Sodium Extrusion by Internally Dialyzed Squid Axons

F. J. BRINLEY, JR., and L. J. MULLINS

From the Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Department of Biophysics, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT A method has been developed which allows a length of electrically excitable squid axon to be internally dialyzed against a continuously flowing solution of defined composition. Tests showed that diffusional exchange of small molecules in the axoplasm surrounding the dialysis tube occurred with a half-time of 2–5 min, and that protein does not cross the wall of the dialysis tube. The composition of the dialysis medium was (mM): K isethionate 151, K aspartate 151, taurine 275, $MgCl_2$ 4–10, NaCl 80, KCN 2, EDTA 0.1, ATP 5–10, and phosphoarginine 0–10. The following measurements were made: resting Na influx 57 pmole/cm²sec (n = 8); resting potassium efflux 59 pmole/cm²sec (n = 4); stimulated Na efflux 3.1 pmole/cm²imp (n = 9); stimulated K efflux 2.9 pmole/cm²imp (n = 3); resting Na efflux 48 pmole/cm²sec (n = 18); Q_{10} Na efflux 2.2 (n = 5). Removal of ATP and phosphoarginine from the dialysis medium (n = 4) or external application of strophanthidin (n = 1) reversibly reduced Na efflux to 10–13 pmole/cm²sec. A general conclusion from the study is that dialyzed squid axons have relatively normal passive permeability properties and that a substantial fraction of the Na efflux is under metabolic control although the Na extrusion mechanism may not be working perfectly.

INTRODUCTION

The interpretation of results from ion permeability experiments on cell membranes is considerably simplified if it is possible to control the concentrations of all solutes on both sides of the membrane. Although ion transfer rates across the squid axon membrane are so small that over a period of 2–3 hr concentrations inside the axon can be considered as reasonably constant, the substrates involved in operating ion extrusion mechanisms can change markedly in times as short as 15 min. This is shown by experiments in which axons were poisoned with CN^- and the Na efflux followed (Caldwell et al., 1960). In these experiments injection of a substrate such as ATP gave a transient increase in Na efflux indicating that the concentration of ATP must have varied greatly

over the time course of the experiment. Injection experiments with squid axons can, therefore, superpose time-variant experimental conditions on an initial set of intracellular substrate concentrations.

A somewhat different approach is possible in the case of mammalian red blood cells where one can remove much of the substrate and salt contained in normal cells and, by reconstituting the ghosts, incorporate wholly new initial conditions with respect to both salt and substrates (Hoffman, 1958). This technique suffers from two disadvantages; during the course of washing and preparation for experimental use, the reconstituted cells act on the incorporated substrate so that initial conditions are not precisely those at the moment of reconstitution. Second, one is now dealing with a population of cells with all the dispersion of membrane properties that a population implies.

With the demonstration by Oikawa et al. (1961) and Baker et al. (1962) that the electrical properties of the squid axon could be maintained during internal perfusion, it appeared that a new method for the study of the ion transport properties of the membrane was at hand. However, the reports presently in the literature indicate that the resting sodium and potassium fluxes of perfused axons are quite different from those of intact or injected axons. Tasaki (1963) has reported on the movements of radioactive Na and K in perfused axons. Calculations from his data indicate that Na influx is about five times larger than normal while K influx is only about 15% of values measured on intact axons. Shaw (1966) measured the net movements of Na and K in perfused fibers. His results showed that Na influx was about twice normal while the K efflux was some twenty times values usually obtained for intact axons.

Our choice of an experimental technique was influenced by the notion that there were difficulties of an unknown origin with internal perfusion techniques which remove most of the axoplasm from a squid fiber. Hence we sought an experimental method that would allow us to control the internal concentrations of ions and other substances generally regarded as substrates for the ion extrusion mechanism of the membrane, while retaining the proteins of the axon. This more conservative approach to internal solute control led to the design of a system that dialyzes a length of axoplasm against a continuously flowing fluid of a given composition. The technical details are given in the Methods section of this paper; the dialysis system utilizes a short (1.5–2.0 cm) length of porous glass tubing connected with longer lengths of impermeable glass tubing on each side of the porous region. With such a tube positioned inside a squid axon, tests showed that the length of axon around the porous region of the dialysis tube had its internal solutes controlled by externally supplied fluids. Since there is no a priori reason for supposing that the internal composition of squid axoplasm can be altered without radically affecting the transport properties of the membrane, it seemed prudent to determine first

how closely the behavior of a squid axon, dialyzed against a fluid closely approximating in composition the normal contents of axoplasm, would correspond to that of an intact or injected axon.

The experiments to be reported were carried out during the years 1965–1967 and show that a dialyzed squid axon has relatively normal passive ion permeability properties and that a substantial fraction of the Na efflux from such axons is under metabolic control.

TABLE I

COMPOSITION OF SOLUTIONS AND OF AXOPLASM

Substance	Seawater	Dialysis fluid*	Axoplasm‡
	mM	*mM*	*mM*
K^+	9	304	344
Na^+	425	75–85	65
Mg^{++}	48	0.1–4§	10
Ca^{++}	9		3.5
Cl^-	496	83–93	151
CN^-	2	2	
$SO_4^=$	25		
Isethionate$^-$		151	165
Aspartate$^-$		151	79
Glutamate$^-$			21
Taurine		275	107
Homarine			20
Betaine			73
EDTA		0.1	
Glucose	2		

* Described in text as "no fuel" (NF); additions made were ATP 5–14 mM (described as "ATP"), phosphoarginine 10 mM (described as "PA"), or [(ATP 5–14 mM) + (PA 10 mM)] (described as "fuel" or "F").

‡ Deffner (1961), Table I (*Loligo*). Unlisted nonionized constituents 53 mM.

§ Early experiments had [Mg] of 0.1–4 mM; in later experiments [Mg] was 4 mM in addition to [ATP].

METHODS

Live specimens of *Loligo pealei* were obtained either at the Marine Biological Laboratory, Woods Hole, Massachusetts (June, July, and August) or from Ocean City, Maryland (October through May). The hindmost giant axon from the stellate ganglion was dissected from the mantle in chilled, flowing seawater and cleaned of connective tissue under a dissecting microscope.

Solutions

EXTERNAL SOLUTIONS The composition of the various seawaters used is given in Table I. The pH of these solutions was adjusted to 7.6 and they were saturated with air after preparation. Cyanide at a concentration of 2 mM was added to these solutions in order to block aerobic phosphorylation in the regions of the axons that were not

dialyzed. Radioactive seawater was made by adding solid Na^{24} Cl directly to seawater. The increase in [Na] produced was less than 5%.

INTERNAL DIALYSIS SOLUTIONS The composition of the internal dialysis fluid used is listed in Table I, together with data from Deffner (1961) on the composition of axoplasm for comparison. In our medium, aspartate was used for the sum of (aspartate + glutamate) and taurine for the sum of all nonionic dialyzable organic constituents. The concentration of Cl^- was made about 80 mM since our own analyses of $[Cl]_i$ were considerably lower than the value reported by Deffner (Brinley and Mullins, 1965 *b*). The [Na] used in most experiments (75–85 mM), although higher than that occurring in the axoplasm of fresh squid, was considered closer to the actual [Na] present in most experiments on intact or injected axons. We wished, further, to have $[Na]_i$ as high as possible in order to activate the Na extrusion mechanism. Cyanide (2 mM) was added to dialysis fluids in order to inhibit oxidative phosphorylation in the axoplasm under study. While dialysis may be expected to remove all substrates from the axoplasm and hence make it impossible for phosphorylation to proceed, there was no certainty that internal compartments in the axoplasm, such as mitochondria, would respond to dialysis in the way axoplasm did. The chelating agent EDTA was added to dialysis fluid at a concentration of 0.1 mM in order to render nontoxic any heavy metals that might be present in the reagents, isotopes, or glassware used. Dialysis fluid was not buffered, mainly because it was not easy to decide on a suitable substance for a buffer. Its pH was carefully adjusted before use to pH 7.1–7.2; these solutions were stored at −90°C.

HIGH ENERGY PHOSPHATE COMPOUNDS The barium salt of phosphoarginine (PA) was obtained as a gift from Professor J. F. Morrison. A commercial source of dilithium phosphoarginine was also used, but subsequent enzymatic anaysis showed that it contained only about 15–20% phosphoarginine. Both the Na and K salts of ATP (Na_2H_2ATP and K_2H_2ATP) were purchased from Sigma Chemical Co.; they were dissolved in appropriate amounts of KOH so that the resulting solution was 0.5 M and about pH 7. Small amounts of acid or base were then added to bring the pH to 7.2. The phosphoarginine was passed through a cation exchange column in the K form to remove Ba^{++} and the effluent from the column tested with K_2SO_4 solution to be sure that it was Ba-free. Stock solutions of ATP made as described above were stored at −90°C because some early experience with storage at the usual deep-freeze temperature (−20°C) suggested that decomposition was not negligible at this temperature. Before use, stock solutions of ATP were analyzed for ATP using test kits capable of detecting 10^{-10} mole ATP. Solutions of ATP that did not assay at least 95% were not used except in the preliminary series of experiments reported below.

