5 mmole/liter causes an increased rate of  $Na^+$ -ion entry. Fifteen min of soaking at 25°C were followed by washing for 10 min at 0°C. Each point represents average of three determinations.

well<sup>91</sup>—in contradiction to the Hodgkin and Huxley doctrine of separation of  $K^+$  and  $Na^+$  channels. Furthermore, as  $[K^+]_{ex}$  increases, its effect on  $Na^+$  permeability reaches a constant nonzero level; about 30% of the total  $Na$  influx is independent of external  $K^+$ , and the remaining 70% is dependent on external  $K^+$  concentration. The  $K^+$  insensitive fraction could be classified as *free diffusion* in Danielli's terms,<sup>96</sup> but what does it represent physically? It certainly cannot be free diffusion through a phospholipid bilayer because ionic permeability through a lipid bilayer is extremely low (i.e.,  $5 \times 10^8$  to  $10^9 \Omega/cm^2$ ; McLaughlan et al.<sup>97</sup>). In terms of the LFC hypothesis, this free diffusion is via the saltatory route through polarized water as shown above (Fig. 2) and as further discussed below (p. 87).

Figure 6, also from Ling and Ochsenfeld,<sup>95</sup> demonstrates yet another facet of the ionic permeability problem. That is, the rate of entry of the strongly binding but slowly permeating  $Rb^+$  is facilitated by external  $K^+$  (as well as  $Na^+$ ) and this effect is not due to anion added together with  $K^+$  but due to  $K^+$  itself (and  $Na^+$  itself). These findings once more show that ion permeation is more complicated than Eq. 18 can predict. They are consistent, however, with a somewhat more complex model in which the desorption step in the adsorption-desorption route involves the participation of a second free cation which, by momentarily forming a *triplet* with the fixed ion-adsorbed cation pair, decreases the activation energy for the dissociation of the adsorbed ion, hence facilitating its entry into the cell. This is illustrated in Fig.

FIGURE 6. Essential independence of the facilitatory effect of K salt on the nature of the anion. The facilitatory action of  $K^+$  ion on Rb ion entry is the same whether the  $K^+$  ion is added as the chloride or the phosphate. All muscles were soaked 30 min at  $0^\circ\text{C}$  except those with the highest  $Rb^+$ -ion concentration (10 mmole/liter), which were soaked 15 min to avoid change in the rate of entry. Washing was in normal Ringer's solution at  $0^\circ\text{C}$  for 10 min. Each point represents a single determination on a single sartorius muscle (CI) or on two sartorius muscles (PO.).



7. In this view the presence of a secondcation exerts a twofold effect: (1) it competes for the surface anionic sites, thus *decreasing* the rate of entry of the entrant ion; and (2) it facilitates the dissociation of the entrant ion, thus *increasing* the rate of its entry.

In summary, the considerable range of data illustrated in Figs. 3, 4, 5, and 6 are in contradiction to the "independence principle" on which the Hodgkin-Huxley theory is built. Instead, they agree well with the concept of the cell membrane as a fixed charge system selectively adsorbing  $K^+$  in much the same way that the cytoplasm does. My conclusion was that a theory of electrical potential based on that fundamental concept might have merit.

#### D. Surface Adsorption Theory of the Cellular Resting Potential

In 1955 I suggested that the cellular resting potential is essentially a surface phenomenon, its magnitude determined by the concentration of adsorbed  $K^+$  on a thin surface layer of the cell bearing fixed anionic sites (Ling<sup>98</sup>).<sup>\*</sup> From this idea, a surface adsorption theory gradually evolved. Cited as a model for the cellular resting potential was the glass electrode, whose surface adsorption properties rather than membrane permeability determine the potential (see p. 70). An equation describing the resting potential,  $\psi$ , was first presented in 1959 (Ling<sup>100</sup>) and elaborated in several later publications (Ling<sup>85,101</sup>). In simplified form, that equation is

$$\psi = \text{constant} - \frac{RT}{F} \ln(K_K[K^+]_{\text{ex}} + K_{Na}[Na^+]_{\text{ex}}) \quad (19)$$

where  $K_K$  and  $K_{Na}$  are the adsorption constant for  $K^+$  and  $Na^+$  respectively on the cell surface anionic sites, and  $[K^+]_{\text{ex}}$  and  $[Na^+]_{\text{ex}}$  are the external concentrations of  $K^+$  and  $Na^+$ . According to this model, the resting potential is related to the logarithm of

<sup>\*</sup>It is interesting that many years later, in an effort to explain the effects of internal perfusion of squid axon with solutions of low ionic strength on the action potential, Hodgkin and Chandler<sup>99</sup> suggested a potential distribution arising from fixed charges on the inner surface of the membrane.

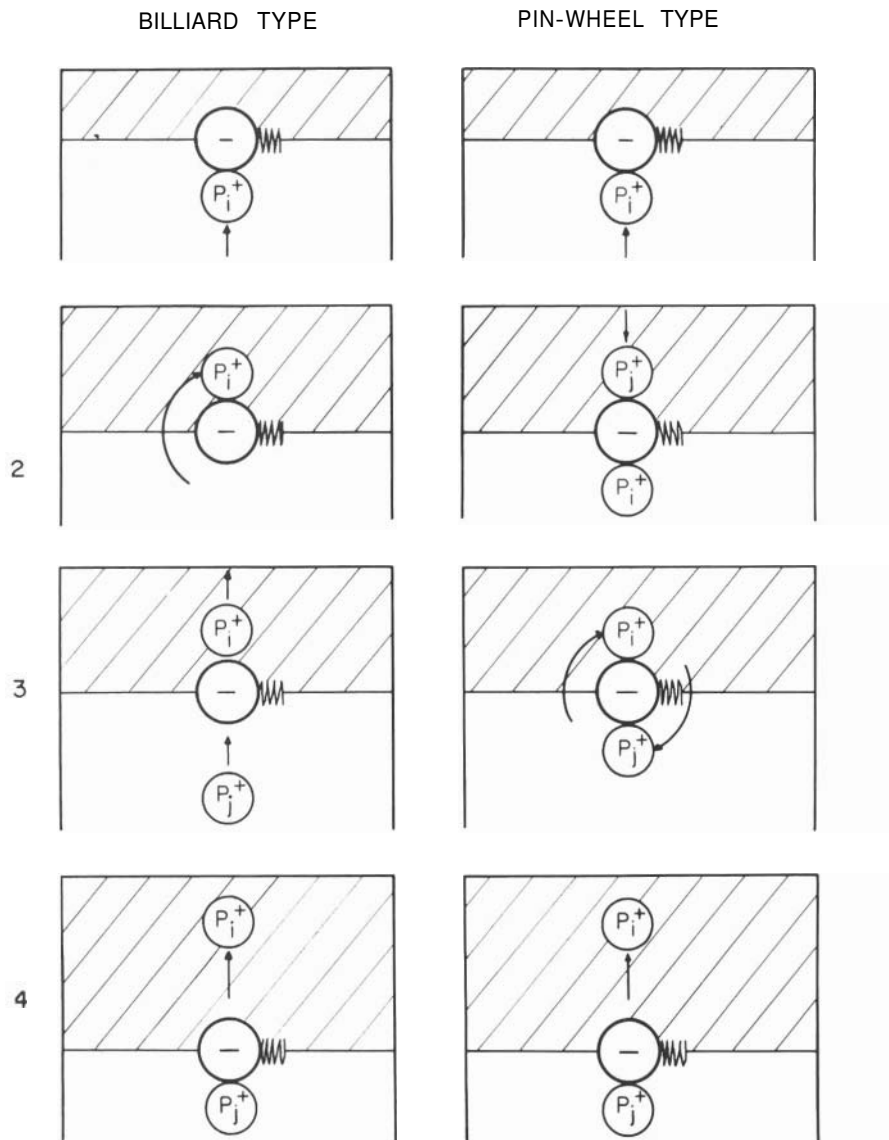


FIGURE 7. Diagram of "billiard" (left) and "pin-wheel" (right) entry modes of  $P_j^+$ . Both are possible varieties of the triplet adsorption-desorption route, so named because the event involves three ions; i.e., the fixed anion and two free cations. The difference between the billiard and the pin-wheel types lies in the origin of the second cation,  $P_j^+$ . In the billiard type,  $P_j^+$  is also from the external solution; in the pin-wheel type,  $P_j^+$  is an ion from within the fixed-charge system (shaded area). The symbols represent hydrated ions in which, for simplicity, the associated water molecules do not appear.

external  $K^+$  and  $Na^+$  as well as the absolute temperature  $T$ . As noted above, the potential does in fact relate to these parameters in a predictable fashion. Equation 19, like its counterpart, the Hodgkin-Katz-Goldman equation, describes the peak value of the action potential during excitation—a subject further pursued later (p. 88). Here let us examine the validity of the concept that the resting potential is essentially a surface phenomenon reflecting (I) the density and nature of anionic

sites on or very close to the surface of the cell, and (2) the concentration of counterions that adsorb onto these surface ionic sites.

1. SUPPORTIVE EVIDENCE FROM INANIMATE MODEL SYSTEMS. The key parameter of the membrane theory in general and the membrane theory of electric potential in particular is selective ionic *permeability*. Historically, three different kinds of inanimate model membranes have been studied: glass membrane, collodion membrane, and lipid layers. Each of these models was thought to contribute to the generation of a potential due to its selective permeability. *Further investigation, however, revealed in each case that the electrical charges inherently present or experimentally introduced on the surfaces are responsible for the electric potential behavior observed.* (For the story of the glass electrode, see Horovitz,<sup>102</sup> Haugaard,<sup>103</sup> Ling.<sup>100</sup> For collodion, see Sollner et al.,<sup>104</sup> Ling.<sup>101</sup> For lipid layers, see Colacicco,<sup>105</sup> McDonald and Bangham.<sup>106</sup>) The cited observations<sup>100-106</sup> are all in harmony with the surface adsorption theory of cellular electrical potentials.

## 2. PROVIDING EXPLANATIONS FOR APPARENTLY CONFLICTING EXPERIMENTAL FINDINGS

From the discussion above, one sees that of the various relations described in the **Hodgkin-Katz-Goldman** equation only a portion is consistently observed experimentally. This portion is enclosed in dashed lines:

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{ex}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} + P_{Cl} [Cl^-]_{in}} \quad (20)$$

Thus *in form* the portion of the **Hodgkin-Katz-Goldman** equation verified consistently is identical to the equation for the cellular potential according to the surface adsorption theory (Eq. 19). This analysis indicates that all the observations supporting the **Hodgkin-Katz-Goldman** model also support the present surface adsorption model. Moreover, as I will next show, the observations contradicting the **Hodgkin-Katz-Goldman** equation are in harmony with the present surface adsorption model.

a. *Relation between external Cl<sup>-</sup> and  $\psi$ .* Failure of attempted demonstrations of the expected relation between external Cl<sup>-</sup> concentration and  $\psi$  in axon and muscle (p. 58) constitutes major evidence against the Hodgkin-Katz theory. In contrast, that failure is predictable from the surface adsorption model if the surface sites on squid axon and frog voluntary muscle cells are primarily anionic. On the other hand, if the surface contains both fixed anionic and cationic sites, a mixed picture emerges (see p. 70), and Cl<sup>-</sup> may then influence the potential.

b. *Relation between  $\psi$  and intracellular K<sup>+</sup>.* First, as noted above, the *Fundulus* egg shows no measurable  $\psi$ . This observation can be explained by the surface adsorption theory on the assumption that at the surface of the egg the fixed anions and/or fixed cations have little or no affinity for the external ions whose concentrations were varied in the study.

Second, since there is no predicted relation between  $\psi$  and total intracellular K<sup>+</sup>

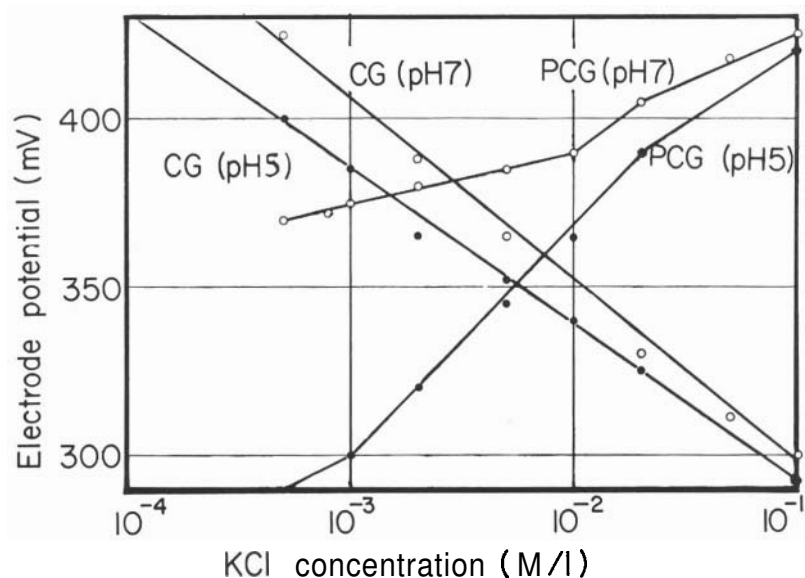


FIGURE 8. Anion and cation sensitivity of a collodion-coated glass (CG) electrode and of a polylysine-treated collodion-coated glass (PCG) electrode at pH 5 and 7.

and  $\text{Na}^+$  concentrations, the sizable collection of data indicating lack of such a relation in nerve and muscle agree well with the surface adsorption theory.

