

Relative Ion Permeabilities in the Crayfish Giant Axon Determined from Rapid External Ion Changes

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ABSTRACT The changes in membrane potential of isolated, single crayfish giant axons following rapid shifts in external ion concentrations have been studied. At normal resting potential the immediate change in membrane potential after a variation in external potassium concentration is quite marked compared to the effect of an equivalent chloride change. If the membrane is depolarized by a maintained potassium elevation, the immediate potential change due to a chloride variation becomes comparable to that of an equivalent potassium change. There is no appreciable effect on membrane potential when external sodium is varied, at normal or at a depolarized membrane potential. Starting from the constant field equation, expressions for the permeability ratios P_{Cl}/P_K , P_{Na}/P_K , and for intracellular potassium and chloride concentrations are derived. At normal resting membrane potential, P_{Cl}/P_K is 0.13 but at a membrane potential of -53 mv (external potassium level increased about five times) it is 0.85. The intracellular concentrations of potassium and chloride are estimated to be 233 and 34 mM, respectively, and it is pointed out that this is not compatible with ions distributed in a Nernst equilibrium across the membrane. It is also stressed that the information given by a plot of membrane potential vs. the logarithm of external potassium concentrations is very limited and rests upon several important assumptions.

The determination of the effect of changes in the ionic composition of extracellular fluid on nerve cells has been the object of many investigations. These experiments have given rise to the ionic theory of the membrane potential which has been formulated in several ways. The constant field equation for the membrane potential (Goldman, 1943) is perhaps the simplest in general form. This equation has also been derived from other assumptions (Hodgkin and Katz, 1949) and can be obtained from the equations derived

for fixed charge membranes (Teorell, 1951, 1953). The constant field equation for the potential difference V_m across a cell surface membrane is formulated as $V_m = -(RT/F) \ln [P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o] / (P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i)$ where P_K , P_{Na} , and P_{Cl} are the ionic permeabilities for potassium, sodium, and chloride; K, Na, and Cl are the ionic concentrations (activity coefficients are assumed constant) with the subscripts i and o referring to inside and outside concentrations. According to this equation, a change in any one of these factors should give rise to a predictable change in the membrane potential. The sensitivity of the membrane potential to variations in external potassium has been demonstrated many times. For sodium ions, it is postulated that sodium permeability is very low compared to that of potassium since variations in external sodium produce ordinarily only very small changes in the membrane potential. For chloride ions, the situation is less clear.

In muscle, Hodgkin and Horowicz (1959) found the resting membrane potential to be quite sensitive to variations in external chloride as well as in potassium and concluded that the permeabilities of chloride and potassium are of the same magnitude. With nerve it has generally been concluded that the cell membrane behaves as if the chloride permeability is low. This conclusion is based in part on observations that at high potassium concentrations the cell membrane appears to behave as a potassium electrode with the membrane potential changing almost 58 mv for a tenfold change in external potassium.

This paper describes a systematic study of the importance of sudden changes in potassium, sodium, and chloride ions to the membrane potential. Results will be presented which support the idea that at resting membrane potential the chloride permeability is low compared to that of potassium. In contrast to this, the chloride permeability in the depolarized nerve is found to be the same order of magnitude as the potassium permeability despite the observation that the steady-state membrane potential appears potassium-dependent. Some tentative conclusions about the distribution of chloride and potassium across the cell membrane will be drawn.

THEORY AND METHODS

A. *Theory*

In these experiments, one of the external ion concentrations is suddenly changed while the others are kept constant and the resulting change in membrane potential is examined. If this process is repeated for each of the external ions some interesting conclusions can be obtained from analysis using the constant field equation. Assuming the constant field equation to be valid we have

$$V_m = -(RT/F)\ln[(K_i + r_2Na_i + r_1Cl_o)/\quad (K_o + r_2Na_o + r_1Cl_i)] = -(RT/F)\ln\xi \quad (1)$$

where $r_1 = P_{Cl}/P_K$ and $r_2 = P_{Na}/P_K$.

For a small variation in external potassium ions (assuming that all other parameter variations are of negligible importance and can be neglected)

$$\partial V_m/\partial K_o = +(RT/F)[1/(K_o + r_2Na_o + r_1Cl_i)]. \quad (2)$$

We now define:

$$T_K = [1/(RT/FK_o)] \cdot (\partial V_m/\partial K_o) = K_o/(K_o + r_2Na_o + r_1Cl_i) \quad (3)$$

where T_K is the potassium-dependent partial potential ratio. It also follows that

$$T_K = [1/(RT/FK_o)](\partial V_m/\partial K_o) = \partial V_m/\partial V_K \cong \Delta V_m/\Delta V_K \quad (4)$$

where the change in the potassium potential ΔV_K is

$$\Delta V_K = (RT/F)\ln(K_{o2}/K_{o1}) \quad (5)$$

where K_{o1} and K_{o2} are the initial and final external concentrations of potassium respectively. Equation 5 does not imply in any way a Nernst relation for the membrane and is only used to formalistically relate K_{o2} and K_{o1} . Similarly

$$T_{Cl} = [1/(RT/FCl_o)] \cdot (\partial V_m/\partial Cl_o) \\ = r_1Cl_o/(K_i + r_2Na_i + r_1Cl_o) \cong \Delta V_m/\Delta V_{Cl} \quad (6)$$

$$T_{Na} = [1/(RT/FNa_o)](\partial V_m/\partial Na_o) \\ = r_2Na_o/(K_o + r_2Na_o + r_1Cl_i) \cong \Delta V_m/\Delta V_{Na}. \quad (7)$$

Utilizing equations (1), (3), (6), and (7) we obtain

$$P_{Cl}/P_K = r_1 = T_{Cl}K_o\xi/T_KCl_o \quad (8)$$

$$P_{Na}/P_K = r_2 = T_{Na}K_o/T_KNa_o \quad (9)$$

$$Cl_i = (1 - T_{Na} - T_K)Cl_o/T_{Cl}\xi \quad (10)$$

$$T_KK_i/K_o + T_{Na}Na_i/Na_o = (1 - T_{Cl})\xi \quad (11)$$

if $T_{Na}Na_i/Na_o$ is small equation (11) gives

$$K_i = (1 - T_{Cl})K_o\xi/T_K \quad (12)$$

from equations (3), (6), and (7)

$$T_K + T_{Na} + T_{Cl} = [\xi(K_o + r_2Na_o) + r_1Cl_o]/\xi(K_o + r_2Na_o + r_1Cl_i). \quad (13)$$

$$\text{If } Cl_o \geq \xi Cl_i, \text{ then } T_K + T_{Na} + T_{Cl} \geq 1. \quad (14 a)$$

$$\text{If } Cl_o \leq \xi Cl_i, \text{ then } T_K + T_{Na} + T_{Cl} \leq 1. \quad (14 b)$$

If $T_K + T_{Na} + T_{Cl} = 1$, equations (1) and (13) give

$$\xi = Cl_o/Cl_i = (K_i + r_2Na_i)/(K_o + r_2Na_o) \quad (15)$$

and chloride is distributed according to the Nernst relation across the cell membrane. If the sodium terms can be neglected in equation (15), potassium is also distributed according to the Nernst relation.

From the above equations, the relative ion permeabilities (equations 8 and 9) and the intracellular chloride concentration (equation 10) can be obtained from variations in external ion concentration when the corresponding membrane potential change is recorded. The determination of intracellular potassium or sodium is not uniquely available since equation (11) contains both ions as variables. Fortunately, however, the contribution of the sodium term is experimentally small in these experiments and therefore intracellular potassium can be calculated from equation (12).

