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Sodium and Potassium Fluxes Across the Dialyzed Giant Axon of *Myxicola*

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Summary. Resting and stimulated fluxes of sodium and potassium across the giant axon of the marine annelid, *Myxicola infundibulum*, have been characterized using the technique of internal dialysis. In most respects the ion movements were found to be similar to those in squid axons. Sodium efflux and potassium influx were found to be active, cardiac glycoside-sensitive fluxes, with a variable coupling ratio. However, when $[ATP]_i$ was lowered to less than 20 μM by treatment with cyanide and continuous dialysis, or to less than 2 μM by dialysis with glucose following injection of hexokinase, Na efflux and K influx were unaltered. The maintained fluxes were not accounted for by an increased passive permeability of the axolemma, although 30–60% of the Na efflux appeared to be due to Na–Na exchange. An altered form of Na pump operation at low $[ATP]_i$ is a more likely explanation than an alternate energy source, or an ATP source proximate to the axolemma. The transient response of ^{22}Na efflux to a change in $[^{22}Na]_i$ was found to be much slower than in squid, $\tau = 360$ sec. The efflux delay could only be accounted for by an extra-axonal diffusion barrier, which is probably the basement membrane surrounding the ventral nerve cord.

The giant axons of certain marine invertebrates, most notably squid (*Loligo* and *Dosidicus*), have been used for studies of the cell membrane for over twenty-five years, their large size enabling the axial insertion of metal wires and capillary tubing. In 1968 Goldman suggested the use of the giant axon of the marine polychaete worm, *Myxicola infundibulum*, as a convenient alternative to squid. In contrast to squid, whose migratory nature and intolerance to handling have limited investigation to a brief season at a few coastal laboratories, *Myxicola* is available year-round, survives shipment well, and can be maintained for months in seawater aquaria. Goldman, Binstock, and Schauf (*see* references of these authors) have characterized the electrical behavior of the *Myxicola* axolemma,

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finding its properties qualitatively similar to those of squid. In an extensive physical and chemical analysis of the isolated axoplasm, Gilbert (1975*a*, *b*) has shown it to be a highly structured gel; the inorganic cation composition is close to that of other marine invertebrates, while the organic ions show substantial variation.

The goal of the present investigation was a characterization of the Na and K fluxes across the axolemma of *Myxicola* to serve as a base for studies of the movements of substances that have been less well characterized in other preparations. It was anticipated that Na efflux and K influx would be coupled, cardiac steroid-sensitive, ATP[1]-driven movements as they are in squid axons (e.g., Hodgkin & Keynes, 1955; Caldwell *et al.*, 1960; Brinley & Mullins, 1967; Baker *et al.*, 1969; Mullins & Brinley, 1969) and other animal cell membranes. It was of particular interest if *Myxicola* would more closely resemble squid than the human red cell in having a variable Na/K coupling ratio (Mullins & Brinley, 1969; Garrahan & Glynn, 1967*b*) and nonsaturating Na efflux with $[Na]_i$ (Hodgkin & Keynes, 1956; Sjodin & Beauge, 1967; Brinley & Mullins, 1968; Garay & Garrahan, 1973). Internal dialysis was chosen as a method because it affords measurement and control of axoplasmic solute concentrations (Brinley & Mullins, 1967). Since the completion of this work, Abercrombie and Sjodin (1977) have reported the results of ^{22}Na microinjection experiments on *Myxicola* axons; the present results concerning Na efflux at normal $[ATP]_i$ are in agreement with theirs. A preliminary report of this study has appeared (Forbush, 1974).

Materials and Methods

Biological Material

Specimens of *Myxicola infundibulum* were received from Marine Research Associates (Lords Cove, Deer Island, New Brunswick) within one week of their collection date. They were maintained at 5–7 °C in 500 gallons of aerated artificial sea water (Instant Ocean). Mean weight of the worms at the time of dissection was 4.4 ± 1.2 g (SD, $n = 115$) in worms collected from July to October, and 3.0 ± 0.7 g ($n = 146$) in those from January through May.

[1] The following abbreviations are used: ID – inside diameter; OD – outside diameter; ASW – artificial seawater; ATP – adenosine 5′-triphosphate; ADP – adenosine 5′-diphosphate; AMP – adenosine 5′-monophosphate; EDTA – ethylene diaminetetraacetic acid; EGTA – ethylene glycol-bis-(β-amino ethyl ether) N-N′-tetraacetic acid; Na, K-ATPase – Na and K stimulated, Mg dependent-adenosine triphosphatase; TES – N-tris (hydroxymethyl) methyl-2-aminomethane sulfonic acid; Tris – tris (hydroxymethyl) aminomethane.

Dissection

Giant axons were dissected using a modification of the procedure of Binstock and Goldman (1969). Thorough fine cleaning was not undertaken in the present study, because even after fine cleaning the *Myxicola* axon remains surrounded by the neuropil of the ventral nerve cord and its basement membrane. While this enabled a faster dissection with less trauma, the relief of intersegmental connective tissue constrictions reported by Binstock and Goldman was not seen. Dissection was complete in 20–30 min with an axon survival rate near 80%. Axon diameters were measured in both constricted and unconstricted regions with an ocular micrometer, and an overall average diameter was estimated; the mean was 566 ± 99 μm ($n=118$). The measurement was imprecise, especially in irregularly shaped axons: numerous axons were seen to be elliptical in cross section, many varied in diameter over their length, and in some the axon outline was partially obscured by remaining body wall muscle.

Dialysis Capillaries

The dialysis techniques of Brinley and Mullins (1967) were used with changes appropriate to *Myxicola* axons and to flexible cellulose capillaries. A 1.0–1.7 cm central region of cellulose acetate capillary tubing (95 μm ID × 140 μm OD, 10 cm length; Fabrics Research, Inc., Dedham, Mass.) was deacetylated in 0.05 M KOH for 24–48 hr to create a porous region. The boundaries of the porous region were marked with water-insoluble dye (red Magic Marker). The capillary was drawn into the axon behind a 7.5-cm length of 125-μm diameter tungsten wire which was guided through the axon and removed.

Solutions

The composition of the external solutions is given in Table 1. The pH of all solutions was adjusted to 7.5–7.6. Unless otherwise noted, all solutions contained 2 mM NaCN or KCN; in early experiments, CN was found to have no effect on Na efflux. Tris and

Table 1. Composition of artificial seawaters (mM)[a]

	NaCl	KCl	$MgSO_4$	$MgCl_2$	$CaCl_2$	LiCl	Glucose	Choline Cl	Tris Cl	TES	EDTA
ASW	429	9	25	23	9	–	–	–	–	5	0.1
OK-ASW	438	–	25	23	9	–	–	–	–	5	0.1
Li-ASW	–	9	25	23	9	429	–	–	–	5	0.1
Glucose-ASW	–	9	25	23	9	–	705	–	–	5	0.1
Choline-ASW	–	9	25	23	9	–	–	430	–	5	0.1
Tris-ASW	–	9	25	23	9	–	–	–	470	5	0.1
OK-OCa-Tris-ASW	–	–	75	75	–	–	–	–	390	5	0.1

[a] All solutions contained either 2 mM NaCN or KCN unless otherwise noted.

Choline concentrations in Table 1 are approximate; solutions were adjusted to desired osmolarity by addition from concentrated stock solutions.

The basic internal dialysate contained 40 mM Na isethionate, 150 mM K isethionate, 151 mM K aspartate, 3.1 mM Mg [aspartate, 120 mM] taurine, and 160 mM glycine. The organic ion composition was modelled after squid, since the *Myxicola* analysis (Gilbert, 1975*b*) was not available at the time of this investigation. K isethionate (Eastman) was freed of 10% Na, Ca, and other impurities by passage over Chelex 100, H^+ form, in series *B*. Na TES (1 mM), and 0.1 mM MgEGTA were included in early experiments and this was increased to 50 mM TES, 5 mM EGTA in later experiments; pH was adjusted to 6.9–7.0. 2 mM KCN was included whenever CN-ASW was used. 15 mM KCl replaced 15 mM K aspartate in experimental series *B*. MgAMP, MgADP, MgATP, phosphocreatine, and glucose were added from 0.4–1.0 M stock solutions, diluting other constituents at most 2%. Osmolarity was checked with freezing point and vapor pressure-dew point osmometers and adjusted by changing the concentration of taurine and glycine.

Flux Measurements

Unless otherwise noted, flux experiments were performed at 10 °C.

Series A1. ^{22}Na efflux experiments: The flux chamber used in this work was a simplified version of those used by Brinley and Mullins (1967). The $5 \times 8 \times 15$ mm bath surrounding the axon was continuously replenished at 1.8 ml/min and the outflow was collected as 2- or 4-min samples. The half time of chamber washout was 15 sec. A correction factor of 0.91 was applied in efflux calculations for fluid withdrawn at 0.1 ml/min from both guard regions and discarded. Dialysate was supplied to the capillary at a rate fixed between 0.3 and 1.8 μl/min; most often 0.59 μl/min. The capillary porous region was 1.7 cm long. Transient changes in Na efflux reported below were not due to poor mixing since ^{22}Na efflux transients longer than 1 sample time were not seen on changing from ASW to various solutions of the same or different density (and vice versa): 0.9 or 1.1 × isosmotic ASW, MgASW, dextrose ASW, Tris ASW, choline ASW, and Li ASW (at high $[ATP]_i$).