Three, this theory can also explain the observed relation between the potential and intracellular concentrations of  $\text{K}^+$  and  $\text{Na}^+$  as reported by Baker et al.,<sup>59</sup> Adrian,<sup>63</sup> and Hagiwara et al.<sup>62</sup> (p. 58). Thus Adrian changed intracellular  $\text{K}^+$  concentration in muscle by exposure to hyper- or hypotonic solutions. His data could be understood if with cell swelling and shrinkage there were a corresponding change in the density of surface anionic sites  $[f^-]$  which, though not explicitly given is represented as  $RT \ln [f^-]$  and included in the constant of Eq. 19.

The studies of Baker et al.<sup>59</sup> and Hagiwara et al.,<sup>62</sup> as well as some of the impressive work of Tasaki and Takenaka,<sup>61</sup> were done on giant axons or barnacle muscle internally perfused. To explain their results, additional information derivable from model studies is needed.

Figure 8 shows that a soft glass electrode having no  $\text{K}^+$  sensitivity acquires it when coated with a very thin layer of collodion (CG) which carries fixed anionic carboxyl groups much as I hypothesized for living cells. At a pH of 7.0 such a CG electrode exhibits a near ideal slope of 54 mV per tenfold increase of external  $\text{K}^+$  concentration. If this electrode is then dipped in a solution of polylysine and allowed to dry in a humidity chamber, it acquires the characteristics of a near ideal anion-sensitive electrode but only at pH 5. At this pH the hydrogen ion suppresses effectively the dissociation of the anionic carboxyl groups. When the pH is 7, both carboxyl groups on the collodion and the  $\epsilon$ -amino groups of the polylysine are ionized. The electrode is now at once sensitive to cation,  $\text{K}^+$ , and to anion,  $\text{Cl}^-$ . As a result, *the  $\psi$  vs. KCl plot becomes flat*. These findings manifest the behavior of an *amphoteric electrode*, which changes with the nature of the surface charges, the relative density of ionized ionic groups, pH, etc.

Although as part of the surface adsorption theory it is proposed that the cell

surface of frog sartorius muscle is covered with isolated fixed anionic  $\beta$ - and  $\gamma$ -carboxyl groups (Fig. 4), the interior of any cell, whether of squid axon or barnacle muscle fiber, must be amphoteric and possess both anionic  $\beta$ - and  $\gamma$ -carboxyl groups and cationic guanidyl  $\epsilon$ -amino groups. Thus variation of  $K^+$  and  $Na^+$  salt concentration in the internal perfusing fluid may reveal fixed anionic as well as fixed cationic sites at the artificially created surface lining the cavities inside of the cell—whether these cavities were brought about by axoplasm extrusion or by solute injection. An internal  $K^+$  concentration vs.  $\psi$  plot with less than ideal slope, as was observed and is mentioned above, may thus be reasonably expected under these conditions.

c. Permeability constants or surface adsorption constants? Equation 19 and the portion of Eq. 20 within the dashed line are formally identical except that the set of quotients  $P_K, P_{Na}$  in one case, and  $K_K$  and  $K_{Na}$  in the other, have totally different physical significance. Edelmann<sup>107</sup> studied the permeability of  $Rb^+$ ,  $Cs^+$ , and  $K^+$  in guinea pig heart muscle. Utilizing a more general form of Eq. 19, he found that as with frog muscle the rank order of permeability was  $K^+ > Rb^+ > Cs^+$ , whereas the rank order of adsorption constant on the cell surface sites was  $Rb^+ > K^+ > Cs^+$ . With this knowledge he studied the relative effectiveness of external  $Rb^+$ ,  $K^+$ , and  $Cs^+$  in depolarizing the cell potential, finding that the effectiveness correlates with the adsorption constant, not the permeability. He concluded that the data do not agree

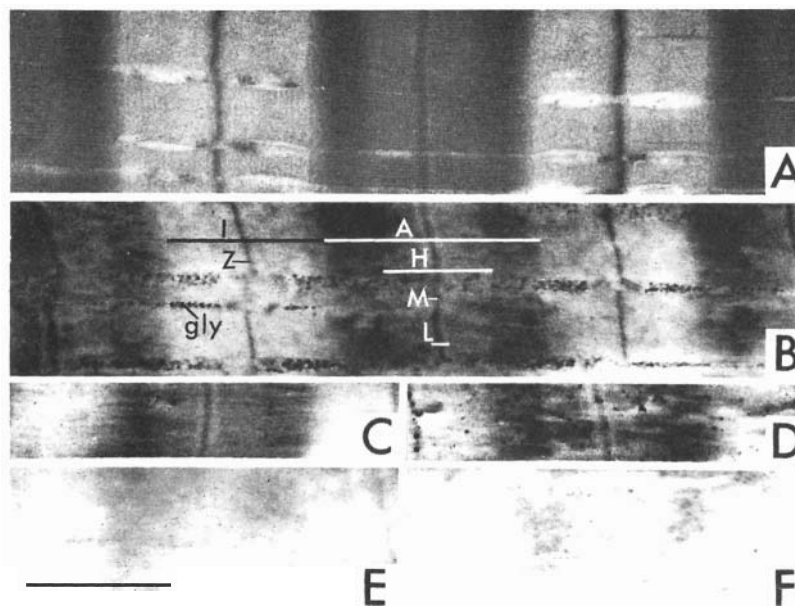


FIGURE 9. Electron micrographs of dry cut, unstained section of freeze-dried frog sartorius muscle. (A) Muscle fixed and stained with uranium-lead by conventional procedure. (B) EM of  $Cs^+$ -loaded muscle without chemical fixation or staining. (C)  $Tl^+$ -loaded muscle without chemical fixation or staining. (D) Same as C after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A-band. (E) Section of central portion of B after loading in distilled water. (F) Normal " $K^+$ -loaded" muscle. (A is partial reproduction of EM from Edelmann, unpublished. B to F from Edelmann<sup>113</sup> by permission of *Physiol. Chem. Phys.*)



with the Hodgkin-Katz model but do agree with the surface-adsorption theory (Edelmann<sup>108</sup>).

#### E. Experimental Establishment of the Bulk of Intracellular $K^+$ in the Adsorbed State

Since the earliest days of the **AI** hypothesis it has held that the bulk of intracellular  $K^+$  is adsorbed on  $\beta$ - and  $\gamma$ -carboxyl groups of certain intracellular proteins (Ling<sup>81</sup>). One may say that this adsorption proposal is the key feature of the **AI** hypothesis, setting it totally apart from the membrane theory, according to which the cell  $K^+$  is all free. From the amino acid analyses of proteins it was shown in 1966 that in voluntary muscle, myosin provides some 60% of the  $\beta$ - and  $\gamma$ -carboxyl groups (Ling and Ochsenfeld<sup>109</sup>). In 1853 Huxley<sup>110</sup> had already reported that myosin is not uniformly distributed in voluntary muscle cells but is found exclusively in the A-bands. If there is selective adsorption of  $K^+$  on  $\beta$ - and  $\gamma$ -carboxyl groups then the bulk of intracellular  $K^+$  must be localized in the A-bands. Furthermore, Hodge and Schmidt<sup>111</sup> had presented arguments in 1960 that in transmission electron microscopy of the proteinaceous material, collagen, the cationic dye uranium binds specifically to the  $\beta$ - and  $\gamma$ -carboxyl groups. Incorporating this idea, the **AI** hypothesis could further predict that  $K^+$  in resting muscle cells should be localized at all those cytological structures that stain dark with uranium.

*The basic assumption of the membrane theory of cellular electrical potential is that the major intracellular cation,  $K^+$ , exists in a free state. Without that assumption, not the Bernstein theory nor the Hodgkin-Katz-Goldman theory nor the Hodgkin-Huxley theory could have been derived.* But this very assumption, and hence all superstructure built on it, have now been disproved. The truth is that the bulk of intracellular  $K^+$  in muscle cells is not free but in an adsorbed state. The experimental evidence for the adsorbed state of  $K^+$  follows.

1. **OBSERVATIONS BY TRANSMISSION ELECTRON MICROSCOPY (TEM).** Using a new freeze-drying technique, in 1977, Edelmann<sup>112,113</sup> published TEM studies of unfixed, unstained frog voluntary muscle sections whose  $K^+$  had been replaced by electron-dense  $Cs^+$  or  $Tl^+$ . He established that the  $Cs^+$  and  $Tl^+$  distributions are not uniform but highly localized and, to the last detail, follow the pattern of standard uranium-stained, chemically fixed muscle preparation (Fig. 9).

2. **OBSERVATIONS BY RADIOAUTOGRAPHY.** Single muscle fibers loaded with radioactive  $^{134}Cs$  or  $^{204}Tl$  were either dried (Ling<sup>114</sup>) or frozen (Edelmann<sup>115</sup>) before being coated with emulsion, then developed at  $-20^\circ C$  or  $-190^\circ C$  respectively. The results are in full harmony with those of the TEM studies.

3. **EVIDENCE FROM ENERGY DISPERSIVE X-RAY MICROANALYSIS.** Using X-ray microprobe analysis of frog muscle cells containing  $Cs^+$ ,  $Tl^+$  or normal  $K^+$ , Edelmann<sup>116</sup> demonstrated the preponderance of each of these ions in the A-bands. Trombitas and Tigyi-Sebes<sup>117</sup> recently confirmed Edelmann's results, using X-ray microprobe analysis of single isolated myofibrils.

4. **OTHER SIGNIFICANT EVIDENCE.** In 1980 Edelmann<sup>118</sup> reinforced the above

findings by use of the new technology of laser microprobe microanalysis. Fresh muscle cells were freeze-dried and infiltrated with Spurr medium at low temperature. Dry-cut sections were then exposed to solutions containing different alkali metal ions and shown to take up  $K^+$  selectively over  $Na^+$  at the A-bands. X-ray microprobe analysis corroborated that result.

*This is a finding of historic importance.* Until Edelmann's results, failure to demonstrate *in vitro* selective  $K^+$  accumulation over  $Na^+$  on cellular proteins was a main factor in the dominance of the membrane theory.

With the localization of  $K^+$  established, the next question is whether it really represents a one  $K^+$  on one anionic site situation or merely  $K^+$  floating around as free counterions in the near vicinity of fixed anions. That the localization is indeed by one-on-one adsorption is indicated by earlier findings that the accumulation of  $K^+$  either in intact muscle cells (Ling and Ochsenfeld<sup>109</sup>) or in cells without a functional membrane pump (Ling<sup>119</sup>) is ion specific and not merely valence specific. That is, the effectiveness of one ion in displacing a preexisting ion in the subcellular structure depends on the *short range* attributes of the ions involved. Such a dependence is not expected if the preexisting ion merely floats around as a free counterion to balance the *long range* electrostatic effect of a fixed anion.

The demonstration that the bulk of intracellular  $K^+$  is in an adsorbed state disproves one of the basic tenets of the membrane theory. *The corollary membrane potential theory of cellular resting potential in its original form therefore is no longer tenable.*

#### IV. FURTHER DEVELOPMENT OF THE ASSOCIATION-INDUCTION (AI) HYPOTHESIS

##### A. Background

In 1952 I described a quantitative theory that could explain the selective preference for  $K^+$  over  $Na^+$ .<sup>87</sup> A major model considered was sulfonate ion-exchange resin which, like most living cells, selectively accumulates  $K^+$  over  $Na^+$ . In 1953 Bregman<sup>120</sup> reviewed his investigation of ion selectivity of ion-exchange resins with different fixed anionic groups: he found that whereas the sulfonate resin selects  $K^+$  over  $Na^+$ , the carboxylic and phosphoric resins actually select  $Na^+$  over  $K^+$ . To explain, Bregman cited earlier ideas of Teunnisen and Bungenberg de Jong<sup>121</sup> who considered reversal of alkali-metal ion selectivity to be due to change in the balance of polarizing "field strength" and the relative polarizability of the fixed anion vs.  $H_2O$ .

In 1955 I suggested that living cells, like the glass electrode, owe their electric potentials to the presence of fixed negative sites on their surfaces.<sup>98</sup> Emphasis was placed on the borosilicate glass that Horovitz<sup>102</sup> and others had demonstrated to be sensitive to  $K^+$  and  $Na^+$  as well as  $H^+$ . At about this time, Rudin and Eisenman<sup>122</sup> saw the need to measure the  $Na^+$  concentration in the "central canal" of mammalian central nervous system and began to manufacture glasses to pursue that aim. They discovered different rank orders of sensitivity to the alkali-metal ions with changed compositions of the glass, and they noted that if the field strength of the anionic groups on the glass increases, the more highly hydrated ions are the ones first

dehydrated by the anionic field. Elaborating on this intuitive idea, they were able to provide a theoretical basis for the eleven different alkali-metal ion selectivity sequences they discovered in their glasses.