B. Methods

MATERIAL Giant axons from the ventral nerve cord of the crayfish *Procambarus clarkii* were used over a period from February through September. The ventral nerve cord was dissected free from the animal, transferred into a plastic chamber containing physiological saline, and mounted so that the medial giant axons lay upward (Fig. 1). The general anatomy of crayfish ventral nerve cord has been described by Johnson (1924) and Furshpan and Potter (1959). The external sheath around the right or left nerve cord was removed between the second to seventh abdominal ganglia (counting distal from the brain). One medial giant axon was isolated from other axons between the second and fifth ganglia. The ganglia themselves were often only partially removed since their complete removal occasionally damaged the axon. The external diameters of the axons were usually between 125 and 175 μ . The length of mounted nerve between holding spring clamps was 3 cm while the cleared region of the axon varied from 9 to 14 mm depending on the animal size.

SOLUTIONS The physiological saline (Van Harreveld, 1936) had the following composition in mM: NaCl 205, KCl 5.4, CaCl₂ 13.5, MgCl₂ 2.6, NaHCO₃ 2.3. pH was adjusted to 7.4 by adding small amounts of HCl. In experiments in which the external potassium concentration was varied, sodium was exchanged for potassium in equal amounts to maintain constant ionic strength and osmotic pressure. For solutions utilizing low sodium, choline or Tris (hydroxymethyl) aminomethane was substituted directly for sodium. In the solutions containing low chloride, glucuronate was substituted in equal amounts for chloride. Chloride was varied in different amounts depending on the concentration of external potassium. Although not necessary for the experiment, the chloride concentration shifts were such that the percentage change in a chloride substitution equaled that being made in a corresponding potassium substitution. Thus if the external potassium concentration was varied

from 5.4 mM to 10 mM keeping chloride concentration constant, the amount that chloride was changed in a chloride shift, with potassium kept constant, was from 242.6 mM to 131 mM. The sodium activity, as measured with a sodium-sensitive glass electrode (Beckman 3927B), was the same in the normal chloride and substituted chloride solutions. This indicated unchanged activities for the unsubstituted ions. In addition, the measured osmotic pressures for the various solutions were in agreement to within 1 to 2% (average osmotic pressure of Van Harreveld's solution was 435 milliosmols as measured by freezing point depression).

ELECTRODES (a) In order to avoid movement artifacts during the rapid exchange of solution, the membrane potential was recorded with a cannulating microelectrode. Long tapering microelectrodes were pulled from Pyrex glass capillaries, bent into an L shape about 1 cm from the tip, and filled with 3 M KCl. A small ball of insulating

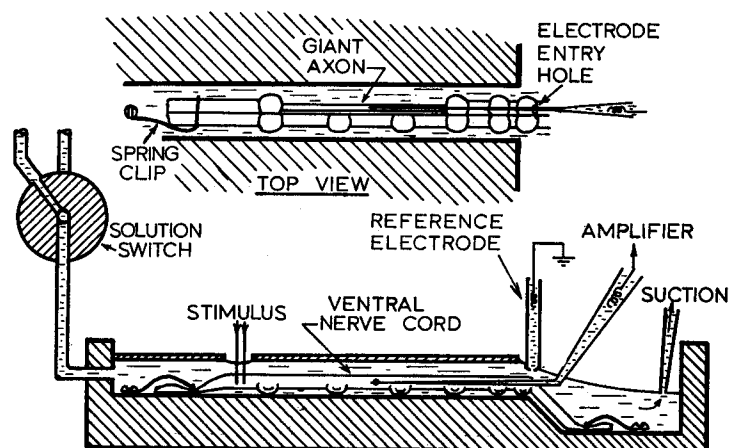


FIGURE 1. Chamber for cannulating axon and rapidly changing external solutions.

lacquer was applied just back of the tip to facilitate entry into the axon and prevent the tip from hitting the cell wall. Microelectrode tip diameters were around 1μ with resistances around 10 megohms. (b) The intracellular chloride activity was sometimes recorded simultaneously with the membrane potential. This was done by a micro-Ag-AgCl electrode introduced into the axon together with the potential recording microelectrode. The manufacture of such double electrodes has been described elsewhere (Strickholm and Wallin, 1965). (c) The reference electrode was a Ag-AgCl electrode brought into contact with the bath via a 3 M KCl conically tapered agar bridge (Appendix 2).

EXPERIMENTAL PROCEDURE The giant axon was cleared from other axons in the chamber shown in Fig. 1. A hole was cut into the axon, usually at ganglion 6 (counting distal from the brain), and the microelectrode was advanced 6 to 10 mm into the axon to its recording position between the third and fourth ganglion. Before the electrode had been inserted 3 mm from the entrance hole, the resting potential had reached 95% of its final value. The length constant for the axons was measured

to range around 1.0 mm. Therefore the cleared region of the axon was at least nine length constants long and potential measurements taken at the center of the cleared region were little affected by potentials at the hole or slowly changing potentials at the uncleared region of the axon. This was important since potential changes were damped and slower to respond if the axon was cleared of adjacent axons for only one ganglion segment. As a rule the entrance hole was filled and sealed off by the widening part of the electrode shank. This helped to minimize the potential contribution of the hole. To the micropipette electrode was connected a Ag-AgCl electrode which led to an input capacity compensating electrometer amplifier of unity gain (Strickholm, 1961). The intracellular potentials were recorded simultaneously on an oscilloscope and a pen recorder.

The resting potential recorded was usually from -79 to -87 mv (at 5.4 mM external potassium) and did not differ from the potentials recorded with a conventional transmural microelectrode. The action potential was in the range of 110 to 130 mv. However, since the microelectrode was long and immersed in the solution, stray capacitance to ground was distributed and not lumped. The true value of the action potential was probably not always recorded due to inherent limitations of electronic compensation for a distributed input capacity. The method utilized here for recording transmembrane potentials proved vastly superior to the conventional transmural microelectrode method. Axon survival time was sometimes in excess of 12 hr if the surface membrane had never been penetrated. The condition of the nerve was considered satisfactory, if at the end of the experiment it still gave action potentials or if depolarized by high potassium, it had a stable steady-state membrane potential.

Different external solutions could be selected via a valve and removed by suction from the distal end of the bath (Fig. 1). A slow flow was maintained to keep the external solution composition constant. If flow stopped, the membrane potential gradually fell several millivolts probably due to potassium leakage from the cut axons of the ventral nerve cord and the reference pipette. The membrane potential recovered if solution flow was resumed. When external solutions were changed, the flow was increased maximally and maintained for some time allowing the whole volume of the bath to be exchanged in 0.2 sec. Experiments were done at room temperature which averaged 20°C .

RESULTS

A. *Variation of External Potassium*

External potassium was suddenly changed stepwise from one of the concentration levels of 2.5, 5.4, 10, 16.7, 25, 40, 75, and 100 mM to another. In each case the sodium concentration was reduced by an equivalent amount. Equation (2) requires that the effect on potential due to a sodium variation be minimal. Large shifts in the external sodium concentration, when sodium was replaced with choline or Tris ions, hardly affected the membrane potential. Therefore these small stepwise changes in sodium concentration during each potassium change were considered unimportant. An example of such a stepwise change is shown in Fig. 2 where K_o is changed from 5.4 to

25 mM. Whenever external potassium was changed, the membrane potential changed in two phases. The first phase was a rapid potential shift requiring 10–30 sec for completion. This was followed by a second slower potential shift requiring 10–20 min before a stable or steady-state potential was reached.

