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# Anomalous Influence of Reduced Internal ATP Levels on Sodium Efflux in *Myxicola* Giant Axons

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**Summary.** Giant axons from the marine annelid, *Myxicola infundibulum*, were internally dialyzed with ATP-free media and with media with lower than normal ATP levels in an attempt to determine quantitatively the ATP requirement of the Na pump in these cells. This was accomplished by using $^{22}Na$ ions to measure Na efflux. When $[ATP]_i$ in dialysis fluid fell to values within the range of 20–40 $\mu$M, a marked stimulation of Na efflux was observed even though an essentially normal ouabain sensitivity of Na efflux persisted; when axons were dialyzed with ATP-free solutions with ouabain present in the external medium throughout the dialysis period, the stimulation of Na efflux still occurred. The stimulation of Na efflux produced by low $[ATP]_i$ levels could be reversed by reintroducing normal ATP levels into the dialysis medium. Reversibility was complete provided axons were not depleted of ATP for periods longer than about 1 hr. Longer periods of ATP depletion led to larger and ultimately irreversible increases in Na efflux. The increases in Na efflux occasioned by ATP depletion either prevented or obscured the decrease in Na efflux expected to occur from unfueling the Na pump. Since $[ATP]_i$ levels required to significantly unfuel the Na pump lie below the levels at which the Na efflux stimulation occurred, it is problematic to quantitatively assess the influence of $[ATP]_i$ levels on Na pump rate by measurements of Na efflux in this preparation. Substitutes for ATP failed to prevent increases in Na efflux. The large increases in Na efflux observed at low $[ATP]_i$ occurred with no important changes in the resting membrane potential, and also occurred in Na-free and Ca-free external media. At least part of the increased Na efflux under these conditions may be due to a Na/Na exchange component, as a significant dependence of Na efflux on $[Na]_o$ appropriate for this kind of exchange was observed in the ATP-depleted axons. Whether the highly reproducible anomalous effect on Na efflux in *Myxicola* axons has some fundamental significance in its own right is a matter for future investigation. A few possible explanations of the anomalous effect of reduced ATP levels are discussed.

**Key Words** ATP depletion · giant axons · internal dialysis · *Myxicola* · sodium efflux · sodium pump

## Introduction

The sodium pump present in giant axons from the marine annelid, *Myxicola infundibulum*, has several properties in common with the sodium pumps in other, well-investigated cells, such as red blood cells, the squid giant axon, and skeletal muscle fibers (Forbush, 1974; Abercrombie & Sjodin, 1977). Notable among these properties are its sensitivity to external K and to internal and external Na, as well as its susceptibility to inhibition by externally applied cardiotonic steroids such as ouabain or strophanthidin. The energy source of the Na pump in several important cell types has been shown to be ATP, which is required inside the cell. The requirement for internal ATP has been most dramatically demonstrated in internally dialyzed squid giant axons, in which Na efflux falls to values around 1 pmol/cm$^2$ · sec when axons are dialyzed with an ATP-free solution, and increases nearly 30-fold when ATP is reintroduced into the dialysis solution (Mullins & Brinley, 1967). Since *Myxicola* giant axons are electrically very similar to squid giant axons (Goldman, 1968; Goldman & Binstock, 1969; Goldman & Schauf, 1973) and possess a similar Na pump, as noted above, it was natural to expect that sufficiently lowering the ATP level inside the *Myxicola* axons would lead to a large reduction in Na efflux. It came as a surprise, therefore, that *Myxicola* giant axons internally dialyzed with ATP-free solutions to produce lower $[ATP]_i$ failed to show a reduced Na efflux (Forbush, 1979). Indeed, some of the axons in the latter experiments showed an increased Na efflux upon lowering $[ATP]_i$.

The purpose of the presently reported work was to further investigate the effects of low internal ATP levels on Na efflux in *Myxicola* giant axons in an attempt to learn why it has not been possible to observe the expected reduction of Na efflux via the

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Na pump consequent on removing internal ATP, the expected energy source of the pump. Failure to explain why the classically expected result is not observed in *Myxicola* giant axons would leave open the possibility that ATP is not the major energy source for fueling the Na pump in *Myxicola* axons (Forbush, 1979). This, in turn, would necessitate a further search for alternative energy sources for the Na pump in *Myxicola* axons.

