COOPERATIVE INTERACTION AMONG CELL SURFACE SITES: EVIDENCE IN SUPPORT OF THE SURFACE ADSORPTION THEORY OF CELLULAR ELECTRICAL POTENTIALS

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• *Resting potentials were studied in frog sartorius muscles equilibrated in Ringer solutions that contained various concentrations of K^+. Cells in solutions that contained mar-zero K^+ showed a rise in porential followed by a slow decline over about 70 hours that paralleled the loss of cellular K^+ in exchange for Na^+. When these cells were placed in 2.5 mM K^+, the re-gain of the porential occurred much more rapidly than the re-gain of cellular K^+. Thus, there was no consistent relation between resting potential and concentration of intracellular K^+. When cells were immersed in solutions containing 100 mM Na^+ and K^+ at concentrations of 2.5 mM and greater, there was a semi-logarithmic relation between porential and $[K^+]_{ex}$, in which potential declined as $[K^+]_{ex}$ increased. However, when cells were equilibrated in $[K^+]_{ex}$ below 2.5 mM, the potential peaked and then declined as $[K^+]_{ex}$ decreased. The results are readily explained by the surface adsorption model of the cellular potential, in which the potential is determined simply by the nature of the fixed surface charges that interact with one another in a cooperative manner. The peak and decline of the potential with decreasing $[K^+]_{ex}$ below 2.5 mM mirrors the autocooperative shift in the affinity of the surface charged sites from one that favors K^+ over Na^+ strongly to one that favors K^+ over Na^+ less strongly.*

INTRODUCTION

Moritz Traube's copper ferrocyanide precipitation membrane (Traube, 1867), with its nearly perfect semipermeable properties, provided the foundation both for Pfeffer's membrane theory of osmotic and solute-distribution properties of living cells (Pfeffer, 1877) and for Ostwaldk suggestion of a membrane origin of cellular electrical potentials (Ostwald, 1890). Ostwald's suggestion was developed first into Bernstein's "membrane theory" (Bernstein, 1902) and then into the "ionic theory" of Hodgkin and Katz (the HKI theory) (Hodgkin and Katz, 1949). These and subsequent workers considered membrane permeability the key parameter determining cellular electrical potentials. The relations between the magnitude of the potential and the temperature and between the potential and the external K' and Na' concentrations have been predicted according to this theory and repeatedly verified. However, although a recent survey shows that a great deal of the experimental evidence collected in the last 25 years confirms the HKI theory. a great deal of evidence contradicts it (Ling, 1979, 1982). In the same reviews it was pointed out that both sets of evidence support the surface adsorption theory of the cellular electrical potential (the SA theory). This theory is an integral part of the association-induction hypothesis (the AI hypothesis) (Ling, 1962; 1967a,b; 1978; 1982).

According to the SA theory, the cellular resting potential bears no direct relation to membrane ion permeability, but is related to adsorption of cations on anionic sites. primarily the β- and γ-carboxyl groups of proteins, on the outer cell surface.

in its simplest form. the SA theory of the resting potential (ψ) can be written as

$$\psi = \text{constant} - \frac{RT}{F} \ln \{\bar{K}_K [K^+]_{ex} + \bar{K}_{Na} [Na^+]_{ex}\}, \quad (1)$$

where R and F are the gas and Faraday constants. respectively; T is the absolute temperature: $[K^+]_{ex}$ and $[Na^+]_{ex}$ are the external K^+ and Na^+ concentrations; and $\bar{K}_K$ and $\bar{K}_{Na}$ are the respective adsorption constants of these ions on the surface anionic sites.

Equation 1 can be put into a more general form:

$$\psi = \text{constant} - \frac{RT}{F} \ln \sum_{i=1}^{n} \bar{K}_i [p_i^+]_{ex}, \quad (2)$$

where $[p_i^+]_{ex}$ is the external concentration of the ith monovalent cation among a total of n kinds. $\bar{K}_i$ is the adsorption constant of the ith species.