The asymptote of the initial rapid phase of the potential change corresponds to ΔV_m in equation (4). Theoretically it should be a very abrupt change completed within milliseconds; i.e. before any changes in intracellular concentrations occur. In the experiments, however, this phase lasted 10 to 30 sec. (Compare Hodgkin and Horowicz (1959) where in muscle the initial potential change occurred in a few seconds.) The crayfish axon anatomy suggests that the initial rapid potential change most likely represents the time required for ions to diffuse around surface satellite cells or through

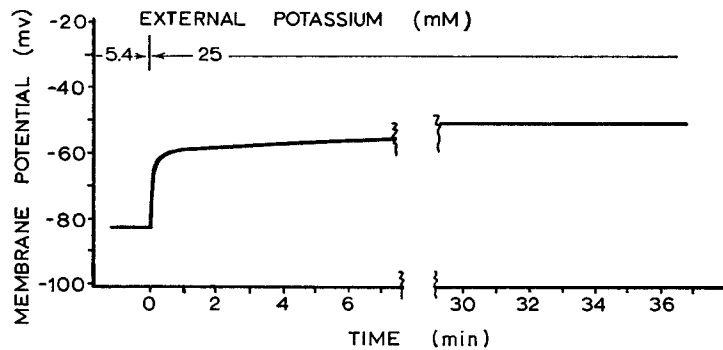


FIGURE 2. The change in membrane potential resulting from a sudden change in external potassium concentration. External chloride concentration kept constant at 242.6 mM. In the two potassium solutions the sodium concentration was adjusted so that the concentration of sodium plus potassium remained constant.

channels to reach the axon membrane surface. If free diffusion to the axon surface were assumed, the experimental data would require an unstirred water layer in excess of 100 μ . Under light microscopy the satellite cell layer appeared to be approximately 10 μ thick. When saline solutions containing sucrose were exchanged for the normal saline, turbulence was seen at the surface of the satellite cells indicating that an unstirred water layer of 100 μ thick could not be present. Similar observations indicating diffusion of ions along channels through surface satellite cells have been reported for the squid axon (Villegas, Caputo, and Villegas, 1962).

If ion diffusion is through channels one obtains (Appendix 1):

$$C_s(t) \cong C_f - (C_f - C_o)e^{-kt} \quad (16)$$

where $C_s(t)$ is the concentration at the axon membrane surface at time t , C_o is the initial external saline concentration, C_f is the final substituted external

solution, and k is a hybrid diffusion constant. The agreement of the postulates of the diffusion model with experiment has been quite good and a comparison with the experimental data and the theoretical calculation is shown in Fig. 3.

The second phase or slow drift in membrane potential has been postulated to result from the movement of ions across the cell surface in order to restore the electrochemical steady states which had been displaced as a result of the external ion shift. That such ionic readjustments occur after an external ion

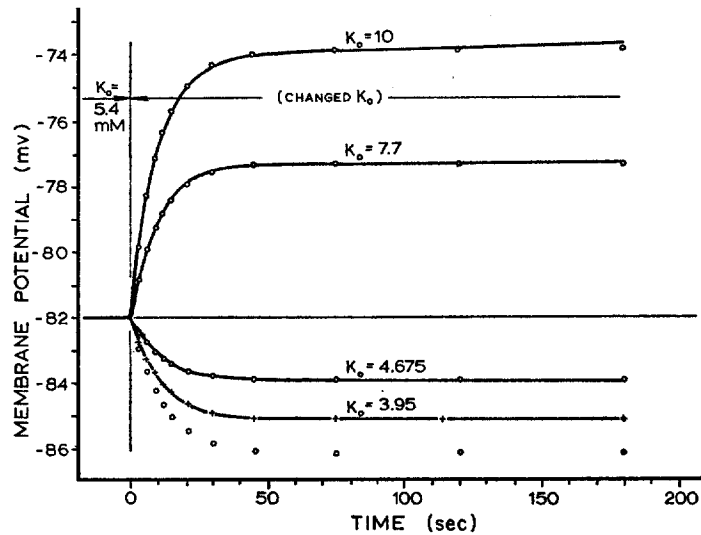


FIGURE 3. The early transient change in membrane potential resulting from external potassium shift (solid lines). The circles are theoretical fits of the expected potential change based on diffusion of ions around surface satellite cells where $T_K = 0.527$ and $k = 0.09$. Agreement is satisfactory except for $K_o = 3.95$ mM and lower potassium changes (see text). If for $K_o = 3.95$ mM, T_K changes to 0.393, agreement of theory and experiment is again obtained without changing the diffusion parameter $k = 0.09$ (crosses).

shift, has been verified by continuous measurements of intracellular chloride activity with a micro-Ag-AgCl electrode (see Fig. 10).

At low external potassium levels (up to 10 mM) the time course of the early diffusion and later ionic readjustment phases is normally separable and the change in membrane potential (ΔV_m) resulting from a potassium shift (ΔV_K) was taken as that immediately following the early phase. An error may be introduced here since the intracellular ion concentrations may already be changing during the early diffusion phase. However, intracellular chloride activity measurements show little change with external ion shifts at the low potassium levels. In Fig. 3, where potassium was shifted from 5.4 to 10 mM, the change in membrane potential ΔV_m was 8.2 mv.

Since the change in potassium potential for this shift (equation 5) was 15.5 mv, the potassium-dependent partial potential ratio T_K was 0.53. At high external potassium levels (25 mm and above), the separation between the two phases was not always distinct. For these situations, the experimental potential changes at the early times were measured and from these data a theoretically expected "zero time" potential difference was calculated from equations (1) and (16). The usefulness of this calculation may, however, be limited since intracellular chloride activities were found to change during the early diffusion phase for ion shifts at the high potassium levels (see Fig. 10).

Equation (4) requires very small incremental ion changes and is independent of the direction of the ion shift. Experiments were performed in which

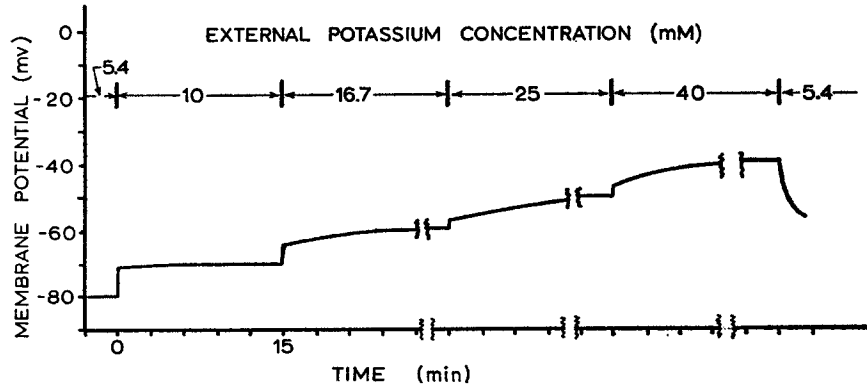


FIGURE 4. The change in membrane potential occurring with stepwise increasing concentrations of potassium. Each potassium shift was made after the membrane potential acquired a steady state. External chloride concentration was kept constant in each of the solutions.

potassium was increased and decreased in various amounts around external potassium concentrations of 5.4 and 40 mm. The experimental T_K , although different for potassium at 5.4 and 40 mm, was the same for small changes around each concentration and for the largest increases used in this experiment. However, deviations occurred with large potassium decreases, a smaller T_K being obtained than with potassium increases. Therefore the experimental procedure of these experiments, in which potassium was suddenly increased in steps from 5.4 to 10 to 16.7 to 25 to 40 to 75 to 100 mm, was felt to be justified in that T_K calculated from these large shifts was experimentally equivalent to T_K determined from small incremental changes around each potassium starting point (see also Fig. 3).