Series A2. ^{22}Na influx experiments: A silicone rubber spacer was used to decrease chamber volume, and flow in the main chamber was greatly reduced or turned off to conserve isotope. The 1-cm capillary porous region was entirely within the main chamber. Dialysate flow was 1.4–1.8 μl/min.

Series B. ^{42}K and simultaneous ^{42}K, ^{22}Na fluxes: A permanent epoxy filler was molded into the chamber leaving a $1.8 \times 2.0 \times 30$ mm slot with inflow at one end and outflow at the other. The decreased volume allowed simultaneous influx/efflux experiments with an external flow $\leqq 0.1$ ml/min.

Radioisotopes

^{22}NaCl and ^{42}KCl (Cambridge Nuclear Corp.) were dried and taken up in dialysate or ASW sufficient to give an activity $\leqq 0.5$ mCi/ml. Up to 1 mM KCl carrier was added to the ASW in influx experiments and up to 20 mM KCl was added to the dialysate in K efflux experiments. In series *A*, ^{22}Na efflux or influx samples were dried and counted in a planchette counter. In series *B*, ^{42}K and ^{22}Na were counted completely independently

($>10^6$ discrimination) in a liquid scintillation counter by counting ^{42}K with the low level discriminator set to reject all ^{22}Na β's (^{42}K at about 10% efficiency); ^{22}Na was counted two weeks later with the spectrometer window optimized for ^{22}Na.

Analysis of Axoplasm

Samples of axoplasm were taken from the section of axon caudal to that used in the flux experiment by slitting the axon along its length with scissors, lifting out the axoplasm with forceps, and collecting it in a 50-μl microcapillary tube for determination of volume (typically several microliters). Samples were diluted into distilled water and freeze-thawed before analysis to disperse the axoplasm.

Determination of axoplasmic constituent concentrations as a function of time during flux experiments was achieved by analysis of the effluent dialysate. The axoplasmic concentration $[ATP]_i$ was calculated from the effluent dialysate concentration $[ATP]_{eff}$ knowing the extent of equilibration of dialysate with axoplasm in passage through the capillary porous region; the latter depends on dialysate flow rate and capillary permeability and was determined empirically by measuring uptake of solute from a bath into dialysis fluid moving through the capillary (B. Forbush, *unpublished*). Thus, at 0.59 μl/min, ATP equilibrated 12% in the porous region of the capillary and measured $[ATP]_{eff}$ was multiplied by 8.3 to get $[ATP]_i$. ATP was determined with the luciferin-luciferase system in the apparatus of Mullins and Brinley (1967) by using 10-μl samples diluted to 1–10 μM. Na and K were determined by atomic absorption spectrophotometry (Model 305b, Perkin Elmer Corp., Norwalk, Conn.).

Introduction of Enzymes into Axoplasm

Series A. 10 μl of a solution containing 0.1 Sigma unit hexokinase and/or in some experiments creatine phosphokinase and 0.4% gelatin was dialyzed against 1 mM TES (pH 7) and applied to the outside of a dialysis capillary as a series of droplets that, when dry, increased the diameter by as much as 60 μm in a series of gentle bumps. On insertion of the capillary, the axon was moved rapidly (~1 cm/sec) over the dry kinases to insure that the enzymes did not dissolve before getting to the region to be dialyzed.

Series B. Less than 1 μl of a solution containing 4 unit/μl hexokinase, 1 unit/μl creatine phosphokinase, 15 mM phenol red, and 0.5 M TES (pH 7.0) was injected, increasing the axon volume by 5–10%. The injection was performed using an oil-filled nonporous length of capillary tubing; after injection was complete the dialysis capillary was drawn into position as the injection capillary was withdrawn.

Extracellular Recording and Axon Condition

Two pairs of Pt–Ir wires in the guard regions were used for extracellular stimulation of the axon (at 2–10 impulses/sec) and measurement of the conducted action potential. The size and shape of the axon potential and conduction time were noted throughout the experiment as an index of axon condition.

Long exposure to ONa sea waters has been found to be deleterious to giant axons (F.J. Brinley, Jr., *personal communication*; Goldman & Binstock, 1969). This observation was confirmed in the present experiments, especially when $[ATP]_i$ was low; in most cases

the propagated action potential indicated deterioration as a result of 20 min in Li-ASW and often the axon was inexcitable following the treatment. Axons generally demonstrated stable fluxes in ONa-ASW's, but there was often a sharp (eg., 10-fold) increase on return to normal ASW (these axons were then inexcitable).

Axon Volume during Continuous Dialysis

In the course of early experiments it was noted that axons swelled during continuous dialysis with isotonic dialysates, increasing their volume as much as 30% in 2 hr, accompanied by loss of electrical excitability. The swelling was found to be due to movement of water from the capillary to the axon driven by a net colloid osmotic pressure difference arising from impermeant axoplasmic proteins. Since the reflection coefficients for low molecular weight solutes at the capillary wall are very low (B. Forbush, *unpublished*), changing the concentration of such solutes in the dialysate did not significantly alter the net osmotic pressure difference across the capillary wall or therefore the water movement out of the capillary.

To balance the flow of water from capillary to axon, the axon was made to lose water to the bath. To this end the dialysate, and therefore the axoplasm, was adjusted to be between 50 and 140 mosM hypotonic. With the dialysate about 100 mosM hypotonic (820 mosM total) the axon volume remained stable for several hours; with more dilute dialysates the axon volume decreased. When axon volume was kept constant through the use of hypotonic dialysates, axon survival increased markedly. A number of experiments were terminated after 6 or 7 hr continuous dialysis with the axon still electrically excitable; generally, 3–4 hr was a "good" survival time. Though expedient, the use of hypotonic dialysates may be undesirable because of the maintained volume flow across the axolemma. Alternative methods would include dialysis using a large negative hydrostatic pressure or addition of an impermeant solute, e.g., inulin, to the dialysate.

Myxicola axons swelled rapidly when placed in isotonic Tris-ASW or choline-ASW, whether or not they had been previously dialyzed. The possibility that the axolemma has a high permeability to organic cations was not tested. It was found that Tris-ASW approximately 100 mosM hypertonic could be used without causing swelling.

Variability

Scatter in the data reported here is expressed by the standard deviation of the population. It will be noted that the variability is considerably greater than in similar data from other species. Nicol and Whitteridge (1955) and Gilbert (1975*b*) have discussed unexpected scatter in conduction velocity and axoplasm composition data from *Myxicola*. Additional sources of error in flux studies include the estimation of the area of irregularly shaped axons, and possibly the degree of axon stretch.

Results

$[Na]_i$, $[K]_i$, and $[ATP]_i$

[Na], [K], and [ATP] were determined in the initial effluent dialysate in a number of experiments with the dialysis flow slowed to

0.29 μl/min. At this rate the dialysate equilibrated with the axoplasm better than 95% for Na and K and to about 23% for ATP, in passage through the capillary (*see Materials and Methods*). Analysis was also performed on axoplasm samples removed from the piece of axon immediately caudal to that dissected for dialysis. As seen in Table 2, $[K]_i \cong 255$ mM, somewhat lower than 280 mM reported by Gilbert (1975*b*). Also, $[Na]_i \cong 38$ mM, significantly higher than the values of 13 and 25 mM reported by Gilbert (1975*b*) and Abercrombie and Sjodin (1977), respectively. Some K loss and Na gain may have resulted from trauma during preparation for dialysis; another possible factor is that the data of Table 2 were obtained from specimens collected in mid-winter when the food supply was low and the worms were small.

$[ATP]_i$ was measured in about half of the axons in this study, including those of Table 2. The mean axoplasmic [ATP] was 1.5 ± 0.5 mM ($n=47$) from axoplasm samples and 1.34 ± 0.37 mM ($n=56$) calculated from $[ATP]_{eff}$. The agreement of these values within experimental error indicates that the bulk of the axoplasmic ATP is freely dialyzable and not compartmentalized in intracellular organelles. This is supported by the finding that the volume of distribution of ATP, as calculated from the rate of decrease of $[ATP]_i$ during dialysis, is roughly equal to the axon volume (B. Forbush, *unpublished*). Abercrombie and Sjodin (1977) have recently reported $[ATP]_i = 1.19 \pm 0.15$ mM (SD, $n=6$).

