K^+-SELECTIVE MICROELECTRODE STUDY OF INTERNALLY DIALYZED SQUID GIANT AXONS

C. N. FONG*§ AND DONALD C. CHANG‡§

*Department of Physiology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada M5S 1A8; ‡Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030; §Department of Physics, Rice University, Houston, Texas 77251; and §Marine Biological Laboratory, Woods Hole, Massachusetts 02453

ABSTRACT Intracellular potassium activity, $(a_K)_i$, and axoplasmic K^+ concentration, $[K^+]_i$, were measured by means of K^+-selective microelectrodes and atomic absorption spectroscopy, respectively, in squid giant axons dialyzed with K^+-free dialysis solution and bathed in K^+-free artificial sea water. $(a_K)_i$ measurements indicated that axoplasmic free K^+ could be depleted by dialysis, whereas $[K^+]_i$ measurements on axoplasm extruded from these axons suggest substantial retention of K^+ (15.5 ± 1.7 mmol/kg axoplasm K^+; $n = 9$). In comparison, $[K^+]_i$ in axoplasm extruded from freshly dissected axons was 330 ± 16 mmol/kg axoplasm ($n = 6$). These data suggest that ~5% of the axoplasmic K^+ ions are not easily removed by dialysis and that these ions are either bound to macromolecular sites or sequestered into membrane-enclosed organelles.

INTRODUCTION

The possible existence of intracellularly bound or sequestered ions is a question that may have a strong bearing on the understanding of membrane excitability and cellular transport. With the exception of Ca^{2+}, it is generally assumed that ions are free in the cytoplasm. This assumption may not always hold. For example, Hinke (1961) and Hinke et al. (1973) have shown by Na^+-selective microelectrodes, whole cell chemical analysis, and radioisotope diffusion analyses that about one-third of the Na^+ ions are not free in the cytoplasm of the squid giant axon and the barnacle giant muscle fiber. Current evidence in nerve cells does not provide clear arguments for intracellularly bound or sequestered K^+ ions. Isotope diffusion measurements by Hodgkin and Keynes (1953) in *Sepia* giant axons indicate that at least 90% of the intracellular K^+ ions exist as free ions. This has been supported by Hinke (1961) in squid giant axons and by Gilbert (1975) in *Myxicola* giant axons.

These studies suggest that only a small fraction of cytoplasmic K^+ may not be free in nerve cells (either bound to macromolecules or sequestered into membrane-enclosed organelles). Nevertheless, a small fraction may play an important role in membrane excitability and ion transport. For example, Chang (1983) has suggested that bound K^+ may significantly alter the ionic environment at the internal surface of the plasmalemma. Bound K^+ at the internal surface of the plasmalemma has been suggested by Landowne and Scruggs (1979) to account for the persistence of a K^+ current in voltage-clamped squid giant axons internally perfused with low K^+ solutions. The suggestion of bound K^+ in neurons is not unreasonable because nonhomogeneous distributions of K^+ have been found in other cellular systems (Armstrong and Lee, 1971; Hinke et al., 1973; Ling and Ochsenfeld, 1973; and Horowitz et al., 1979).

To determine if a small fraction of K^+ was bound or sequestered in the squid giant axon, internal dialysis was combined with K^+-selective microelectrodes and atomic absorption spectroscopy. Dialysis with K^+-free internal dialysis solution and continuous superfusion with K^+-free artificial sea water were used to deplete the axoplasm of free K^+. Free K^+ depletion was monitored continuously with K^+-selective microelectrodes. Bound K^+ was determined by atomic absorption measurements of total K^+ in axoplasmic samples extruded after the depletion of free K^+.

The results of these experiments suggest that most of the axoplasmic K^+ ions can be rapidly removed but there exists a small fraction (~5%) of axoplasmic K^+ ions that are either bound to macromolecules or sequestered by membrane-enclosed organelles.

METHODS AND MATERIALS

Materials

Hindmost stellar giant axons were dissected from live squids (*Loligo pealei*) obtained from the Marine Biological Laboratory, Woods Hole, MA, and cleaned of connective tissue under a dissecting microscope.

Address correspondence to C. N. Fong, School of Optometry, University of California Berkeley, 360 Minor Hall, Berkeley, CA 94720

Internal Dialysis

The principles and methods of internal dialysis have been described by Brinley and Mullins (1967). Briefly, the cellulose acetate tubing (a gift from Dr. J. M. Russell) was made porous over a defined 2.5-cm length by exposure to a 100-mM KOH solution for ~22 h. The porosity of the dialysis tubing was checked by visually examining its ability to pass chlorophenol red. The axons were cannulated with the porous dialysis tubing according to the method of Baker and Carruthers (1981).