This sort of information stimulated me to expand the basic concepts introduced in the original LFC hypothesis<sup>81</sup> and thus develop the association-induction hypothesis, now a general theory of living phenomena.

### B. The *c*-Value and Ionic Selectivity

Bregman's data<sup>120</sup> indicates cationic selectivity order reversal when one proceeds from sulfonate resin to carboxylic and phosphoric resins. It is well known, of course, that sulfonic acid is a strong acid with a  $pK_a$  value of 1 or less whereas carboxylic acid, like acetic acid, is a weak acid with a  $pK_a$  value larger than 4. It is also well known that acid strength in general largely depends on the atoms attached to the functional oxyacid group. For example, substituting the 3 H on the methyl group with chlorine atoms converts the weak acetic acid to the very strong trichloroacetic acid. The much more electronegative Cl atoms draw electrons toward themselves and by the propagated inductive effect reduce the electron density at the oxygen atom of the anionic site. With this in mind, a parameter called the *c*-value was introduced. It represents a displacement in angstrom units of a single electronic charge along the axis joining the singly charged oxygen to the interacting cation in order to *simulate* the effect of varying charge density at the oxyacid group (for details, see Ling<sup>85</sup>). A high *c*-value means a high  $pK_a$  value; a low *c*-value means a low  $pK_a$  value.

With the *c*-value defined, I then outlined a linear model in which the amount of hydration was not arbitrarily assigned as in the earlier model shown in Fig. 1. Instead, 0, 1, 2, or 3 water molecules (called configuration 0, I, II, and III respectively) may be interspersed between the oxyacid and the interacting cations (Fig. 10). All ion-ion, ion-dipole, dipole-dipole, Born repulsion, and van der Waal interacting energies were taken into account, and by minimizing the total energy the statistical distribution of the different configurations for each cation at different *c*-values was calculated. In the final result, a plot of the theoretical adsorption energy against the *c*-value was obtained as illustrated in Fig. 11, in which the polarizability of the anionic group used was  $1.25 \times 10^{-24}$  cm. The work, briefly reported in 1957<sup>123</sup> and 1960,<sup>101</sup> was described in more detail in 1962.<sup>85</sup>

In 1962 Eisenman also presented a quantitative model. In this model, as in the 1952 model I had first constructed, all energies other than the coulombic term were ignored and the ions were either fully hydrated (existing as free ion) or completely dehydrated (in an associated state exclusively; i.e., configuration 0 of Fig. 10).<sup>124</sup> Neither of these two basic assumptions used in Eisenman's simplification was easily justifiable in view of the more complete knowledge already existing.

My linear model of 1960, presented above, is far from being entirely satisfactory since it incurs many simplifying assumptions. But the theoretically calculated changes in the relative selectivity for a pair of ions like  $K^+$  and  $Na^+$  with changes in *c*-value offers an explanation for the order reversal observation of Bregman.<sup>120</sup> Moreover, these calculated changes will be found useful in understanding various cell activities discussed below (p. 89).

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### C. The Polarized Multilayer Model of Cell Water

The idea that water in living cells may somehow differ from water in a dilute solution has a long history. Water associated with various biomaterials was referred to as "Schwellungswasser" or imbibition water for many years. Both Pfeffer,<sup>7</sup> who founded the membrane theory, and Overton,<sup>17</sup> who introduced the lipoidal membrane theory, argued for the presence of *Schwellungswasser* in living cells. Fisher,<sup>125</sup> Moore and Roaf,<sup>126</sup> Lepschkin,<sup>127</sup> Ernst,<sup>128</sup> Gortner,<sup>129</sup> Nosonov and Aleksandrov,<sup>130</sup> and more recently Troschin<sup>131</sup> vigorously objected to the then popular membrane theory and suggested some or all water in living cells may be "bound" in some way.

In 1965 the polarized multilayer model of cell water was proposed as part of the AI hypothesis (Ling<sup>132</sup>). According to the hypothesis<sup>133</sup> the normal resting living cell contains throughout its volume a matrix of proteins that exist in an extended conformation with backbone NHCO groups directly exposed to bulk-phase water. This matrix of chains thus contains positively charged NH groups (P) and negatively charged CO groups (N) at distances roughly one water-molecule diameter apart. The

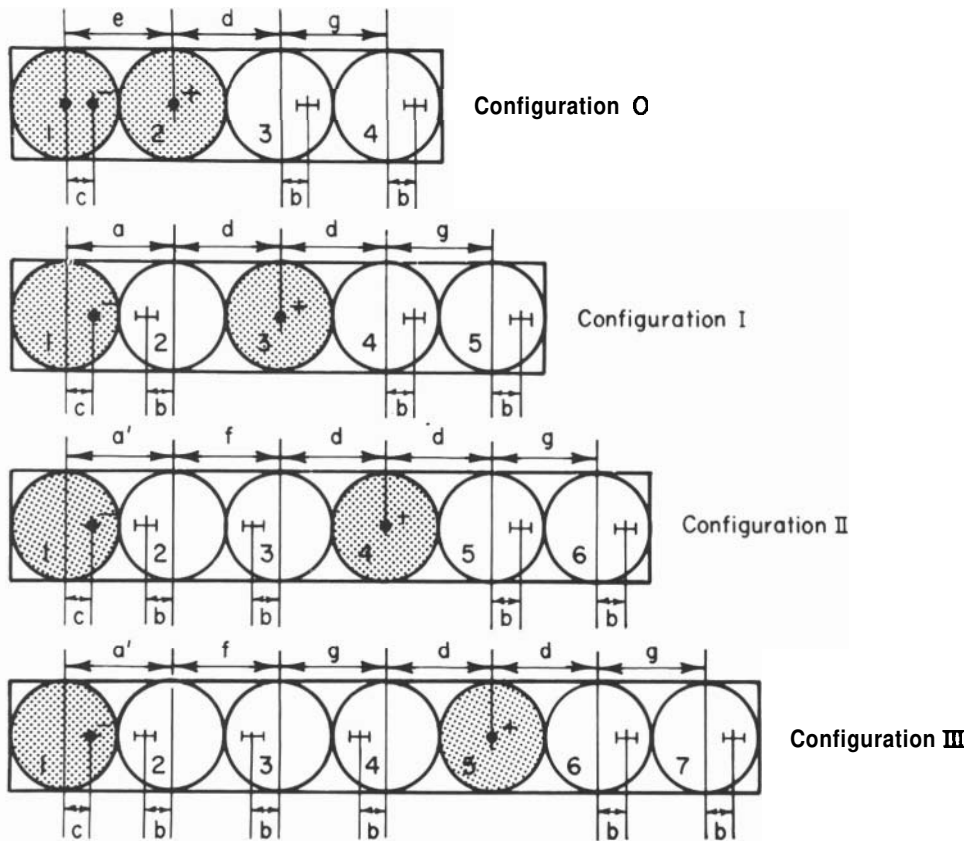


FIGURE 10. The linear model. Shaded circle on the left in each configuration represents the negatively charged oxygen atom of an oxyacid (e.g., carboxyl); shaded circle on the right represents its counterion. Empty circles represent water. The various letters denote distances used in the computations.

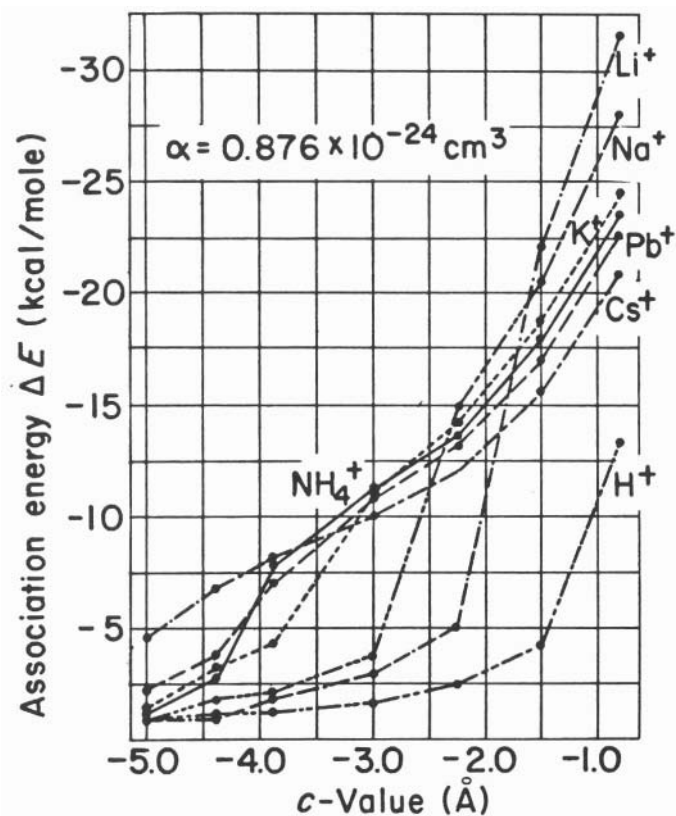


FIGURE 11. Relation between the calculated association energy,  $\Delta E$ , of various cations and the  $c$ -value of the anionic group. The polarizability of the anionic site,  $\alpha$ , is  $0.876 \times 10^{-24} \text{ cm}^3$ .

matrix of chains called an NP-NP-NP system provides the boundary condition for the multilayer polarization of virtually all the cell water (see below, p. 76). The number of layers of water molecules polarized between pairs of extended chains is not more than ten or counted on a per chain basis not more than five, water molecules deep.

Water in the state of polarized multilayers cannot, rigorously speaking, be called "bound" because the molecules are quite free to diffuse; nor can it be called "nonsolvent," because in this theory there is no categorically nonsolvent water. Instead, its solvency differs depending on the solute involved: spherically symmetrical small uncharged molecules are quite soluble in the polarized, multilayered water; large molecules are excluded in degree roughly proportionate to their size and complexity.

In this model, cell water in the state of polarized multilayers accounts for (1) the maintained low level of  $\text{Na}^+$ , sugar, and amino acids as well as foreign but permeable substances,<sup>134,135</sup> (2) the preserved swelling and shrinkage of cells devoid of an intact membrane,<sup>136</sup> and (3) the semipermeable properties of the cell surface (Ling<sup>137</sup>).

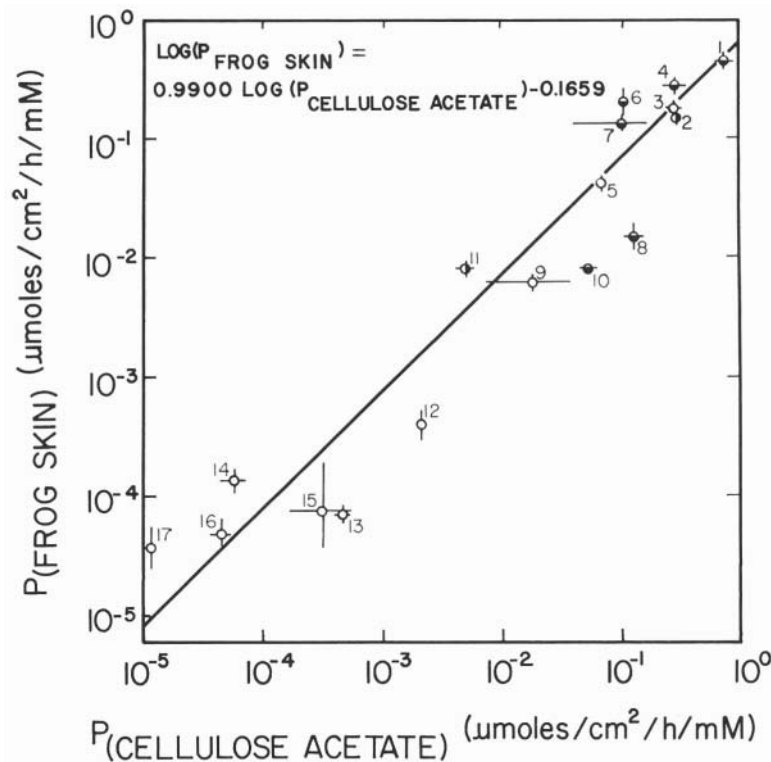
Above all, the polarized water model allows each of these basic physiological properties of the living cell to be brought under the control of drugs, hormones, ATP,  $\text{Ca}^{2+}$ , etc. Collectively called *cardinal adsorbents*, they interact with key

cardinal sites on proteins that control both the physical state of water and the selective adsorption of ions (Ling<sup>138</sup>).