Table 2. Axoplasmic concentrations of Na, K, and ATP

Axon	$[Na]_i$ (mM) Estimated from dialysate samples	$[K]_i$ (mM)		$[ATP]_i$ (mM)	
		Axoplasm samples	Dialysate samples	Axoplasm samples	Based on dialysate samples
1207	30	260	–	1.7	1.4
1210	32	228	–	1.2	1.2
0130	40	226	225	1.3	1.7
0204	43	242	230	1.2	1.2
0205	60	243	240	2.0	1.8
0211	45	304	245	1.8	1.5
0212	29	250	275	1.4	1.8
0215	40	309	227	1.2	–
0220	32	–	273	–	1.3
2228	40	–	272	–	–
0302	34	–	280	–	1.6
Mean ± SD	38 ± 9	258 ± 32	251 ± 23	1.5 ± 0.3	1.5 ± 0.2

Lowering $[ATP]_i$

In the course of these studies it was desired to lower $[ATP]_i$ as far as possible. In the past this has generally been accomplished by treatment of the axons with metabolic inhibitors for several hours (*cf.* Caldwell, 1960) or by means of dialysis with an ATP-free dialysate (Brinley & Mullins, 1967). In Fig. 1*A*, $[ATP]_i$ calculated from $[ATP]_{eff}$ is plotted as a function of dialysis time. It is seen that with the latter method about 2 hr is required to lower $[ATP]_i$ to 100 μM in an average axon (580 μM diameter). The rate constant for ATP washout was found to be inversely proportional to axon volume (B. Forbush, *unpublished*) so with larger axons more time was required (*see* Fig. 1*B–C*). While DiPolo (1974) reported μM levels of ATP in squid axons after 1 hr CN and dialysis, he

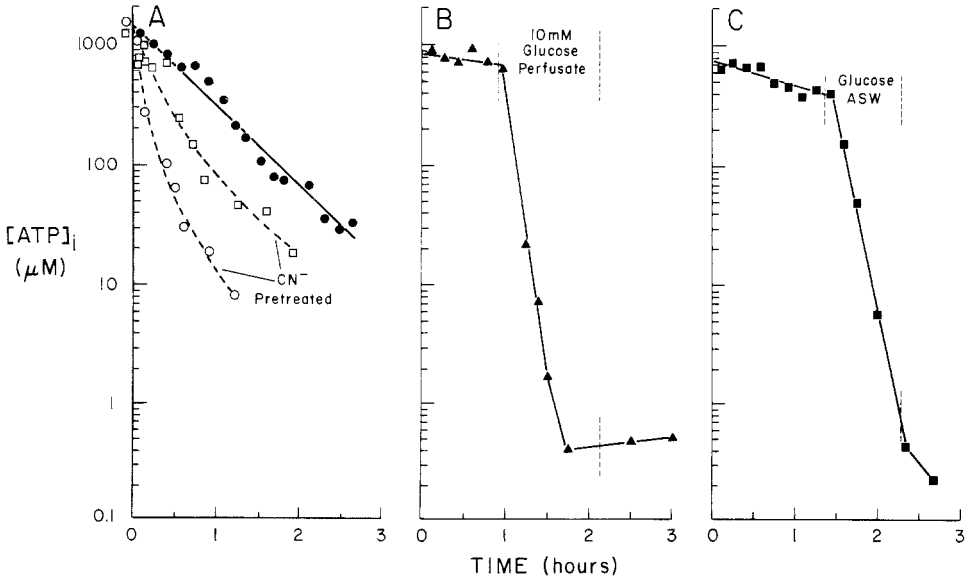

Fig. 1. $[ATP]_i$ during continuous dialysis. $[ATP]_i$ was determined from consecutive effluent dialysis samples. (*A*): Effect of pretreatment in CN on the washout of ATP. –●–, ATP washout from normal axon (axon reference 1105). –□–, –○–, ATP washout from axons pretreated 8 hr in CN without dialysis (axon references 0913, 1026). Symbols to the left of the ordinate indicate $[ATP]_i$ determined in a brief dialysis period prior to the 8-hr treatment. Axon diameters were all 520–555 μM; $T = 10$ °C. (*B*) and (*C*): Effect of glucose on $[ATP]_i$ in axons containing exogenous hexokinase. $[ATP]_i$ was determined from consecutive effluent dialysis samples. In *B*, 10 mM glucose was included in the dialysate between $t = 54$ and 128 min. Axon reference 0823; diameter 800 μm. In *C*, the axon was bathed in 600 mM glucose ASW between $t = 81$ and 136 min. Axon reference 0831, diameter 900 μm. $T = 10$ °C

equated $[ATP]_i$ with $[ATP]_{eff}$, failing to take incomplete dialysate-axoplasm equilibration into account, and thus underestimated $[ATP]_i$ by about 10-fold.

The effect of prolonged 2 mM CN treatment on $[ATP]_i$ was examined in five experiments (Table 3). In the first two of these, the axons were kept in CN-ASW in a refrigerator near 0 °C for 4.5 hr prior to transfer to the flux chamber. In three others, the axons were initially dialyzed for 2–5 min, sufficient time to determine initial $[ATP]_i$ without altering it significantly; they were then kept in the flux chamber at 10 °C for 6–8 hr in CN-ASW without being dialyzed. After treatment, dialysis was begun or resumed and $[ATP]_i$ was determined from $[ATP]_{eff}$. As shown in the last column, CN treatment reduced $[ATP]_i$ in *Myxicola* axons by only 40–50% in 6–8 hr at 10 °C, dramatically less than the 90% reduction in 1 hr at 14–20 °C reported in *Loligo* (Caldwell, 1960). Higher temperatures would presumably have speeded ATP utilization; however, *Myxicola* axons do not survive well for extended periods above 15 °C. In one experiment (axon reference 0205) 2 mM Na iodoacetate was included in the CN-ASW to test the possibility of ATP production through glycolysis; no additional reduction in ATP level was observed.

Figure 1*A* illustrates that prolonged treatment with CN did increase the rate of washout of ATP in a subsequent period of continuous dialysis. Since the rate constant for washout is determined by capillary properties and axon volume, neither of which are affected by CN treatment, this rate increase must reflect ATP removal by a route other than the dialysate. Most likely, as the CN-depleted high energy store of phosphagens is removed by dialysis and is no longer available to maintain a high ATP/ADP ratio, ATP is dephosphorylated to ADP.

Table 3. The effect of CN treatment on $[ATP]_i$

Axon	Diameter (μm)	$[ATP]_i$ (mM) initial		Δt (hr) in CN	T °C	$[ATP]_i$ (mM) after Δt
		Axoplasm sample	From $[ATP]_{eff}$			From $[ATP]_{eff}$
2704	430	–	–	4.8	0–4	1.6
2720	500	–	–	4.5	0–4	2.0
0913	525	1.4	–	7.5	10	0.7
1026	580	2.1	1.7	7.6	10	0.9
0205[a]	570	2.0	1.8	6.2	10	1.4

[a] ASW contained 2 mM Na iodoacetate as well as CN.

To achieve more effective control of $[ATP]_i$, DeWeer's (1970) approach using exogenous kinases and substrates was extended to the dialyzed axon. Hexokinase and sometimes creatine phosphokinase were applied to the outside of the dialysis capillaries or injected prior to insertion of the capillary in the axon. Subsequently, at any desired time in the course of dialysis, the ATP level could be lowered or raised by the incorporation of glucose or ATP+creatine phosphate, respectively, in the dialysate. Figure 1*B* illustrates the effectiveness of the method in lowering the internal ATP concentration. The axon, which contained exogenous hexokinase, was dialyzed continuously and $[ATP]_i$ was determined from $[ATP]_{eff}$. 10 mM glucose was included in the dialysate in the period between the dashed time markers; it reduced $[ATP]_i$ to less than 1 μM within 45 min. Note that when glucose was later removed from the dialysate the ATP concentration did not rebound, indicating that it was reduced throughout the axon and not just near the capillary.

In early experiments the lowering of $[ATP]_i$ with glucose-hexokinase resulted in a deterioration of the axons as judged by increase in conduction time of the action potential and by an increase in both Na influx and Na efflux. The changes were reversed by the addition of ATP and creatine phosphate to the dialysate in place of glucose. In later experiments dialysate buffering was increased from 1 mM TES, 0.1 mM EGTA to 50 mM TES, 5 mM EGTA to buffer the proton release of the hexokinase reaction and Ca^{++} release at low $[ATP]_i$; deterioration was less marked. Coincident with the increase in buffering, the effectiveness of the glucose-hexokinase system decreased and glucose dialysis reduced $[ATP]_i$ only to 10–20 μM rather than $\leqq 1$ μM.

With exogenous hexokinase inside an axon, the ATP concentration could also be lowered by means of glucose substitution in the ASW, as shown in Fig. 1*C*. From the time taken for reduction in $[ATP]_i$, it is possible to place a lower limit on the glucose permeability of the axolemma of $>10^{-8}$ cm/sec. Unfortunately, the use of glucose ASW with hexokinase-containing axons proved fatal to the axons probably due to the effects of lowered $[ATP]_i$ in the unbuffered end regions.