More recently. a refined version of the SA theory was presented (Ling, 1979) in which the surface adsorption sites for K^+ or Na^+ arc no longer considered to be independent of one another but show an autocooperative interaction, similar to that demonstrated for the bulk-phase adsorption of K^+ and Na^+ in a variety of living cells (Ling, 1966, Ling and Bohr, 1970; Jones, 1970; Karreman, 1972; Gulati. 1973; Negendank and Karreman, 1979). The equation for the resting potential in this newer version of the SA theory is as follows:

$$\psi = \text{constant} - \frac{RT}{F} \ln \frac{1}{[K^+]_{ex}} \left(1 + \frac{\xi - 1}{\sqrt{(\xi - 1)^2 + 4\theta\xi}} \right), \quad (3)$$

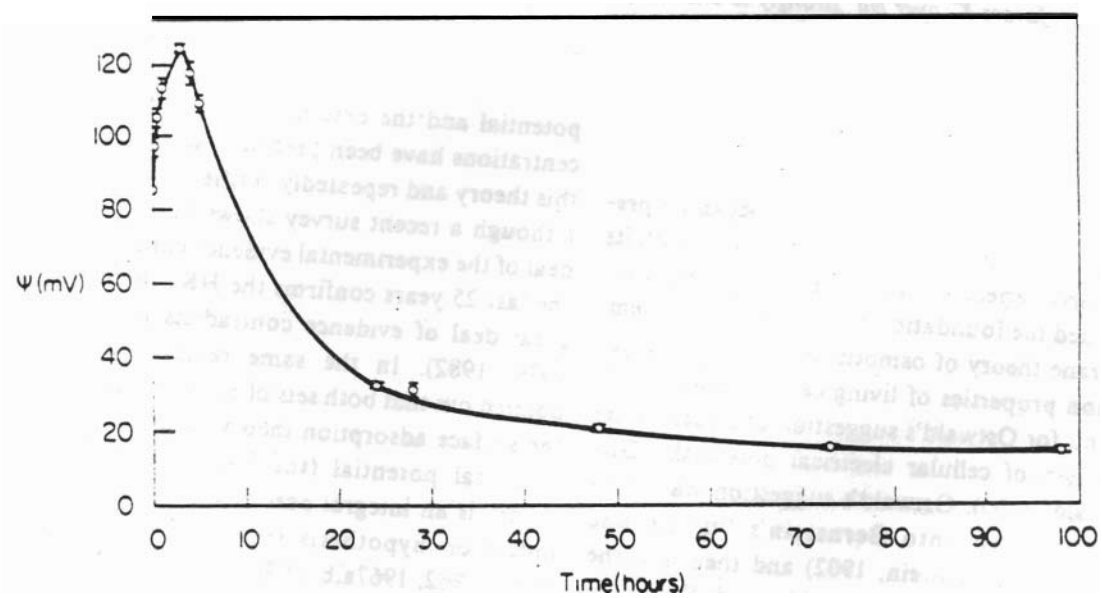

FIGURE 1. Time course of depolarization of resting potential of frog sartorius muscles in "K-free" Ringer solution. Sartorius muscles were incubated in 500 ml of a sterile Ringer solution containing only a trace of K^+ (i.e., 5 μM), and resting potentials were measured at intervals. Data are means ± SEM of potentials measured in 6 cells at each time.