## Materials and Methods

### Biological Specimens

Giant axons were dissected from specimens of *Myxicola infundibulum* obtained from Marine Research Associates, New Brunswick, Canada. They were kept at 5–7°C in aerated artificial seawater reconstituted from Instant Ocean (Aquarium Systems, Mentor, OH).

### Internal Dialysis Method for *Myxicola* Giant Axons

A modification of the method of Brinley and Mullins (1967) was employed. The main modification of significance was a reduction in the length of the dialysis region to 0.5 cm. This was achieved by reducing the dimensions of the central region of the chamber used for isotope collection to 0.5 cm, and by reducing the porous region of the dialysis capillary to the same dimension. The reason for the modification was to confine the flux measurement to one nodal segment of the axon. The main anatomical difference between *Myxicola* and squid giant axons is that *Myxicola* provides segmented axons with nodal or constricted regions, which are shorter than the internodal regions. The dissection of *Myxicola* axons is more difficult than that for squid axons, and blood vessels must be picked off the surface of the axons with fine forceps. The nodal regions are more difficult to clear of blood vessels and are more likely to undergo injuries. In preliminary experiments with a conventional chamber, nodal regions often coincided with the region of dialysis. Those with marginal injuries that did not lead to collapse of the axon often showed up as leaky regions during dialysis. It was this experience that led us to adopt the shorter dialysis region within which a very short, healthy, and node-free region of axon could be centered. Such centering or positioning of the axon was accomplished by dyeing the porous region of the capillary faintly with a red dye and positioning the porous region optically in the central region of the chamber.

The absolute values of the Na effluxes reported in this work fall at the low end of the range of values previously observed in *Myxicola* giant axons when microinjected (Abercrombie & Sjodin, 1977) or dialyzed (Forbush, 1979). A possible explanation for this is that flux measurement in our dialysis chamber was mainly over one internodal segment of the axon, whereas in the previous microinjection and dialysis experiments flux measurements were conducted over a longer region that presumably contained entire nodal regions of membrane as well. The density of pump sites may vary over the surface of the axon, but a more likely explanation is that the nodal regions are leakier to ions because of the much greater difficulty in removing blood vessels from these regions during dissection. That this is the correct explanation is suggested by the previously reported dialysis data (Table 7, Forbush, 1979). Axons showing large increases in Na efflux consequent to lowering of $[ATP]_i$ had on the average, lower values of initial Na efflux than those showing either no change or small increases in Na efflux. Those axons showing greater than a doubling of Na efflux had fluxes in the range observed in the present investigation when account is taken of differences in the value of $[Na^+]_i$ (20 mM in our normal dialysis medium *vs.* 40 mM in the medium of Forbush, 1979).

The other details of the method and chamber, including guard regions on both sides of the dialysis region, were the same as in the method of Brinley and Mullins (1967) and Forbush (1979). Capillary tubing used in all of the experiments (except for two ATP washout experiments; *see below*) consisted of a stretch of cellulose acetate tubing (95 μm i.d. × 140 μm o.d., Fabrius Research Inc., Dedham, MA) that had been made porous (i.e., in a 0.5-cm stretch) by treatment for 2–3 hr in 2N $HNO_3$ (40°C), and then deacetylated for 14–18 hr in 50 mM NaOH. All experiments were conducted at 13–15°C.

### ATP Washout Curves

In order to verify that washout of ATP by dialysis was adequate and within an acceptable time range in the experiments, washout curves had to be developed for the various dialysis regimes. Dialysis using glass capillaries and plastic capillaries, with or without the additional assistance of metabolic poison (CN), was performed on axons, and the dialysate that washed out was analyzed for ATP levels by a luciferin-luciferase ("firefly") assay using the apparatus of Mullins and Brinley (1967). The results in terms of [ATP] are shown in Fig. 1. Experiments were performed in LiSW (*see* Table 1) to which was added NaCN (2 mM) and ouabain ($10^{-4}$ M); dialysis fluid was used that was high Na (*see* Table 2) and contained either NaCN (2 mM) or no NaCN (glass capillary experiment).

### Radioactive Na

$^{22}NaCl$ (New England Nuclear, Boston, MA) was obtained in a carrier-free solution. It was added to dialysis fluid to yield specific activities in the range 2–5 cpm/pmol. Counts were made using an automatic gamma-counter (Searle Model 1185, Searle Analytic, Silver Spring, MD).