Resting and Stimulated Fluxes of Na and K

Unidirectional cation fluxes were studied in two series of experiments. The results are presented in Table 4 where they may be compared to similar data for dialyzed squid axons. The data from the two experi-

mental series have been separated because of a significant difference in mean Na efflux. The origin of this difference is unknown and none of the experimental parameters that were altered between series *A* and *B* appears as a most likely cause. As detailed in *Materials and Methods*, these include chamber geometry, radioisotope counting technique, presence of ^{42}K in ASW or dialysate, presence of 15 mM KCl in dialysate, purification of K isethionate, and the lot of worms. The value of Na efflux in series *A* (18 ± 6 pmol/cm^2 sec, $n = 55$) is in good agreement with that reported recently by Abercrombie and Sjodin (1977) for injected *Myxicola* axons: 15.8 ± 3.2 (SEM) pmol/cm^2 sec.

A typical Na efflux experiment is shown in Fig. (2). The axon was dialyzed successively with dialysates having ^{22}Na activities of 6×10^4 (*I*), 0 (*II*), and 6×10^3 (*III*) cpm/μl; following each change of dialysate,

Table 4. Comparison of Na and K fluxes in giant axons

		Myxicola axons[a]	Squid axons	
Resting fluxes (pmol/cm^2 sec)				
Series A)	Na efflux	18 ± 6 ($n = 55$)	24*[b], 13.3*[i], 29*[h]	
	Na influx	25 ± 6 ($n = 5$)	57[b], 61[i], 42[h]	
Series B)	Na efflux	37 ± 9 ($n = 14$)		
	K efflux	54 ± 14 ($n = 6$)	59[b], 38[f]	
	K influx	64 ± 25 ($n = 11$)	19[c], 29[d], 16[f], 17[i]	
Stimulated fluxes (pmol/cm^2 impulse)				Squid *Theoretical*[k]
	Na efflux	1.0 ± 0.4 ($n = 20$)	1.85–3.0*[ej], 2.4*[i]	5
	Na influx	18 ± 1 ($n = 3$)	3.4–8.4[e], 10.3[i]	20
	K efflux	20 ± 8 ($n = 4$)	5–9[g], 4.7[i], 8.5[f]	20
	K influx	5 ($n = 1$)	0.2[g], 0.4[i]	5

* Extrapolated to $[Na]_i = 40$ mM, assuming linear dependence of Na efflux on $[Na]_i$.

[a] This work, $T = 10$ °C, *Myxicola infundibulum*.

[b] Brinley and Mullins, 1967, $T = 13$–15 °C, *Loligo pealii*.

[c] Mullins and Brinley, 1967, $T = 16$ °C, *Loligo pealii*.

[d] Sjodin and Beauge, 1967, $T = 17$ °C, *Loligo pealii*.

[e] Cohen and Landowne, 1974, $T = 2$–6 °C, *Loligo pealii and forbesi*.

[f] Caldwell and Keynes, 1960, $T = 13$–18 °C, *Loligo forbesi*.

[g] Landowne and Scruggs, 1976, $T = 6$–8 °C, *Loligo pealii*.

[h] Caldwell *et al.*, 1960*a*, $T = 18$ °C, *Loligo forbesi*.

[i] Keynes, 1951, $T = 14$ °C, *Sepia*.

[j] Landowne, 1978, $T = 6$ °C, *Loligo pealii*.

[k] Hodgkin and Huxley, 1952.

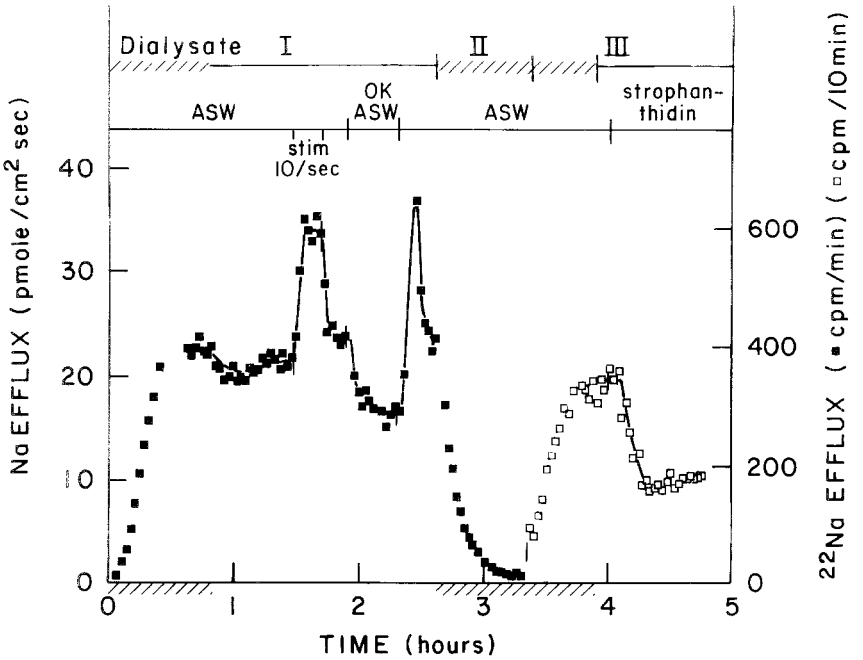

Fig. 2. ^{22}Na efflux from a giant axon as a function of time in a continuous dialysis experiment. Dialysates of three different isotopic activities were employed in succession. 6×10^4 (*I*), 0 (*II*), and 6×10^3 (*III*) cpm/μl. Note that the left ordinate (pmol/cm^2 sec) is not applicable during the periods of equilibration to new dialysate concentrations, indicated by hatching on the dialysate line and abscissa. The effects of electrical stimulation at 10 imp/sec, OK-ASW, and 5×10^{-5} M strophanthidin are shown. CN was not included in the dialysate or ASW. Axon reference: 0524; diameter 470 μm; $T = 5$ °C

equilibration of the axoplasm with the dialysate required about 30 min, as reflected in the time course of ^{22}Na efflux. Note that although in this and in subsequent figures, the unidirectional flux is correctly monitored only after this plateau is reached, some figures present the ^{22}Na efflux data from the start of dialysis.

The "leak" fluxes, or movements of Na and K down their electrochemical gradients were found to be similar in magnitude to those in squid axons (Table 4). As in other preparations, K efflux was very sensitive to axon condition and increased rapidly following minor mechanical trauma or more slowly during continuous dialysis. If the downhill movements of Na and K are through simple leaks involving free diffusion, the contribution of the same leaks to Na efflux and K influx can be calculated from the flux ratio equation (Ussing, 1949). Assuming a membrane potential of -50 mV, $[Na]_i = 40$ mM, $[Na]_o = 429$ mM, and

Na influx $\simeq 25$ pmol/cm^2 sec, then passive Na efflux is $\simeq 0.25$ pmol/cm^2 sec, about 1% of the observed efflux in Table 4. Similarly, passive K influx is calculated to be about 30% of the observed flux. Thus 99% of unidirectional Na efflux and 70% of K influx must be due to an active transport or mediated exchange process. Note that the magnitude of the "active" fluxes is approximately sufficient to balance loss through the "leaks" in the dialyzed axon.

The stimulated extra Na influx, K efflux, and K influx are significantly greater in *Myxicola* than in squid (Table 4) and are therefore much closer to the theoretical values for squid predicted by the equations of Hodgkin and Huxley (1952, at 6 °C, *cf.* Cohen & Landowne, 1974; Landowne & Scruggs, 1976; Landowne, 1977). However, the agreement is probably fortuitous as net ionic currents during computed action potentials are several-fold *lower* in *Myxicola* than in squid (*compare* Goldman & Schauf, 1973; Goldman *et al.*, 1975, to Hodgkin & Huxley, 1952). It thus appears that the discrepancy in magnitude between theoretical and observed stimulated fluxes (excepting Na efflux) may be reversed in *Myxicola,* relative to squid.

The Effect of Cardiac Steroids on Na Efflux and K Influx

Cardiac steroids are well known inhibitors of the Na−K pump and in squid axons they decrease Na efflux to about 10–30% of the resting level at 12–20 °C (Caldwell & Keynes, 1959; Baker *et al.*, 1969) or to about 10 pmol/cm^2 sec (Brinley & Mullins, 1967; $[Na]_i = 80$ mM). In excitable *Myxicola* axons 5×10^{-5} M strophanthidin reduced the Na efflux to 15–85% of the resting level (*see,* eg., Fig. 2) with a mean of $54 \pm 14\%$ ($n = 15$) and 10^{-4} M ouabain reduced Na efflux to $44 \pm 4\%$ ($n = 5$) and K influx to $51 \pm 20\%$ ($n = 4$) of the resting efflux; a similar reduction of Na efflux has recently been reported by Abercrombie and Sjodin (1977). The drop in Na efflux after application of strophanthidin proceeds more slowly in *Myxicola* (half time of 5–20 min; *see* Fig. 2) than after application of ouabain to squid axons at 12 °C (Baker & Manil, 1968; Baker & Willis, 1972); this could be due to extraaxonal diffusion barriers in *Myxicola* or to a difference in the rates of binding of ouabain and strophanthidin.