K^+-Activity Measurements

The axoplasmic K^+ activity was measured by means of K^+-selective microelectrodes. The K^+-selective microelectrodes were prepared by silanizing micropipettes pulled from nonfilamented Kwik Fill capillary tubings (World Precision Instruments, New Haven, CN) to a typical resistance of 2 M ohms when filled with 3 M KCl. These micropipettes were placed on a Teflon block and oven dried for at least 1 h at 200°C then silanized with *N*-trimethylsilydimethyamine (TMSDMA) (Fluka Chemical Co., Hauppauge, NY) for at least 1 h at 200°C.

Silanized micropipettes were stored for up to 1 wk before use. K^+-selective microelectrodes were made by pressure injection of a 100 mM KCl solution into the lumen of the silanized micropipettes until the tips were filled. A column of K^+ liquid ion-exchanger (Fluka Chemical Co.) was introduced into the tip by suction.

K^+-selective microelectrodes were calibrated before and after an experiment in 500 mM, 50 mM, 5 mM, and 0.5 mM KCl solutions, in 200 mM KCl + 200 mM NaCl, and in K^+-free dialysis solution, and standard dialysis solution. These microelectrodes were used only if their response was at least 56 mV for a 10-fold change in K^+ activity from 0.5 mM KCl to 500 mM KCl. The typical selectivity of these microelectrodes for K^+ over Na^+ determined by the separate solution method (i.e., response to pure KCl solutions was compared with response in 200 mM KCl + 200 mM NaCl) was 80–100:1. Calibration with standard and K^+-free dialysis solutions was used to compare $(a_K)_i$ measurements in dialyzed axons with (a_K) values of the dialysis solutions.

A K^+-selective microelectrode inserted into an axon measures the sum of the membrane potential, E_m, and the potential due to the intra-axonal K^+ activity, $(E_K)_i$, i.e.,

$$E = (E_K)_i + E_m, \tag{1}$$

where E is the total intracellular potential measured by the K^+-selective microelectrode. $(E_K)_i$, was determined from E by subtracting E_m measured by microelectrodes pulled from filamented Kwik Fill capillary tubing (World Precision Instruments, Inc.) filled with a 4 M Na formate + 100 mM NaCl solution.

The tip potential of the membrane potential–measuring microelectrodes was determined in 500 mM KCl solution, normal artificial sea water, and K^+-free dialysis solution before and after an experiment. These microelectrodes were used only if their tip potentials did not vary by >3 mV in the solutions just described. The typical resistance of the membrane potential–measuring microelectrodes was 2 M ohms.

$(a_K)_i$ values were calculated from $(E_K)_i$ values according to the method of Hinke and McLaughlin (1967) by assuming negligible Na^+ interference on the K^+-selective microelectrode.

For microelectrode calibrations and intra-axonal recordings, both the K^+-selective microelectrode and the membrane potential microelectrode were referred to a Ag/AgCl pellet (E. W. Wright, Guilford, CN) connected to the bath solution via a 3 M KCl agar bridge contained in a piece of PE 50 tubing. The microelectrode potentials were measured with a FD-223 electrometer (World Precision Instruments, Inc.). The experiments were accepted only if the calibration of both microelectrodes before and after an experiment were within 3 mV.

Calculation of Free Axoplasmic K^+ Concentration

The free axoplasmic K^+ concentration, $(K^+)_i$, at the termination of dialysis with K^+-free internal dialysis solution and superfusion with K^+-free artificial sea water was calculated according to the relation

$$(a_K)_i = \gamma_K(K^+)_i \tag{2}$$

(Robinson and Stokes, 1955) using a value of 0.65 for γ_K, the activity coefficient of K^+ in the K^+-free internal dialysis solution. The value of γ_K was calculated from the extended Debye-Hueckel equation (Robinson and Stokes, 1955)

$$\log \gamma_K = \frac{-A(I)^{1/2}}{1 + Ba(I)^{1/2}} + bI, \tag{3}$$

where the values at 20°C from Robinson and Stokes (1955) for A, B, and a are 0.5046, 0.3276, and 3.04, respectively, the value for b at 20°C from Parsons (1959) is 0.0187, and I is the ionic strength of the K^+-free internal dialysis.