#### D. Experimental Verification of the Polarized Multilayer Model of Cell Water

1. IN MODEL SYSTEMS. Confirmation that bulk water has reduced solubility for  $\text{Na}^+$ , sugars, and amino acids when this water is in the presence of a matrix of extended protein chains comes from (1) studies of aqueous solutions of isolated proteins and (2) studies of synthetic polymers which, like proteins existing in an extended conformation, contain oxygen at distances roughly two water-molecules apart. Native proteins with most of their backbone CONH groups locked in helical H-bonds have little effect on the solvency of the bulk-phase water. However, when for one reason or another the proteins exist in an extended conformation, polarization of bulk-phase water follows as a result (Ling et al.<sup>134,135</sup>).

2. IN LIVING CELLS. According to the AI hypothesis, free  $\text{Na}^+$ , sugars, amino acids, etc. exist in living cells at a lower concentration than in the surrounding medium due to a simple unitary mechanism—the reduced solubility of all these solutes in polarized water. In support of this view, it has been shown that (1) alteration in the level of one normally excluded solute is accompanied by a similar



**FIGURE 12.** Plot of permeability to eleven hydroxylic compounds ranging from water (1) to sucrose (17) at three different temperatures ( $0^\circ$ ,  $4^\circ$ ,  $25^\circ\text{C}$ ) of reversed frog skin against the permeability of heat-treated cellulose-acetate membrane. Straight line described by equation and shown in graph was obtained by method of least squares (from Ling<sup>137</sup> by permission of *Biophys. J.*).

change in the distribution of other normally excluded solutes,<sup>139,140</sup> and (2) the equilibrium level of a solute accumulated in the cell water varies inversely with the size and complexity of the solute molecule in accordance with theoretical predictions and behavior of model systems (Ling et al.<sup>135,141</sup>).

#### E. Polarized Water at the Cell Surface as the Seat of Selective Permeability

1. EVIDENCE AGAINST THE LIPOIDAL MEMBRANE THEORY. A major part of this counter-evidence has been reviewed in detail elsewhere.<sup>137,142</sup> Among the many reasons for rejecting the concept of the lipid barrier are:

(1) Near total removal of lipids from cell and mitochondrial inner membranes causes no change in the thickness of the layered structure of the "unit membrane" (Fleischer et al.,<sup>143</sup> Morowitz and Terry<sup>144</sup>).

(2) The surface of "microspheres" prepared from pure proteinaceous material show a trilaminar structure resembling the unit membrane (Fox<sup>145</sup>).

(3) Electron microscopic studies led Sjostrand and Bernhard<sup>146</sup> to conclude that the inner membrane of liver mitochondria does not contain a continuous lipid layer.

(4) Specific K<sup>+</sup> ionophores fail to induce an increase of K<sup>+</sup> permeability in a variety of living cells and isolated mitochondria. For example, specific K<sup>+</sup> ionophores increase drastically the K<sup>+</sup> permeability of lipid bilayer membranes. Yet those same ionophores have no effect on the K<sup>+</sup> permeability of (a) squid axon (monactin; Stillman et al.<sup>147</sup>), (b) mouse liver mitochondria (valinomycin; Maloff et al.<sup>148</sup>), (c) frog egg and frog muscle (valinomycin, monactin, nonactin; Ling and Ochsenfeld, in preparation).

These findings invalidate any hypothesis based directly on the assumption of the universal existence of a lipid layer as the permeability barrier of living cells.

2. EVIDENCE THAT POLARIZED WATER IS THE SEAT OF SURFACE SELECTIVE PERMEABILITY. Figure 12 shows the permeability at three different temperatures of eleven hydroxylic compounds, including water, through a synthetic membrane of hydrated cellulose acetate plotted against the permeability through inverted frog skin. The correlation coefficient is +0.96 (Ling<sup>137,149</sup>). The slope of the line obtained by the method of least squares is 0.99. This is not merely a significant correlation but an exact correspondence, because the ordinate and abscissa are in the same units. Since the cellulose acetate active surface has pores with diameters (45 Å) several times larger than the nearly impermeant sucrose (9.4 Å), and since cellulose derivatives have been shown to polarize water (Ling et al.<sup>135</sup>), I drew the conclusion that water similarly polarized by extended protein chains at the cell surface is the seat of selective permeability. A similar explanation can be offered for the semipermeability of Traube's copper ferrocyanide gel membranes.<sup>8</sup>

#### F. The Inductive Effect, Cooperativity, and a Molecular Mechanism for Physiological Control

Selective adsorption of the major cation, K<sup>+</sup>, and multilayer polarization of the bulk of cell water represent the "association" aspect of the association-induction

model. The inductive effect, in this model, accounts for the coherent behavior of the protein-ion-water system.

In recent years, study of the inductive effect has evolved beyond its historical empiricism. Thus the theory of **Chiáng** and **Tai**<sup>150</sup> contributed deductive rather than empirical methods for describing molecular properties in terms of molecular structure and known atomic properties.

The inductive effect provides the mechanism for one amino acid residue to influence the property of the functional group of a near-neighbor amino acid residue. Hence the **c-value** of a  **$\beta$ -carboxyl** group of an **aspartic** residue would be much higher if the two nearest-neighbor amino acid residues were glutamic residues carrying ionized  $\gamma$ -carboxyl groups rather than neutral glycine or positively charged **lysine** residues. Actually, the inductive effect is a group of constituent effects. Some of these are direct electrostatic effects (D-effect) mediated through space. A more significant component is the inductive effect proper (I-effect) mediated through the intervening **atoms**.<sup>85</sup> The combined D- and I-effects have been referred to as the F-effect or, in the language of the **AI** hypothesis, the "direct" F-effect.

This direct F-effect can only influence target groups a short distance away. On the other hand a long-range effect mediated by the polypeptide backbone ("indirect" F-effect) was described by Ling in 1962.<sup>85</sup> A year later the term "allosteric effect" was coined by Monod, Changeaux, and **Jacob**<sup>151</sup> to describe long-range interaction. The indirect F-effect offers an explanation of why proteins mediating allosteric effects usually undergo changes of conformation. The detailed step-by-step **domino**-like scheme has been reviewed several times in the recent **past**.<sup>152-154</sup>

#### G. Cooperative Adsorption and Desorption of Water and Ions, and Control by Cardinal Adsorbents

According to the **AI** hypothesis, coherent all-or-none types of physiological behavior of living cells, as illustrated in nerve excitation and muscle contraction, reflect cooperative—or more correctly, autocoperative—behavior of the **protein**-ion-water system. The direct F-effect provides the nearest neighbor interaction energy.

In 1964 Yang and Ling, using **Ising's** one-dimensional model, derived an isotherm for cooperative adsorption on protein chains (**Ling**<sup>153,154</sup>):

$$[P]_{\text{ad}} = \frac{[f]}{2} \left( 1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma/RT)}} \right) \quad (21)$$

where  $[P]_{\text{ad}}$  represents the concentration of adsorbed  $i$ th solute on sites whose total concentration is  $[f]$ ,  $-\gamma/2$  is the nearest neighbor interaction energy, and  $\xi$  is defined as follows:

$$\xi = \frac{[P]_{\text{ex}}}{[P]_{\text{ex}}} \cdot K_{j \rightarrow i}^{\circ \circ} \quad (22)$$

where  $[P]_{\text{ex}}$  and  $[P]_{\text{ex}}$  are the concentrations of the  $i$ th and  $j$ th solute in the

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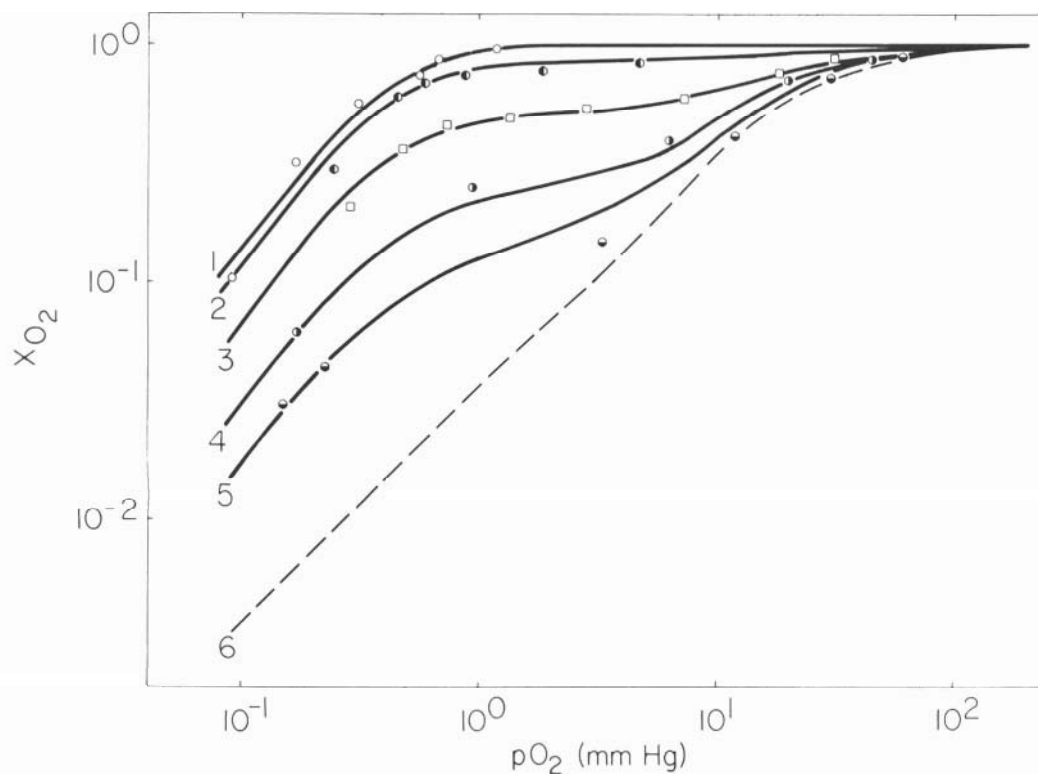


FIGURE 13. Oxygen uptake by hemoglobin (stripped) in the presence and absence of inositol hexaphosphate. Hemoglobin solution was 0.3%, pH = 7.0, 10°C. (1) No IHP. (2)  $1.2 \times 10^{-5}$  M IHP. (3)  $2.4 \times 10^{-5}$  M IHP. (4)  $3.6 \times 10^{-5}$  M IHP. (5)  $4.8 \times 10^{-5}$  M IHP. Points are experimental data of Benesch and Benesch.<sup>157</sup> Lines are theoretical, calculated according to an equivalent version of Eq. 21 with the following values for the different parameters:  $K_0^{\circ} = 3.33$ .  $K_c^{\circ} = 0.067$  (mm Hg)<sup>-1</sup>.  $-(y^{\circ}/2) = 0.39$ .  $-(\gamma^{\circ}/2) = 0.21$  kcal/mole.  $K_c^{\circ} = 4.2 \times 10^{-4}$  (M)<sup>-1</sup>.  $-\Gamma/2 = 0.68$  kcal/mole. Dashed line is theoretical curve at  $DPG_{ex} = 0$  (Ling,<sup>198</sup> by permission of Natl. Acad. Sci.).

surrounding medium, and  $K_j^{\circ}$  is the intrinsic equilibrium constant for the jth to ith solute adsorption exchange.

When  $-\gamma/2 > 0$ , the isotherm is autocoooperative. When  $-\gamma/2 < 0$  the isotherm is heterocoooperative. When  $-\gamma/2 = 0$ , the isotherm is a Langmuir isotherm with no cooperativity. An autocoooperative isotherm is sigmoid in linear plots. In a plot of  $\log ([P]_{ad}/[P]_{ad})$  against  $\log ([P]_{ex}/[P]_{ex})$ , a straight line with the slope of the curve at the point of equal adsorption by the ith and jth solute is formally identical to Hill's empirical equation and Hill's coefficient  $n$  is explicitly equal to  $\exp(\gamma/RT)$ .<sup>155</sup>

Equation 21 can accurately describe the oxygen binding of hemoglobin where the sites of adsorption are the heme prosthetic groups (Ling<sup>157</sup>). It can accurately describe dodecyltrimethylammonium bromide binding on bovine serum albumin where the binding sites are the  $\beta$ - and  $\gamma$ -carboxyl groups (Ling<sup>153</sup>). It can also accurately describe phenol binding on collagen where the binding sites are the backbone peptide groups (Ling<sup>154,156</sup>). These findings affirm the basic assumption of the AI hypothesis: the inductive effect provides the nearest neighbor interaction energy mediated primarily through the backbone of partially resonating polypeptide chains.