Comparing data from K influx experiments on the one hand and Na efflux experiments on the other, Mullins and Brinley (1969) have demonstrated that in squid axons the ratio of glycoside-sensitive Na efflux to K

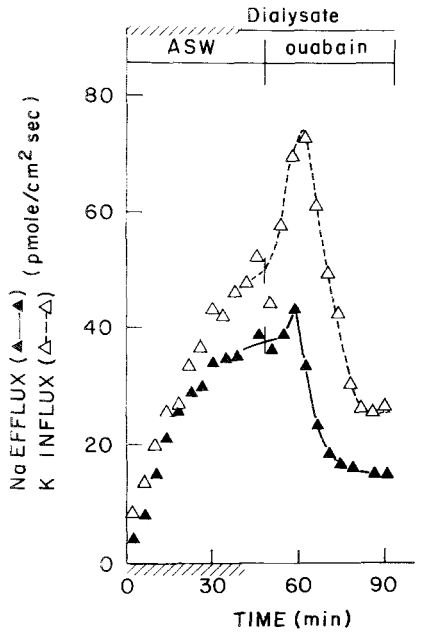

Fig. 3. Double labeling experiment showing simultaneous Na efflux and K influx in a *Myxicola* axon. Ouabain (5×10^{-5} M) was added to the ASW after $t = 39$ min. Hatching indicates the period of dialysate-axoplasm disequilibrium when the ordinate is inapplicable. Axon reference 2848; diameter 500 μm; $T = 10$ °C

influx varies between 1:1 at low $[Na]_i$ to about 3:1 at high $[Na]_i$. In this work (series *B*) double labeling with ^{42}K and ^{22}Na enabled simultaneous determination of Na efflux and K influx, as shown in Fig. 3. Exposure to ouabain decreased Na efflux by 23 pmol/cm^2 sec and K influx by 24 pmol/cm^2 sec in that experiment, giving a coupling ratio of 0.96. In two other experiments coupling ratios of 0.46 and 1.78 were observed. Thus, although the results from three axons must be considered preliminary, Na−K pump coupling as determined by ouabain inhibition is remarkably variable. Alternatively, the possibility exists that the uninhibited Na−K pump is coupled at a fixed ratio but that Na and K pump fluxes exhibit different, and variable, ouabain sensitivity in *Myxicola*.

Note that in the experiment of Fig. 3, as in about one third of such experiments, a transient increase in flux preceded inhibition. Transients in Na efflux from squid axons have been occasionally observed at low concentrations of cardiac steroids, but no explanation has been advanced (Baker & Manil, 1968; Baker & Willis, 1972; DeWeer, 1970). The present finding that K influx is increased as well as Na efflux suggests that a

transient stimulation of the Na–K pump is involved; such a stimulation is in accord with the recent finding of an increase in $[K]_i$ in Purkinje fibers at low ouabain concentrations (Cohen, Dout & Noble, 1976) and with a stimulation of microsomal Na, K-ATPase (for review *see* Lee & Klaus, 1971).

The Effect of K_o, Na_o, and Na_i on Na Efflux

Replacement of normal ASW with K-free ASW results in a 50–70% reduction in Na efflux in *Loligo forbesi* (Baker *et al.*, 1969) and a 0–82% reduction in *Loligo pealii* (DeWeer, 1970). In *Myxicola* the effect was found to be similar but smaller in magnitude, with a mean decrease of $22.5 \pm 11.4\%$ ($n=33$); Abercrombie and Sjodin (1977) have reported a drop of 36% in injected axons. A typical result is shown in Fig. 2. The transient overshoot of efflux on returning to (9 K) ASW was a consistent feature; its origin is uncertain, although it may result from an external diffusion barrier discussed below.

Replacement of external Na with glucose-ASW ($n=4$) or choline-ASW ($n=2$) did not affect Na efflux, while Li-ASW reduced it $22 \pm 6\%$ ($n=3$). Abercrombie and Sjodin (1977) found no change on replacement of Na with Li at $[K]_o = 10$ mM, but did report a decrease using Mg-mannitol. These authors have pointed out that in this respect *Myxicola* axons appear similar to squid axons with an elevated ADP/ATP ratio.

Hodgkin and Keynes (1956), Sjodin and Beauge (1967), and Brinley and Mullins (1968) described a linear relationship between Na efflux and $[Na]_i$ in squid axons, and Abercrombie and Sjodin (1977) observed linearity up to $[Na]_i \simeq 90$ mM in *Myxicola*. In this study, in two experiments in which dialysates of the same isotopic activity (cpm/ml) but containing either 20 or 150 mM $[Na]_i$ (K replacement) were used in succession, isotopic ^{22}Na efflux was virtually unchanged (+5%, −10%), indicating that Na efflux is linear to at least $[Na]_i = 150$ mM.

Temperature Dependence of Na Efflux

Hodgkin and Keynes (1955) noted that the temperature coefficient of Na efflux from *Sepia* axons decreases with increasing temperature from a $Q_{10} \simeq 5$ between 1 and 10 °C, to a $Q_{10} \simeq 2$ between 19 and 27 °C. Similarly, Arrhenius plots of activity of Na, K-ATPase from various sources exhibit discontinuities of slope, and the location of the break

Table 5. Temperature dependence of Na efflux

	T_1 (°C)	T_2 (°C)	Q_{10}[a]
A. Resting efflux	2	5	3.6 ± 0.4 ($n=2$)
	2	10	3.0 ± 1.0 ($n=5$)
	5	17	1.5 ± 0.2 ($n=5$)
	10	25	1.7 ± 0.1 ($n=3$)
B. Stimulated efflux	5	10	1.0
	5	17	0.95

[a] $Q_{10} = \left[\frac{\text{efflux } (T_2)}{\text{efflux } (T_1)}\right]^{\frac{10}{(T_2 - T_1)}}$.

point correlates with the lipid composition of the membrane and the body temperature of the animal (Tanaka & Teruya, 1973; Solomonson, Liepkalns & Spector, 1976). *Myxicola*, which lives between 2 and 10 °C (on the east coast of North America) would thus be expected to have a low break point. That this is true is shown in Table 5 by the results of 15 experiments in which a single temperature jump was performed early in dialysis. The Q_{10} of Na efflux is greater than 3 below 5 °C and less than 2 above 10 °C.

Cohen and Landowne (1974) have reported that the extra Na influx and efflux associated with electrical activity has a Q_{10} near unity, in contrast to a $Q_{10} \leqq 0.5$ predicted by Hodgkin and Huxley (1952). Recently Landowne (1977) has accounted for part of the discrepancy with the finding of a temperature-dependent component of Na efflux that requires external Na and therefore deviates from the principle of independence of unidirectional fluxes. In two experiments in which the temperature was raised from 5 to 10 or 17 °C and then returned to 5 °C, the Q_{10} of stimulated Na efflux from *Myxicola* axons was only slightly different from 1.0 (Table 5*B*). Since a temperature coefficient of 0.55 is predicted (Goldman *et al.*, 1975) from the temperature coefficients of voltage-clamp parameters determined by Schauf (1973), it is likely that stimulated Na fluxes deviate from theory based on independent movements in *Myxicola* as they do in *Loligo*.

Effect of $[ATP]_i$ on Fluxes

It is now generally agreed that the Na−K pump in a wide variety of animal tissues requires ATP for its operation. In squid giant axons

metabolic inhibitors such as CN or dinitrophenol first render Na efflux insensitive to external K and then cause it to drop to near the strophanthidin inhibited level when ATP disappears completely (Caldwell *et al.*, 1960*b*; DeWeer, 1970). Using dialyzed axons, Brinley and Mullins (1968) have shown that the dependence on $[ATP]_i$ is complex: Na efflux decreases to 50% as $[ATP]_i$ is lowered from 5 mM to 200 μM and to 20% as $[ATP]_i$ is lowered to 10 μM. It was expected that Na efflux from *Myxicola* axons would show a similar dependence on $[ATP]_i$.