Solutions

The composition of normal artificial sea water was 420 mM NaCl, 10 mM KCl, 20 mM $CaCl_2$, 50 mM $MgCl_2$, 2 mM NaOH, and 2.5 mM Hepes buffer. The composition of K^+-free artificial sea water was 420 mM NaCl, 20 mM $CaCl_2$, 50 mM $MgCl_2$, 4 mM NaOH, and 5 mM Hepes buffer. The composition of the standard dialysis solution was 380 mM KF, 30 mM NaF, 40 mM K-phosphate buffer, and 200 mM sucrose. The composition of K^+-free dialysis solution was 432 mM NaF, 20 mM Na-phosphate buffer and 200 mM sucrose. The pH of the artificial sea waters and the internal dialysis solutions were 7.9 and 7.2, respectively.

Experimental Protocol

All experiments were carried out at room temperature (19–22°C). Axons were cannulated with the dialysis tubing in a Plexiglass chamber such that the length of axon dialyzed corresponded with the length of axon superfused with artificial sea water (Fig. 1). The axons were first dialyzed with standard dialysis solution and superfused with the normal artificial sea water for at least 30 min before microelectrode recordings were attempted. When both the K^+-selective microelectrode and the mem-

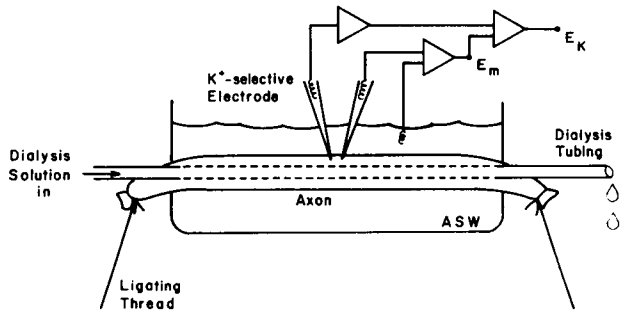

FIGURE 1 Schematic drawing of a dialyzed squid giant axon superfused with artificial sea water (ASW) in the experimental chamber. The axon is ligated at the ends, stretched gently across the experimental chamber, and cannulated axially with the dialysis tubing. The portion of the axon dialyzed corresponds with the portion of the axons superfused with artificial sea water. Microelectrodes used to record $(a_K)_i$ and E_m were inserted transversely through the axolemma into the axoplasm just beneath the axolemma. The microelectrodes were separated typically by ~5 mm.

brane potential measuring microelectrode had recorded stable potentials for at least 10 min and $(E_K)_i$ was within 3 mV of E_K in the standard dialysis solution the standard dialysis solution and normal artificial sea water were replaced simultaneously with K^+-free dialysis solution and K^+-free artificial sea water, respectively. The axon was considered to be depleted of free K^+ when $(E_K)_i$ approached an asymptotic value within 3 mV of E_K measured in K^+-free dialysis solution. Dialysis was terminated and the dialysis tubing withdrawn from the axon. The remaining axoplasm was extruded onto parafilm and transferred to a preweighed Eppendorf vial for weight determination. The axoplasm was then digested in 25 μl of concentrated HCl, neutralized with 20 μl of concentrated NH_4OH and diluted to 5 ml with ultrapure water for total K^+ analysis by means of atomic absorption analysis on a Perkin-Elmer Model 2380 atomic absorption spectrophotometer.

RESULTS

Fig. 2 is a sample of a continuous $(E_K)_i$ recording in a squid giant axon. The axon was first dialyzed with standard dialysis solution and superfused with normal artificial sea water. At point *A* the internal dialysis solution was changed to K^+-free dialysis solution and the external medium was changed to K^+-free artificial sea water. The rapid fall in $(E_K)_i$ illustrates that free K^+ was rapidly removed by internal dialysis and superfusion with K^+-free solutions. The mean $(a_K)_i$ value when axons were dialyzed with standard dialysis and superfused with normal artificial sea water was 278 ± 4.5 mM (mean ± SE; $n = 11$). This value is close to the value of (a_K) measured in the standard dialysis solution (271 mM). It should be noted that interference on the K^+-selective microelectrode from the Na^+ in the internal dialysis solution should only cause an overestimation in $(a_K)_i$ of ~0.2 mM (calculated from selectivity determined by the separate solutions method). Thus it was reasonable to assume negligible Na^+ interference on the K^+-selective microelectrode when the axons are dialyzed with standard dialysis solution and superfused with normal artificial sea water. The good agreement between the K^+ activity values suggests that internal dialysis can control the free axoplasmic K^+. When the axon was depleted of free K^+ (see Methods for criteria) the final value of $(a_K)_i$ was 0.474 ± 0.05 mM (mean ± SE; $n = 11$). This value of $(a_K)_i$ is similar to the measured error in K^+ activity produced by the Na^+ interference of the K^+-free dialysis solution on the K^+-selective microelectrode. Thus it seems safe to assume that internal dialysis and superfusion with K^+-free solutions had depleted the axon of free K^+.