### Solutions

External and dialysis solutions that were used in the experiments are described in Tables 1 and 2; certain details are omitted from the tables, such as information on metabolic poisons when used, and are given in the legends to the figures. The osmolarity of external solutions ranged from 940–1000 mosM, and that of dialysis fluids from 890–936 mosM.

### Measurement of Na Efflux

The flux chamber used to measure Na fluxes was similar to one previously used by Brinley and Mullins (1967) to study Na fluxes

**Table 1.** Composition of artificial seawater solutions (concentrations in mM)

| | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | LiCl | Mannitol | HEPES |
|---|---|---|---|---|---|---|---|---|
| ASW | 430 | 10 | 10 | 25 | 25 | 0 | 0 | 5 |
| 10 K, 0 Ca ASW | 435 | 10 | 0 | 25 | 25 | 0 | 0 | 5 |
| 0 K ASW | 445 | 0 | 0 | 25 | 25 | 0 | 0 | 5 |
| LiSW | 0 | 20 | 10 | 25 | 0 | 410 | 0 | 5 |
| 10 Ca Mg mannitol | 0 | 0 | 10 | 189 | 0 | 0 | 450 | 5 |
| 0 Ca Mg mannitol | 0 | 0 | 0 | 192 | 0 | 0 | 458 | 5 |

**Table 2.** Composition of internal dialysis solutions (concentrations in mM)

| | NaAsp | KAsp | $CaCl_2$ | $MgCl_2$ | Glycine | $K_2TES$ | $KH_2PO_4$ |
|---|---|---|---|---|---|---|---|
| Normal Na (20 mM) | 20 | 300 | 0 | 3 | 354 | 5 | 10 |
| High Na (100 mM) | 100 | 200 | 0 | 3 or 10[a] | 354 | 50 | 0 |
| Low Na (2–3[a] mM) | 0 | 300 | 0 | 3 | 354 | 50 | 0 |
| Very low Na (85 μM) | 0 | 300 | 0 | 3 | 433 | 50 | 0 |

[a] *See* legends to the figures for specific values; exact [Na] in low Na solutions was contributed by NaCN alone or NaCN plus Na pyruvate.

in squid giant axons, and by Forbush (1979) to study Na fluxes in *Myxicola* axons. After beginning the dialysis with fluid containing $^{22}Na$, a minimum of 15 min was allowed before making measurements of Na efflux; this was done to allow equilibration of the dialysis fluid with the cellular compartment. Equilibration times in excess of 15 min appear blank in the figures that follow (total equilibration time = 15 min + blank time), unless otherwise accounted for in the legends to the figures.

## Measurement of Membrane Potential

In cases where the resting membrane potential was measured and monitored during the period of Na efflux measurements, measurement was via a calomel electrode inserted into the aqueous system in contact with the dialysis effluent. Since the polyethylene dialysis tubing used was a perfect insulator for our purposes, measurement of electrical potential was confined to the porous region of the dialysis capillary, which is assumed to be equipotential with the axoplasm in that region. The difference in electrical potential between the axon interior and the external solution was obtained by measuring the potential difference between the calomel electrode within the capillary system and a calomel electrode placed in the central external solution surrounding the axon. The potential difference was measured with a high-input-impedance Grass P16 D.C. Amplifier and a digital voltmeter. In practice, the Grass amplifier was set to a gain of 10×, and the D.C. voltage at its output was measured with the digital voltmeter set to an appropriate range. Measurements of electrical potential difference were accurate to within ±0.5 mV. The possible influence of asymmetry potentials in the potential-measuring circuit was checked by shorting the two solution pools with which the calomel electrodes were in contact, by use of a 3M KCl salt bridge. This procedure produced a reading of 0.0 mV at the digital voltmeter. A small junction potential between axoplasm and the interior of the dialysis capillary is not ruled out, however, which could affect the absolute values of $E_m$. This junction potential would not be expected to change during the experiment as only the value of [ATP] was changed in dialysis fluid.