Figure 4 shows Na efflux from a giant axon in which $[ATP]_i$ was lowered by continuous dialysis and determined from $[ATP]_{eff}$. It is seen that Na efflux rose 20% as $[ATP]_i$ was lowered from about 300 μM at $t=70$ min to 70 μM at $t=150$ min and then declined again as $[ATP]_i$ fell towards 20 μM. The results of this and 13 similar experiments performed with or without CN are presented in Table 6. Surprisingly, there was not a significant change in Na efflux when $[ATP]_i$ was lowered to less than 100 μM. Even in the single experiment in which there was a large decrease

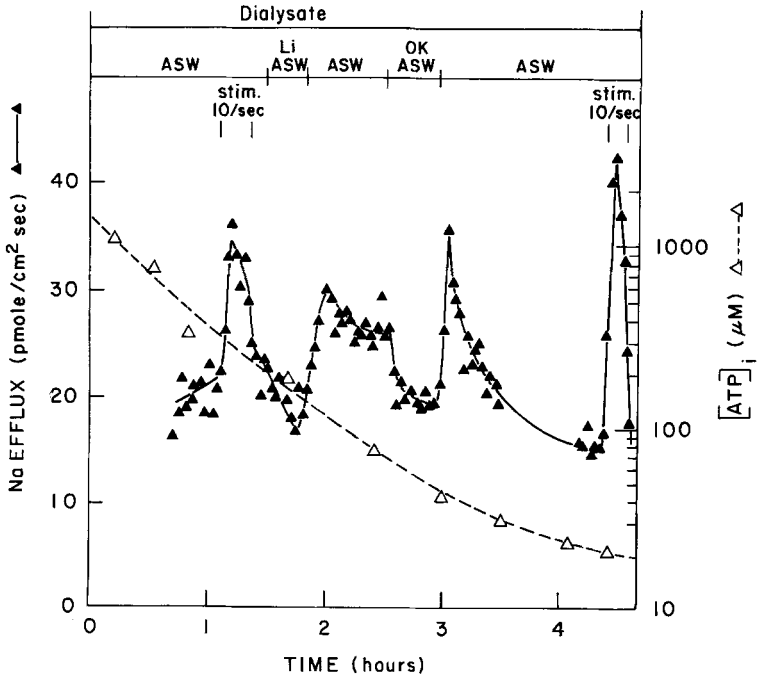

Fig. 4. Na efflux experiment in which $[ATP]_i$ was lowered by continuous dialysis. $[ATP]_i$ (right ordinate) was determined from the concentration in the effluent dialysate. The effects of Li-ASW, OK-ASW, and stimulation are shown. Axon reference 0726; diameter 650 μm; $T=10$ °C

in efflux (axon reference 0704), subsequent dialysis with 5 mM MgATP failed to restore normal efflux, indicating that lowered $[ATP]_i$ was not the cause of the change.

In order to bring about a more rapid and complete drop in $[ATP]_i$, the glucose-hexokinase enzyme system was used, as described above. Figure 5 shows the effect of the rapid decrease in $[ATP]_i$, following dialysis with glucose, on the Na efflux: rather than the expected drop in efflux, a marked increase was seen. When the axon was later dialyzed with 20 mM MgATP a return to near the original efflux level occurred. In

Table 6. Effect of $[ATP]_i$ on resting Na efflux

	Axon reference	Axon diameter (μm)	t_1 (min)	t_2 (min)	$[ATP]_i$ (μM)		Na efflux (pmol/cm² sec)		Efflux (t_2) / Efflux (t_1)
					(t_1)	(t_2)	(t_1)	(t_2)	
I. Dialysis and CN									
−CN	0418*	600	30	160[a]	≃600[e]	≦30[e]	20	28	1.4
	0426*	400	40	180[a]	≃400[e]	≦30[e]	44	40	0.9
	0514*	600	160[c]	240	≃100[e]	≦30[e]	27	27	1.0
	0524**	470	35	240	≃400[e]	≦30[e]	22	20	0.9
	0601	490	80	260	≃150[e]	≦30[e]	38	38	1.0
	0606	550	140	240	≃100[e]	10,000[b]	29	20	0.7[b]
	0607	520	90	340	≃100[e]	≦30[e]	13	15	1.2
	0720	450	50	160[a]	350	30	24	12	0.5
	0726	620	50	260	300	20	20	16	0.8
Mean (±SD)									1.0 (±0.3)
+CN	0621	525	80	250	≃150[e]	≦30[e]	25	31	1.2
			250	350	≦30[e]	10,000[b]	31	28	0.9[b]
	0702	520	40	260	≃500[e]	≦30[e]	15	11	0.7
	0704	420	30	250	700	6	29	9	0.3
			250	320	6	5,000[b]	9	11	1.2[b]
	0713	580	60	360	380	10	12	18	1.5
	0718[d]	400	60	290[a]	230	10	17	17	1.0
Mean (±SD)									0.9 (±0.5)

Experiments were performed at 10 °C, except * at 17 °C and ** at 5 °C.

[a] Last measurement before a final rise in efflux.

[b] 10 mM MgATP added to dialysate. Not included in mean.

[c] Axon was at 5 °C from $t=0$ to $t=150$ min; there was a 13% increase in efflux between $t=50$ min and $t=100$ min.

[d] 1 mM IAA was included in the dialysate after $t=160$ min.

[e] $[ATP]_i$ estimated from axon volume and permeability of capillary to ATP.

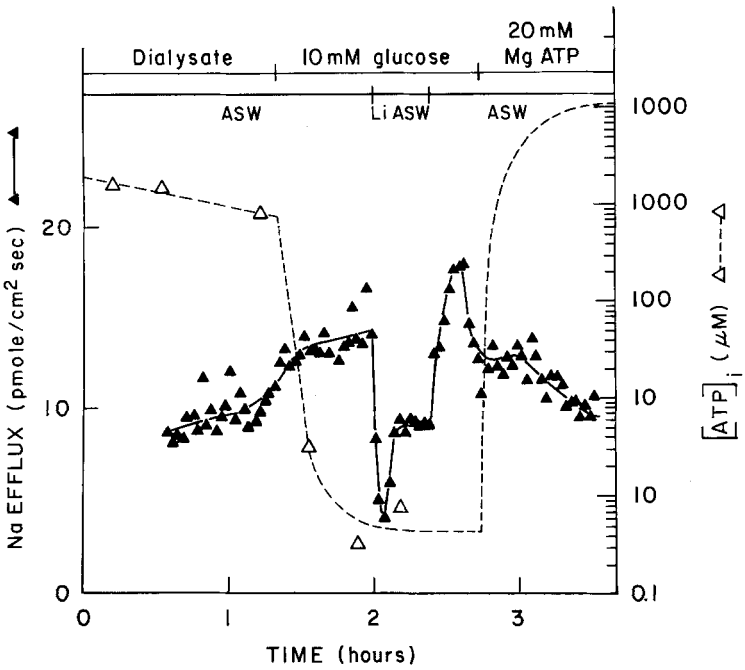

Fig. 5. The effect of lowered $[ATP]_i$ on Na efflux in a weakly buffered axon. $[ATP]_i$ (right ordinate) was determined from the concentration in the effluent dialysate and was lowered by dialysis with glucose; the axon contained exogenous hexokinase and gelatin. 20 mM ATP was included in the dialysate at $t = 165$ min and after this the time course of $[ATP]_i$ is estimated from the rate of ATP washout during the first 80 min. The effect of Li-ASW at low $[ATP]_i$ is shown. [TES] = 10 mM; [EGTA] = 1 mM. Axon reference 0821; diameter 750 μm; $T = 10$ °C

17 experiments summarized in Table 7, efflux increased to a mean of 1.4 times the resting level, and in 5 of these when MgATP was subsequently dialyzed into the axon the efflux returned towards the initial value. When the pH/Ca buffering was increased in later experiments, only a slight increase, if any, was observed when $[ATP]_i$ was lowered (Table 7). Thus, although $[ATP]_i$ was reduced to less than 10 μM in many of these experiments, it was not possible to demonstrate that ATP was required for Na extrusion.

In one case cardiac steroid sensitivity of Na efflux was retained when $[ATP]_i$ was lowered by continuous dialysis (axon reference 0607): Na efflux was inhibited 60% by strophanthidin after 5.8 hr of dialysis when $[ATP]_i$ must have been under 30 μM (also, *see* Fig. 2). However, in 4 experiments in which $[ATP]_i$ had been lowered further using the enzyme systems, strophanthidin had no effect on Na efflux. The change was irreversible, as sensitivity was not restored by dialysis with MgATP.

Table 7. Effect of $[ATP]_i$ on resting Na efflux

II. Hexokinase – Glucose							
Axon reference[a]	Axon diameter (μm)	Dialysate [TES][b] (mM)	Flux (pmol/cm² sec) (1) Before glucose	(2) After glucose	After ATP	$[ATP]_i$ After glucose (μM)	Flux 2 / Flux 1
Na Efflux							
0815	680	1	14	14	–	0.4	1.0
0816	550	1	8	9	–	0.3	1.1
0821	750	1	10	14	10	0.3	1.4
0823	750	1	8	19	12	0.4	2.4
0907	500	1	16	25	–	8	1.6
0910	675	10	13	18	13	5	1.4
4916	650	10	9	18	–	25	1.4
0918	650	10	14	40	27	–	2.8
2920	–	10	9[e]	17[e]	10[e]	2.5	1.9
1210	585	50	32	36	32	1	1.2
0211	600	50	13	13	–	120	1.0
0220	520	50	16	16	–	10	1.0
0228	620	50	26	26	–	14	1.0
2862[c]	700	50	30	50	–	<4	1.7
0864[c]	720	50	20	24	–	<6	1.2
0866[c]	500	50	50	62	–	<0.7	1.2
2866[c]	600	50	40	42	–	<2	1.0
0949[d]	620	50	25[d]	29[d]	–	30[d]	1.2

[a] All experiments were at 10 °C.
[b] [EGTA] = [TES]/10.
[c] Double labeling experiments. Enzymes were injected into the axon. Na iodoacetate (1 mM) was included in the dialysate.
[d] Creatine – CPKase used to lower $[ATP]_i$.
[e] Assuming diam. = 500 μm.