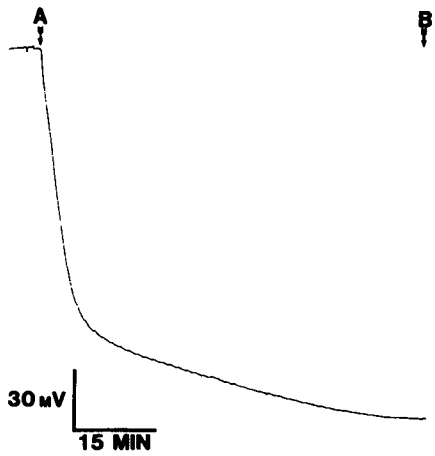

FIGURE 2 The intracellular K^+ potential, $(E_K)_i$, measured by the K^+-selective microelectrode is plotted vs. time. At the start of the experiment the axon was superfused with normal artificial sea water and dialyzed with standard internal dialysis solution. A downward change in $(E_K)_i$ corresponds to a decrease in $(a_K)_i$. At point *A* the superfusate was changed to K^+-free artificial sea water and the dialysate was changed to K^+-free dialysis solution. At point *B* dialysis was stopped and the dialysis tubing was withdrawn.

TABLE I

VALUES OF $(a_K)_i$ IN AXONS DIALYZED WITH STANDARD DIALYSIS SOLUTION AND SUPERFUSED WITH NORMAL ARTIFICIAL SEA WATER, AND VALUES OF $(a_K)_i$, $(K^+)_i$, AND $[K^+]_i$ IN THE SAME AXONS AFTER DIALYSIS WITH K^+-FREE DIALYSIS SOLUTION AND SUPERFUSION WITH K^+-FREE ARTIFICIAL SEA WATER

Axon No.	$(a_K)_i$*	$(a_K)_i$‡	$(K^+)_i$‡§	$[K^+]_i$‡
		mM		*mmol/kg axoplasm*
7-2-1	292	0.46	0.71	16.7
7-2-2	279	0.48	0.74	—
7-14-1	252	0.49	0.75	12.0
7-23-1	294	0.46	0.71	16.3
7-29-1	275	0.47	0.72	—
7-30-1	283	0.46	0.71	12.2
7-31-1	256	0.48	0.74	24.5
7-31-2	292	0.47	0.72	10.7
8-6-1	291	0.45	0.69	9.4
8-7-1	263	0.50	0.77	20.1
8-8-1	284	0.49	0.75	17.9
mean‖	278 ± 4.5	0.474 ± 0.05	0.728 ± 0.007	15.7 ± 1.7

*Values from axons dialyzed with standard dialysis solution and superfused with normal artificial sea water. ‡Values at the end of dialysis with K^+-free dialysis solution and superfusion with K^+-free artificial sea water. §Calculated from $(a_K)_i$ values after dialysis with K^+-free dialysis solution and superfusion with K^+-free artificial sea water as described in Methods. ‖Values are mean ± 1 SE.

The individual $(a_K)_i$ values measured during dialysis with standard dialysis solution and superfusion with normal artificial sea water and the corresponding individual $(a_K)_i$ and $[K^+]_i$ values measured at the termination of dialysis with K^+-free dialysis solution and superfusion with K^+-free artificial sea water along with the calculated individual $(K^+)_i$ values are summarized in Table I. The mean calculated free K^+ concentration, $(K^+)_i$, at the termination of dialysis, the corresponding mean $[K^+]_i$ value measured in axoplasmic samples extruded from these axons, and the mean measured $[K^+]_i$ in axoplasmic samples extruded from freshly dissected axons using atomic absorption spectroscopy are summarized in Table II for comparison. The mean calculated $(K^+)_i$ at the termination of dialysis was 0.728 ± 0.007 mM (mean ± SE, $n = 11$). In comparison, the mean measured $[K^+]_i$ for axoplasmic samples extruded from these axons was 15.7 ± 1.7 mmol/kg axoplasm (mean ± SE, $n = 9$). The measured mean $[K^+]_i$ value for axoplasmic samples extruded from freshly dissected axons was 330 ± 16 mmol/kg axoplasm (mean ± SE, $n = 6$). Comparison of the mean $[K^+]_i$ values (Table II, columns 2 and 3) suggests that ~5% of the axoplasmic K^+ was not removed easily when free K^+ was depleted by dialysis and superfusion with K^+-free solutions.