The ATP and PA concentrations in preliminary experiments were set at 10–15 mM because Caldwell et al. (1960) had shown that a restoration of the Na efflux from CN-treated axons was only obtained by the injection of ATP such that the resulting concentration immediately after injection was several times greater than normal. In our later experiments, when it became obvious that a metabolically dependent component of the Na efflux was present in dialyzed axons, ATP concentrations were reduced to 5 mM because our analyses of the ATP concentration of axoplasm gave a mean value of 4.4 mM.

Because Mg^{+2} and ATP^{-4} strongly associate to form $MgATP^{-2}$, the free Mg^{++} concentration will vary with [ATP]. In early experiments, before the importance of free [Mg^{++}] was appreciated, the total [Mg] in dialysis fluids was the same whether or not they contained ATP. In later experiments, the [Mg] of the dialysis fluid was adjusted to contain 4 mM Mg^{++} in addition to the [ATP]. No adjustment was made for added PA since this compound does not bind Mg strongly.

ISOTOPES For Na efflux measurements, dried $Na^{22}Cl$ (specific activity 60 c/mole) was added to the dialysis fluid. Aliquots of stock solutions (about 0.5 M) of PA and/or ATP were then added and appropriate additions of distilled water or K

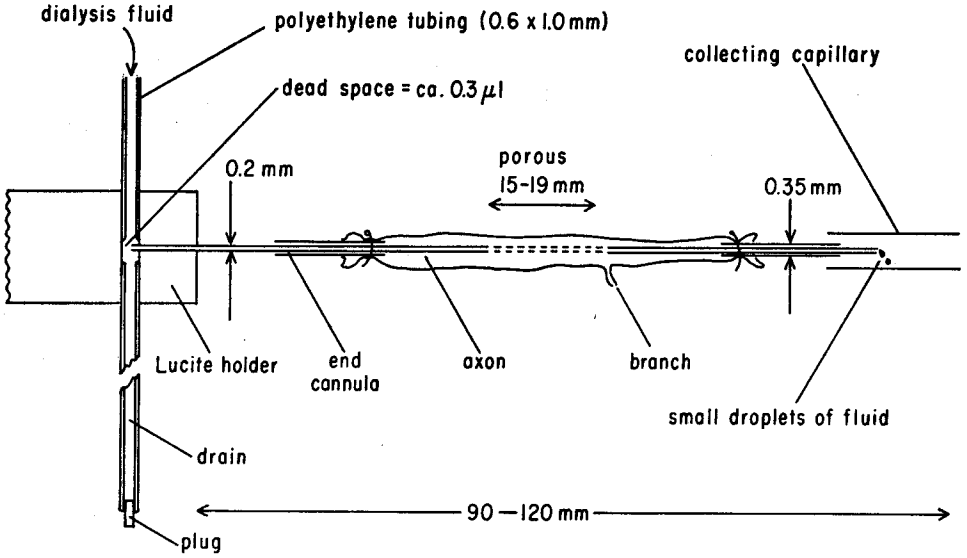

FIGURE 1. A diagram (not to scale) of the Lucite holder for the porous capillary is shown on the left. This Lucite holder is mounted on a micromanipulator. In the center of the diagram the porous capillary is shown inserted in a squid axon and on the right-hand side of the diagram is the capillary which collects the effluent fluid being pumped through the porous capillary by a motor-driven syringe.

isethionate were then made to keep the specific activities of Na constant for all solutions. Slight inequalities in the concentration of other ions were unavoidable but were less than 5%. For K efflux experiments, the procedure was the same as listed above except that dried $K^{42}Cl$ (specific activity on arrival 60 c/mole) was used.

Internal Dialysis

PRINCIPLE OF THE METHOD A glass capillary tube 12–14 cm long, 175–225 µ in diameter, and with a wall thickness 50–75 µ was inserted longitudinally into a cleaned squid axon. The capillary extended beyond either end of the axon (see Fig. 1). Although the capillary will be referred to as "glass," it was in fact a special type of porous silica (Corning 7930) which contained over 96 weight % silica on a dry basis. Most of the capillary tube had been consolidated by heating to render it impervious.

The central 15–20 mm of the capillary was not heated and therefore remained porous. Approximately 10% of the surface area of the capillary walls in the porous length consisted of pores 20–40A in diameter. These pores permitted essentially unhindered diffusion of small ions and molecules but severely restricted the passage of protein. The preparation of these capillaries and their permeability properties are more fully described below but may be summarized by saying that the product (diffusion coefficient × porous surface area) for material of molecular weight less than 1000 was about 5–20% of that in free solution.

If fluid is continually passed through the porous capillary, then the axoplasm outside the porous region will continuously exchange solute with the flowing fluid and will assume, as rapidly as diffusion permits, the composition of the flowing fluid. Since diffusional equilibrium is reached within a few minutes over the radial distances occurring in squid axons, this method allows rapid and reversible control of the concentration of any diffusible species of molecule or ion in the axoplasm. It provided, therefore, a technique for studying the effects of various internal environments of arbitrary composition on the unidirectional ion fluxes.

Efflux can be measured by perfusing a radioactive species through the porous capillary and collecting the radioactivity which crosses the membrane. Influx can be studied by placing the radioactive species outside the axon and collecting, by dialysis, the radioactivity entering the axon. Since diffusion within the axoplasm is much more rapid than permeation across the axon membrane, an essentially steady state of tracer movement across the membrane was rapidly attained. At the ends of the porous region the situation becomes extremely complicated because isotope now can diffuse longitudinally as well as radially. The magnitude of these effects is considered in the theoretical section (below) and certain arrangements that can minimize these effects are described in a section on efflux chamber design.

PREPARATION OF POROUS CAPILLARIES We are indebted to Dr. M. E. Nordberg and Mr. Thomas Elmer of the Research Laboratory, Corning Glass Works, for much information about porous glass and for fabricating an initial lot of porous glass capillaries. Our thanks are also due to Mr. Al Kaiser of the Corning Glass Works who was the engineer in charge of production of most of the capillaries used. The porous length of the capillaries was sharply delimited; the transition from porous to nonporous glass occurred over less than 1 mm of length. The porous region could be located by characteristic optical properties at the junction or, with greater precision, by the use of phenol red which readily stained the walls of the porous region.

The porosity of the capillaries delivered was further increased by treatment with HNO_3 at 90°C. This procedure also caused pronounced etching of the surface and great loss of mechanical strength. Capillaries thus treated frequently proved to be highly toxic when placed inside axons, causing rapid, irreversible loss of the action potential followed by obvious precipitation of the axoplasm on the surface of the capillary. Correspondence with the manufacturer indicated that the glass capillaries supplied to us had been treated to remove heavy metal used in their manufacture. The acid treatment we used apparently exposed additional toxic heavy metals. After considerable experimentation, the following sequence of procedures for preparing glass capillaries was adopted. (*a*) Capillaries were treated for 9–17 hr with 0.5 M HNO_3 at

90°C and then thoroughly rinsed in distilled water. (*b*) The capillaries were then placed in 50 mM EDTA pH 6.5 for 48 hr to chelate heavy metals. Deviation from pH 6.5–7.0 markedly accelerated the dissolution of the porous region, as did higher concentrations of EDTA. (*c*) Prior to each experiment, the capillaries, previously stored in distilled water, were replaced in EDTA for 12 hr and then rinsed in water. Capillaries treated in this manner proved to have a useful life, barring an accident, of several weeks, during which time the porosity and mechanical fragility gradually increased.

EVALUATION OF POROSITY TO LOW MOLECULAR WEIGHT SUBSTANCES Specifications by the manufacturer state that the porous area of porous glass is about 10–20% of the geometrical area. This was checked on two samples by measuring the amount of water adsorbed by the porous regions of the capillary. The results indicated that these samples, which were probably more porous than those routinely manufactured,

TABLE II

PERMEABILITY PROPERTIES OF POROUS GLASS CAPILLARIES

		Percent removed in one transit*						
Capillary reference	Date of assay	Na	ATP	Phenol red	Hemoglobin	Myokinase	Aldolase	Water filtered
1E20	033065	90	~95					
1D9NA-20-2	062966		38		1.1×10^{-5}		$<10^{-5}$	
1D9NA-20-1	070766		37–43		1.4×10^{-5}		$<10^{-5}$	2.1
1D9NA-20-3M	071966		25	~5	8.6×10^{-6}	$<10^{-5}$		
2D8NA-6-1	072966		20					
1D7NA-20-4	120166				1.0×10^{-7}			0.8
1D13NA-20-3	120166			~20				

* Flow rate ≅ 0.5 μl/min. Pressure at porous region about 40 cm H_2O. Dialysis of all substances against distilled water, or seawater.

had 18% of their area porous. Selected capillaries were evaluated in a semiquantitative manner for their permeability to substances such as Na and ATP in the following manner. A capillary tube about 5 cm in length and 0.05 cm in internal diameter was positioned on the stage of a dissecting microscope and filled with distilled water. The porous capillary was placed inside the distilled water capillary with the porous region surrounded by distilled water. A polyethylene tube connected the porous capillary tube to a motor-driven syringe and a solution of ^{22}Na or ATP was allowed to flow through the porous capillary tube at 0.5 μl/min. The effluent from the porous capillary was collected in small calibrated lengths of capillary tubing (either 2 or 5 μl) and these were analyzed for either ^{22}Na or ATP. A second method of evaluating porosity was to mount the porous capillary in a chamber (described below) and to collect the efflux coming from the capillary in the same manner as used in studying axons. In both methods, it is possible to calculate the per cent of material removed in one transit of dialysis fluid through the capillary and the data obtained are shown in Table II.