Fig. 4 shows an experiment in which external potassium is changed from 5.4 to 10 to 16.7 to 25 to 40 mm. Each potassium shift was made after the membrane potential had acquired a steady state. The shape of the potential

change is apparently different at different levels of membrane potential. When potassium was changed from 5.4 to 10 mM, the initial rapid potential change was relatively large compared with the later slow change. The later slow change usually did not exceed 1–2 mv and was sometimes almost absent. In contrast to this, when potassium was changed at higher levels the rapid initial potential change was reduced and the later slow potential change was as large or larger. The appearance of the slow potential change was correlated with changes in intracellular chloride activity. The axon

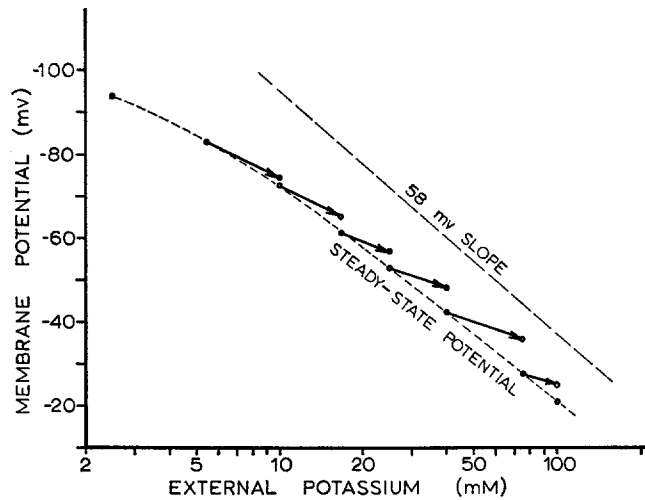


FIGURE 5. The initial membrane potential change at different external potassium levels of 5.4, 10, 16.7, 25, 40, and 75 mM where potassium was suddenly shifted to the next higher concentration. The arrows show the immediate change in membrane potential after each potassium shift while the dashed line through the circles represents the steady-state membrane potential. External chloride concentration and the sum of external potassium and sodium concentrations were kept constant.

membrane behaves so that as external potassium is increased, the immediate response of the membrane to a potassium change becomes proportionately smaller. However, if the *steady-state membrane potential* is plotted against $\log K_o$, the membrane acts as if it were potassium-dependent (Fig. 5). Thus when external potassium is changed from 25 to 40 mM, the initial slope at early time is typically 23 mv per tenfold change in potassium concentration, and only after a lapse of time does it become 58 mv. The experimental data tabulating the variation of T_K with external potassium are in Table I and Fig. 7. No corrections were made for changes in liquid junction potentials at the reference pipette since these potential changes are minimal for each potassium shift.

B. Variation of External Chloride

At each different potassium level, chloride was varied, substituting glucuronate for chloride, while potassium was kept constant. Fig. 6 shows a series of such chloride variations. The membrane potential changes show two phases, an early rapid change and a later slower potential change. The early potential change is complex and consists of the membrane potential change and two additional phase boundary potentials of 3–4 mv. The phase boundary potential appears between the low chloride and the normal chloride solutions at the axon satellite cell surface and at the tip of the reference electrode.

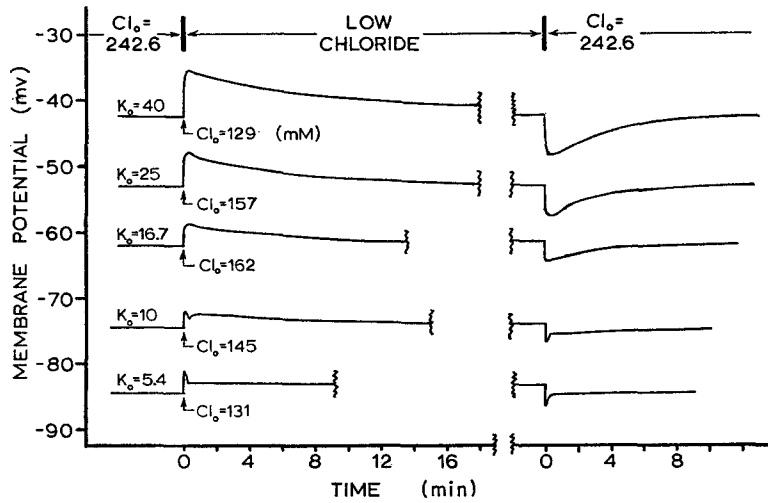


FIGURE 6. The effect of sudden external chloride concentration changes on the membrane potential at different potassium levels. Chloride ion was replaced by glucuronate ion. Sodium and potassium concentrations were kept constant during each chloride shift. The early potential oscillations (seen best at $K_0 = 5.4$ and 10) result from liquid junction potential transients at the axon surface and reference electrode (see Appendix 2).

With cylindrical reference electrodes, this phase boundary potential can last many minutes and the time course depends on how long the reference electrode has stayed in the normal solution before a chloride shift. This long lasting artifact of 3–4 mv seriously altered the apparent membrane potential dependence on chloride. Its reduction was achieved by using a reference pipette of large solid angle taper and small tip diameter (Appendix 2). With reference pipette tip diameters less than 0.1 mm and half-angle tapers of 20 to 30 degrees, the time required to approach a stable reference electrode potential has been around 10 sec. The phase boundary artifact across the axon satellite cells could not be reduced and lasted as long as the early potential change.

With a rapidly responding reference electrode, the curves are all of the same character, namely an initial 10–30 sec rapid change in potential and a later exponential drift back toward a potential near the starting value. Analogous to the potassium case, the completion of the initial rapid potential change corresponds to ΔV_m of equation (6) and the time it takes for completion is explained as diffusion time around satellite cells to the axon surface. The later potential change is again interpreted as due to transmembrane ionic movements as a result of displacing the steady-state concentration gradients of the axon. Analogous to the potassium shifts, the later slow potential phase is separable from the early phase for chloride variations at low potassium levels (Fig. 6). At high potassium levels, the initial change merges with the later slow potential change and the membrane potential dependence, ΔV_m , on chloride ions was difficult to determine. To overcome this uncertainty the following approaches were used:

(a) External chloride was returned to its initial value early in the later potential phase and T_{Cl} calculated from the early potential change, ΔV_m , of the returning membrane potential. Justification for this procedure rests first on observations showing that if chloride is returned to its initial value soon after the early diffusion period, intracellular chloride activity has not shifted much. Upon return to normal chloride, the later potential change which involves intracellular ion adjustments is therefore almost absent. The early potential phase is thus better separable during this return to normal chloride than when chloride was initially decreased. Second, were observations showing that at lower potassium concentrations where T_{Cl} could be accurately measured, T_{Cl} was the same for the initial change and return in external chloride provided the return was made soon after the initial potential transient period.

(b) It was found that the later part of the slow potential change after a Cl shift could be approximated as an exponential decay curve. Therefore the logarithm of $V_m(\infty) - V_m(t)$ was plotted against time and extrapolated to give the desired zero time membrane potential from which ΔV_m was obtained.

Agreement between the two methods was good. At each of the potassium levels, the observed change in potential after a chloride shift was corrected for the change in steady-state liquid junction potential at the reference electrode using Henderson's equation (Henderson, 1907). The following corrections were added to the voltage changes observed at each potassium level, $K_o = 5.4$, + 0.8 mv; $K_o = 10$, + 0.7 mv; $K_o = 16.7$, + 0.6 mv; $K_o = 25$, + 0.7 mv; $K_o = 40$, + 0.8 mv.