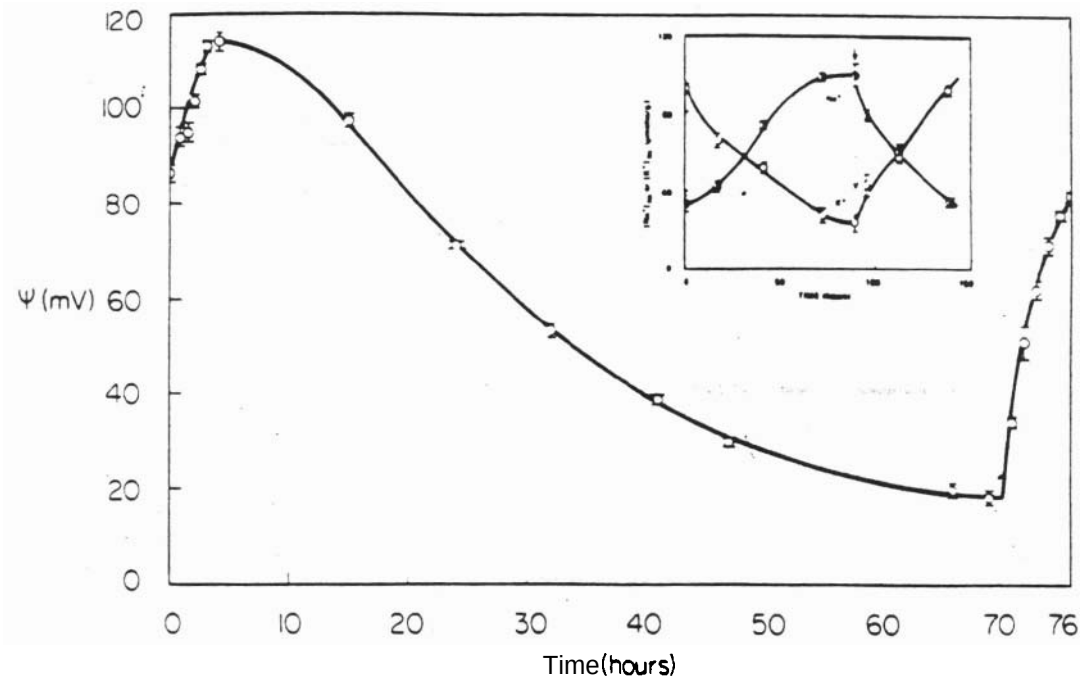

FIGURE 2. Time course of depolarization of resting potential of a frog sartorius muscle in "K-free" Ringer solution and repolarization on subsequent return to a Ringer solution containing the normal K^+ conantration (2.5 mM) marked by the arrow. Inset is from Ling and Bohr (1970). For details of inset, see text. Data are means ± SEM of potentials measured in 6 cells at each time.

where

$$\theta = \exp(\gamma/RT), \quad (4)$$

where $-\gamma/2$ is the nearest neighbor interaction energy and

$$\xi = \frac{[K^+]_{ex}}{[Na^+]_{ex}} \cdot K^{\infty}_{Na-K} \cdot \quad (5)$$

K^{∞}_{Na-K} is the intrinsic equilibrium constant in the Na–K exchange adsorption.

This paper reports experimental studies designed to test the SA theory in general and predictions of Equation 3 in particular.

MATERIALS AND METHODS

We used isolated sartorius muscles of Northern American leopard frog (Rana *pipiens pipiens*, Schreber) from Vermont. The technique of measuring the resting potential of single muscle fibers was essentially the same as that described by Ling and Gerard (Ling and Gerard, 1950).

The basic Ringer solution contained the following ingredients: 2.5 mM K^+, 100 mM Na^+, 1.0 mM Ca^{++}, 1.2 mM Mg^{++}, 86.7 mM Cl^-, 15.7 mM HCO_3, 2.7 mM PO_4, 0.1 mM NO_3, and 23.5 mM glucose. In addition it contained 14 vitamins and 21 amino acids as well as penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) (see Ling and Bohr, 1969). As a rule the Ringer solutions were in equilibrium with a gas phase containing 95% O_2 and 5% CO_2. In virtually all cases in which incubation lasted longer than a few hours, sterility was strictly maintained. Unless otherwise stated, the incubation solutions were gently shaken in a room maintained at a constant temperature of 25° ± 1°C.