Bulk water flow across the porous wall was measured by jacketing the porous region of the capillary with a close fitting glass capillary partially filled with water. The motion of the meniscus in the jacketing capillary tube was followed by observing it under a microscope fitted with an ocular micrometer. A measurement of meniscus motion in the absence of fluid flow through the porous capillary gave the rate of evaporation of water from the jacket, and the change in rate of meniscus motion when fluid was driven through the porous capillary at a rate of 0.5 μl/min together with the known dimensions of the annulus of water made it possible to calculate water filtration. The volume flow of water required a pressure of about 40 cm H_2O at the porous region and the extent of this water flow is given in Table II. It is to be noted that, unlike the measurements with ATP and ^{22}Na, the concentration of H_2O on both sides of the porous capillary was the same and the net water flow was a purely hydraulic one.

THE POROSITY TO PROTEINS Rather concentrated solutions of the proteins hemoglobin, myokinase, and aldolase were placed in the tube surrounding the porous capillary as described for low molecular weight substances. Distilled water was driven through the porous capillary at 0.5 μl/min and the effluent collected and analyzed for the appropriate protein substance. In order to be certain that the water flow described above was not interfering with movement of protein, some experiments were done in which the water flow through the porous capillary was stopped for a fixed period of time; the fluid was then washed out of the porous region and analyzed. The results obtained are given in Table II. The general conclusion about the behavior of the porous glass which we used is that the product (diffusion constant $\times$ area) for substances with a molecular weight of less than 1000 is of the order of 10% of that of free diffusion. For protein, this product is very much less than 0.1% of that of free diffusion.

BEHAVIOR OF THE POROUS CAPILLARY IN THE AXON In order to insert the porous capillary into the axon it must be mounted on some sort of a rigid holder. Second there must be some provision for rapid changes of dialysis fluid with a minimum of dead space. These requirements are met with the capillary holder shown in Fig. 1.

The Lucite body of the capillary holder is attached to the horizontal arm of a micromanipulator which controls the advance of the capillary through the axon cannula and into the axon.

The chamber into which the end of the porous capillary protrudes can be completely flushed through the polyethylene tubes entering and leaving the Lucite holder. The dead space of the system consists of the volume of the capillary itself which is about 0.3 μl. At the flow rates employed in this study, this volume was washed out in 1–3 min. Because the resistance to flow in the capillary was much greater than in the tubing, solutions could be flushed through the system relatively rapidly without forcing fluid through the capillary. Several small bubbles of air were used to separate different dialysis solutions as they were pushed through the tubing. About 75 μl of solution sufficed to flush and refill the plastic tubing, the free end of which was attached to a Hamilton gas-tight syringe mounted on a motor-driven pump. A

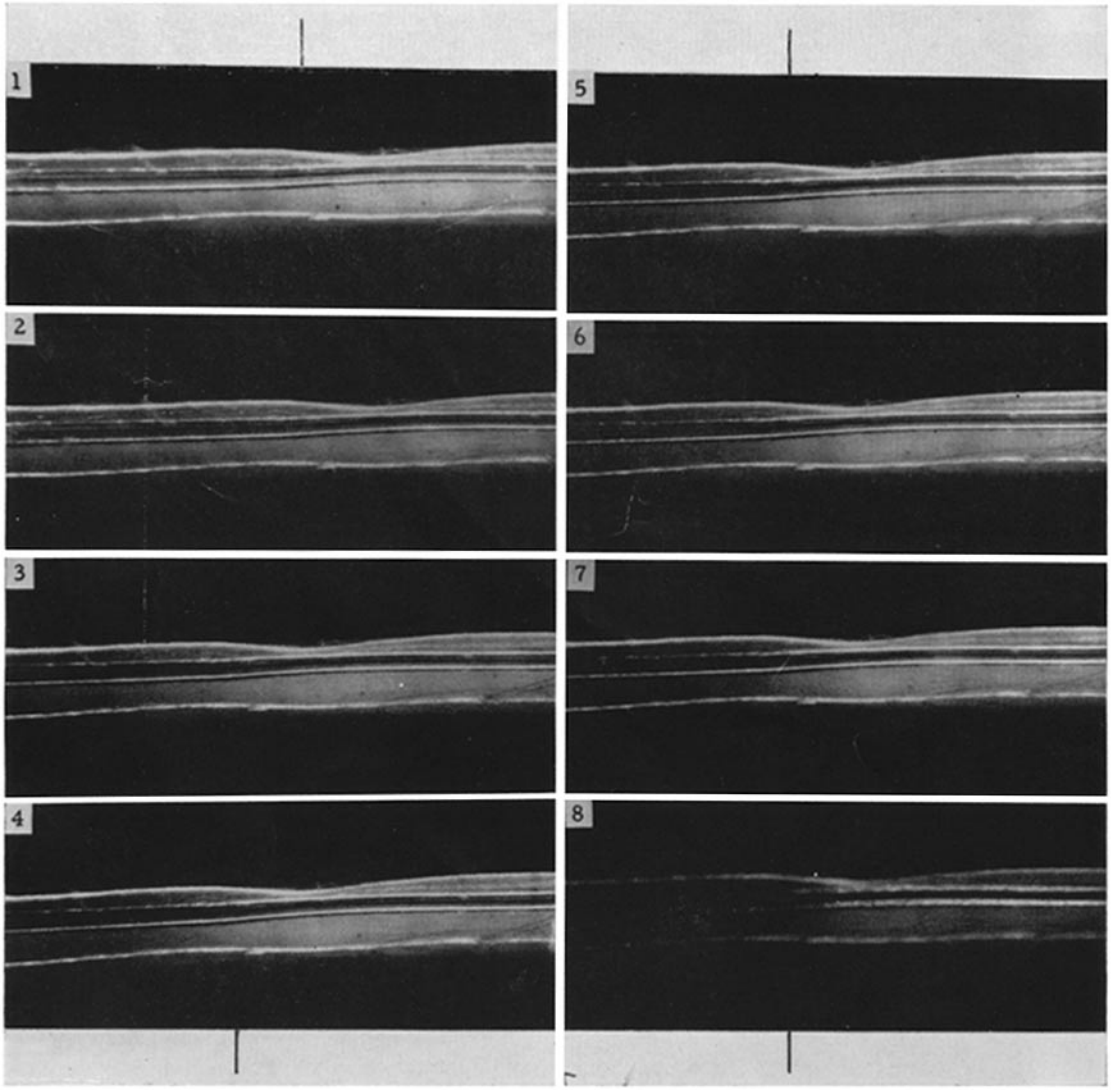

FIGURE 2. A photomicrograph of a squid axon with a porous glass capillary inserted in the axon. The photographs show the junction between porous and nonporous regions of the capillary and the location of this region is approximately given by a line shown on the margins of the photograph. In frame 1, the capillary is filled with K isethionate while frame 2 was taken 10 sec after a solution of phenol red had been introduced into the dialysis capillary. The porous region is to the left of the photograph and the nonporous to the right. The axon is 0.5 mm diameter and the transition between porous and nonporous regions is not more than 1 axon diameter. Frame 3 was taken 44 sec after the start of phenol red perfusion and a darkening of the axoplasm on the left-hand side is appreciable. Frames 4 through 7 were taken 90, 120, 150, and 330 sec after the start of phenol red perfusion. In frame 7 the equilibration of phenol red throughout the axoplasm in the porous region is apparent, while the axoplasm remains entirely clear on the nonporous (right-hand) side of the junction. Frame 8 was taken 400 sec after phenol red dialysis had begun. A blue filter was introduced into the optical system in order to enhance the contrast.

complete solution change was effected in about 4 min; this interruption was far too short to reduce the specific activity of sodium in the dialyzed axoplasm.

The tests for porosity which were all done in vitro do not necessarily guarantee an identical behavior inside the axon, since it can be argued that the axoplasm may occlude some of the pores and thus reduce the permeability. To meet this objection the following experiments were done with the capillary inside intact axons. Fig. 2 shows a series of photomicrographs of a squid axon with a porous capillary in place.

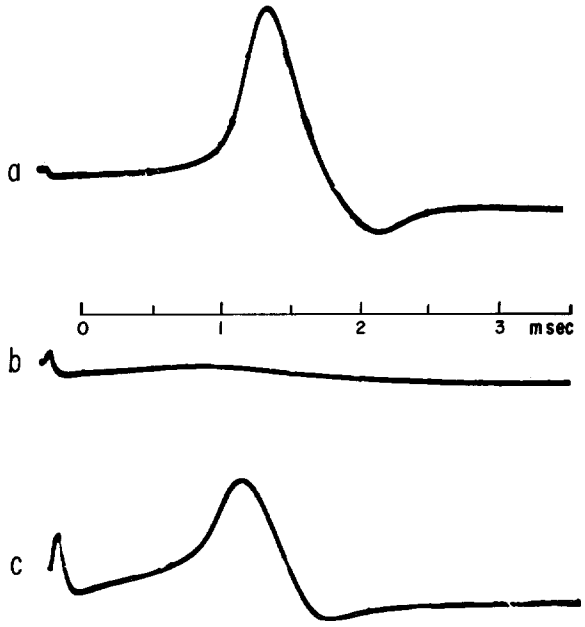

FIGURE 3. This shows an extracellularly recorded action potential from a squid axon undergoing internal dialysis. In trace *a*, the internal dialysis fluid was 0.5 M K isethionate. Trace *b* shows the abolition of the action potential 53 sec. after the internal perfusion solution had been changed to 0.5 M Na isethionate, while trace *c* shows the recovery of the action potential after the internal perfusion with Na had gone on for 40 min, followed by a change of the internal dialysis medium to K isethionate for about 1 min.