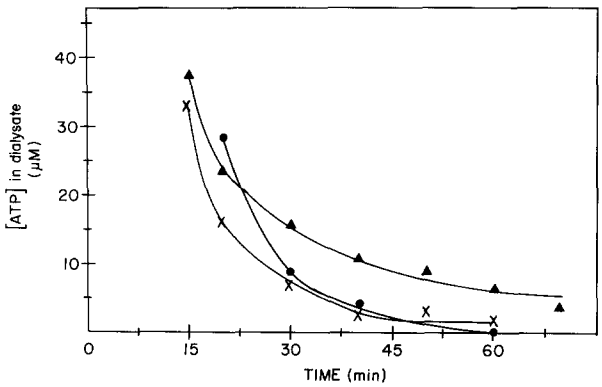


**Fig. 1.** ATP concentration in effluent dialysate during washout of ATP with ATP-free dialysis fluid containing metabolic inhibitors. External media were LiSW containing ouabain ($10^{-4}$ M), and 2 mM NaCN during dialysis. The high Na (100 mM) ATP-free dialysis fluid containing metabolic inhibitors was used (2 mM NaCN, 1 mM arginine, 1 mM pyruvate, and 5 μg/ml oligomycin). Three different capillary protocols were used: (●) a porous glass capillary; (▲) a plastic capillary pretreated at 20°C for 2 hr in 2N $HNO_3$ and then deacetylated for 20 hr in 50 mM NaOH; and (×) a plastic capillary pretreated at 40°C for 2 hr in 2N $HNO_3$ and then for 14 hr in 50 mM NaOH. The latter type of capillary was used in all other experiments in this study. Capillaries were 0.141–0.143 i.d. and all axons were about 500 μm in diameter. Washout was allowed to proceed for 15 min before taking samples of the dialysate

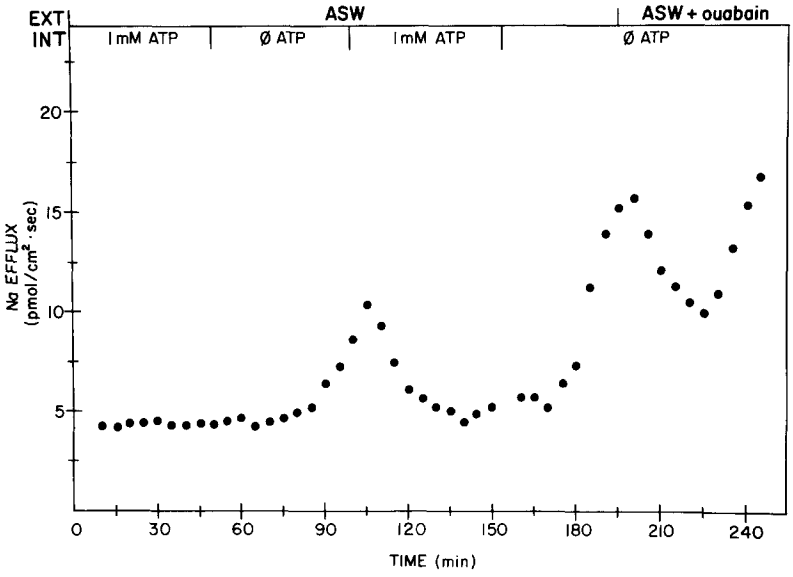


**Fig. 2.** Anomalous effect of reduction in $[ATP]_i$ on Na efflux in *Myxicola* giant axons in ASW. $[Na^+]$ was 20 mM ("Normal Na") throughout. [ATP] in the dialysis fluid was 1 mM at the beginning of the experiment and upon subsequent restorations of ATP to the fluid. Ouabain ($10^{-4}$ M) was added after a significant increase in Na efflux was produced by the removal of ATP from the dialysis fluid; it caused a temporary reduction of Na efflux before resumption of the previously produced increase in the efflux. Composition of dialysis fluid: normal Na solution (additional details: internal, 3 mM $MgCl_2$, 1 mM EGTA, with or without 1 mM ATP; external, with or without $10^{-4}$ M ouabain). Axon diameter was 743 $\mu$m