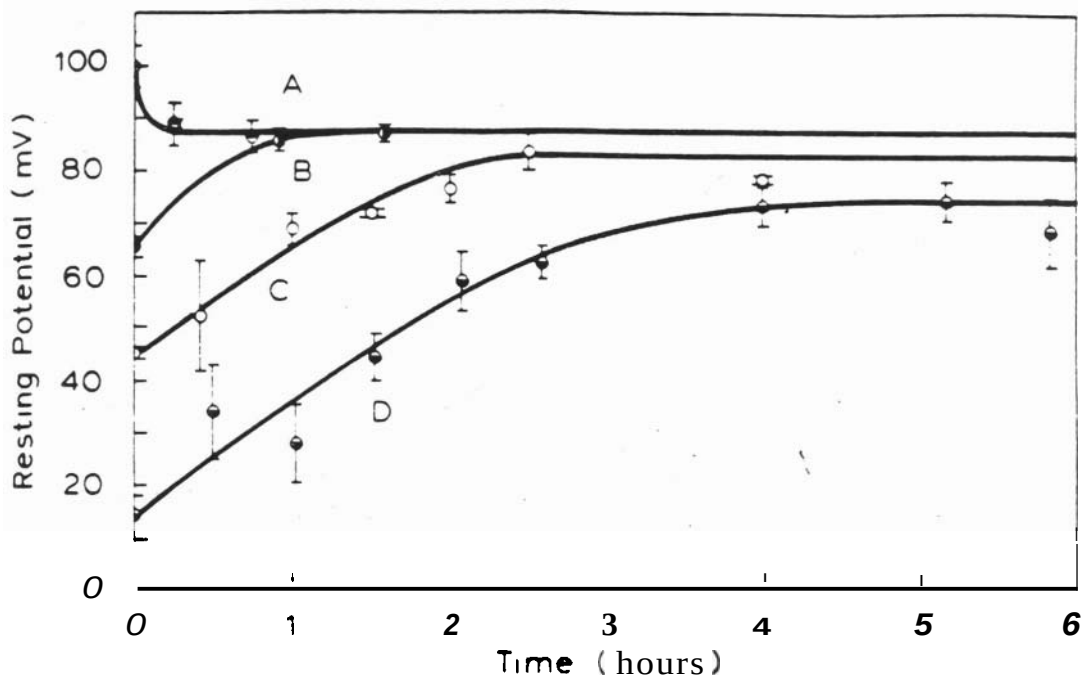

FIGURE 3. Recovery of resting potentials following return to a normal 2.5 mM K^+ Ringer solution after t h m days of prior incubation in various low-K^+ Ringer solutions; the concentrations of K^+ in these low-K' incubation solutions were 0.93 mM (A), 0.60 mM (B), and 0.34 mM (C) and 0.02 mM (D). Each point represents the mean ± S.E. from at least t h m individual measurements.

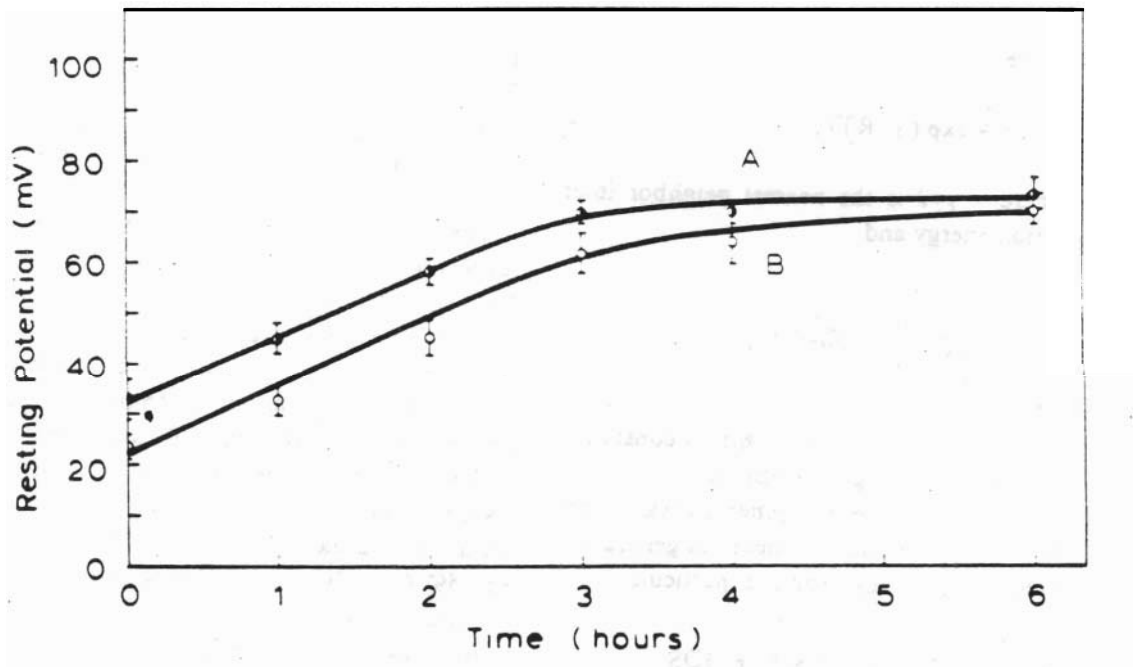

FIGURE 4. Recovery of mting potentials following return to a normal 2.5 mM K^+ Ringer solution at 25° C with gentle shaking. Each point represents a total of 16 readings from four different muscles. The average weights of the large muscles (A) were 164.3 ± 1.3 mg; that of the small muscles (B) 93.5 ± 5.7 mg.