The junction between porous and nonporous glass, determined optically, is indicated by the vertical line. In frame 1 of Fig. 2, the capillary is empty, while frame 2 shows the same region 10 sec after starting the perfusion of a solution of phenol red through the capillary. The dye is clearly visible inside the capillary and, in the original photograph, faint staining of the axoplasm outside the porous length was also visible. The remaining photographs in the figure were taken at progressively longer intervals after dialysis had been begun and they show progressively deeper staining of the axoplasm. Frame 8 was taken through a blue filter to increase contrast. The length of the transition zone between porous and nonporous capillary (as indicated by staining of the axoplasm) is about 1 axon diameter or 500 μ.

Baker, Hodgkin, and Shaw (1962) have reported that impulse conduction in perfused axons is lost when the internal sodium concentration is raised to about 220 mM. Fig. 3, showing externally recorded action potentials, illustrates how this observation was used to determine the rapidity of axoplasm dialysis by the capillary. Trace *a* was taken during a preliminary dialysis with isotonic potassium isethionate. 53 sec after a sudden change to isotonic sodium isethionate, excitability was lost, as shown in trace *b*. The conclusion from this experiment was that the internal sodium rose from zero to about 40% of its final concentration in 53 sec, corresponding to a half-time for dialysis of 1 min. Trace *c*, taken 40 min after the start of perfusion, shows the recovery of the action potential when the internal dialysis solution was again changed to isotonic K isethionate.

During the course of the efflux experiments, a number of determinations of the half-time for tracer equilibration following the sudden introduction of tracer into or removal from the inside of the capillary were made. These experimental half-times were in the range 2–5 min, in rather good agreement with the theoretical calculations for diffusion equilibration (see Theoretical section).

The general conclusion from these three types of experiments was that the permeability of porous capillaries was not significantly altered by placing them inside axons, and that a prolonged contact of several hours with axoplasm does not reduce the porosity.

Theoretical Analysis of Diffusion

In order to compare the observed tracer diffusion in the axoplasm with that to be expected on theoretical grounds, numerical or analytical solutions of the appropriate diffusion equation were obtained for two sets of boundary conditions corresponding to the situation obtaining in efflux and influx experiments. Since substrates such as ATP may be expected to be consumed by metabolism during the course of diffusion from the outer surface of the porous capillary to the membrane, an appropriate modification of the diffusion equation has been made to permit calculation of concentration profiles when solute consumption is taking place.

RADIAL DIFFUSION IN THE POROUS REGION—END EFFECTS NEGLECTED A solution to this problem was obtained to determine whether the experimental half-time of 2–5 min obtained for tracer equilibration of axoplasm agreed with theoretical expectations. The experimental arrangement of a porous glass capillary inside the axon was approximated by two hollow concentric homogeneous cylinders, 1 and 2, each characterized by a diffusion coefficient D_i. The inner cylinder represented the porous glass, the inner surface of which was kept at a constant concentration of solute. The outer cylinder represented the axoplasm, and was bounded by the membrane. Because the actual efflux of Na^+ across the membrane is very small compared to that entering the axoplasm by diffusion, the outer surface of cylinder 2 was considered impermeable. The efflux of tracer from the axon at any time, although trivial in amount, will be proportional to the specific activity present at the membrane so that the experimental time-efflux relation after onset of dialysis reflects the time-concentration relation at the axolemma and this can be compared with the theoretical calculations.

The set of equations which allows distributed consumption of the diffusing species in the axoplasm is as follows:—

$$\frac{\partial C}{\partial t} = \frac{D}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C}{\partial r}\right) - kC^n \quad \text{for} \quad r_1 \leqq r \leqq r_3$$

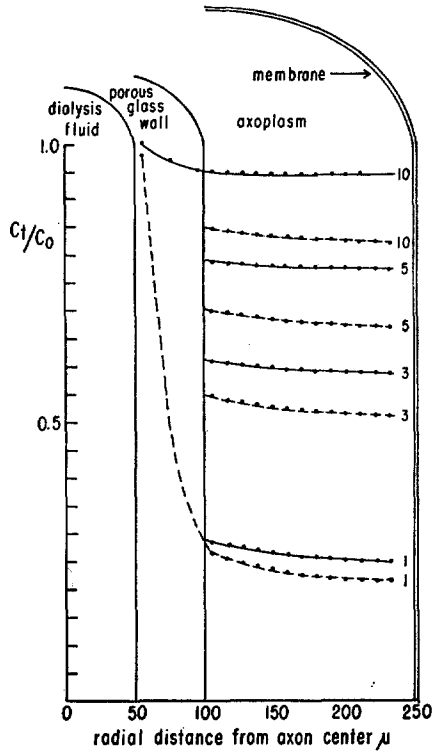

FIGURE 4. This is a diagram of the porous glass capillary inside a squid axon; the superimposed ordinate shows the concentration of a solute (C_t) as a fraction of its concentration in dialysis fluid (C_o) for the radial distances from the center of the axon, shown on the abscissa. The curves shown are solutions to the diffusion equation given in the text for times in minutes shown on the curves. Solid lines represent the no solute consumption case (and are therefore appropriate for indicating ^{22}Na distribution) while broken lines represent a solute consumption by axoplasm of 0.056 μmole/mM-g-min (and are therefore approximately indicative of ATP distribution).

with boundary conditions

$$C(r_1, t) = C_0 \qquad \frac{\partial C(r_3, t)}{\partial r} = 0$$

$$D_1 A_1 \frac{\partial C_1}{\partial r}\bigg|_{r = r_2} = D_2 A_2 \frac{\partial C_2}{\partial r}\bigg|_{r = r_2}$$

where C = concentration, t = time, D = diffusion constant, r = radial distance from cylinder axis, k = constant relating solute concentration to consumption, n = exponent to allow for solute consumption ($n = 0, 1$ for these calculations), A = area. Subscripts 1 and 2 refer to porous glass and axoplasm respectively, r_1, r_2, r_3 are distances in r of the inner and outer surface of the porous glass and the axon membrane.

Solution of this system of equations was obtained by numerical integration of the differential equation using an IBM 7094 computer. The program for the computer was written by Mr. Richard Thomas and supervised by Dr. W. H. Huggins; it was checked by comparing the results with certain special cases for which analytical solutions could be obtained. We believe that the curves shown in Fig. 4 are correct to within 10%.

Fig. 4 shows the theoretical radial concentration profile in the porous glass and axoplasm for parameters appropriate to small molecules such as ATP (see figure legend for constants used). Fig. 4 also shows the calculated profiles for the same diffusion parameters when the diffusing species is being consumed uniformly in the axoplasm at a rate appropriate for ATP at 17°C. Fig. 5 shows the theoretical time-con-

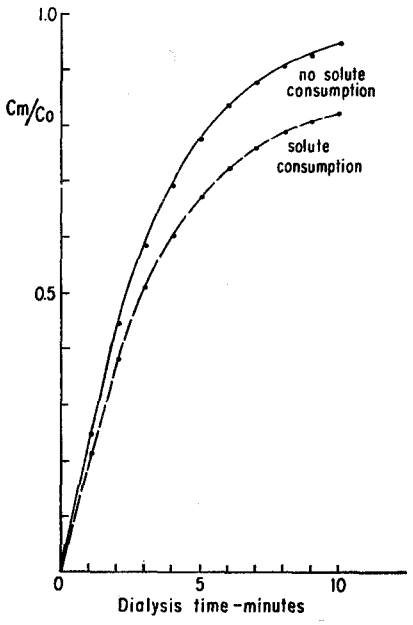

FIGURE 5. The solute concentration at the membrane (C_m) is plotted as a fraction of the concentration of the solute in dialysis fluid (C_o) for various times shown on the abscissa. The solid line represents the no solute consumption case and the dashed line a consumption shown on the curve. The times for half-equilibration in both cases are about 2.5 min.

centration relations at the surface of the outer cylinder (axon membrane) for diffusion with and without consumption. In either case the time to reach half-maximum is about 2–3 min. The maximum surface concentration reached in the case of consumption, however, is only about 85% of that supplied at the inner surface of the porous capillary. The computed half-times are somewhat smaller than those experimentally observed; they are probably satisfactory if one considers that experimentally, the change of concentration at the inner face of the porous glass is not likely to be instantaneous, as is assumed in the theoretical treatment.

The analysis above provides no information about diffusion near the end regions of the porous capillary, where the gradients will have both longitudinal and radial components. Because the contribution of these end regions to the total efflux is difficult to assess even approximately, and because it may vary with experimental circumstances, a guard system was designed to exclude the efflux from the end regions

from the collecting system. This guard system is described in the section dealing with efflux chamber design.