Experiments were performed to determine whether T_{Cl} values determined from the chloride shifts utilized here were equivalent to small chloride

shifts as required by equation (6). These experiments were possible with accuracy only at low external potassium ($K_o = 5.4$ mM) where the initial early and later potential changes were clearly separable. To increase or decrease external chloride without changing osmotic pressure or ionic strength, the axon was equilibrated at an intermediate chloride concentration of 187 mM where glucuronate was substituted for chloride. External chloride was changed in various amounts up to its normal concentration of 242.6 mM and also down to 131 mM. The observed potential changes, when corrected for the liquid junction potential at the reference electrode, gave identical T_{Cl} 's. Similarly, if the axon was equilibrated in normal chloride

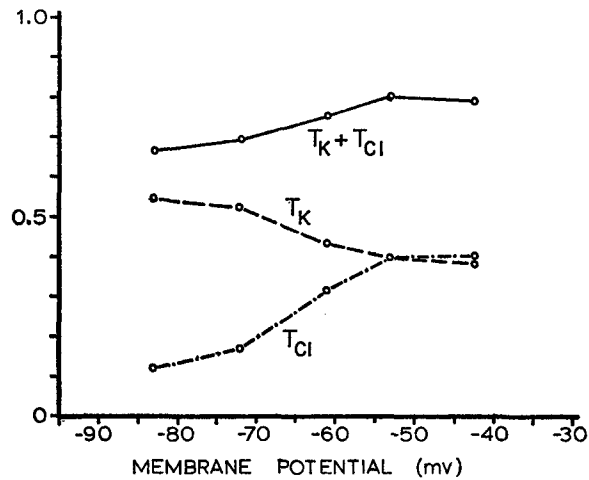


FIGURE 7. The variation of the ion-dependent partial potential ratio T with the membrane potential.

of 242.6 mM and chloride was decreased in various amounts, T_{Cl} for small and large chloride decreases was constant. These experiments support the supposition that the results obtained with the large chloride shifts in these experiments are equivalent to those with small chloride shifts around each initial level.

Fig. 6 shows that the sensitivity of the membrane to changes in chloride concentration varies considerably depending on the external potassium concentration. At normal resting potential ($K_o = 5.4$ mM) the membrane was initially depolarized only 1–2 mv when chloride was decreased to 53% of the normal value. In contrast to this, at an external potassium level of 25 mM, the initial depolarization was 4–5 mv when chloride was reduced to 63% of the normal. The collected results showing the variation of T_{Cl} with external potassium are given in Table I and Fig. 7.

C. Variations of External Sodium

Sodium was reduced to 115 mM by direct replacement with Tris (hydroxymethyl) aminomethane or choline ions at potassium levels of 5.4 and 25 mM. Results with these ions at either of these potassium levels were essentially the same. When external sodium was suddenly reduced, the membrane potential became more negative by about 0.4 mv after an initial transient. This corresponds closely to the potential change expected for the liquid junction potential at the reference pipette as calculated from Henderson's equation (Henderson, 1907). The observed change in membrane potential was estimated therefore as no more than 0.7 mv. Since the change in the sodium potential ΔV_{Na} was 14.6 mv, T_{Na} was estimated as being not more than 0.05.

TABLE I

K_o	$-V_m \pm (sd)$	$T_K \pm (sd)$	$T_{Cl} \pm (sd)$	T_{Na}^*	P_{Cl}/P_K	P_{Na}/P_K^*	Cl_i	K_i
mM	mv						mM	mM
2.5	94.0±2.7 (5)							
5.4	83.0±3.8 (45)	0.55±0.09 (43)	0.12±0.05 (37)	0.05 (6)	0.14	24×10 ⁻⁴	34	233
10	72.1±2.6 (7)	0.52±0.08 (7)	0.17±0.03 (7)		0.24		39	279
16.7	61.1±2.1 (7)	0.44±0.06 (7)	0.32±0.06 (7)		0.57		38	292
25	53.1±1.8 (16)	0.40±0.06 (14)	0.40±0.05 (14)	0.05 (5)	0.85	17×10 ⁻³	44	309
40	42.3±2.1 (15)	0.39±0.06 (12)	0.40±0.04 (4)		0.93		69	330
75	27.8±2.3 (5)	0.38±0.07 (4)						
100	21.0 (1)							

The number of measurements are given in parentheses.

sd = one standard deviation.

* These values represent an upper limit.

D. Compiled Results and Other Observations

When the results obtained for the membrane potential V_m , and for the ion-dependent partial potential ratios, T_K , T_{Cl} , and T_{Na} were utilized, the permeability ratios P_{Cl}/P_K and P_{Na}/P_K were calculated (equations 8 and 9). The variation of the permeability ratios with membrane potential and external potassium, is shown in Table I and Fig. 8. It is seen in Fig. 8 that the chloride permeability becomes equal to the potassium permeability in that part of the curve where the slope is close to a 58 mv difference for a tenfold change in potassium.

From equations (10) and (12) the intracellular concentrations of chloride and potassium were calculated and compared with values expected if these ions were distributed in electrochemical equilibrium (Fig. 9). The results suggest that potassium and chloride ions are not in electrochemical equi-

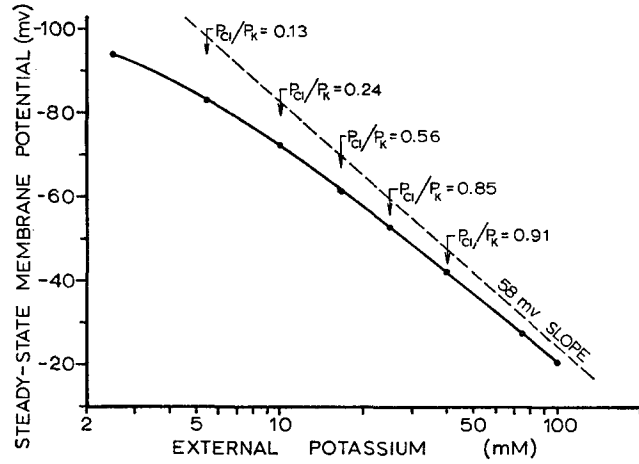


FIGURE 8. The relation of the steady-state membrane potential, and the permeability ratio of chloride to potassium, to the external potassium concentration.

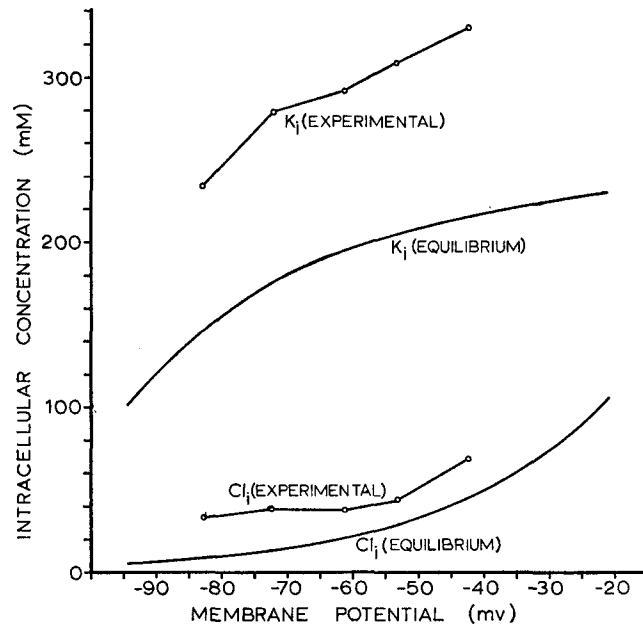


FIGURE 9. Comparison of the expected equilibrium concentrations of intracellular potassium and chloride with the experimentally calculated concentrations as a function of membrane potential. Initial starting conditions were at $K_o = 5.4$ mM and at -83 mv membrane potential. The membrane was depolarized by increasing external potassium concentration. External chloride concentration and the sum of external potassium and sodium concentration were kept constant. The equilibrium concentrations were calculated from the external potassium and chloride concentrations and the observed membrane potential at each potassium concentration.

librium across the membrane at normal resting potential and that with each change in extracellular potassium there is a change in intracellular concentrations. In order to establish the latter condition by a direct measurement, experiments were performed in which a micro Ag-AgCl electrode for recording chloride activity was inserted into the axon together with the potential-recording micropipette. Axon survival was, for unknown reasons, rarely more than 15–20 min in these experiments and therefore usually only one change in external concentration was possible in each experiment. Axon failure was readily recognized by a loss of the action potential, a decreasing membrane potential, and a steady increase in intracellular chloride activity which approached that of extracellular chloride. The results obtained before axon failure gave an intracellular chloride concentration of 35 mM at normal resting potential (Strickholm and Wallin, 1965), a result which compares favorably with the value of 34 mM calculated with equation (10) from these experiments. When external potassium was shifted from 5.4 to 10 mM, the intracellular chloride activity showed a small increase varying from 0 to 2 mM. At higher initial external potassium levels, the intracellular chloride activity showed a larger increase for a corresponding increase in extracellular potassium. These results are in qualitative agreement with the results of Fig. 9 and substantiate the view that intracellular concentrations do change with changes in the external potassium level.