DISCUSSION

This study shows that most of the axoplasmic K^+ was rapidly exchangeable. This study also provides evidence that ~5% of the K^+ in the squid giant axon may be bound and not easily removed by internal dialysis with a K^+-free solution. This is illustrated by comparing the $(K^+)_i$ and $[K^+]_i$ values (Table I) after dialysis with K^+-free dialysis solution and superfusion with K^+-free artificial sea water. The difference between the $(K^+)_i$ and $[K^+]_i$ values shows that the axoplasm can retain bound K^+ (almost 16 mmol/kg axoplasm) even when free K^+ had been depleted. Comparison of the values for $[K^+]_i$ in axoplasmic samples extruded from K^+-depleted axons and in axoplasmic samples extruded from freshly dissected axons shows that ~5% of the K^+ in the squid axon exists in a bound state. This finding is consistent with the conclusion of Hodgkin and Keynes (1953) that only a small fraction of the internal K^+ may be bound. However, the experiments presented here provide more direct evidence for the existence of a small fraction of bound K^+. The term "bound" is used very loosely here because we do not know if this K^+ is sequestered into membrane-enclosed organelles or bound to axoplasmic macromolecules by chelation or by binding to charged sites.

TABLE II
COMPARISON OF MEAN VALUES OF $(K^+)_i$ AND $[K^+]_i$ FROM AXONS AFTER DIALYSIS WITH K^+-FREE DIALYSIS SOLUTION AND SUPERFUSION WITH K^+-FREE ARTIFICIAL SEA WATER WITH MEAN VALUES OF $[K^+]_i$ FROM FRESHLY DISSECTED AXONS

$(K^+)_i$*	$[K^+]_i$*	$[K^+]_i$‡
mM	*mmol/kg axoplasm*	
0.728 ± 4.5 (11)	15.7 ± 1.7 (9)	330 ± 16 (6)

*Values from axons after dialysis with K^+-free dialysis solution and superfusion with K^+-free artificial sea water. ‡Values from freshly dissected axons. All values are mean ± 1 SE. Values in brackets are the number of determinations.

The observation that $(a_K)_i$ fell rapidly when the axon was exposed to internal and external K^+-free solutions confirms the $[K^+]_i$ measurements that most of the internal K^+ must be relatively mobile and easily removed by dialysis. Evidence that most of the K^+ removed was by diffusion across the dialysis tubing and not across the axolemma is derived from the recordings of E_m (not shown) which invariably showed a rapid depolarization when the normal artificial sea water and standard internal dialysis solution were replaced simultaneously with K^+-free artificial sea water and K^+-free internal dialysis solution, respectively. In contrast, experiments in which the normal artificial sea water was replaced by K^+-free artificial sea water without a similar replacement of the internal dialysis solution invariably showed a hyperpolarization. We calculate that $(a_K)_i$ fell with a half time of 2–5 min. This is in good agreement with Brinley and Mullins (1967) for free exchange of small molecules across a dialysis tubing. It should be noted that the easy removal of free K^+ does not prove that all of the K^+ easily removed by dialysis (95%) must be thermodynamically free or that our K^+ fractions are directly comparable with K^+ fractions measured by other means such as nuclear magnetic resonance or radioisotope diffusion. The results presented can only tell us that ~5% of the K^+ in the squid axoplasm has restricted exchange with the bulk free axoplasmic K^+.

The present data cannot determine precisely the site or the nature of the K^+ binding, but we believe that this K^+ is most likely bound to axoplasmic macromolecules. Only two major membrane-enclosed organelles exist in the squid giant axon: the endoplasmic reticulum and the mitochondria. Somlyo et al. (1981) have shown by x-ray microanalysis that mitochondria do not sequester K^+. We are not aware of any comparable data for the endoplasmic reticulum. However, internal dialysis removes both ATP and arginine-phosphate from the axon (Baker, 1984), thereby eliminating K^+ sequestration by energy-dependent processes (presumably membrane-enclosed organelles) in the dialyzed squid giant axon.