RESULTS

Time Course of Resting Potential Change in Low and Normal K^+ Ringer Solutions at 25° C. Figure 1 shows the time course of changes in the resting potential when one set of sartorius muscles were incubated in a Ringer solution containing not the usual 2.5 mM K^+ but virtually no K^+. It took about two to three hours for the resting potential to reach a new high level. If a large volume of "K^+-free" Ringer solution (i.e., one or two muscles in 500 ml) was used. the potential then began to fall slowly until it finally reached a new low level. When the muscle was transferred back to a normal Ringer solution containing 2.5 mM K^+, as in the set of muscles in Figure 2, the resting potential rose rapidly. approaching its original high level of 85 mV or so within 6 to 8 hours (Figure 2). The inset of Figure 2. taken from Ling and Bohr (1970) shows the time course of changes in the total K^+ and Na' contents of sartorius muscles during similar exposure, first to low-K' and later to normal-K* Ringer solution. Thus the data shown in the main pan of Figure 2 and in the inset represent different aspects of the same experiment: in the inset, total K^+ and Na' contents were recorded and in the central graph. resting potentials wen recorded. Let us now compare the similarities and differences between the two.

In the low-K' Ringer, the time it took for the total K^+ level of the cells to fall to a final low level was about 80 hours; it also took approximately the same time (70 hours) for the resting potential to fall to the new low level.

On returning to normal K^+ Ringer. it also took nearly the same length of time (60 hours) for the total K^+ content to regain its normal value; in sharp contrast. the resting potential required only about 6 to 8 hours to regain its normal value.

In Figure 3, the recovery of the potential in a normal 2.5 mM K^+ Ringer was compared in muscles that had been exposed for 3 days to four different low-K' concentrations (0.02, 0.34. 0.60, and 0.93 mM). The time it took for the potential to return to its final level varied with the K^+ concentration to which the muscles had been exposed. At concentrations of 0.60 and 0.93 mM, the return was fast (1 hr.); at concentrations of 0.02 and 0.34 mM, the return was slower (>2.5 hr).

Figure 4 shows that the return to a higher potential in normal 2.5 mM K^+ Ringer solution following exposure to low-K* Ringer does not depend on the size of the muscles. This independence of muscle size and hence depth of the extracellular spaces shows that the slow return to a normal resting potential is not due to a delay in diffusion through the extracellular space.

The Resting Potential at Varying External K^+ Concentrations and a Constant Na^+ Concentrarion of 100 mM. The fact that the resting potential of muscles exposed to low-K^+ Ringer solution required a long time to reach its new steady level (Figures 1 and 2) shows that much of the earlier data on the effect of below-normal external K' concentration (normal K^+ concentration is 2.5 mM) on the resting potential, carried out after a short equilibration time, were not related to equilibrium levels; in contrast, time course studies show that the effects of external K^+ concentration at a K^+ concentration equal to or above 2.5 mM do represent equilibrium values (see below) (Ling, 1962; Ling and Gerard, 1950). When frog sartorius muscles are incubated in 100 mM Na^+ and K^+ is added at concentrations as high as 100 mM, cellular volume remains unchanged (Ling, 1977b).

Figure 5 presents a plot of the steady resting potentials of frog sanorius muscles at low external K^+ concentration after 3 days' incubation at 25°C and at high external K' after only 10 to 15 minutes of equilibration.