Since it was experimentally found that the sodium terms in equation (13) can be neglected, equations (13) and (15) predict that if potassium and chloride are in equilibrium across the membrane, the sum of T_K and T_{Cl} should equal unity. The collected experimental data of Table I and Fig. 7 show that they add up to only 0.67 indicating also that the concentrations of intracellular potassium and chloride are not in electrochemical equilibrium. An additional check to test whether potassium and chloride are in electrochemical equilibrium would be to change external potassium and chloride simultaneously, keeping the product $K_o \times Cl_o$ equal to a constant. This follows from the equilibrium relation $K_o \times Cl_o = K_i \times Cl_i$, where a simultaneous change in external chloride and potassium should not change the intracellular concentrations if the product of the external ions is kept constant and potassium and chloride are in equilibrium. Also the change in membrane potential should remain constant after the initial transient and should equal the change in the Nernst potential $(RT/F) \ln (K_{of}/K_{oi})$, where K_{of} and K_{oi} are the final and initial external potassium concentrations.

Fig. 10 shows one of several experiments in which external chloride and potassium were simultaneously exchanged, with the product $K_o \times Cl_o$ kept constant. After the external solution was exchanged, the membrane potential depolarized and then recovered by a few millivolts. Simultaneous with this potential recovery phase was a loss of intracellular chloride as detected by

an intracellular Ag-AgCl electrode. This chloride loss was in contrast to the chloride gain observed whenever extracellular potassium alone was increased. Occasionally seen much later in time was an additional slow depolarization of a few millivolts. In Fig. 10, the initial change in membrane potential was from -87 to -57 mv, or a 30 mv change. The calculated change in the ion potentials of external chloride and potassium was 37.1 mv,

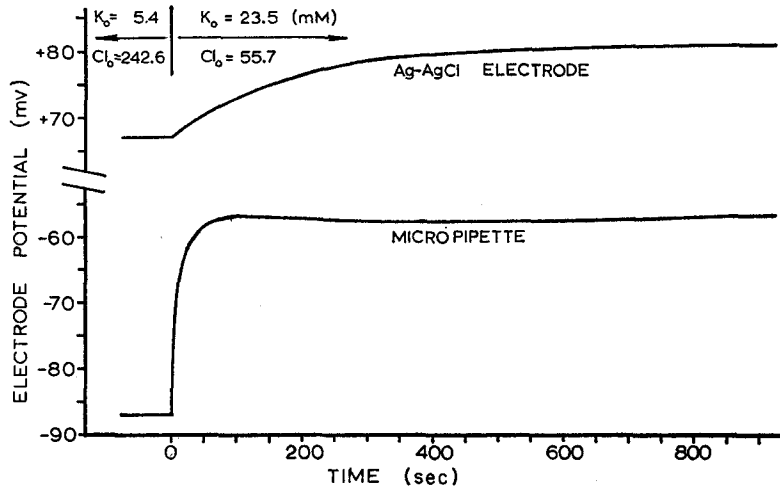


FIGURE 10. The variation of membrane potential and intracellular chloride activity as a result of a sudden simultaneous change in external chloride and potassium concentrations. The product of external chloride and potassium was kept constant. Chloride ion was replaced by glucuronate ion and the sum of potassium and sodium was kept constant. The potentials were recorded with an intracellular micropipette electrode and an intracellular Ag-AgCl electrode. The micropipette potentials are referred to the external bath reference electrode while the reference for the Ag-AgCl electrode was the intracellular micropipette. Zero potential reference for the Ag-AgCl electrode was taken as the potential when the Ag-AgCl and micropipette electrodes were immersed in crayfish saline ($Cl_o = 242.6$ mM). From the potentials of the Ag-AgCl electrode, initial intracellular chloride concentration was calculated as 16.9 mM which changed to 9.7 mM after the external ion substitution. At $K_o = 5.4$ mM, $T_K = 0.58$ and $T_{Cl} = 0.19$. Using this data and equation (10), Cl_i is found to be 16.6 mM which agrees with the Ag-AgCl electrode determination of intracellular chloride.

which is greater than the observed 30 mv change in membrane potential. These constant product experiments therefore additionally substantiate the observation that potassium and chloride ions are not in electrochemical equilibrium across the cell membrane.

DISCUSSION

The effect of variation of external ions on nerve resting potential has been described many times (Curtis and Cole, 1942; Hodgkin, 1951; Huxley and

Stämpfli, 1951; Dalton, 1959). It has generally been concluded that the potential is controlled mainly by potassium, whereas sodium, chloride, and other ions are of little importance. Thus, internal perfusion of squid axons with chloride-free solutions does not alter the membrane potential more than a few millivolts (Baker et al., 1962). The experiments described here confirm that sodium and chloride do not contribute much to the normal resting potential. However, if the membrane is depolarized by increasing the outside concentration of potassium, the sensitivity of the membrane potential to chloride variations increases markedly (Figs. 6 and 7), while the sensitivity to potassium decreases and the sensitivity to sodium remains low. The experiments clearly illustrate that one cannot use the 58 mv slope per tenfold concentration change observed here in a plot of steady-state membrane potential vs. $\log K_o$, to claim that the membrane is *predominantly* potassium-permeable. It must also be stressed that a 58 mv slope in such a plot is valid only when the intracellular ion concentrations are constant throughout the experiment. This seems to be fairly well established in striated muscle cells (Boyle and Conway, 1941; Adrian, 1956; Conway, 1957) but it cannot be taken for granted in all cell types. Thus in the crayfish axons used here, the internal chloride activity, as measured with a micro Ag-AgCl electrode, increases after a potassium increase on the outside. In heart muscle cells Carmeliet and Janse (1965) have shown that the intracellular chloride concentration increases when the external potassium concentration is raised. They were also unable to find any large increase in intracellular water at high extracellular potassium and conclude that the results are at variance with the observations in frog skeletal muscle. Lieberman (1966) measured the diameter of crab axons while varying the outside concentration of potassium and recorded no consistent change in diameter until the potassium concentration was raised three to five times. If this is applicable to the crayfish axon, it probably means that intracellular concentrations increase at the lower potassium levels because of transmembrane ion movements and not because of changes in cell volume. To clarify these points, measurements of intracellular concentrations and osmolarity are in progress.