EFFECT OF END REGIONS OF THE POROUS CAPILLARY ON INFLUX The method used for making influx measurements does not exclude isotope from regions of the membrane beyond the ends of the porous region of the capillary. During influx experiments, isotope can enter the axoplasm in regions outside of the porous length and then diffuse longitudinally from the axoplasm into the porous region. The following calculation serves to estimate the magnitude of the error introduced from this source.

The geometry assumed for the problem was that of a semiinfinite circular cylinder ($0 \geq z \geq \infty$) with the circular face, $z = 0$, kept at zero concentration. The isotope diffusion into one end region is equal to the flux across the circular face. The contribution from both end regions into the porous region would therefore be twice this amount. The end regions were considered to be sufficiently far apart so that they did not interact.

The solution of this problem for the stated boundary conditions is given in Carslaw and Jaeger (1959) as:

$$\frac{C(r,z,t)}{C_0} = \frac{h}{a}\sum \frac{J_0(r\alpha_n)}{(h^2+\alpha_n^2)J_0(a\alpha_n)}\left[2e^{-\alpha_n z} + e^{\alpha_n z}\operatorname{erfc}\left(\alpha_n\sqrt{Dt} + \left(\frac{z}{2\sqrt{Dt}}\right)\right) - e^{-\alpha_n z}\operatorname{erfc}\left(\alpha_n\sqrt{Dt} - \left(\frac{z}{2\sqrt{Dt}}\right)\right)\right]$$

At the face $z = 0$, and for large t, the transport of material can be written as

$$M = -4\pi D C_0 h^2\left[\sum_0^\infty \frac{1}{(h^2+\alpha_n^2)\alpha_n}\left(1 + \frac{e^{-\alpha_n^2 Dt}}{\alpha_n\sqrt{\pi Dt}} - \operatorname{erfc}\alpha_n\sqrt{Dt}\right)\right]$$

where α_n are the roots of: $\alpha P J_1(\alpha a) - D J_o(\alpha a) = 0$, $h = P/D$, and P is the permeability coefficient for material crossing the axolemma.

Numerical evaluation of this equation indicates that for a 15 mm porous capillary, the steady-state diffusion into the ends of the porous regions may contribute as much as 50% to the total influx. However, this steady state is approached with a half-time of about 10 hr. The steady-state contribution, moreover, is overestimated considerably because the actual exposure of the axon to tracer extends only to a few millimeters beyond the porous region and not for an infinite distance as assumed in the model. The conclusion from this analysis is that edge effects will not significantly affect influx experiments lasting at most 2–4 hr.

Efflux Chamber

The salient design considerations for this piece of apparatus were as follows: (*a*) Each end of the axon must be independently movable in both vertical and horizontal planes to facilitate insertion of the porous capillary without scraping the inside of the membrane. (*b*) Efflux from the regions of axon at the junction between porous and nonporous glass must be excluded from the collecting system. (*c*) There must be provision for extracellular stimulation and recording of action potentials.

These requirements were satisfactorily met by the chamber shown in Fig. 6. A 4–5 cm length of cleaned squid axon was cannulated at both ends and mounted horizontally. The porous capillary was passed through the left-hand end cannula, which acted as a collimator, and was slowly advanced along the length of the axon. This procedure was viewed from above by a binocular dissecting microscope. A front surface mirror mounted at an angle of 45° to the direction of observation enabled one to view the tip

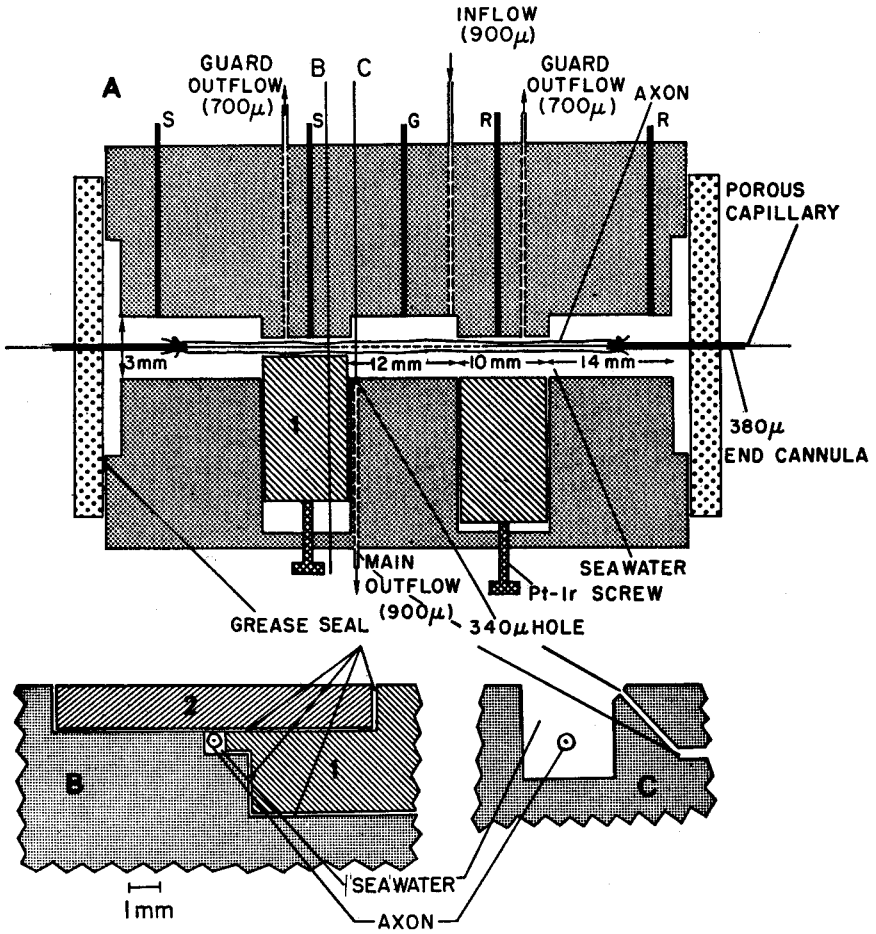

FIGURE 6. Schematic drawing of apparatus used to hold the cannulated axon during dialysis. A is a top view (not to scale); B and C are cross-sections (to scale) at the indicated levels. Labels *S*, *R*, and *G* refer to stimulating, recording, and ground leads, respectively, which are recessed slightly to avoid contact with the axon. The cannulae, to which the ends of the axon are tied, are seated in the shaded blocks which are attached to micromanipulators to allow positioning of the axon in the chamber or in the slot. After the axon has been lowered into the slot, the Lucite pieces labeled *1* are advanced with the thumbscrew to form part of the guard chamber. The piece labeled *2* is then placed on top to complete the guard compartment. The clearance between the axon and any side of the guard compartments is 50–100 μ.

of the porous capillary within the axon simultaneously in the vertical and horizontal planes.

The two end cannulae were carried in separate movable Lucite blocks attached to micromanipulators. These blocks were held against the main chamber by grease seals. Alignment of the axon relative to the tip of the capillary was accomplished by independent adjustment of these movable blocks.

The porous capillary was slowly steered first through the axon and then through the right-hand end cannula until the porous region lay in the center of the axon and the capillary tip extended from the right-hand end cannula. The final position of the capillary was set by reference to vernier scales on its manipulator drive. The axon with the porous capillary inside was then lowered into a slot cut in the bottom of the chamber. Simultaneous movement of the porous capillary and end cannulae was not required because the flexibility of the porous capillary allowed it to be as much as 1 cm off axis without danger of breakage.

After the axon was maneuvered into the slot, a system of greased blocks converted the guard compartments into watertight channels 700 μ square in cross-section (see Fig. 6).

EVALUATION OF GUARD SYSTEM If the guard system is operating properly, none of the flux originating from the dialyzed regions in the guard compartments should reach the center compartment, although a portion of the radioactivity coming from the center compartment will exit through the guard compartments. The ratio of flux collected to total flux coming from the dialyzed region is given by the following equations. If V_c is the volume of fluid collected from the center compartment and V_g, the volume removed by each guard syringe, then the radioactivity originating from the length of axon in the center compartment is:

$$\left(\frac{V_c + V_g}{V_c}\right) X_c$$

where X_c (cpm) is the radioactivity collected from the center. (Note that the fluid withdrawn through the guard compartment nearest the inflow does not pass over the axon and hence does not remove radioactivity from the center compartment.) If L_1 and L_2 are lengths of the center compartment and the total porous length of the capillary and X_T the total radioactivity coming from the dialyzed region, then the ratio, L_1/L_2, should equal the ratio:

$$\frac{(V_g + V_c)}{V_c} \cdot \frac{X_c}{X_T}$$

for a perfect fractionation of the flow.

An experimental test of these theoretical relations was made using a porous capillary placed in the chamber. The capillary was perfused with phenol red dye to which the porous region is permeable. The flow system was operated as described above and the amount of dye collected from the center compartment was measured in a spectrophotometer. The ratio of lengths, L_1/L_2, was 0.78, and $(V_g + V_c)X_c/V_cX_T$ was 0.81 at the flow parameters used. At other flows the agreement was less satisfactory, presumably because of uncontrolled eddy currents.