In conclusion our data show that most of the intra-axonal K^+ ions must be relatively mobile because depletion of K^+ can occur rapidly by diffusion across a dialysis membrane. Our estimate that 5% of the total intra-axonal K^+ is bound must be regarded as a minimum value because the depletion of free K^+ by dialysis can be expected to remove a fraction of the bound K^+ ions. Further work will be required to determine the site and nature of the K^+

binding and to establish its possible role in membrane excitability and ion transport.

We wish to thank the following people for their useful input and the loan of materials and equipment: Drs. H. L. Atwood, S. Barry, M. P. Charlton, H. Gainer, P. E. Gallant, H. C. Pant, J. M. Russell, S. J. Smith, and I. Tasaki. We also thank Drs. H. L. Atwood and M. P. Charlton for reading the manuscript. Special thanks to Mr. P. Byrne for modifications of the experimental chamber.

C.F. was supported by a Medical Research Council of Canada Postdoctoral Fellowshiop and D.C. was supported by the National Science Foundation (BNS-8406932) and by the Office of Naval Research (NOOO14-85-K-0424).

Received for publication 26 March 1987 and in final form 23 October 1987.

REFERENCES

Armstrong, W. McD., and C. O. Lee. 1971. Sodium and potassium activities in normal and "sodium rich" frog skeletal muscles. *Science (Wash. DC).* 171:413–415.

Baker, P. F. 1984. Intracellular perfusion and dialysis: application to large nerve and muscle cells. *In* Intracellular Perfusion of Excitable Cells. P. G. Kostyuk and O. A. Krishtal, editors. IBRO Handbook Series: Methods in Neurosciences. Vol. 5. John Wiley & Sons, New York. 1–17.

Baker, P. F., and A. Carruthers. 1981. 3-*o*-Methylglucose transport in internally dialyzed giant axons of *Loligo. J. Physiol. (Lond.).* 316:503–525.

Brinley, F. J., Jr., and L. J. Mullins. 1967. Sodium extrusion by internally dialyzed squid giant axons. *J. Gen. Physiol.* 50:2303–2331.

Chang, D. C. 1983. Ion-selectivity and "gating" properties of the conductance pathways in squid axon. The view of a membrane-cortex model. *In* Structure and Function in Excitable Cells. D. C. Chang, I. Tasaski, W. J. Adelman, Jr., and H. R. Leuchtag, editors. Plenum Press, New York.

Gilbert, D. S. 1975. Axoplasm architecture and physical properties as seen in the *Myxicola* giant axon. *J. Physiol. (Lond.).* 253:257–301.

Hinke, J. A. M. 1961. The measurements of sodium and potassium activities in the squid giant axon by means of cation-selective microelectrodes. *J. Physiol. (Lond.).* 156:314–335.

Hinke, J. A. M., and S. G. A. McLaughlin. 1967. Release of bound sodium in single muscle fibres. *Can. J. Physiol. Pharmacol.* 45:655–667.

Hinke, J. A. M., J. P. Caille, and D. C. Gayton. 1973. Distribution and state of monovalent ions in skeletal muscle based on ion electrodes, isotopes, and diffusion analyses. *Ann. NY Acad. Sci.* 204:274–296.

Hodgkin, A. L., and R. D. Keynes. 1953. The mobility and diffusion coefficient of potassium in giant axons from *Sepia. J. Physiol. (Lond.).* 119:513–528.

Horowitz, S. B., P. L. Paine, L. Tluczek, and J. K. Reynhout. 1979. Reference phase analysis of free and bound intracellular solutes. I. Sodium and potassium in amphibian oocytes. *Biophys. J.* 25:33–44.

Landowne, D., and V. Scruggs. 1979. Slow changes in the magnitude of potassium current associated with changes in the internal perfusion solution in squid giant axons. *Biol. Bull. (Woods Hole)* 157:377.

Ling, G. N., and M. M. Ochsenfeld. 1973. Mobility of potassium ion in frog muscle cells, both living and dead. *Science (Wash. DC).* 181:78–81.

Parsons, R. 1959. Handbook of Electrochemical Constants. Academic Press, New York.

Robinson, R. A., and R. H. Stokes. 1955. Electrolyte Solutions. Butterworths, London.

Somlyo, A. V., H. Gonzales-Serratos, H. Shuman, G. McCelland, and A. P. Somlyo. 1981. Calcium release and ionic changes in the sarcoplasmic reticulum: an electron probe study. *J. Cell Biol.* 90:577–594.