The theory of this paper depends on the validity of the constant field equation (equation 1) which is based on a zero net membrane current. If nonzero currents flow during an ion shift, the derived equations may not be meaningful except perhaps the operational definitions ($\partial V_m / \partial V_K$, etc.) for T_K , T_{Na} , and T_{Cl} . At resting membrane potentials, the observed intracellular chloride change, after potassium was changed from 5.4 to 10 mM, was small. The results obtained here for intracellular chloride with equation (10) and from chloride activity measurements (Strickholm and Wallin, 1965) are in good agreement which supports the theory of this paper at normal potassium level. At high potassium elevations, the calculated intracellular

potassium (equation 12) appears to be in error since an osmotic pressure difference would be required which could not be supported by the cell. Additional measurements of intracellular concentrations (Wallin, 1966) seem to agree with concentrations calculated from equations (10) and (12) at normal potassium levels. However, considerable deviations occur between calculated and measured intracellular concentrations at elevated external potassium. Since it was observed that intracellular chloride changed almost as fast as the early potential change at high potassium levels, the applicability of the equations appears limited for those situations in which membrane permeabilities are known to be high and net currents may not be zero (Hodgkin and Huxley, 1952). The requirement of constant permeabilities places other restrictions on the equations. Experimentally T_K was found constant for various small potassium shifts at $K_o = 5.4$ and 40 mM. However T_K does change with time during the later slow potential shift. This suggests that for short times T_K is independent of small potential variations. Similar results were obtained for T_{Cl} . Equation 8 gives $P_{Cl}/P_K = T_{Cl}K_o\xi/T_KCl_o$, which suggests that if T_K and T_{Cl} are constant around each potential point, the permeability ratio P_{Cl}/P_K , and perhaps the permeabilities, remain constant during the early period of the rapid ion shifts. On the other hand voltage clamp studies (Hodgkin and Huxley, 1952) have shown that permeabilities change within milliseconds with sufficient membrane depolarization. The interpretations of this paper may therefore not be valid for those situations in which membrane conductances are known to undergo rapid changes.

The experiments do not give any explanation for the change in membrane characteristics between the normal and the depolarized nerve. Several factors could be responsible, e.g. the lowered potential field itself or the increased K concentration on the outside. Tobias (1959) suggests that K in addition to its depolarizing effect possibly could change membrane structure and thus alter permeabilities. Such a possibility does not seem altogether unreasonable in view of experiences with cation and anion effects on protein structure and solubility.

The data suggest that neither potassium nor chloride is distributed in equilibrium with the membrane potential. Intracellular potassium is calculated to be 233 mM but, if potassium were in equilibrium the concentration would have been 145 mM. Similarly, intracellular chloride was found to be 34 mM while its equilibrium concentration is 9 mM (see Fig. 9). This is in accordance with other experimental findings. Mauro (1954) and Keynes (1963) have presented evidence that indicates that chloride is not in electrochemical equilibrium in squid axons. Similar findings are reported for heart muscle by Carmeliet and Janse (1965) and for intestinal smooth muscle by Goodford (1964). These results are compatible with the findings of this paper and support the idea that chloride is actively transported. The general

conclusion would be that ion permeability ratios or intracellular ion concentrations of sodium, potassium, or chloride cannot be estimated from a knowledge only of steady-state membrane potentials and external concentrations. Therefore, studies on cell membranes which show a 58 mv change in steady-state membrane potential for a tenfold external potassium change do not necessarily indicate a membrane which is predominantly potassium-permeable.

Appendix 1

DIFFUSION OF IONS TO THE AXON SURFACE

This model assumes that diffusion occurs along channels or paths around the surface satellite cells and connective tissue which surrounds the axon (Fig. 11). For the small potassium changes such as 5.4–10 mM, the phase boundary or liquid junction potential between the two potassium solutions is well below a millivolt. Consequently, the effect of the voltage field on diffusion may be ignored and ion diffusion may be approximated as that of a nonelectrolyte. With the large chloride shifts, the junction potentials may amount to 3 or 4 mv. For these conditions the derivation represents an approximation.

Initially, the concentration at the immediate axon surface and for the external solution is C_o . When the external concentration C_o is suddenly changed to C_f , a concentration gradient is produced from the external solution to the axon surface where the surface concentration C_s is still C_o . The assumption is made, which has experimental support, that the diffusion path volume Aa is small relative to the volume V just at the axon surface. The approach to a steady-state concentration along the diffusion paths is therefore more rapid than the change in surface concentration C_s . Consequently, the concentration gradient along the access paths is almost linear.

The diffusion equation for flow at the axon surface is

$$dn/dt = -DA dc/dx \quad (1)$$

For a linear concentration gradient along the diffusion paths, equation (1) becomes

$$dn/dt = -DA(C_s - C_f)/a \quad (2)$$

where a is the average path length and A the cross-sectional area of the paths. The change in surface concentration dC_s equals dn/V giving $dn/dt = VdC_s/dt$. Equation (2) then becomes

$$dC_s/(C_s - C_f) = -(DA/Va)dt \quad (3)$$

Integrating this equation over time and utilizing the boundary conditions that $C_s = C_o$ for $t = 0$, and $C_s = C_f$ for $t = \infty$ give

$$C_s(t) = C_f - (C_f - C_o)e^{-kt}, \text{ where } k = DA/Va \quad (4)$$

Experimentally, k has averaged around 0.1/sec, which supports the supposition that the diffusion path volume Aa is small relative to the volume V immediately above the axon surface. The ratio of these volumes is $Aa/V = a^2k/D$. Assuming a diffusion path length of 10 to 20 μ and taking $D = 1.6 \times 10^{-5}$ cm²/sec, the ratio Aa/V is around 0.01 which supports the supposition.

It should be emphasized that although the diffusion model may not be correct, equation (4) has given good agreement with the experimental observations (see Fig. 3). Therefore equation (4) could equally well be considered as no more than a good curve fit for describing the concentration of ions at the axon surface. Consequently equation (4) is not necessarily dependent on any specific diffusion model.

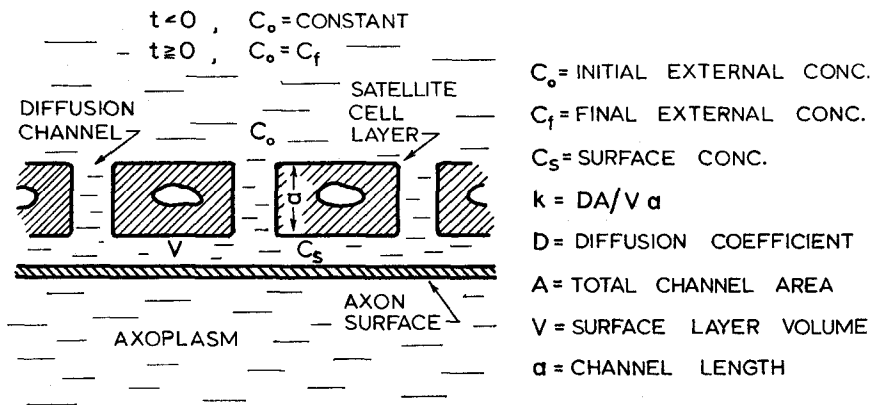


FIGURE 11. Diffusion access model for the axon surface.