### H. Control of Cooperative Adsorption *In Vitro* and *In Vivo*

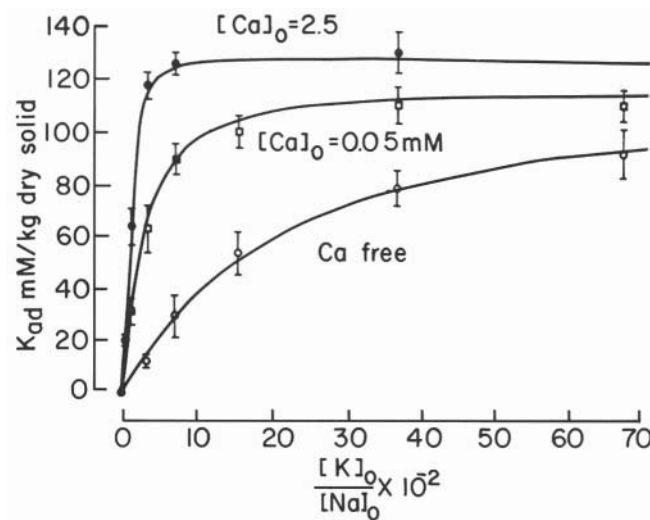
Figure 13 shows oxygen binding by hemoglobin as influenced allosterically by inositol hexaphosphate. The points are experimental from Benesch and Benesch.<sup>157</sup> The lines are theoretical, based on Eq. 21. Even more significant is the finding that ATP can exercise a similar allosteric influence on oxygen binding (Chanutin and Curnish<sup>158</sup>).

Figure 14, taken from Jones,<sup>159</sup> shows that like the cardinal adsorbents inositol hexaphosphate and ATP,  $\text{Ca}^{2+}$  has a controlling influence on the total  $\text{K}^+$  concentration in canine vascular smooth muscle. Jones found that increase of  $\text{Ca}^{2+}$  resulted in increase of the intrinsic equilibrium constant  $K_{\text{Na}^+\rightarrow\text{K}^+}^{\circ\circ}$  in favor of  $\text{K}^+$ . Decrease of  $\text{Ca}^{2+}$  had the opposite effect—increasing the relative preference for  $\text{Na}^+$ . A similar effect of  $\text{Ca}^{2+}$  in enhancing  $\text{K}^+$  accumulation in rat liver cells was later observed by Gilbert.<sup>160</sup>

The powerful effect of cardinal adsorbents in general and  $\text{Ca}^{2+}$  in particular is illustrated in the data of Fig. 14. We see that in the same external medium containing the normal concentration of  $\text{K}^+$  and  $\text{Na}^+$ , all 125 mM of the cell sites are occupied by  $\text{K}^+$  in the presence of a normal concentration of  $\text{Ca}^{2+}$ ; i.e., 2.5 mM. Virtually all  $\text{K}^+$  is replaced by  $\text{Na}^+$  when the  $\text{Ca}^{2+}$  is removed from the medium.

### V. THE MOLECULAR MECHANISM OF RESTING AND ACTION POTENTIAL ACCORDING TO THE AI MODEL

As previously remarked (p. 66), I suggested in 1955<sup>98</sup> that it is the fixed anionic  $\beta$ - and  $\gamma$ -carboxyl groups in a macroscopically thin layer of the cell surface that generate the cellular resting potential. (For comments on similar models proposed



**FIGURE 14.** Slowly exchanging K in steady state, with various ratios of  $[\text{K}]_0/[\text{Na}]_0$  at  $37^\circ\text{C}$  in carotid artery ( $[\text{K}]_0 = [\text{Na}]_0 = 150 \text{ mM}$ ). Three levels of  $[\text{Ca}]_0$  were employed: 2.5 mM (closed circles; 0.05 mM (open squares); and Ca-free (open circles). Vertical bars indicate  $\pm$  mean SE (ten, nine, and eight dogs were used respectively). Curves were computed theoretically. (Jones,<sup>159</sup> by permission of *Ann. NY Acad. Sci.*)

by others, see APPENDIX.) In the years following, the concept was developed further. It was found that during excitation, in response to the removal of the cardinal adsorbent  $\text{Ca}^{2+}$  there is an electronic conformation change of the cell surface proteins (Ling<sup>85,123</sup>). The  $c$ -value of these surface anionic sites shifts transiently in an all-or-none manner from one state in which  $\text{K}^+$  is preferred over  $\text{Na}^+$  (low  $c$ -value) to another in which the relative  $\text{Na}^+$  preference is greatly increased (high  $c$ -value) (Ling<sup>85,101,123</sup>). Concomitantly a transient depolarization of cell surface water occurs, creating an increase of nonspecific membrane conductance (Ling<sup>137,161</sup>). The molecular event during the propagation of an action potential was described in terms of the indirect F-effect, and the transition from the resting to the active state was described as autocoperative in nature (Ling<sup>85</sup>), with the inductive effect providing the major component of the nearest neighbor interaction energy.

#### A. Equation for the Cellular Resting Potential

The original simple equation of the cellular resting potential (Eq. 19) was developed from my assumptions that (1) surface anionic sites do not interact, and (2) ionic adsorption on these  $\beta$ - and  $\gamma$ -carboxyl groups follows a Langmuir adsorption isotherm. This equation can explain a variety of observations both explicable and not explicable on the basis of the Hodgkin-Katz-Goldman equation. However, Eq. 19 does not take into account cooperative interaction, an essential premise of the **AI** hypothesis. Therefore I subsequently derived a more appropriate equation for the cellular resting potential,<sup>162</sup> taking the form

$$\psi = \text{constant} - \frac{RT}{F} \ln \left\{ \frac{1}{[\text{K}^+]_{\text{ex}}} \left( 1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma/RT)}} \right) \right\} \quad (23)$$

where  $\xi$  and  $\gamma$  have the same meanings as in Eq. 21, from which Eq. 23 is derived.

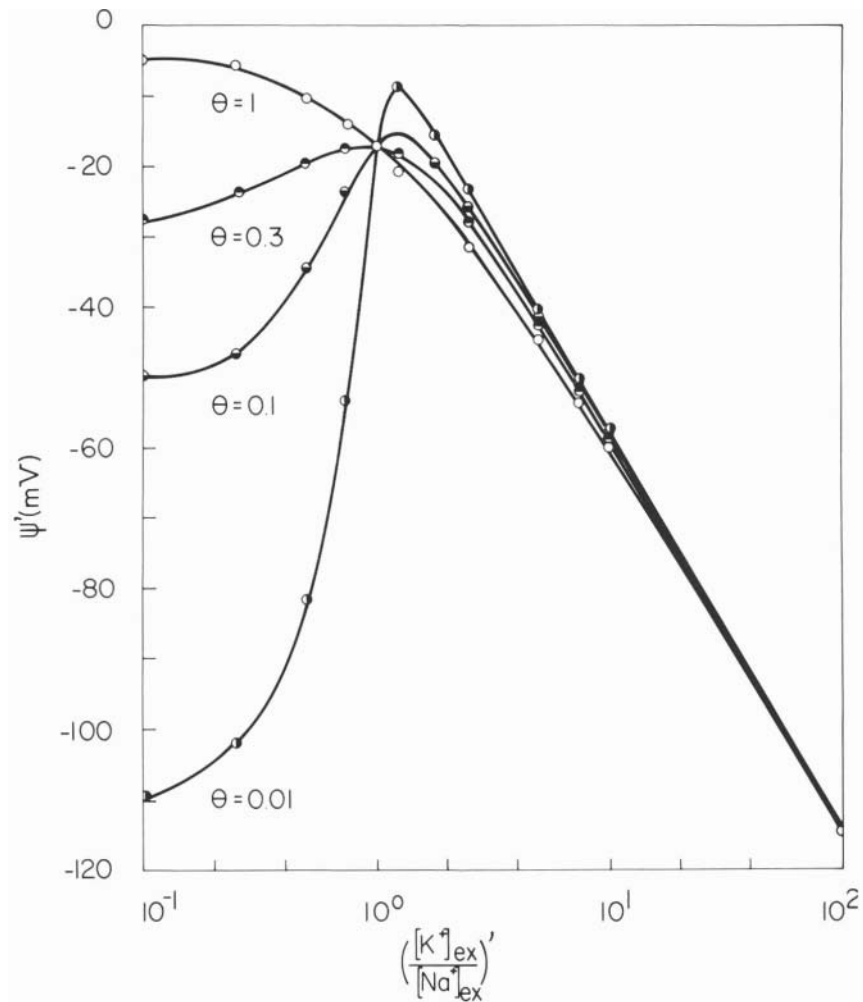
1. VARIATION OF  $\psi$  WITH CHANGES IN NEAREST NEIGHBOR INTERACTION ENERGY. In Fig. 15, theoretical curves are plotted with different values of the nearest neighbor interaction energy,  $-\gamma/2$  (or  $\theta = \exp \gamma/RT$ ). When no near-neighbor interaction occurs ( $-\gamma/2 = 0, \theta = 1$ ), the theoretical curve is identical to that predicted by both Eq. 19 and Eq. 6. That is, in an external environment of constant or nearly constant  $\text{Na}^+$  concentration, decrease of external  $\text{K}^+$  concentration to below its normal level in a Ringer's solution does not cause further change of  $\psi$ . Instead, a flattening of  $\psi$  at low  $[\text{K}^+]_{\text{ex}}$  has been widely **observed**.<sup>23,24,28,46,148</sup>

However, in a number of living tissues including heart muscle, ionic permeability appears to be much higher. Thus although it took some 72 hours at room temperature for  $\text{K}^+$  to reach a new equilibrium in frog skeletal muscle (Ling and Bohr<sup>163</sup>), similar equilibrium in heart muscle can be reached in 40 minutes (Ruzylo and Vick<sup>164</sup>). In mammalian heart muscle it was found that in the presence of near constant  $[\text{Na}]_{\text{ex}}$ , decreasing  $[\text{K}^+]_{\text{ex}}$  causes a change in  $\psi$  entirely like that shown in Fig. 15 with  $-\gamma/2 = 0$ .<sup>164-167</sup>

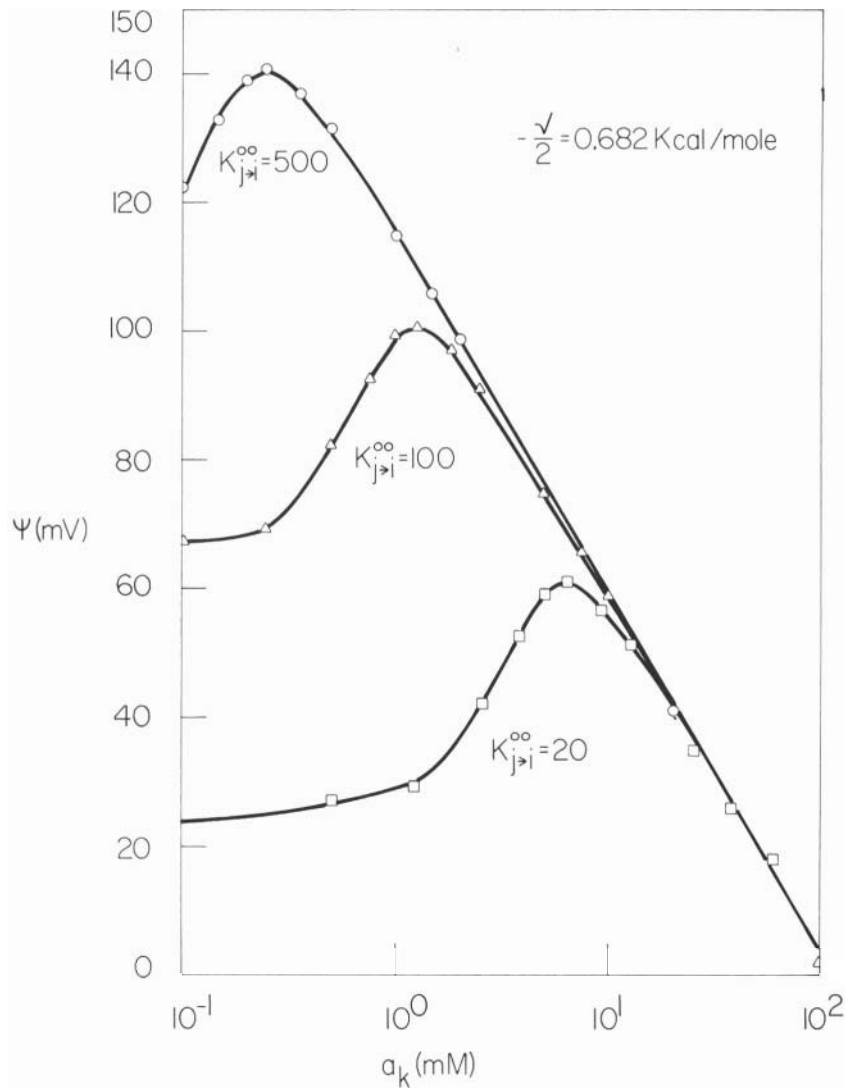
This experimental agreement with theoretical expectations suggests that the cell surface anionic sites responsible for the resting potential have properties quite similar to those of the myosin  $\beta$ - and  $\gamma$ -carboxyl groups adsorbing the bulk of **intra-**

cellular  $\mathbf{K}^+$ . If the surface adsorption shows a similar autocoperative transition between a  $\mathbf{K}^+$  and a  $\mathbf{Na}^+$  state, then this basic information becomes of importance in understanding and explaining the action potential.