Under conditions of low $[ATP]_i$, unidirectional Na efflux might be maintained by exchange diffusion of internal for external Na; this appears to be the situation in partially poisoned squid axons in which $[ATP]_i$ is still fairly high but $[ATP]_i/[ADP]_i$ is lowered (Caldwell *et al.*, 1960*b*; DeWeer, 1970). In nine experiments of which Fig. 5 is typical, with low $[ATP]_i$, substitution of Na with Li resulted in a brief reduction to 40 % ± 20 % of the initial level, followed by a "steady state" efflux into Li-ASW equal to 70 % ± 30 % of the initial level; return to (429 Na)-ASW was accompanied by an overshoot of efflux. In a single experiment

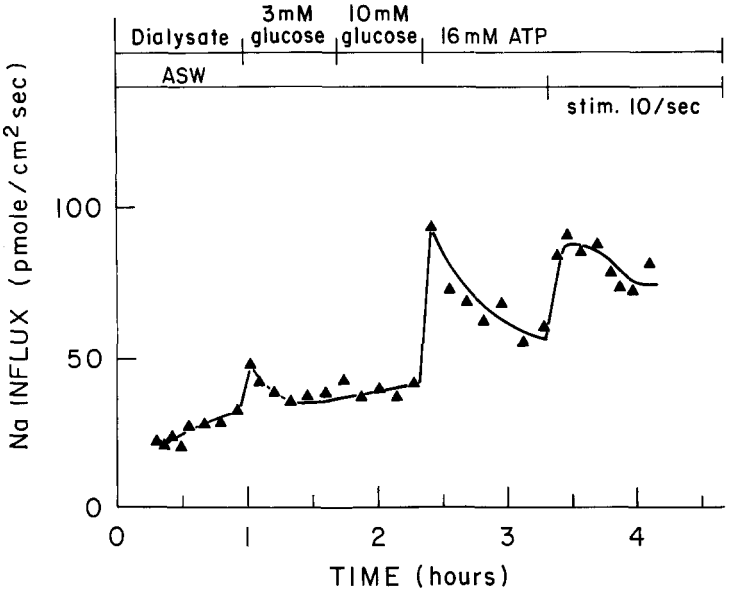

Fig. 6. The effect of lowered $[ATP]_i$ on Na influx in an axon buffered with 50 mM TES, 5 mM EGTA. $[ATP]_i$ was not measured; it is presumed to have been lowered (<20 μM) by dialysis with glucose and restored by dialysis with 16 mM ATP. The axon contained exogenous hexokinase and gelatin. The line was fit by eye. Axon reference 1016; diameter 450 μm; $T=10$ °C

glucose ASW reduced Na efflux to 40% of the initial level. From these data it appears that a significant fraction of the Na movement could be due to Na−Na exchange. On the other hand, Na efflux into Tris-ASW ($n=2$: 120%, 95%) or choline-ASW ($n=3$: 100%, 100%, 60%) was not significantly different from that into (429 Na)-ASW, so an alternate mechanism must be responsible under these conditions. A similar difference in the effect of various Na substitutes on Na efflux from squid axons has been seen by Baker *et al.* (1969). Na−Ca exchange does not appear to play a role: in two experiments efflux into OK-OCa-Tris-ASW was 80% and 120% of the control Na efflux at low $[ATP]_i$.

The possibility that an increased Na leak pathway might be compensating for a lowered pump flux at low $[ATP]_i$ was investigated by examining Na influx. As seen in Fig. 6 and in one other experiment, Na influx remained below 50 pmol/cm^2 sec when $[ATP]_i$ was lowered with the axoplasm well buffered. The passive Na efflux through this leak would be less than 1 pmol/cm^2 sec, a small fraction of the total Na efflux.

In the experiment of Fig. 7, simultaneous Na efflux and K influx were measured while $[ATP]_i$ was lowered by glucose-hexokinase. Here, as in one other experiment, Na efflux and K influx increased slightly;

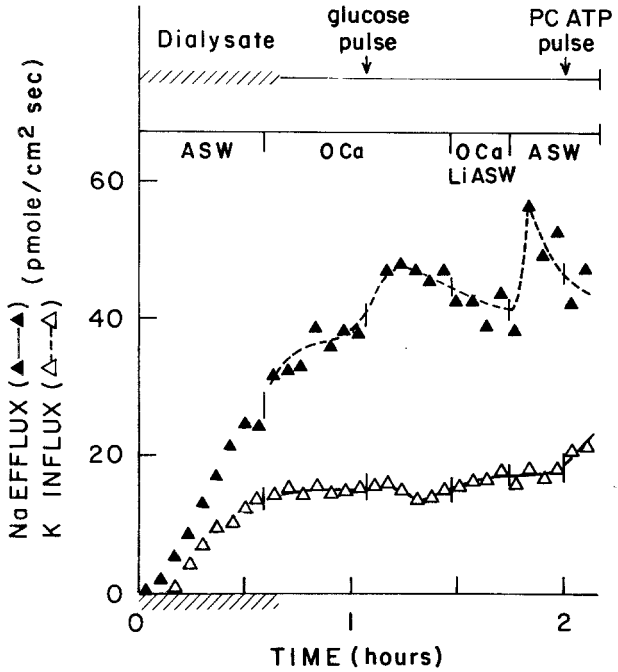

Fig. 7. The effect of lowered $[ATP]_i$ on simultaneous Na efflux and K influx. $[ATP]_i$ (not shown) was lowered to <6 μM by dialysis for 45 sec with 750 mM glucose at $t=140$ min and raised by dialysis for 2 min with 700 mM PC (phosphocreatine) and 100 mM MgATP. The effects of OCa-ASW and OCa-Li-ASW are shown. Prior to the experiment the axon had been injected with 1 μl containing hexokinase and creatine phosphokinase (1.7 Sigma units each), 500 mM TES, 50 mM EGTA, 15 mM phenol red, and 10 mM Na iodoacetate. The dialysate contained 50 mM TES, 5 mM EGTA, and 1 mM Na iodoacetate as well as CN. Hatching indicates the period of dialysate-axoplasm disequilibrium when the ordinate is inapplicable. Axon reference 0864; diameter 720 μM; $T=10$ °C

subsequent dialysis with MgATP and phosphocreatine had little effect. Thus it was not possible to demonstrate an ATP requirement for either Na or K pump fluxes.

Transient Behavior of ^{22}Na Efflux

During the course of this work, it was noted that the response of ^{22}Na efflux to changes in $[^{22}Na]_i$ occurred more slowly than expected on the grounds of known diffusion barriers. This was most evident when the axon was dialyzed briefly with isotope and the dialysis capillary was then quickly rinsed and blown empty so that no further isotope could enter the axoplasm. Figure 8*A* shows an experiment in which four pulses

of dialysate were used, the first two containing ^{22}Na and the last two with no isotope. After each pulse, ^{22}Na efflux is seen to take at least 10 min to reach a new steady state. The approaches to steady state are replotted in Fig. 8*B*; the time constant for this approach had a mean of 370 ± 195 sec in 12 experiments. Such a delay does not occur in squid axons: Hodgkin and Keynes (1956) found that ^{22}Na efflux rose to a steady state within 1 min following injection of isotope into the axoplasm. Also, during continuous dialysis of *Myxicola* axons, ^{22}Na efflux was found to approach a steady state more slowly than the approach to equilibrium of ^{22}Na between the axoplasm and the dialysate *(unpublished)*. The possibility of a diffusion barrier external to the *Myxicola* axolemma will be discussed below.