A second test of the efficacy of the guard system was made as follows. The porous capillary was withdrawn until the porous region was entirely inside one guard compartment with the edge of the porous region about 0.5 mm from the center compartment. The guard syringe was turned off and the amount of dye reaching the center compartment by diffusion from the guard compartment was determined. The guard syringe was then turned on and the amount of dye collected under these circumstances was measured. The results showed that the guard syringe kept at least 95% of the "diffusion flux" out of the center compartment.

EFFLUX EXPERIMENTS The excess seawater was removed from the chamber and the motor-driven syringes were turned on. Fluid was delivered into the center compartment at 1.2 ml/min and was removed by a suction from the 340 μ standpipe as shown in Fig. 6. Ordinarily the level of fluid in the center compartment remained near the top of the Lucite block. Brief fluctuations in the fluid level in the groove during sample changing were of no consequence since the axon was at all times held motionless beneath the meniscus. The standpipe was attached via a polyethylene catheter to a stoppered test tube connected to a vacuum line. Sampling of the efflux fluid was accomplished by changing the test tubes at minute intervals. Two additional motor-driven syringes withdrew fluid from each guard flow line at a rate of 0.07 ml/min causing a stream of solution to flow from the center compartment into each guard compartment, thus preventing any isotope leaving the axon in the guard compartment from being included in the efflux collection system. Experimental tests of the performance of the guard system have already been described. The rapidity of the center compartment washout was tested by rapid injection of a few microliters of phenol red dye into the center compartment when all syringes were operating. The slot washout was at least 95% complete in 1 min.

INFLUX EXPERIMENTS For influx experiments the same chamber was used, except that there were no guard syringes and the entire porous length of the capillary was located in the center compartment. Under these conditions the dialysate collected by the porous capillary included not only the ^{24}Na traversing the membrane in the porous region but also that which had crossed the membrane lying beyond the porous region and which had diffused longitudinally through the axoplasm. However, as discussed in the Theoretical section, such contribution to the collected radioactivity will be only about 10% for the relatively short duration experiments (30–60 min) reported here.

COLLECTION OF DIALYSATE FLUID As shown in Fig. 1, the tip of the porous capillary is covered by a longer glass tube attached to a manipulator. Fluid emerging from the porous capillary fills the tube by capillary action. Samples of the dialysis fluid were routinely taken for determination of isotope specific activity. Collection of samples could be as often as every 2 min, but was usually every 4–5 min.

RESULTS

In presenting the results obtained with internal dialysis experiments the following criteria for a satisfactory experiment were considered important: (*a*) the Na efflux should be stable, (*b*) the magnitude of the efflux should be

within the range of values obtained with injected squid axons, and (*c*) there should be evidence that the Na efflux is sensitive to the presence or absence of ATP since this substance is considered to be an energy source for Na extrusion.

Na Efflux Stability and Magnitude Some idea of the stability of the Na efflux

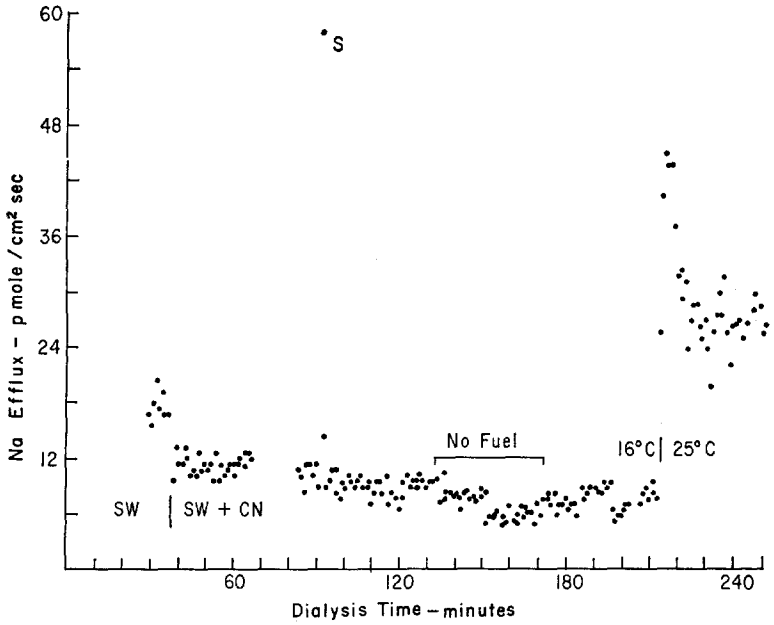

FIGURE 7. The sodium efflux from a dialyzed squid axon is plotted as a function of the time during which dialysis with a fluid containing 8.3 mM ATP + 10 mM PA was carried out. The axon was initially in seawater and this was changed to 2 mM CN seawater at the point indicated on the figure. At 91 min the axon was stimulated for 1 min at 30/sec and the response of the Na efflux to this stimulation is shown by the point near the top of the curve marked *S*. At 122 min the internal dialysis fluid was changed to one without ATP or PA. This is shown as "no fuel." This led to a decline in the Na efflux. Fuel was readmitted at 172 min and the efflux increased somewhat. A change in temperature at 214 min as shown on the chart yielded a considerable increase in the Na efflux and a transient change in efflux that lasted for more than 5 min

in a typical experiment is shown in Fig. 7. This axon was dialyzed with a medium containing ATP 8, PA 10, and Mg 2 mM and showed an efflux of Na that was entirely stable over some 4 hr of internal dialysis, aside from those periods when the experimental conditions were deliberately altered. The mean increase in base line Na efflux in 13 preliminary experiments was only 0.1%/min and it is difficult to be certain in these experiments whether such a change is an increase in passive ion leak or a change in rate of active Na extrusion. In any case, the efflux stability compares favorably with results obtained from

injected squid axons and can be taken as evidence that dialysis per se does not result in any great deterioration of the axon over the periods of time which are normally used for experimentation with intact axons. The mean duration of these preliminary experiments was 124 min; several experiments were terminated for reasons other than fiber deterioration. The magnitude of the Na efflux in the experiment shown in Fig. 7 is 12 pmole/cm² sec at 16° C. This value is clearly lower than values obtained in later experiments even when scaled to the temperature used. We believe that three factors contributed to

TABLE III

Na EFFLUX FROM DIALYZED AXONS

(Preliminary series)

					Dialysis fluid				Na efflux	
					[Mg]	[Na]	[ATP]	[PA]	Resting	Stimulated
Axon reference	Axon diameter	Dialysis time	Dialysis rate	Temperature					pmole/cm²sec	pmole/cm²imp
	μ	min	μl/min	°C	mM	mM	mM	mM		
032265	540	50	~2.5	12	0	75	1		19	3.4
040265	532	110		9	0	90	1		8	
040665	450	85		10	0	100	10		11	
060365	500	150	0.5–2.5	13	0	100	10	10	8	1.0
070165	450	100		16	0	75	10		46	
071465	475	180	1.3	17	0	60	10		16	0.5
071765	500	250	1.0	15	0	45	8.3	10	12	1.4
072465	450	160		18	0.1	103	7	8.1	15	
072765	450	90	1.3	13	0.1	103	7	8.1	73	
102765	501	160	0.3	13	4	89	4	10	35	1.1
120765	617	133	0.3	10	4	89	4	10	22	2.6
120865	660	45	0.5	11	4	89	4	10	16	1.2
032366	584	100	0.3	12	7.4	132	10		18	1.3
Mean ± SD		124							23±5	1.6±0.3
n =		13							13	8

this lower Na efflux: (*a*) the Mg^{++} concentration was too low, (*b*) we had only about one-half of the nominal ATP concentration in the dialysis medium because improper storage of ATP stock solutions resulted in considerable hydrolysis to ADP, and (*c*) we were using a commercially obtained source of phosphoarginine which subsequent enzymatic analysis showed to contain only 10–15% phosphoarginine. The data obtained in 13 preliminary experiments are summarized in Table III where the mean Na efflux under fueled conditions is 23 pmole/cm² sec. A change of $[Mg^{++}]$ to 4 mM (in addition to that chelated with added phosphonucleotide) and improved methods for the preparation of ATP solutions and their storage at low temperature yielded a mean value for Na efflux of 48 pmole/cm² sec at 13–15°C as shown in Table IV. In

some of these experiments ATP was used as the sole source of pump fuel while in others phosphoarginine supplied by Professor Morrison was used. While there may be some small difference in level of Na efflux, it is clear that ATP alone yields an efflux that is well within physiological limits.

TABLE IV

Na EFFLUX FROM DIALYZED AXONS
(Final series)

Axon reference	Axon diameter	Dialysis time	Temperature	Dialysis fluid mM				Na efflux	
								Resting	Stimulated
				[Mg]	[Na]	[ATP]	[PA]	pmole/cm²sec	pmole/cm²imp
	μ	min	°C						
062366-2	585	50	14	4	89	4	4	70	0.9
062466	568	100	15	4	89	4	4	75	1.5
070166	534	140	14	4	89	10	10	65	3.9
070766	468	50	14	4	89	10	0	75	
072266	475	90	14	4	89	10	30	~70	
100566	550	110	9	4	80	14	10	70	
101266	500	120	14	4	80	14	10	30	
101866	500	160	11–14	4	80	12	10	38	
102566	500	150	12–13	4	76	5	10	27	
110866-1	743	76	13	4	76	5	10	41	
110866-2	601	110	12	4	76	5	10	46	
111066-2	501	85	14	4	76	5	10	57	
111566-1	668	130	9–12	4	76	5	10	26	2.0
111566-2	584	110	10–13	4	76	5	10	23	4.3
111666	618	92	11	4	76	5	10	25	5.5
112266-1	568	120	15	4	85	5	10	40	
112266-2	575	113	14–16	4	85	5	10	41	4.5
010467-1	584	121	15–16	4	85	5	10		1.0 4.0
012067*	500	90	16–18	4	85	5	10	52	
Mean ± SD		106						48±4	3.1±0.6
n =		19						18	9

* Dialysis fluid contained 10 μM atractyloside.