2. VARIATION OF  $\psi$  WITH CHANGING  $K_{\mathbf{Na}^+\rightarrow\mathbf{K}^+}^{\circ\circ}$ . Figure 16 presents theoretical plots of  $\psi$  against the logarithm of external  $\mathbf{K}^+$  concentration at a constant external  $\mathbf{Na}^+$  concentration (100 mM) with different values of  $K_{\mathbf{Na}^+\rightarrow\mathbf{K}^+}^{\circ\circ}$  (the intrinsic equilibrium constant) and a constant  $-\gamma/2$  value of 0.682 kcal/mole, a value close to that observed for bulk-phase  $\mathbf{K}^+$  and  $\mathbf{Na}^+$  adsorption in living cells (Jones,<sup>159</sup> Ling and Bohr<sup>163</sup>). Note that a 5-fold reduction of  $K_{\mathbf{Na}^+\rightarrow\mathbf{K}^+}^{\circ\circ}$  without reversal of selectivity would change  $\psi$  to a lower value and even reverse polarity. Equation 23, as illustrated in Fig. 16, provides a theoretical basis for interpretation of the electrical responses of



**FIGURE 15.** Plot of resting potential against external  $\mathbf{K}^+$  and  $\mathbf{Na}^+$  concentration ratio. Ordinate represents  $\psi'$ , which is equal to  $\psi - \text{constant}$ . Abscissa represents  $(\frac{[\mathbf{K}^+]_{\text{ex}}}{[\mathbf{Na}^+]_{\text{ex}}})'$ , which is  $(\frac{[\mathbf{K}^+]_{\text{ex}}}{[\mathbf{Na}^+]_{\text{ex}}} \cdot K_{\mathbf{Na}^+\rightarrow\mathbf{K}^+}^{\circ\circ})$ . For experiments carried out in the presence of a constant concentration of  $\mathbf{Na}^+$  (e.g., 100 mM) the abscissa is then  $[\mathbf{K}]_{\text{ex}} \cdot (K_{\mathbf{Na}^+\rightarrow\mathbf{K}^+}^{\circ\circ}/0.1)$ .



**FIGURE 16.** Plot of resting potential against external  $K^+$  concentration in the presence of a constant external concentration (100 mM) of  $Na^+$  according to Eq. 23.  $-\gamma/2$  is constant at 0.682 kcal/mole while  $K_{Na^+ \rightarrow K^+}^{\infty}$  represented here as  $K_{j>i}^{\infty}$ , varies as indicated.

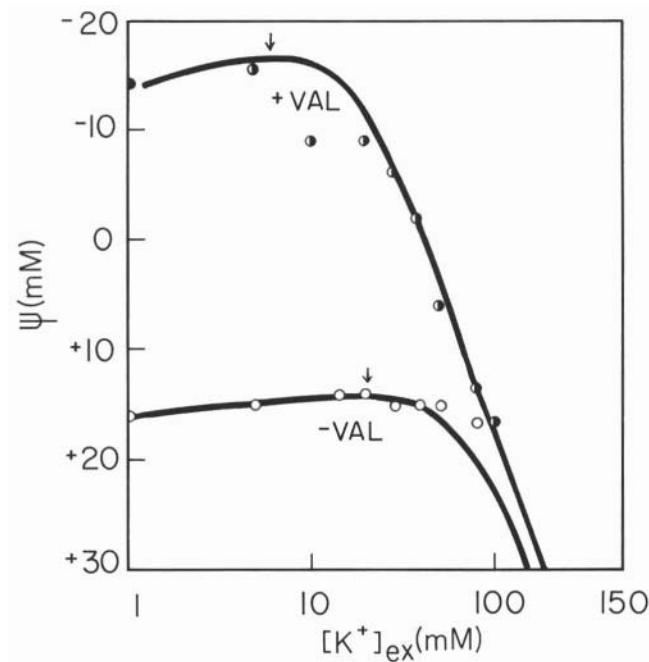
living cells to endogenous drugs, hormones, and other cardinal adsorbents. For example, adrenaline causes hyperpolarization in frog muscles (Ling<sup>51</sup>) whereas azide (Ling and Gerard<sup>168</sup>) or 2,4-dinitrophenol (Koketsu<sup>169</sup>) cause prompt depolarization. According to the AI hypothesis these changes are due to the action of these cardinal adsorbents on cardinal (receptor) sites on the cell surface proteins, causing a shift of  $K_{i \rightarrow j}^{\infty}$  in one or the other direction. These changes of potential are created by surface adsorption changes and are not directly connected with the concentration of intracellular  $K^+$  which, as I have clearly shown (p. 71) is adsorbed and not free. One recalls that it was the independence of  $\psi$  with respect to changes in  $[K^+]_{in}$  that led to the postulation of the electrogenic pump hypothesis. For a criticism of that hypothesis see Ling.<sup>142</sup>

Figure 17 illustrates the resting potential of giant mitochondria from **cuprizone**-treated mouse as reported by Maloff et al.<sup>170</sup> Very little  $K^+$  sensitivity was demonstrable in the untreated sample but in response to valinomycin ( $10^{-7} M$ ) the mitochondrial potential developed the typical  $K^+$  sensitivity seen in many normal living cells. Yet Maloff et al., whose experimental data are presented in ref. 148, showed clearly that valinomycin does not increase  $K^+$  permeability of the mitochondria. I have **suggested**<sup>142</sup> that the effect of valinomycin is to increase  $K_{X^+}^{\infty}$  of the outer membrane surface of the inner membrane by a factor of 3. If such increase is assumed, the solid lines in Fig. 17 are theoretically predicted from Eq. 23. The X cation is presumably  $H^+$ .

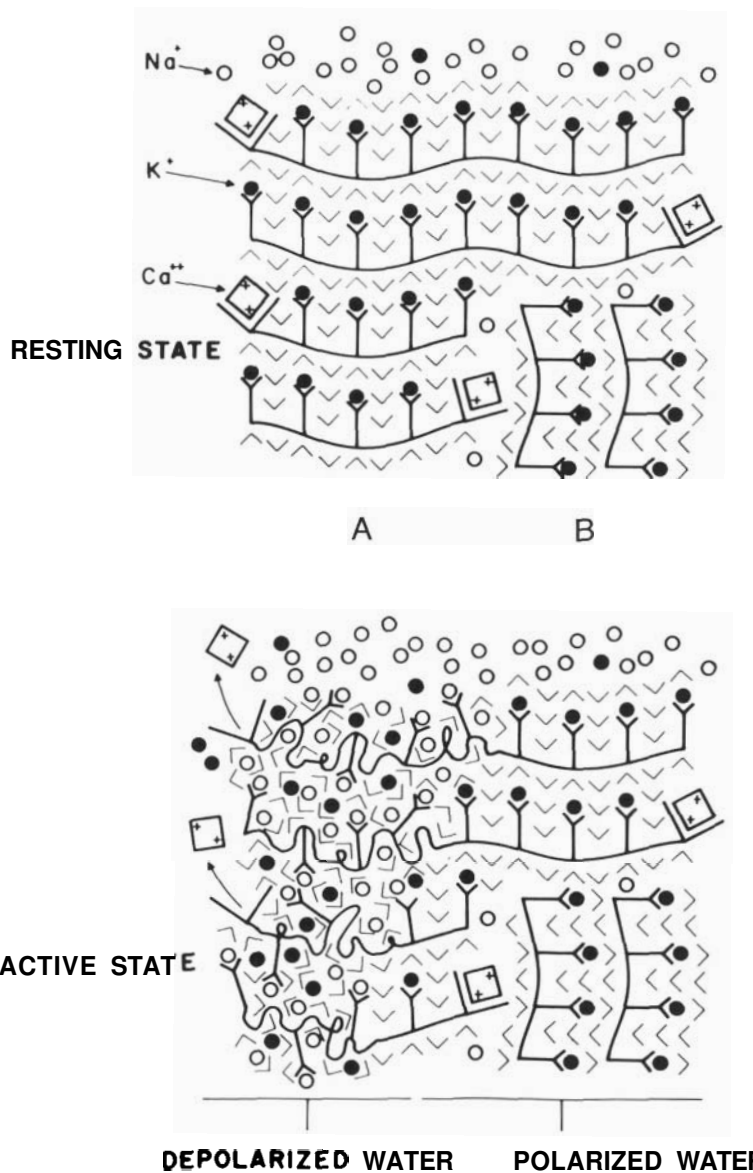
Although the above examples demonstrate the ability of the **AI** model to explain many observations of this type, its usefulness is not limited to interpreting the resting potential. Indeed, I believe that the controlled cooperative transitions implicit in this model provide the "gating" process during excitation. In this connection it seems worthy to recall that the bulk-phase  $K^+ \rightarrow Na^+$  preference change in canine vascular muscle is under the control of the cardinal adsorbent  $Ca^{2+}$  (Fig. 14).

### B. Molecular Events Underlying Excitation

Bernstein<sup>5</sup> suggested that nerve and muscle excitation involve transient increase in membrane permeability. Squid giant **axons** were the means for confirmation of that



**FIGURE 17.** Resting electrical potential of isolated giant mitochondria in cuprizone-fed mice in the presence and absence of valinomycin. Data points are from Maloff, Scordilis, Reynolds, and Tedeschi.<sup>148</sup> Curves are theoretically calculated. The intrinsic equilibrium constant for the exchange of an unidentified cation (possibly  $H^+$ ) and  $K^+$  increased by a factor of 3.3 in response to valinomycin treatment. The nearest neighbor interaction energy ( $-\gamma/2$ ) remained at 0.201 kcal/mole.



**FIGURE 18.** Diagram of a resting region of the cell surface (top) and a partially activated region (left of bottom figure). (•) = K<sup>+</sup>, (◦) = Na<sup>+</sup>, V = water molecules, (Ca) = Ca<sup>++</sup>. Left side of diagram represents cells or cell regions where underlying protein chains run parallel to cell surface; right side represents protein chains running perpendicularly to cell surface.

view by Cole and Curtis.<sup>30,31</sup> Indeed, in these axons the resting resistance attributed to cell membrane fell from 1000 Ω/cm<sup>2</sup> to 20 Ω/cm<sup>2</sup> (ref. 31). As mentioned earlier (p. 54), this transient permeability increase offered the basis for the theory of excitation and action potential presented by Hodgkin, Huxley, and Katz in terms of opening and closing of specific Na<sup>+</sup> and K<sup>+</sup> gates. Some workers go so far as to consider the closing of the Na gate and K gate due to Ca<sup>2+</sup> ion physically blocking Na<sup>+</sup> and K<sup>+</sup> from their respective pores; the gates open when this blocking Ca<sup>2+</sup> is removed. However, there is reason to doubt so simple a pore-blocking hypothesis, as we learn below.

**1. THE  $\text{Na}^+$  CHANNEL AND ITS OPENING AND CLOSING ACCORDING TO THE AI HYPOTHESIS.** In the **AI** model, the functional cell surface (Fig. 18) is a two-dimensional matrix of proteins carrying fixed anionic sites in the form of an array of  $\beta$ - and  $\gamma$ -carboxyl groups each under the influence of a cardinal site. In a resting cell, the cardinal site is occupied by a  $\text{Ca}^{2+}$  ion. This ion allosterically controls the  $c$ -value of the  $\beta$ - and  $\gamma$ -carboxyl groups, maintaining each at the relatively low  $c$ -value where  $\text{K}^+$  is preferred over  $\text{Na}^+$ . As a result,  $\text{K}^+$  is the predominant counter-cation at the cell surface.

The same or similar protein chains offering the  $\beta$ - and  $\gamma$ -carboxyl groups for  $\text{K}^+$  adsorption may in fact also exist in extended conformation, thereby lending their exposed backbone  $\text{NHCO}$  groups to polarize deep layers of water between the proteins at the cell surface. (For earlier diagrams see Ling<sup>153</sup>).

Under the cell surface the protein chains may be arrayed parallel to the cell surface (sector A) or perpendicular to the cell surface (sector B).

a. The resting cell surface of muscle and nerve. The physiological properties of such a cell surface model, according to the **AI** hypothesis, could be summarized as follows:

(1) The cell surface is semipermeable—meaning that it is most permeable to water but progressively less permeable to larger and more complex molecules. This is supported by data shown in Fig. 12.

(2) The cell surface behaves more or less like a  $\text{K}^+$  electrode, because at low  $c$ -value  $\text{K}^+$  is preferentially adsorbed over  $\text{Na}^+$  (Fig. 11).

(3) The cell surface potential shows no sensitivity to chloride because the surface sites are primarily anionic (see Fig. 8, also Ling, ref 85, p. 278).

(4) The cell surface potential shows no sensitivity to external  $\text{Mg}^{2+}$ -concentration (Ling and Walton, unpublished) because  $\beta$ - and  $\gamma$ -carboxyl groups are isolated and not in pairs or clustered (for support from model study, see Ling<sup>154</sup>).