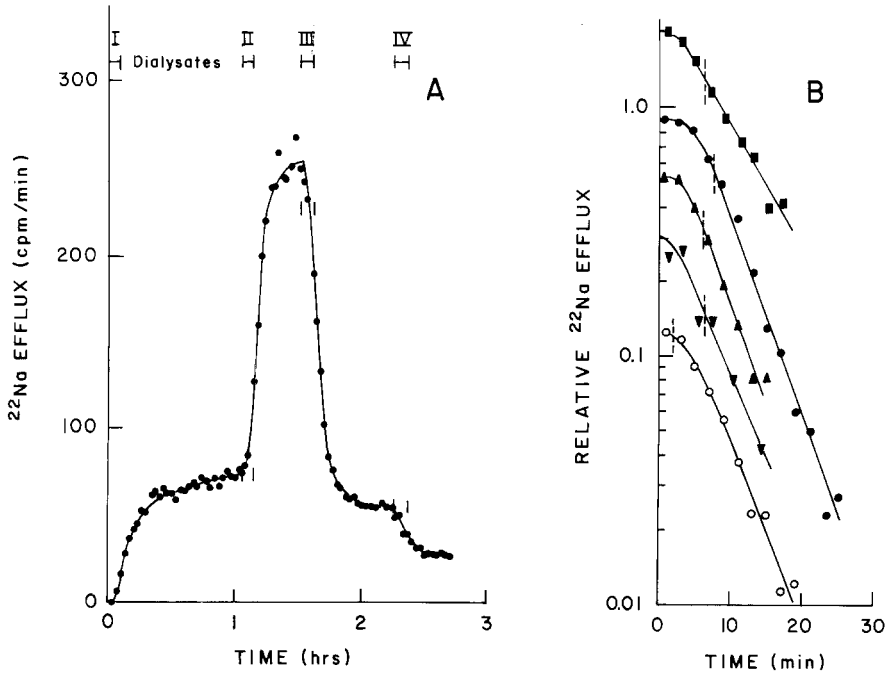

Fig. 8. (*A*): ^{22}Na efflux from a giant axon as a function of time in an experiment in which four dialysate pulses were used. ^{22}Na activity was 6×10^4, 6×10^4, 0, and 0 cpm/ml in dialysates *I*, *II*, *III*, and *IV*, respectively. The axon was dialyzed only during the time periods indicated at the top of the figure. Axon reference 0522; diameter 550 μm; $T = 5$ °C. (*B*): Approaches to steady state ^{22}Na efflux during and after dialysis pulses. Data from *A* are shown by solid symbols and additional data (axon reference 2228) by open circles. The curves are arbitrarily displaced along the logarithmic ordinate for clarity. On the abscissa, the start of the pulses is at $t = 0$, and the ends of the pulses are marked by vertical dashes. The data are plotted, with the undisplaced $t = 0$ intercept equal to 1.0, as follows: (■, *I*): (62.5-efflux)/62.5; (▲, *II*): (250-efflux)/176; (●, *III*): (efflux-55)/195; (▼, *IV*): (efflux-29)/26; (○, Axon 2228): (971-efflux)/971

Discussion

Insensitivity of Fluxes to $[ATP]_i$

Na and K fluxes across the giant axon membrane of *Myxicola* appear very similar to those across the axolemma of *Loligo*, with one striking exception: the lack of a demonstrable dependence of the active fluxes on $[ATP]_i$. The sensitivity of these fluxes to ouabain and strophanthidin, highly specific inhibitors of the Na, K-ATPase, and the widespread occurrence and interspecies similarity of that enzyme are compelling reasons to consider alternatives to the conclusion that [ATP] is not the immediate energy source for Na and K pumping in *Myxicola*. (i) The ATP concentration at the axolemma may be higher than measured $[ATP]_i$. (ii) An altered form of the pump enzyme may catalyze exchange diffusion at low $[ATP]_i$.

While DeWeer (1970) has argued that axially injected enzymes should diffuse radially to the axolemma quite rapidly, the gel state of the axoplasm (Gilbert, 1975*a*) might prevent this. If so, a radial concentration gradient would result from dephosphorylation of ATP by hexokinase only near the dialysis capillary and could be maintained by a rephosphorylation of ADP towards the axon membrane. Although this cannot be ruled out, it appears unlikely in the presence of 2 mM CN, and in view of the large capacity of the glucose-hexokinase system, and the expected rapid diffusion of ATP in axoplasm (*see* Gilbert, 1975*a*). Furthermore, when $[ATP]_i$ and presumably $[ADP]_i$ and $[AMP]_i$ were lowered by continuous dialysis alone, Na efflux was unaffected. Proverbio and Hoffman (1977) have presented evidence for a membrane-bound pool of ATP that can serve as a local source for the Na^+, K^+ ATPase in human red cells; however, this pool is small and is depleted as cytoplasmic ATP is depleted. To be consistent with the present results, a membrane source of ATP would have to be insensitive to CN and iodoacetate, fueled by a nondialyzable substance or by a constituent of the dialysis medium, and the ATP produced would be diffusionally restricted from the axoplasm.

Unchanged unidirectional Na efflux and K influx when $[ATP]_i$ is lowered could be due to Na−Na and K−K exchange catalyzed by an uncoupled Na−K pump. Although exchange diffusion of Na and K have been reported in other preparations, their characteristics are different from those required here; most notably, Na−Na exchange requires internal ADP (Glynn & Hoffman, 1971; DeWeer, 1970), and K

−K exchange in red cells is small, requires inorganic phosphate in addition to nucleotide, and occurs under different conditions from Na −Na exchange (Glynn, Lew & Luthi, 1970). In *Myxicola* at low $[ATP]_i$ the presence of Na−Na exchange can be inferred from the lowered Na efflux on substitution of Na_o with Li or choline; however, in Tris or dextrose ASW Na efflux remains high, and it becomes necessary to postulate still another mode of Na efflux into these media. Clearly, further studies will be needed to understand the mechanism of Na and K movement in *Myxicola* at low $[ATP]_i$.

Transient Behavior

The transient behavior of Na efflux will be considered in terms of a barrier to free diffusion external to the axolemma. The delay in response of ^{22}Na efflux to a change in $[^{22}Na]_i$ cannot be due to diffusion within the axoplasm since, with an axoplasmic Na diffusion coefficient $D \simeq 6 \times 10^{-6}$ cm^2/sec (Gilbert, 1975*a*), Na would be distributed to $>90\%$ of steady state within 30 sec [from Hodgkin & Keynes, 1956, Eq. (14)]; this is consistent with observations of ^{22}Na efflux from squid axons (Hodgkin & Keynes, 1956). Neither would free diffusion in a very large unstirred layer external to the axon be sufficient to account for the delay: for a 300 μm unstirred layer, the time taken to reach $1/e$ efflux equilibration is $\simeq 25$ sec (by differentiation of Eq. (5), Carslaw & Jaeger, 1959, p. 113; and $D \simeq 10^{-5}$ cm^2/sec). Note also that, although the axons were not fine-cleaned in this study, attached body wall musculature covered less than 30% of the nerve cord surface and could not be responsible for the homogeneous delay seen in Fig. 8.

A likely location of a region of restricted diffusion is the periaxonal tissue layer comprising the balance of the ventral nerve cord; it is roughly equal to the giant axon in volume. The entire nerve cord is surrounded by a thick ($\simeq 2$ μM) basement membrane which is permeable to peroxidase but not to ferritin (E. Gfeller & L. Goldman, *unpublished*; L. Goldman, *personal communication*). A simple limiting case of restricted diffusion is that of a homogeneous space of volume V surrounded by a thin barrier of area A and permeability P. As discussed by Frankenhauser and Hodgkin (1956), the time constant for equilibration of this space with the bath is given by $\tau = V/PA$. A lower limit for permeability $P \simeq 5 \times 10^{-5}$ cm/sec can be calculated from the series resistance of 4.6 Ωcm^2 attributed to the periaxonal tissue layer by Binstock *et al.*

(1975), assuming all of the resistance to be in an unselective barrier (*see* Frankenhauser & Hodgkin, 1956, p. 365, with $P = 22\ \Omega$cm, $D = 10^{-5}$ cm^2/sec). If the basement membrane were the barrier, and if in the limit the entire volume of the nerve cord were in isotopic equilibrium in the time range of interest, then $V/A \simeq 0.015$ cm and $\tau \simeq 300$ sec, within bounds of the observed delay in Na efflux ($\tau = 370 \pm 195$ sec). While this is entirely speculative, it is useful to examine the limits of the system; in particular, if most of the small cell bodies and processes comprising the periaxonal space are not in isotopic equilibrium, it becomes difficult to reconcile the long time constant with the measured series resistance.

Transient behavior is of considerable interest because if there is an extra-axonal barrier to diffusion several features of isotopic flux studies would be affected. (i) As is seen here with ^{22}Na, observed efflux lags behind efflux at the axolemma by several minutes. (ii) Changes in solute concentration in the bathing medium will be delayed in reaching the axolemma. Here it may be noted that Goldman (1968) found that changes in $[K]_o$ were fully effective in changing the resting potential only after several minutes, and he ascribed the delay to diffusion through extracellular space. (iii) Solute concentrations external to the axolemma may be significantly different from those in the bath, even in the steady state. For instance, in OK-ASW a net K efflux of 50 pmol/cm^2 sec would raise $[K]_{ext}$ to 1 mM within the above barrier ($P = 5 \times 10^{-5}$ cm/sec). (iv) In efflux experiments a significant quantity of isotope may be stored in the extra-axonal compartment. Changes in the condition of the extra-axonal tissue may alter P or V. Not only will τ be changed, but also if V is increased or decreased, a transient drop or increase in measured efflux will be recorded that may be incorrectly interpreted as originating at the axolemma. Transient phenomena that may fall in this category include the overshoot in Na efflux after exposure to K-free ASW (Fig. 4) and the transients with low $[ATP]_i$ and Li-ASW (Fig. 1).

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