After resting potential measurements were

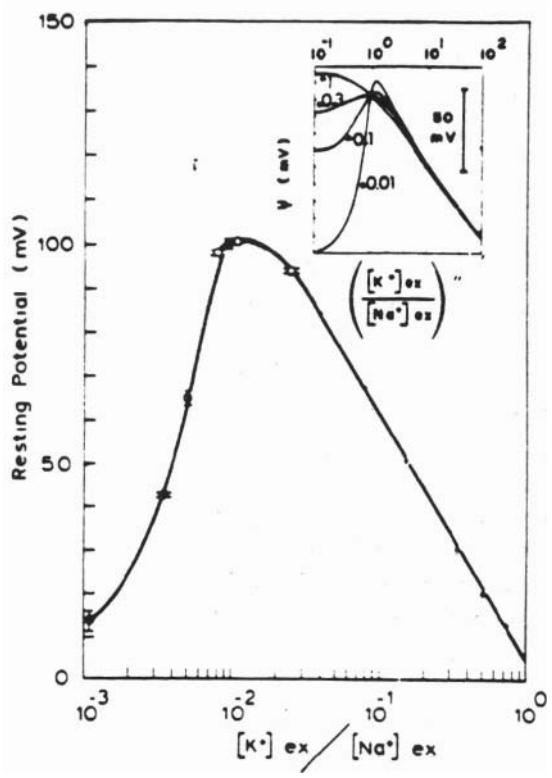

FIGURE 5. Variation of the equilibrium resting potential of frog sartorius muscles at different external K^+ concentrations and constant external Na^+ concentration (100 mM). All points corresponding to an external K^+ concentration of 4 mM or higher (small solid points) were obtained by the conventional procedure: measurements were made 10 minutes after the application of each higher K^+ concentration. In this range. for4 mM to 100 mM external K^+, each point represents the average of four single determinations. The standard errors are smaller than the width of the points and are not expressed. For points corresponding to 2.5 mM or lower concentrations of K^+, the muscles were incubated for three days at various K^+ concentrations. Each point represents average and S.E. of six reading. The inset shows theoretical curves plotted from Equation 3 for various levels of θ; the abscissa, $(K^+_1/Na^+_{ex})^n$, represents $(K^+_{ex}/Na^+_{ex}) \cdot K^{\infty}_{Na-K}$.

made, each muscle was returned to a normal 2.5 mM K' Ringer solution. All muscles then regained a normal resting potential similar to that already shown in Figure 2. This precaution assures that the measured resting potentials were not from dead muscles. The inset shows a set of theoretical curves calculated from Equation 3, published before the set of experiments cited here were undertaken (Ling, 1979). The data would fit a curve with θ equal to 0.03, and K^{∞}_{Na-K} equal to 210.

DISCUSSION

The Significance of the Widely Different Time Courses for Depolarization and .Repolarization. In 1960 Ling showed that the resting potential of frog sartorius muscle, dropped to a new low level almost instantly following the application of a Ringer solution containing 30 mM K^+, and that this low level of potential was maintained for at least 10 hours at room temperature (Ling, 1960, see also Ling 1962). in spite of the fact that there was a steady gain of intracellular K^+ during this period of time (Ling and Ochsenfeld, 1966). This constancy of the resting potential in the face of a steadily rising internal K^+ concentration offered one early set of evidence against the membrane theory, predicting that the resting potential depends directly on the intracellular K^+ concentration.

It was also shown that a Corning 015 glass electrode is not sensitive to K^+. Yet, application of a very thin layer of oxidized and partially dried collodion rendered the glass electrode sensitive to the K^+ concentration in the envionment (Ling, 1962; 1967a, 1967b). These findings led to the conclusion that it is not the ionic permeability through the membrane that determines the ion to which the electrode is sensitive. Rather, the selective adsorption on the surface anionic sites underlies the sensitivity exhibited by the 015 glass electrode to H^+ and not to K^+, by the collodion-coated glass electrode to both H^+ and K^+, or by the frog sartorius muscle to K^+ but less to Na^+. Only a few layers of anionic sites at the surface of the living cells or their glass electrode model determine the potential.

On the basis of this theory one can anticipate that the resting potential of living cells would change abruptly when the cells are

plunged into a Ringer solution containing a K^+ concentration higher than 2.5 mM because all that is involved is the occupancy of a very small number of vacant sites at the surface. Similarly, when the muscle is plunged into a rather small volume of Ringer solution containing 0.6 to 1.0 mM of K, one observes a fairly rapid attainment of a new higher level of potential. Again, this is not difficult to understand, because it involved primarily a decrease in the number of K^+ ions adsorbed at the surface. However. when the muscle is shaken in a large body of Ringer solution containing virtually no K, the muscle as a whole loses K^+ slowly and steadily. In the process, the surface anionic sites will continue to receive K from the inside of the cell and thus maintain a high potential until eventually intracellular K^+ is exhausted, at which time all the surface anionic sites will become occupied by Na^+, and the potential will approach a level of nearly zero (Figure 2). The similar time courses for a loss of total cell K and a gain of total cell Na on the one hand, and for a fall of resting potential, on the other, suppon this view.