(5)  $\text{K}^+$  entry shows competition and saturability because most  $\text{K}^+$  traffic is via the adsorption-desorption route (Fig. 2) on  $\beta$ - and  $\gamma$ -carboxyl groups with a  $pK_a$  of 4.6 (Fig. 4). Also,  $\text{K}^+$  and  $\text{Na}^+$  activate  $\text{Rb}^+$  entry due to triplet formation (Figs. 6, 7).

(6)  $\text{Na}^+$  entry occurs (a) partly by the same adsorption-desorption route utilized by  $\text{K}^+$ , and thus shows partial though much less favorable competition with  $\text{K}^+$  (Fig. 5); and (b) partly by the saltatory route, jumping through the interstices. The saltatory path offers relatively high resistance to the passage of  $\text{Na}^+$  and other cations because of the electric fields of the fixed  $\beta$ - and  $\gamma$ -carboxyl groups as well as the relatively low permeability of the polarized water to large hydrated ions (Fig. 12).

(7) In muscle cells the  $\text{K}^+$  adsorbing sites directly below the cell surface are arrayed in rows parallel to the cell surface (Fig. 18A). High activation energy for  $\text{K}^+$  migration between chains reduces outward  $\text{K}^+$  conductance (Ling<sup>153</sup>). This elevated activation energy may account for the anomalous rectification phenomenon first observed by Katz<sup>172</sup> (see also Adrian<sup>173</sup>). In other types of cells the chain may be more random or perpendicular. In those cases  $\text{K}^+$  may jump from one site to another, offering high conductivity (for details of mechanism and evidence, see Ling<sup>152</sup>), hence normal rectification. The same mechanism can account also for the much higher (radial) cytoplasmic resistance and membrane capacitance of muscle cells in comparison with nerve cells (see Katz<sup>29</sup>).



### C. Molecular Basis for Sudden, Transient Permeability Increase During Excitation

1. COMPARISON WITH HODGKIN-KATZ THEORY. The Hodgkin-Katz model of the resting potential described by Eq. 5 differs fundamentally from the surface adsorption model of the resting potential described by Eqs. 19 and 23. However, some common ground is shared by the corresponding models of the action potential. Before comparing the two, let me briefly describe the action potential model according to the **AI** hypothesis.

In the **AI** model the front end of an action potential causes a local depolarization. When this depolarization reaches a certain level, the cardinal adsorbent  $\text{Ca}^{2+}$  is detached. As a result, the protein or proteins that the cardinal adsorbent  $\text{Ca}^{2+}$  controls undergo a propagated cooperative change (the indirect F-effect). The change manifests itself in at least two major ways:

First, an *ion-specific* permeability increase occurs. This proceeds from a c-value shift of the  $\beta$ - and  $\gamma$ -carboxyl groups on the microscopically thin surface layer of the cell to a higher value, as a result of which the preponderant preference for  $\text{K}^+$  is lost and the surface anionic sites locally begin to adsorb  $\text{Na}^+$ . The phenomenon is envisioned as a local version of the gross shift, seen in whole muscle cells, to adsorbed  $\text{Na}^+$  rather than adsorbed  $\text{K}^+$ , a shift initiated by loss of  $\text{Ca}^{2+}$  (Fig. 14). The effect on the surface potential is described by Eq. 23 with a  $\text{Ca}^{2+}$ -dependent change of  $K_{\text{Na}^+\rightarrow\text{K}^+}^{\circ\circ}$ . (A detailed theoretical treatment of the ionic currents is given by Karreman<sup>174</sup>).

Second, a nonspecific permeability increase occurs. Along with the propagated shift to  $\text{Na}^+$  adsorption on the  $\beta$ - and  $\gamma$ -carboxyl groups, there is a step-by-step propagated change from the backbone's extended water-polarizing conformation to a folded (e.g.,  $\alpha$ -helix) or other peptide-to-peptide water-nonpolarizing conformation. With this depolarization, water abruptly loses its property of excluding hydrated  $\text{Na}^+$ , whereupon sudden increase of permeability and conductance occurs. Transient inward diffusion of  $\text{Na}^+$  follows, creating the inward  $\text{Na}^+$  current. This inward diffusion of  $\text{Na}^+$  is ion-specific because the entrant ion must successfully compete for the surface anionic sites now at a high  $\text{Na}^+$ -favoring c-value. However, additional driving force is provided by the sudden depolarization and normalization of water solvency near the cell surface and the nonspecific permeability increases that ensue.† Thus the final metastable equilibrium state potential has an additional component equal to

$$\psi = \frac{RT}{F} \ln \frac{[\text{Na}^+]_{\text{in}}^{\text{free}}}{[\text{Na}^+]_{\text{ex}}} \quad (24)$$

If now we compare the **AI** action potential model with that of Hodgkin and Huxley, we see that Eq. 24 resembles Hodgkin and Huxley's  $\text{Na}^+$  potential except that only free intracellular  $\text{Na}^+$ , rather than total  $\text{Na}^+$  concentration, is involved. It

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†An important distinction of this model is that water depolarization not only reduces the resistance of  $\text{Na}^+$  permeation but also increases the effective total surface area for  $\text{Na}^+$  permeation. This new facet makes it much more reasonable to visualize all-or-none surge of  $\text{Na}^+$  current through a membrane already rapidly permeable to  $\text{Na}^+$  (see p. 57).

follows that the general equation for the action potential (as well as the resting potential) is

$$\psi = \text{constant} - \frac{RT}{F} \ln \left\{ \frac{1}{[\text{K}^+]_{\text{ex}}} \left( 1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma/RT)}} \right) \right\} + \frac{RT}{F} \ln \frac{\gamma_{\text{in}}^{\text{Na}} [\text{Na}^+]_{\text{in}} + \gamma_{\text{in}}^{\text{K}} [\text{K}^+]_{\text{in}}}{\gamma_{\text{ex}}^{\text{Na}} [\text{Na}^+]_{\text{ex}} + \gamma_{\text{ex}}^{\text{K}} [\text{K}^+]_{\text{ex}}} \quad (25)$$

where  $\gamma_{\text{in}}^{\text{Na}}$ ,  $\gamma_{\text{in}}^{\text{K}}$  and  $\gamma_{\text{ex}}^{\text{Na}}$ ,  $\gamma_{\text{ex}}^{\text{K}}$  are the activity coefficients of  $\text{Na}^+$  and  $\text{K}^+$  in the cell surface layer water and external solution respectively. The second term on the right hand side of Eq. 25, a diffusion potential term, vanishes when the cell is at rest because under this condition  $\gamma_{\text{in}}^{\text{Na}}/\gamma_{\text{ex}}^{\text{Na}} = [\text{Na}^+]_{\text{ex}}/[\text{Na}^+]_{\text{in}}$  and  $\gamma_{\text{in}}^{\text{K}}/\gamma_{\text{ex}}^{\text{K}} = [\text{K}^+]_{\text{ex}}/[\text{K}^+]_{\text{in}}$ . Now

$$q_{\text{Na}} = \gamma_{\text{ex}}^{\text{Na}}/\gamma_{\text{in}}^{\text{Na}}; \quad q_{\text{K}} = \gamma_{\text{ex}}^{\text{K}}/\gamma_{\text{in}}^{\text{K}} \quad (26)$$

where  $q_{\text{Na}}$  and  $q_{\text{K}}$  are the equilibrium distribution coefficients of  $\text{Na}^+$  and  $\text{K}^+$  in the surface cell water. Substituting Eq. 26 into Eq. 25, and taking into account of the fact that  $\gamma_{\text{ex}}^{\text{Na}} [\text{Na}^+]_{\text{ex}} + \gamma_{\text{ex}}^{\text{K}} [\text{K}^+]_{\text{ex}}$  is a constant and that  $\gamma_{\text{ex}}^{\text{Na}} = \gamma_{\text{ex}}^{\text{K}}$ , Eq. 25 can be simplified as follows:

$$\psi = \text{constant} - \frac{RT}{F} \ln \left\{ \frac{1}{[\text{K}^+]_{\text{ex}}} \left( 1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\xi \exp(\gamma/RT)}} \right) \right\} + \frac{RT}{F} \ln \left( \frac{[\text{Na}^+]_{\text{in}}^{\text{free}}}{q_{\text{Na}}} + \frac{[\text{K}^+]_{\text{in}}^{\text{free}}}{q_{\text{K}}} \right) \quad (27)$$

During excitation  $K_{\text{Na} \rightarrow \text{K}}^{\text{oo}}$  falls due to the c-value increase of the surface anionic sites;  $q_{\text{Na}}$  (and  $q_{\text{K}}$ ) concomitantly increases. The inrush of  $\text{Na}^+$  brings about not only a cancellation of the resting potential but an overshoot. The absorption of  $\text{Na}^+$  displaces  $\text{K}^+$  from adsorption sites at and near the cell surface, causing an increase of free  $\text{K}^+$  (i.e.,  $[\text{K}^+]_{\text{in}}^{\text{free}}$ ) and hence the delayed outward  $\text{K}^+$  current.

It is clear that due to autocoperativity both  $K_{\text{Na} \rightarrow \text{K}}^{\text{oo}}$  and  $q_{\text{Na}}$  (and  $q_{\text{K}}$ ) shift between two discrete states (Ling<sup>85,101,123</sup>). The value of  $\psi$  at the active state should be approximated by the value of the peak height of the action potential. The time course of transition between the two autocoperative states is S-shaped with steep slope and demonstrable from the one-dimensional Ising model, as successfully treated by Huang,<sup>175</sup> Negendank and Karreman,<sup>176</sup> and Huang and Negendank.<sup>177</sup>

In the Hodgkin-Huxley theory, the late outward  $\text{K}^+$  current of an action potential was attributed to the opening of a second  $\text{K}^+$  gate. In the AI hypothesis, no such additional  $\text{K}^+$  gate is required or suggested. Indeed, the outward K current is seen as due fractionally to the  $\text{K}^+$  displaced by the in-moving  $\text{Na}^+$  but primarily to the shift of intracellular  $\text{K}^+$  from its normal resting adsorbed state to the free state which

increases momentarily the outward  $K^+$  flux. Loss of the excess positive charge accumulated during inward  $Na^+$  movement is reversed.  $Ca^{2+}$  once more returns to its cardinal sites and a reversal of the  $K_{Na^+K}^{\circ}$  and  $\gamma_{in}^{Na}$  change leads to reestablishment of the resting polarized condition.

2. COMPARISON WITH EXPERIMENTAL OBSERVATION. In suggesting that the inward  $Na^+$  current is driven by a suddenly activated  $Na^+$  potential described by Eq. 24, the present model partially agrees with the Hodgkin-Huxley model. However, since  $Na^+$  adsorption is on sites not absolutely unavailable to  $K^+$ , the movement of  $Na^+$  as well as  $K^+$  are as a rule not independent of each other. The surface adsorption model is therefore in accord with earlier cited data (p. 57) that contradict the independence principle of the Hodgkin-Huxley theory.

(I) The basic concept that cell surface anionic  $\beta$ - and  $\gamma$ -carboxyl groups undergo a transient increase of  $c$ -value, brief to be sure but lasting throughout the duration of the inward  $Na^+$  current, is in harmony with the following observations:

(a) These surface anionic sites before excitation have a relatively low  $pK$  value (4.6) (see Fig. 4).

(b) The rank order of selectivity of resting surface sites is  $Rb^+ > Cs^+ > K^+ > Na^+$ , roughly corresponding to a  $c$ -value of  $-4.5 \text{ \AA}$  according to the theoretical data of Fig. 11.

(c) The anionic sites essential for the inward  $Na^+$  current have a higher  $pK$  value. Thus the  $pK_a$  value for the anionic site mediating  $Na^+$  entry for squid axons was given as 5.2 by Hille<sup>51,52</sup> and 6.5 by Stillman.<sup>54</sup> The  $pK_a$  value of frog node of Ranvier was given by Drouin and The as 5.15 and for *Myxicola* axon 4.8.<sup>53</sup>

(d) The rank order of ion selectivity of the anionic site essential for the  $Na^+$  current is  $Li^+ = Na^+ > K^+ > Rb^+ > Cs^+$  (Hille<sup>52</sup>) which roughly corresponds to a  $c$ -value of  $-1.5 \text{ \AA}$  from the theoretical curve of Fig. 11.

(e) A large body of evidence has indicated that the Na channel is primarily proteinaceous (for review, see Hucho and Schiebler<sup>43</sup>). Proteins contain anionic groups mostly in the form of  $\beta$ - and  $\gamma$ -carboxyl groups. Thus the anionic sites with high  $pK$  value can reasonably be expected to be those same  $\beta$ - and  $\gamma$ -carboxyl groups when the proteins exist in the activated conformation. (It is worth mentioning that in 1952 Hodgkin and Huxley briefly alluded to a change of channel selectivity in the reverse direction from a  $Na^+$  channel to a  $K^+$  channel but preferred the two-channel concept).