Effects of an ATP-Free Dialysis Medium Fig. 7 also shows the effect on Na efflux of omitting both ATP and PA from the perfusion medium. Under this no-fuel dialysis, the Na efflux declines slowly to about 50% of its initial value and it rises toward control levels when fuel is reapplied later in the experiment. This change was made with a number of the axons in the preliminary series with the result that the Na efflux declined to a mean value of about 40% of the control flux. A final experimental variation shown in Fig. 7 was a change in temperature from 13° to 26°C. This change produced a mean steady-state change in efflux level of 2.2 times (see Table V) for a

10°C temperature increase. The transient response of Na efflux to temperature was seen in two out of three axons in which this temperature step experiment was performed. The transient could not have been due to a slow thermal response of the fluid in the chamber, since direct monitoring of the temperature in the slot showed the changes to be complete within 1–2 min. Calculations of the temperature response of axons from appropriate formulae in Carslaw and Jaeger (1959), assuming the axon to be an infinite cylinder and the thermal diffusivity of axoplasm to be the same as water (1.44×10^{-3} cm²/sec at 25°C), indicate that the transient is not due to slow warming of the fibers.

Effect of Strophanthidin The cardiac glycoside ouabain has been shown by Caldwell and Keynes (1959) to reduce markedly Na extrusion from squid

TABLE V
TEMPERATURE COEFFICIENT OF SODIUM EFFLUX IN DIALYZED SQUID AXONS

Axon reference	Temperature			Na efflux (T_2) / Na efflux (T_1)	Q_{10}
	T_1	T_2	ΔT		
	°C	°C	°C		
071765	16	25	9	2.3	2.5
072465	18	25	7	1.9	2.4
072765	13	25	12	2.3	2.0
102765	13	26	13	1.9	1.7
120765	8	27	19	5.2	2.4
Mean ± SD					2.2±0.1
n=					5

axons. This substance has a similar inhibitory action on the ATPase of squid nerve membrane fragments (Bonting and Caravaggio, 1962) so that its action is presumed to be one of interference with the energy supply to the Na pump. We have used strophanthidin, the aglycone of ouabain, to test whether the Na efflux of a dialyzed axon would respond to this inhibitor. An experiment is shown in Fig. 8 in which an axon fueled with ATP + PA was treated with seawater containing 10 μM strophanthidin. There ensued a prompt fall in Na efflux and shortly after the Na efflux had reached its fully inhibited value, the internal dialysis solution was changed to one containing no fuel. Strophanthidin was then removed but the Na efflux continued at a low level until fuel was resupplied to the internal perfusion medium. This led to a gradual recovery of Na extrusion toward control levels.

The Na efflux of this axon was 23 pmole/cm²sec and with strophanthidin

the efflux fell to 10 pmole/cm²sec. Although the control value of Na efflux is about half that for injected axons at 13°C, the inhibited value is virtually identical with values reported by Caldwell and Keynes (1959). It would appear, therefore, that in dialyzed axons strophanthidin decreases the Na efflux to values similar to those found in other experiments with intact axons.

Sodium Influx Because the manipulations involved in internal dialysis might a priori be expected to cause an increased passive ion leakage of the membrane, it seemed necessary to measure the flux of an ion that is presumed

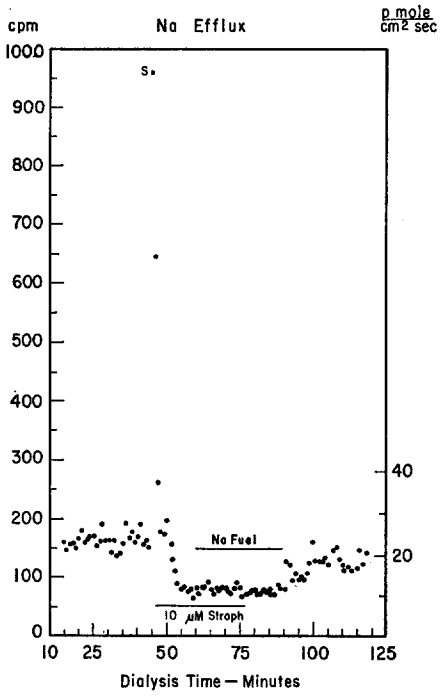

FIGURE 8. This axon was dialyzed with fuel containing 4 mM ATP, 10 mM PA, and 4 mM Mg^{++}. After 42 min of control dialysis, the axon was stimulated for 1 min at a frequency of 25/sec. A peak response to this stimulation is shown by the efflux point labeled *S*. A concentration of 10 μM strophanthidin was applied and as the figure shows, there was a prompt reduction of Na efflux. Fuel was removed from the dialysis fluid at 60 min and overlapped the period in strophanthidin. When fuel was reapplied, there was a recovery of the Na efflux.

to move passively across the membrane. By introduction of ^{24}Na into the seawater flowing outside of the axon (as described in the Methods section) while dialyzing the axon internally with a nonradioactive medium, it was possible to collect the ^{24}Na entering the axon in the porous region of the dialysis capillary and thus to evaluate the rate of entry of Na^+. An experiment of this sort is shown in Fig. 9. The sample interval for this experiment was 2–4 min and the radioactivity collected per sample rises to a plateau with a time constant that is expected from the performance of our flow system. In eight such experiments the mean value of Na influx was 57 pmole/cm² sec (see Table VI).

The primary purpose of these experiments was not to determine the stability of the influx; however, in two experiments there was no essential change

in the influx in 80–90 min of perfusion. The remaining six experiments were terminated before a reliable assessment of stability could be made.

Potassium Efflux Another check on the passive ion permeability properties of the axon membrane in dialyzed squid axons was to measure the potassium efflux with ^{42}K. Fig. 9 shows a representative experiment. The mean K efflux from four axons was 59 pmole/cm²sec, which is about 50% higher than values reported for injected squid axons (45 pmole/cm² sec) (Brinley and Mullins, 1965 *a*). The mean flux increase was about 1.1%/min, although there was considerable scatter in the data.

Effects of Stimulation on Sodium and Potassium Efflux During the course of the experiments, axons were occasionally stimulated at 10–50 impulses per

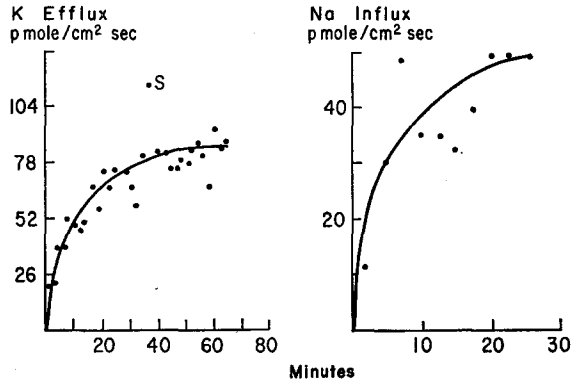

FIGURE 9. The diagram on the left shows the efflux of K from a dialyzed squid axon when the internal dialysis medium contained no fuel and $[Mg]_i$ = 4 mM. The response to stimulation of the axon for 30 sec at 50/sec is shown by the point marked *S*. On the right, the influx of Na is plotted as a function of time for an axon internally dialyzed with a medium consisting of 440 mM K isethionate and 100 mM Na isethionate.

sec for 30–60 sec to check on the rapidity of washout of the chamber, since all of the extra efflux should be confined to one, or at most two, collecting intervals. The extra efflux associated with each impulse was 3.1 pmole/cm² imp for sodium(Table IV) and 2.9 pmole/cm² imp for potassium (Table VII). In addition, axons were given occasional test stimuli to check on excitability during the course of flux measurements.

DISCUSSION

The measurements reported in this paper were made to examine the extent to which a squid axon, subjected to internal solute control by dialysis, can extrude Na^+. Secondary objectives were to examine the passive movements of Na^+ and K^+ during rest and during bioelectric activity. These latter measurements were important because the ability of the experimenter to see

an active extrusion of Na^+ depends on the existence of a relatively low membrane permeability for this ion.