Appendix 2

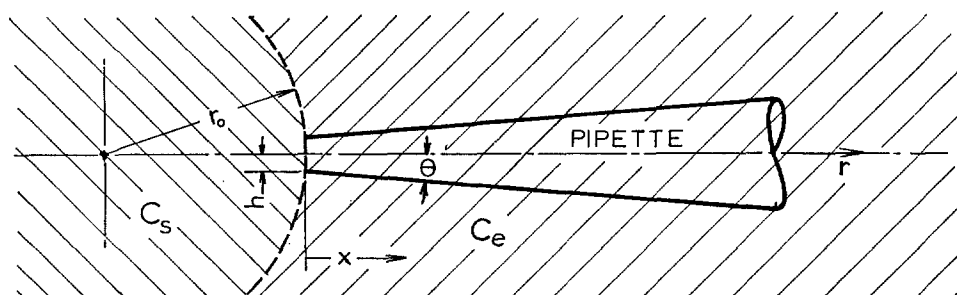
DIFFUSION RESPONSE TIME IN SALT BRIDGE REFERENCE ELECTRODES

A. Strickholm

When one ion is substituted for another ion of different mobility as in these experiments, a transient potential artifact appears at the liquid junction of the salt bridge reference electrode. This potential transient results because the concentrations are distributed over a length of the electrode from the tip into the shank. When the solution at the tip is changed, two liquid junction potentials appear. The first is at the electrode tip between the new and old bath solutions. The second is the original liquid junction potential which is distributed along the concentration gradients of the old solution and the 3 M KCl of the electrode. The potential transient time depends on the rapidity with which the old bath solution can dissipate itself by diffusion and a

single liquid junction potential can be created. In these experiments, when chloride was replaced with glucuronate or propionate, this potential artifact was 3–4 mv and lasted minutes to hours depending on how long before an ion exchange the cylindrical reference electrode was placed in Ringer's solution. This potential transient was often unrecognized and caused large errors in the membrane potential dependence on an ion (see Fig. 6).

One solution to this problem was to use a flowing 3 M KCl junction which was, however, impractical because of the physiological effects of KCl. Another solution would be to have a small hole of short length separating large volumes of the bath and 3 M KCl solutions. The potential transient time would then depend on the diffusion



$$(1) \frac{1}{D} \frac{\partial C}{\partial t} = \frac{1}{r} \frac{\partial^2 (rC)}{\partial r^2}$$

$$(2) C(r,0) = C_s, \quad r \leq r_0 \\ = C_e, \quad r > r_0$$

$$(3) C(r,t) = C_s, \quad r \leq r_0 \\ = C(r,t), \quad r > r_0$$

$$(4) \text{Limit } C(r,t) = C_e \\ r \rightarrow \infty$$

D = Diffusion Coefficient

h = Pipette Tip Radius

θ = Half Angle of Pipette Taper (radians)

C_e = Initial Pipette Concentration

C_s = Bath Concentration

$C(r,t)$ = Concentration at Distance r and Time t

FIGURE 12. Model for diffusion in pipettes.

time required to approach a steady state across this hole. An approximation to this situation was made in these experiments by using reference electrodes of small tip diameter and large solid angle taper. The ideal situation is approached with an infinitely small hole and solid angle taper of 2π .

An exact solution for the potential transient in a reference electrode is difficult. However, if the small potential gradients can be neglected, one can determine the speed of approach to a steady-state ion distribution for conical electrodes with the cylindrical electrode being a limiting case of zero taper. The diffusion equation for a conical reference electrode is most easily solved in spherical coordinates (Fig. 12). In Fig. 12, equation (1) represents the diffusion equation while equations (2) to (4) are the appropriate boundary value conditions. The solution of the equations gives

$$C(r, t) = C_e - (r_0/r)(C_e - C_s)\{1 - \text{erf}[(r - r_0)/2\sqrt{Dt}]\}. \quad (5)$$

The steady-state concentration is

$$C(r, \infty) = C_e - (r_0/r)(C_e - C_s) \tag{6}$$

If the reference pipette electrode taper θ is small, $h = r_0\theta$, $x = r - r_0$, and equation (5) gives

$$C(x, t) = C_e - [1/(1 + x\theta/h)](C_e - C_s)[1 - \text{erf}(x/2\sqrt{Dt})] \tag{7}$$

Solutions to equation (7) are plotted in Fig. 13. Shown are diffusion response times for pipette tapers θ of 0.0175 radian (1°) and 0.35 radian (20°). It is evident that for a

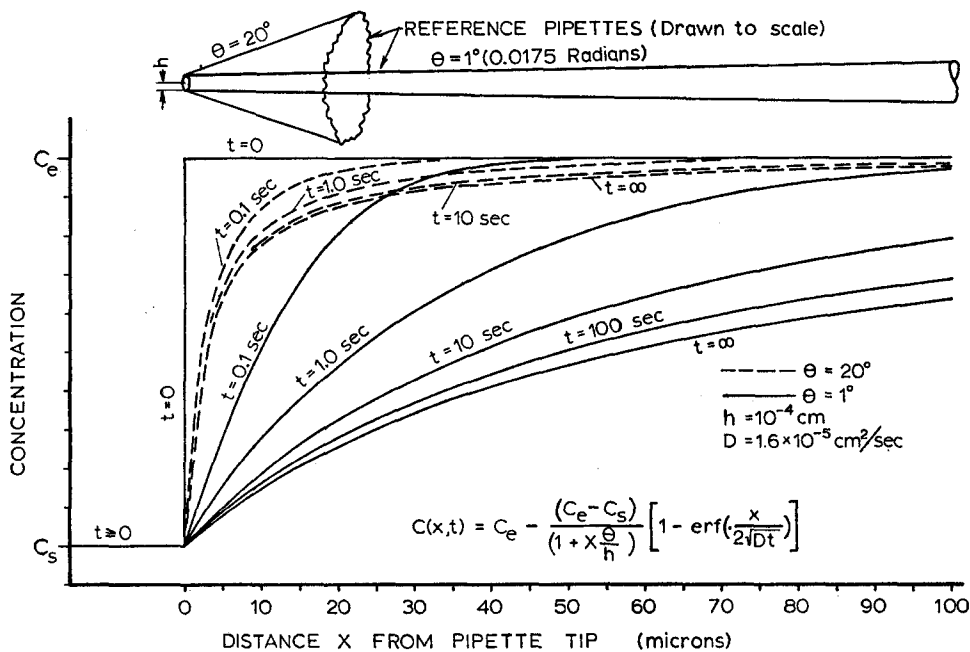


FIGURE 13. External reference electrode. Diffusion response times for reference pipette of two different tapers.

cylindrical reference electrode ($\theta = 0^\circ$), the concentrations will never approach a steady state. The equations also indicate that the higher the ratio of θ/h , the more rapidly equilibrating the reference electrode will be.

Since the reference electrode has been discussed, it may be of interest that micro-pipette electrode resistances can be determined from this concentration distribution. If conductivity σ is proportional to concentration, the pipette resistance R_e in the steady state is

$$R_e = \int_{r_0}^{\infty} dr/\sigma(r, t)A = [1/\pi h\theta(\sigma_e - \sigma_s)]\ln(\sigma_e/\sigma_s) \tag{8}$$

where σ_e and σ_s are the pipette and solution conductivities respectively. Equation

(8) indicates that micropipette electrode resistance is not only a function of tip diameter $2h$, but that pipette taper θ and the concentrations of the pipette and bath solution are also important. Equation (8) also explains why micropipette resistances change upon pipette entry into a cell, where σ_s changes on cell penetration (Strickholm, 1957).

The diffusion equilibrium time of a reference pipette immersed in one solution and then changed to another has been of importance in this paper. The solution to this diffusion problem can be obtained by replacing the boundary value conditions of equation (2) with the steady-state solution described in equation (6). This is the situation in which the reference electrode is equilibrated in a saline of concentration C_s and then placed into a new solution of concentration C_s' . The solution to this new boundary problem is

$$C(r, t) = C_s - (r_0/r) \{ (C_s - C_s') - (C_s - C_s') \operatorname{erf}[(r - r_0)/2\sqrt{Dt}] \}. \quad (9)$$

The steady-state solution to equation (9) is as obtained before in equation (6)

$$C(r, \infty) = C_s - (r_0/r)(C_s - C_s') \quad (10)$$

Again it is seen in equation (9) as in equations (5) and (7) that in order to obtain a rapidly equilibrating reference electrode the ratio of θ/h must be large. The potential transient will therefore be quite long in a cylindrical electrode ($\theta/h = 0$), in contrast to an electrode with a large value of θ/h .

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