(2) The theoretical model of propagated depolarization of cell surface water coinciding with the switching from  $K^+$  to  $Na^+$  adsorption is supported by the finding of Villegas et al.<sup>178</sup> whose data are reproduced in Table I. Sugar and sugar alcohols, normally excluded and virtually impermeant through a polarized water model (Fig. 12) suddenly became permeant and then only in the presence of  $Na^+$ , in accord with the AI model. (For other evidence of increase of nonspecific permeability during activation, see Luxoro et al.<sup>179</sup>)

Can the present model quantitatively match the 50-fold decrease of membrane resistance accompanying the action potential discharge (from  $1000 \text{ \Omega/cm}^2$  to  $20 \text{ \Omega/cm}^2$ ) and the estimated increase in sucrose permeability of more than 40-fold

(Table I)? To answer, let us return to Fig. 12. Here the sucrose permeability is more than ten thousand times lower than permeability to water. Now, permeability through a thin membrane is equal to  $D_i q_i$ , where  $D_i$  is the diffusion coefficient and  $q_i$  is the equilibrium distribution of the  $i$ th solute between the membrane phase and external solution. Thus the ratio of sucrose permeability to water permeability through a thin layer of normal water (in which  $q_{\text{sucrose}} = q_{\text{water}} = 1$ ) would be approximately  $D_{\text{sucrose}} q_{\text{sucrose}} / D_{\text{water}} q_{\text{water}} = D_{\text{sucrose}} / D_{\text{water}} = 5.23 \times 10^{-6} / 2.35 \times 10^{-5} = 22.2$ , where  $5.23 \times 10^{-6}$  and  $2.35 \times 10^{-5}$  cm<sup>2</sup>/sec are the respective diffusion coefficients of sucrose in water and the self-diffusion coefficient of water in water (Dodgman et al.,<sup>180</sup> Wang et al.<sup>181</sup>). Thus if the cell surface water is entirely covered with water as found in the activated layer of the cellulose acetate membrane, and if during excitation all of the cell surface water turns into normal water, there would be a  $10^4 / 2.22 \times 10 = 450$ -fold increase. (I have chosen water permeability as a reference because the normal resting cell membrane is extremely permeable to water as is also the cellulose acetate membrane (Ling<sup>131</sup>). However, permeability of the resting nerve and muscle cell membrane to sucrose is considerably higher than that of frog skin or an activated cellulose acetate layer. With this in mind, one must anticipate a very large area of the excited cell-membrane water to have become totally depolarized.

(3) The present model does not prescribe the existence of a second  $K^+$  channel that opens and closes specifically for  $K^+$  during the passage of an action potential. It is the membrane theory with its basic tenet of free  $K^+$  in the cell—a tenet now disproven—that demands a gate opening and closing model (see p. 56). Instead, the reason for outward  $K^+$  movement is desorption from cell surface  $\beta$ - and  $\gamma$ -carboxyl groups.

The presence throughout the cytoplasm of fixed carboxyl groups with relatively low  $c$ -values at which  $K^+$  is normally preferred is substantiated by the observation of acidic groups at the muscle cell surface with  $pK_a$  value (4.6) by Ling and Ochsenfeld<sup>95</sup> very similar to the value from in vitro measurements of isolated proteins (Tanford<sup>182</sup>). That early finding<sup>95</sup> has been repeatedly confirmed. Thus in frog Ranvier node Drouin and The<sup>53</sup> observed acidic groups with  $pK_a$  value of 4.63

TABLE I. Penetration of <sup>14</sup>C-Erythritol, Mannitol, and Sucrose in Resting and Stimulated Squid Axons

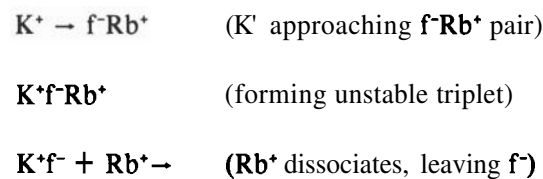
Molecule	Number of nerve fiber pairs	Permeability in 10 <sup>-3</sup> cm/sec <sup>a</sup>				
		Resting axon (axon a)	Stimulated at 25/sec (axon b)	Net increase stim. 25/sec (b - a)	p of net increase <sup>b</sup>	Calculated permeability during activity <sup>c</sup>
Erythritol	10	3.6 ± 0.4	6.1 ± 1.0	2.5 ± 0.8	0.02	110
Mannitol	10	2.3 ± 0.4	4.0 ± 0.5	1.7 ± 0.3	0.001	75
Sucrose	10	0.9 ± 0.1	1.8 ± 0.3	0.9 ± 0.3	0.02	40

<sup>a</sup>The values are mean ± standard error. <sup>b</sup>Statistical significance calculated according to Student's method for correlated samples. <sup>c</sup>Calculated permeability during activity obtained by assuming that the permeability change per impulse lasts 1 msec. [From Villegas et al., *J. Gen. Physiol.*, 48, 37 (1965).]

● 0.05. Stillman<sup>54</sup> observed acidic groups for  $K^+$  channels in squid axon to have a  $pK_a$  value lower than  $pK_a$  5.0. Schauf and Davis<sup>56</sup> discovered, in *Myxicola* giant axon, acidic groups with  $pK$  value of 4.4. These data clearly show that the inside of the cell has the same type of  $\beta$ - and  $\gamma$ -groups that the cell surface has and that like the surface groups, the inner groups function at once as channels and barriers to ionic movement. Just as  $Rb^+$  and  $Cs^+$  slow down  $K^+$  inward permeation (Fig. 3A), those same ions block delayed  $K^+$  current as Chandler and Meves<sup>47</sup> demonstrated in 1965. Similarly the reduction of the delayed current by  $Na^+$  (Armstrong<sup>42</sup>) can be compared with the blocking of  $Na^+$  entry by  $K^+$  (Fig. 5).

(4) Regarding inactivation, the delayed current normally observed under the voltage clamp condition shows no such inactivation. Yet tetraethylammonium introduced into the squid axon blocks the delayed  $K^+$  current. Similarly  $Cs^+$  and  $H^+$  have blocking or channel inactivation effects.

To explain, the data of Fig. 3A are useful. Here the entry of  $K^+$  is competitively inhibited by the presence of  $Rb^+$ ; yet  $Rb^+$  entry is facilitated by the presence of  $K^+$  (Fig. 6). Clearly this shows that the migration is not simply by sorption on a vacant site followed by desorption leaving behind another vacant site. It is not that this does not occur, it does, but infrequently. The predominant route is via the transient formation of a triplet with the participation of a second counteranion as illustrated in Fig. 7. Thus if  $f^-$  represents a fixed anion, and a movement to the right describes the entry into the cell, the facilitation of  $Rb^+$  entry by  $K^+$  follows the sequence:



Thus the approaching  $K^+$  weakens the interaction between  $f^-$  and  $Rb^+$ , leading to dissociation of the  $Rb^+$ . It is likely that the optimal condition for maximal conductance would be a population of counterions of the same kind that assume a higher configuration (Fig. 10) at equilibrium. If instead of  $Rb^+$  a tetraethylammonium ion (or better, its C9 derivative) occupies  $f^-$ , then oncoming  $K^+$  has no chance of weakening the  $C9f^-$  bond sufficiently to cause its departure; ion migration or conductance must then be arrested. These concepts, first described in 1962 (Ling<sup>48</sup>) do not differ fundamentally from the proposals of Bezanilla and Armstrong,<sup>49</sup> with the exception that in my model it is polarized water rather than a lipid layer that provides the required barrier.

With this consideration in mind, it seems reasonable to assume that the  $Na^+$ -channel inactivation is due to the occupancy of the high  $c$ -value  $Na^+$ -preferring sites by  $K^+$ , just as  $K^+$  channel inactivation is due to TEA,  $Cs^+$ , and  $H^+$ .

(5) Local transient swelling occurs during passage of an action potential, as Tasaki<sup>183</sup> has elegantly demonstrated in squid axon. He offered no specific explanation. However, in an earlier publication<sup>184</sup> I showed that isotonic  $KCl$  causes cell swelling by dissociating restraining salt-linkages between protein chains. I

suggest that during the conformation change in the course of the action potential, some salt-linkages are dissociated transiently and reversibly, thereby creating the transient swelling. In support, I mention another paper of Tasaki and associates (Pant et al.<sup>185</sup>), which showed that **perfused** squid axons release proteins into the perfusate in response both to high concentrations of K<sup>+</sup> salt and to repetitive electrical stimulation. It is obvious that if the salt-linkages holding a protein molecule in place are all broken, the protein will be released; if only some of the salt-linkages are broken, swelling will follow as a consequence. □

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## APPENDIX

### Brief Summaries of Theories of Cellular Electric Potentials Other Than the Hodgkin-Katz-Huxley Theory and the Present Association-Induction Model

1. **TASAKI'S THEORY.** Following demonstration of two stable potential states in the squid giant axon (Tasaki and Hagiwara<sup>186</sup> and Tasaki<sup>187</sup>), Tasaki<sup>188</sup> published in 1963 a theory of action potential which in general outline bears much similarity to my own. Thus Tasaki postulated that negative sites occupy a critical layer at the cell surface determine the potential, and that excitation involves cooperative transition from one state to another with accompanying changes in the ionic preference of these negative sites (Tasaki,<sup>187,189</sup> Tasaki and Singer<sup>190</sup>).

The Tasaki<sup>191</sup> theory, however, does differ from mine in several ways. Here are three examples. (a) Tasaki considered the shift of the two cooperative states to be between a Ca<sup>2+</sup>-state and a monovalent ion state. (b) Tasaki relied on a three-dimensional zeolite model from the work of Barrer and Falconer,<sup>192</sup> whereas mine used a one-dimensional Ising model based on the assumption that the nearest neighbor interaction energy is mediated through the polypeptide chains. (c) Tasaki considered the

role of Na<sup>+</sup>, K<sup>+</sup>, etc. in terms of their different abilities to dissociate divalent and macromolecular complexes.

2. **THEORY OF FISHMAN, CHODOROV, AND VOLKENSTEIN.**<sup>57</sup> This is a quantitative treatment, based on first physical principles, of the empirical findings and the formal mathematical treatments of the action potential by Hodgkin and Huxley. It is too complex to be briefly explicated. What is of interest here is that these investigators were not convinced that Ca<sup>2+</sup>-detachment opens the Na<sup>+</sup> gate as proposed in the membrane theory. Rather, they believed that the depolarizing electric field produces a transition in some activation particles and that this transition then produces changes in Ca<sup>2+</sup>-adsorption.

3. **THEORY OF CHIMADZHEV, MULER, AND MARKIN.**<sup>193</sup> Chimadzhev and coworkers assumed that a set of globular-lipoprotein complexes can exist in two conformational states designated P and A, and that P and A have different affinities for Ca<sup>2+</sup>. In the P state the membrane is impermeant to Na<sup>+</sup>; in the A state it has permeability equal to g. As in my theory, and that of Fishman et al., cooperativity with positive nearest neighbor interaction was assumed to underly the steep rise of g<sub>Na</sub> with potential change.

4. CHANG'S **THEORY**.<sup>194</sup> Like the **Hodgkin-Huxley** theory, Chang's construct is primarily a phenomenological one, presenting little or no molecular mechanisms. The cell is considered to be covered with a uniform lipid membrane of finite thickness—in Chang's own words, "with a unit membrane or a lipid bilayer with pores."<sup>194</sup> This membrane is not to be confused with a "physiological membrane." The axoplasm, however, has the property of an ion exchanger as proposed in the LFC hypothesis (Ling<sup>25</sup>).

Chang's equation for the resting potential is

$$\psi \cong -\frac{RT}{F} \ln \left( \frac{A}{[\text{K}^+]_{\text{ex}} Y_{\text{K}} + [\text{Na}^+]_{\text{ex}} Y_{\text{Na}} + \frac{[\text{Cl}^-]_{\text{ex}} Y_{\text{Cl}}}{A}} \right)$$

where  $A$  is the density of anionic charged groups in the whole cell, and

$$Y_{\text{K}} = \exp\{-\phi_{\text{K}}/RT\}$$

$$Y_{\text{Na}} = \exp\{-\phi_{\text{Na}}/RT\}$$

$$Y_{\text{Cl}} = \exp\{-\phi_{\text{Cl}}/RT\}$$

where the  $\phi$ 's and hence  $Y$ 's are themselves functions of the resting potential  $\psi$  and of gross intracellular ion concentration  $[\text{K}^+]_{\text{in}}$ ,  $[\text{Na}^+]_{\text{in}}$ , and  $[\text{Cl}^-]_{\text{in}}$ .

The theory suggests that during excitation the ion selectivity of the axoplasm changes. This reference to the axoplasm later became restricted to a thin layer of cortex directly beneath the lipid membrane which undergoes unfolding or uncoiling, thus offering less resistance to ion movements (**Chang**<sup>58</sup>).

□ □