## Results

The first experiments performed were for the purpose of verifying that the dialysis technique used in our laboratory was adequate for our purposes. These experiments were aimed at demonstrating the following in two groups of experiments: (*i*) the efficiency of the lowering of $[ATP]_i$ levels when *Myxicola* axons are dialyzed with ATP-free solutions; and (*ii*) the stability of the axon preparation, as well as the reproducibility in the dialyzed axons of results previously obtained by microinjection (Abercrombie & Sjodin, 1977). The first concern is dealt with in Materials and Methods and the results are summarized in Fig. 1. The second set of results is not detailed here, but is summarized as follows. Sodium efflux in axons internally dialyzed with normal (high Na) dialysis fluid containing 1 mM ATP showed a sensitivity to $[K]_o$ (K-free effect averaged 40% reduction in dialyzed axons *vs.* 50–60% in intact axons), and a percent inhibition by ouabain (range = 60–70%), that were within the range of the effects previously observed in this laboratory in microinjected *Myxicola* axons (Abercrombie & Sjodin, 1977) and in internally dialyzed axons in another laboratory (Forbush, 1979). Axons dialyzed with normal dialysis fluid also showed a Ca-dependent Na efflux that was in good agreement with results previously obtained in microinjected axons (Sjodin & Abercrombie, 1978). It was evident to us, therefore, that the present investigation was made using an adequate internal dialysis technique.

The first experiments in the area of ATP effects were performed by dialyzing *Myxicola* axons internally with normal dialysis fluid containing ATP (1 mM) for a period long enough to ensure a stable baseline for $^{22}Na$ efflux. In practice, axons were dialyzed with the normal dialysis fluid containing $^{22}Na$ ions for a period of 15 to 30 min prior to the taking of efflux samples. Efflux samples were then taken for a period of at least 30 min. If the baseline $^{22}Na$ efflux was reasonably stable during this period, the axon was used in the experimental program, which at this point consisted of dialysis with an ATP-free medium followed by reintroduction of normal ATP levels. The results of a typical experiment are shown in Fig. 2. Approximately 20 min after the beginning of dialysis with the ATP-free medium, Na efflux began to rise. After 50 min of dialysis with the ATP-free medium, dialysis with the initial ATP-containing medium was resumed. A prompt restoration of Na efflux values to near the initial baseline values occurred. A second period of ATP withdrawal was effected. The Na efflux again increased; and finally, $10^{-4}$ M ouabain was applied externally. A rapid inhibition of Na efflux followed addition of ouabain. After maximal inhibition of the Na pump, as could be expected after 30 min in ouabain, the Na efflux resumed its rise at approximately the rate noted before addition of ouabain.

The experiment of Fig. 2 and others like it are of major consequence to the problem of unfueling the Na pump in *Myxicola* giant axons. The experiment shows clearly that lowering the internal ATP level in *Myxicola* giant axons leads to an increase in the Na efflux by some as yet unknown mechanism operating at values of $[ATP]_i$ well above those that would be required to unfuel the Na pump. The increase in Na efflux usually begins at around 25 min after beginning dialysis with an ATP-free medium.

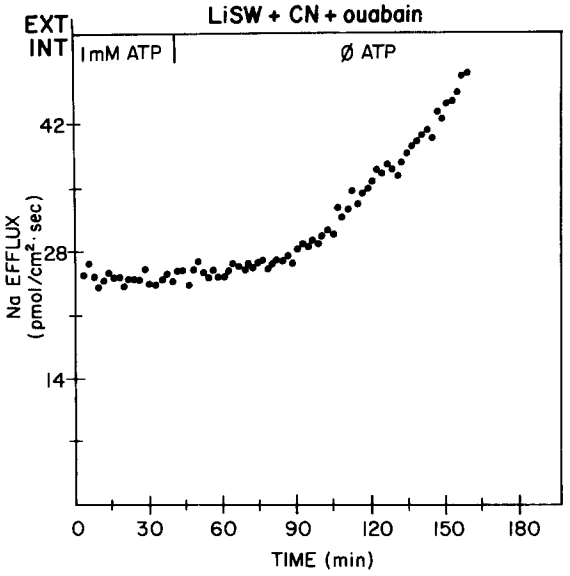


**Fig. 3.** The anomalous effect occurs in the absence of external Na and in the presence of cyanide. The external medium was LiSW containing $10^{-4}$ M ouabain and 2 mM KCN. Composition of dialysis fluid: high Na (100 mM) solution (3 mM $MgCl_2$, with or without 1 mM ATP). Starting [ATP] in the fluid was 1 mM (preparation had been allowed to stabilize for 2½ hr before $t = 0$ in the graph). Axon diameter was 600 $\mu$m