When a depolarized muscle is returned to a normal 2.5 mM K^+ Ringer solution, reoccupancy of the cell surface anionic sites again involves only adsorption of K to the surface layer of sites and can be expected to be rapid. However, in contrast to the exposure of normal muscle to a higher than normal K concentration (e.g., 30 mM), this return to normal potential involves not only simple site occupancy but also a cooperative transition of the surface anionic sites from the Na^+ state to the K state. Like all similar cooperative, or more correctly, stochastic, processes, this transition is timedependent (Negendank and Karreman, 1978; Huang and Negendank, 1980), which, we believe, explains why it would still take some 6 to 8 hours for the resting potential to reach the new equilibrium value even though this is still about 8 times faster than the time required for total intracellular K^+ to return to its normal level. Indeed, according to the inset in Figure 2, one would expect that by the time the resting potential has risen to its full value, intracellular K^+ concentration could not have recovered more than a fraction of its initial concentration. These findings offer additional support for the SA theory of the cellular resting potential, which predicts that there is no direct dependence of ψ on the bulk-phase intracellular K^+ concentration but that only adsorption on the surface anionic sites determines ψ.

Autocooperarivity Among Surface Anionic Sites. As mentioned in the Introduction, there is now widely confirmed evidence that cooperative interaction exists among the K^+- and Na^+-adsorbing sites *within* a variety of living cells. Recently, three laboratories using a total of four different methods (autoradiography, transmission electron-microscopy, x-ray microprobe analysis, and laser microprobe mass spectrometry (LAMMA)), all showed that K^+ in frog muscle is not free and evenly distributed inside the cells but is adsorbed within the A bands and Z lines (Ling, 1977a; Edelmann, 1977, 1978a, 1978b, 1980; Trombitas and Tigyi-Sebes, 1979). This conclusion is further supported by the observation of Huang et al (1979) of the X-ray absorption edge fine structure of K in frog red blood cells. Their data strongly suggest that K is in a state of complex binding. Taking all the findings together we feel that the cooperative adsorption isotherms of K and Na* originate from interaction between intracellular K^+-adsorbing sites that exist primarily on cell proteins, as described by the association-induction hypothesis (Ling, 1966; Ling and Bohr, 1970; Jones, 1970; Karreman, 1972; Gulati, 1973).

With this point firmly established, one recalls that for frog sartorius muscles, the average intrinsic equilibrium constant at 25°C for the bulk-phase K^+- and Na^+-

adsorbing sites (K^{∞}_{Na-K}) is equal to 135, and the nearest-neighbor interaction energy ($-\gamma/2$) is equal to 0.54 Kcal/mol. The data presented in Figure 5 permit us to estimate that K^{∞}_{Na-K} equals roughly 210 and $-\gamma/2$ is 1.0 Kcal/mole.

The demonstration that the surface anionic sites are autocooperatively linked is of considerable importance. According to the AI hypothesis this autocooperativity provides the basis for the action potential, during which there is an all-or-none shift of electron density of the surface anionic sites from a state of overwhelming preference for K^+ over Na^+ to one in which there is a greater preference for Na^+. Concommitant with this shift, depolarization of water at the cell surface leads to the inward Na^+ current followed by a return to the K^+ state (Ling, 1962, 1971, 1982, see also Ling, 1973).

The fact that during the passage of an action current there is an increase not only of permeability of Na^+, but of uncharged molecules like erythritol and sucrose, adds support to the concept that depolarization of cell surface water increases permeability to all large, complex molecules and hydrated ions during the activated state (Villegas et al., 1965).

It is also interesting to ponder the basic similarity in the values of K^{∞}_{Na-K} and $-\gamma/2$ for the bulk-phase adsorption sites for K^+ and Na^+ and for the surface sites, which would suggest that similar sites are involved. We have already shown that the surface anionic sites have a pK value around 4.6, which is characteristic of the β- and y-carboxyl groups (Ling and Ochsenfeld, 1966). Furthermore, the bulk-phase K^+-adsorbing sites are those β- and y-carboxyl groups concentrated in the A bands (Ling, 1977a; Edelmann, 1977). These are in fact the same sites that adsorb uranium ion in an EM preparation —as confirmed by the autoradiographic study of Ling (1977a) and by the transmission electron microscope and X-ray microprobe analysis of Edelmann (Edelmann. 1977, 1978a, 1978b). Thus the dark uranium-stained double-lines referred to as the unit membrane may correspond to the β- and γ-carboxyl groups concentrated at the cell surface.

HKI Theory, A I Hypothesis, and the *Cellular* Resting Potential When Normal External K^+ Concentration is Below Normal. A logarithmic relation between external K^+ concentration and the resting potential of isolated nerves was' reported in 1900 by MacDonald (MacDonald, 1900). Curtis and Cole (1942) studied the effect of a wide range of external K^+ concentrations on the resting potential of squid axon; they noted that at external K^+ concentrations below that in the normal environment the resting potential did not continue to rise with decreasing K^+ concentration, as predicted by the Nernst equation. Instead, the potential became stabilized at a more or less constant level. Similar observations were made by Ling and Gerard (Ling and Gerard, 1950).and many others (cited in Ling, 1962).