Composition of the Perfusion Medium Since our objective in these preliminary experiments was to obtain evidence for active sodium extrusion, no effort was made to change the composition of the dialysis medium except for

TABLE VI

SODIUM INFLUX IN DIALYZED SQUID AXONS

Axon reference	Axon diameter	Temperature	Na influx, pmole/cm² sec	Duration of experiment
	μ	°C		min
080466	450	11	66	15
080566-1	450	~10	90	25
080566-2	~450	11	105	80
080566-3	400	10	45	90
020167-1	801	13	24	56
020167-2	718	~13	37	20
020167-3	701	~13	37	30
020267	567	11	~56	30
Mean ± SD			57±9	
n=			8	

TABLE VII

POTASSIUM EFFLUX FROM DIALYZED SQUID AXONS

Axon reference	Axon diameter	Temperature	K efflux, Resting, pmole/cm² sec	K efflux, Stimulated, pmole/cm² imp
	μ	°C		
061566-1	450	16.5	87	2.3
061566-2	600	13.0	47	4.2
061666-1	490	~15	36	2.1
061666-2	450	13	67	
Mean ± SD			59±11	2.9±0.9
n=			4	3

a moderate increase in magnesium concentration above that initially used. Two papers have recently appeared (Tasaki et al., 1965; Adelman et al., 1966) indicating that perfused axons remain electrically excitable longest when perfused with a simple mixture of sodium and potassium fluorides. Unfortunately, fluoride forms insoluble magnesium salts and might be expected to inhibit the Na+K–activated ATPase presumably associated

TABLE VIII

COLLECTED FLUX DATA FOR SQUID AXONS

	Present data dialyzed axon 12–14 °C	Perfused axon 17–23 °C	Intact axon 13–15 °C	Intact axon 18–19 °C	Intact axon 22–23 °C	Species	Reference
Na influx, resting, *pmole/cm²sec*	57 (8)		17–40			L.p.*	Brinley and Mullins (1965 *a*)
				49		L.f.	Keynes (unpublished)
					63	L.p.	Shanes and Berman (1955)
		170–280				L.p.	Tasaki‡ (1963)
		133				L.f.	Shaw‡ (1966)
K efflux, resting, *pmole/cm²sec*	59 (4)		45			L.p.	Brinley and Mullins (1965 *a*)
				38		L.f.	Keynes (unpublished)
		942				L.f.	Shaw‡ (1966)
Stimulated fluxes, *pmole/cm² imp*							
Na efflux [500–750]§	1.6–3.1 (17)		5.3			L.p.	Brinley and Mullins (1965 *a*) [26,000]§
				5.4		L.f.	Hodgkin and Keynes (1955) [15,000]
K efflux [750]	2.9 (3)		ca. 5			L.p.	Brinley and Mullins (1965 *a*) [26,000]
			8.5			L.f.	Caldwell and Keynes (1960) [30,000]
Na efflux, *pmole/cm²sec*							
Resting		16		125		D.g.	Canessa and Rojas‖ (1966)
				18		L.f.	Hodgkin and Keynes (1956)
	48 (18)		{47}	70		L.f.	Caldwell et al. (1960)
					72	L.p.	Shanes and Berman (1955)
Glycoside-insensitive	10 (1)		{8}	12		L.p.	Caldwell et al. (1960)
		16		25		D.g.	Canessa and Rojas‖ (1966)
CN-insensitive	13 (4)¶			11		L.f.	Caldwell et al. (1960)
Q_{10} (sodium efflux)	2.2 (5) 8–27°C		3.3 10–20°C			S	Hodgkin and Keynes (1955)

Braces indicate measurement reduced to another temperature using $Q_{10} = 2.2$.
Parentheses indicate Numbers of measurements.
* L.p., *Loligo pealei*; L.f., *Loligo forbesi*; D.g., *Dosidicus gigas*; *S*, *Sepia*.
‡ $SO_4^=$ perfusion fluid.
§ Numbers in brackets indicate total impulses conducted for the measurement.
¶ No PA or ATP.
‖ F^- perfusion medium.

with sodium transport, as this requires magnesium (Canessa and Rojas, 1966; Skou, 1957). Opit et al. (1966) have investigated the effects of fluoride on kidney ATPase and conclude that fluoride may have a direct inhibitory effect on the enzyme as well, since as little as 2 mM fluoride may reduce ATPase activity as much as 23% although the solubility product of MgF_2 is not exceeded at such a concentration of fluoride.

Passive Fluxes A comparison of the values for ion fluxes obtained in this work with those reported by other investigators is shown in Table VIII, and enables one to judge the extent to which dialyzed squid axons resemble intact or injected axons. Our value for Na influx is about 1.5 times the largest mean value we have reported previously and, if the membrane potential of the axons we used is assumed to be —60 mv, then P_{Na} for dialyzed squid axons corresponds to 5 A/sec. It might be noted that the usual method of measuring Na influx in axons is to treat them with seawater containing radioactive Na for a period of time and then to measure the radioactivity found in the axoplasm. There is no need, in experiments of this sort, for internal capillaries. In our measurements with a dialysis capillary in place, it seems not unreasonable that some increase in P_{Na} necessarily follows from the mechanical trauma of inserting an internal capillary. This explanation for the somewhat increased P_{Na} of the fiber may also apply for K efflux where there is an increase of about 1.5 times over values for injected squid axons. Calculation of P_K from this flux yields a value of 70 A/sec. Thus the ratio P_K/P_{Na} in dialyzed axons appears to be 14, while the ratio for intact or injected axons is about 25 (Brinley and Mullins, 1965).

During the course of efflux experiments we frequently measured the extra efflux of Na or K resulting from stimulation of the axon. These measurements were made primarily to check on the performance of the efflux-collecting system; the number of impulses delivered to the stimulating electrodes was kept as small as possible in order to minimize any possible electrode-produced toxicity or deleterious effects on the axon due to overstimulation. The results obtained both for Na and K efflux per impulse are about one-half to one-third of previously reported values. However, the experiments are not really comparable since the number of stimuli delivered was about one-thirtieth of that used in previous investigations.

Evidence for Active Transport The passive ion permeability properties of the membrane for Na are such that with $[Na]_i$ of 80 mM and a membrane potential of —60 mv one would expect passive Na efflux to be about one-fortieth of Na influx on the basis of flux ratio calculations. As our mean value for Na influx was 57 pmole/cm² sec, efflux should be about 1.4 pmole/cm² sec. Our mean value for Na efflux when internal [ATP], [Mg], and [PA] are all presumed to be optimal is 48 pmole/cm² sec, a value manyfold that expected

for a purely passive outward Na movement. It happens to agree very closely with the mean value obtained by Caldwell et al. (1960) when scaled from a temperature of 19°C to our usual temperature of 13°C (see Table VIII). When axons are treated with cardiac glycosides such as ouabain, Na efflux declines to about one-sixth of normal values. In our single experiment, strophanthidin reduced Na efflux in the axon to about one-half, but the magnitude of fueled efflux from this axon was only 22 pmole/cm² sec. Presumably, if the Na efflux had been more adequately fueled, there would have been a correspondingly larger fractional reduction. What is interesting is that Na efflux declines to about the same absolute level of flux whether injected or dialyzed axons are being considered. This value for efflux is very much larger than that calculated for a purely passive leakage of Na; it is not clear what the nature of the glycoside-insensitive Na efflux may be.

If injected axons are bathed in CN seawater for an hour, their internal stores of ATP and PA fall to very low levels (Caldwell et al., 1960) and Na efflux declines to about one-sixth of control values. The dialyzed axons reported on in this paper were also completely cyanided, but fuel was provided by including ATP and PA in the dialysis fluid continuously supplied to the axoplasm. One would expect, therefore, that if ATP and PA were withdrawn from the dialysis fluid, the Na efflux would decline and the axons would then resemble those studied by Caldwell et al. (1960). This is approximately true, as the data in Table VIII show, since Na efflux from axons dialyzed first with "fuel" and then changed to a fuel-free dialysis fluid showed a mean efflux of 13 pmole/cm² sec or a reduction in efflux to almost one-fourth of its mean value under fuel conditions. Both these values for Na efflux are much larger than that calculated for a purely passive Na movement. It seems reasonable, however, to suppose that an injected axon subjected to treatment with CN has an $[ATP]_i$ that is not zero and indeed Caldwell et al. (1964) give analytical data showing that the ATP concentration in CN-poisoned axons is about 120 μM. For dialyzed axons, if one assumes that 10 mM ATP was supplied to the axon and the inflowing fluid in the dialysis tube was suddenly changed to an ATP-free solution, it would require almost five time constants for the axoplasm to reach a concentration of 100 μM in ATP. Furthermore, the ATP supplied in the region of the porous/nonporous junction of the capillary will have diffused longitudinally into the ends of the axon and now constitutes a reservoir from which the axoplasm can be resupplied with ATP even though none is included in the dialysis fluid. A possible explanation for the residual efflux in axons dialyzed with fuel-free solutions would be that the axoplasm was not completely free of ATP. Methods of obtaining high energy phosphate bond–free conditions in axoplasm are considered in a subsequent paper (Mullins and Brinley, 1967).

If there were a substance with a molecular weight similar to that of ATP

which was necessary as a cofactor for the operation of the Na pump, it would clearly have suffered a marked decrease in its concentration during the dialysis of several hours. In such a case, one might expect to see a declining base line of Na efflux. There is no evidence to support such a finding; indeed, from Fig. 7 it is clear that the Na efflux is capable of responding to a temperature change after 200 min of continuous dialysis. This experiment can be used to argue against an alternative explanation that the apparent constancy of Na efflux is a compound effect of a declining Na extrusion and an increasing Na leak. The experiments tell nothing about the possibility that some molecule of high molecular weight might be acting as a cofactor in operating the Na extrusion mechanism because the dialysis technique will not remove such substances. The experiments were not designed to test whether ATP or PA or both substances were required as fuel for the Na extrusion mechanism; however, in several experiments ATP alone was used in the dialysis fluid without an obvious loss of Na extrusion.

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