In 1949 Hodgkin and Katz (Hodgkin and Katz, 1949), adopting Goldman's constant field theory, introduced their equation for the cellular electrical potential:

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{ex}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex} + P_{Cl} [Cl^-]_{in}}, \quad (6)$$

where P_K, P_{Na}, and P_{Cl} are the permeability constants of the cell membrane for K^+, Na^+, and Cl^-, respectively. $[Cl^-]_{ex}$ and $[Cl^-]_{in}$ are the extracellular and intracellular chloride ion concentrations, respectively. Other symbols are as defined earlier.

Later. this equation was modified and took the following form (Katz, 1966):

$$\psi = \frac{RT}{F} \ln \frac{P_K [K^+]_{in} + P_{Na} [Na^+]_{in}}{P_K [K^+]_{ex} + P_{Na} [Na^+]_{ex}}. \quad (7)$$

The theoretical justification for dropping the chloride terms was seriously challenged (Ling, 1978). although the experimental basis for this elimination was unquestioned (Hodgkin and Horowicz, 1959). For short-term experiments. at least, Equation 6 can be further simplified and generalized into the following form:

$$\psi = \text{Constant} - \frac{RT}{F} \ln \sum_{i=1}^{n} P_i [p_i^+]_{ex} \quad (8)$$

where P, is the permeaility of the ith permeant cation P_i^+. Equation 8 is identical in form to Equation 2, derived on the basis of the AI hypothesis; however, the coefficients, P_i's on the one hand. and K_i's on the other, have quite different physical bases. The results of experiments to test the predictions of each model support Equation 2 but not Equation 8 (Edelmann and Baldauf, 1971; Edelmann, 1973; Ling, 1978) and will be discussed next.

As mentioned above, both theories predict a simple logarithmic relation between ψ and external K^+ concentration at or above a normal concentration, and both explain the stabilization of ψ at K' concentration below normal, because in these experiments $[Na^+]_{ex}$ is more or less constant.

Weidemann observed that in canine Purkinje muscle fibers the resti'ng potential did not stabilize at a constant value as $[K^+]_{ex}$ continues to decrease below its normal value and as [Na'],, was held more or less constant at near its normal value in the Ringer solution (Weidemann, 1956). Instead, the potential decreased at very low external K', just as we have observed and as is shown in Figure 5. This observation was later confirmed and extended by Ruzyllo and Vick in canine Purkinje muscle (Ruzyllo and Vick, 1974) and by Gorman and Marmor in molluscan neurons (Gorman and Marmor. 1970).

In the experiments reported here, a profound difference existed in the time needed for the resting potential to attain a new equilibrium level in muscles plunged into a Ringer solution containing more or less K^+ than that in the normal Ringer solution. For high external K', the equilibrium is reached almost instantly (Ling, 1960); for low K^+, it took many hours. In sharp contrast, it took only 15 minutes of equilibration time for Ruzyllo and Vick (Ruzyllo and Vick, 1974) to observe the same low equilibrium potential at very low external K^+ concentration.

Earlier. we explained that the slow attainment of the new equilibrium level of the resting potential in low $[K^+]_{ex}$ is probably due to slow loss of K' from within the cells and the continued supply of K' to the surface potentialdetermining sites. If this interpretation is correct, there can be only one explanation for the rapid attainment in cardiac muscles of the new equilibrium level of the resting potential in low $[K^+]_{ex}$: the time needed for the loss of intracellular K^+ must be much faster. To our knowledge, there is no exact counterpart of the data shown in the inset of Figure 2 for canine heart muscles. However. Edelmann. Pfleger. and Matt did report labeled K^+ efflux of guinea pig heart muscles; the time of 90% exchange ($t_{0.9}$) at 37°C is about 50 minutes (Edelmann et al., 1971). In contrast, $t_{0.9}$ for labeled K^+ exchange at 25°C in frog muscle is about 2000 minutes (25°C)! The difference is far beyond what one would anticipate with a difference in temperature of 12°C. Thus it seems that indeed a much more rapid exchange of cell K^+ exists in mammalian cardiac muscles than in amphibian voluntary muscles.

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