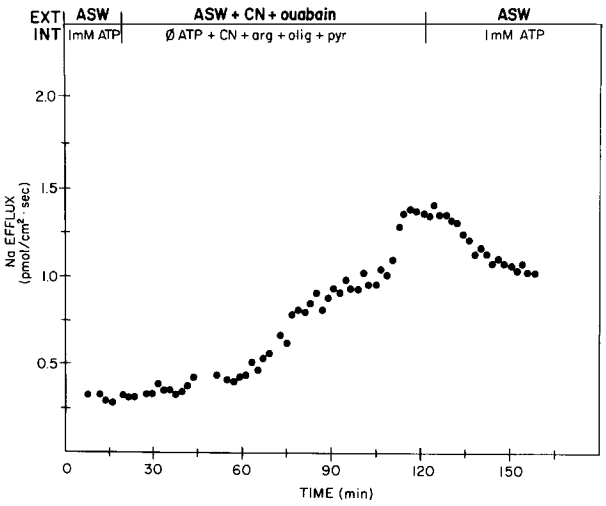


**Fig. 4.** Effect of metabolic inhibitors applied externally or internally on the anomalous increase of Na efflux induced by decreased $[ATP]_i$. Entire experiment was carried out in ASW containing CN (2 mM) and ouabain ($10^{-4}$ M). The dialysis fluid contained Na at a concentration of 3 mM (low Na) and either contained ATP (1 mM) or was ATP-free and contained CN (2 mM, as KCN), arginine (1 mM), pyruvate (1 mM) and oligomycin (5 mg/ml)

At this time, [ATP] in the collected dialysate samples is in the range 10 to 20 $\mu$M. The actual concentration of ATP at the inner plasma membrane surface at this time may, of course, be considerably beyond this range (Forbush, 1979). The point is that a large and steady increase in the Na efflux is in progress at the lowered value of $[ATP]_i$ at a time when considerable ouabain sensitivity of Na efflux still exists. Therefore, it can be concluded that still lower ATP levels inside the axon would be required to unfuel the Na pump.

In the squid giant axon it has been shown that at $[ATP]_i \cong 1$ $\mu$M no measurable pumped Na efflux occurs, whereas with $[ATP]_i = 12$ $\mu$M a small but measurable pumped Na efflux of a few pmol/cm$^2$ · sec occurs (Mullins & Brinley, 1967). Assuming that the Na pump in *Myxicola* axons is similar to that in squid axons, $[ATP]_i$ would have to be lowered to around 1 $\mu$M to insure a completely unfueled condition. It can be seen from Fig. 1 that the dialysate in *Myxicola* giant axons dialyzed with an ATP-free medium reaches a value below 2 $\mu$M after 1 hr of dialysis. Given the rates of diffusional equilibration of small molecules in axoplasm, an additional hour of continuous dialysis would be expected to produce an unfueled condition. The data in Fig. 3 show that continuing the ATP washout for a second hour of dialysis results in a continuation of the rise in Na efflux. Thus the expected unfueled effect on Na efflux via the pump is not seen even when dialysis with an ATP-free medium has been continued to the point where it can be safely concluded that $[ATP]_i$ is uniformly 1 $\mu$M or lower. Figure 3 also demonstrates that external Na is not required for the anomalous rise in Na efflux to occur.

The rise in Na efflux occurring at lowered values of $[ATP]_i$ clearly would obscure any effect of unfueling on the Na pump. The increased Na efflux obtained at low $[ATP]_i$ apparently has nothing to do with any normal operational mode of the pump, as it occurs in the presence of $10^{-4}$ M external ouabain in the same manner as in the absence of ouabain; the results shown in Fig. 4 were obtained in the presence of $10^{-4}$ ouabain and are seen to be similar to results in the absence of ouabain (Fig. 2). The results in Fig. 2 and in Fig. 4 also demonstrate that the effect can be reversed by restoring ATP to the dialysis medium; reversibility occurs whether or not external ouabain is present. Reversibility was the rule rather than the exception and other cases will be described subsequently.

Some experiments were performed in which axons were dialyzed with solutions containing lower than normal but non-zero values of [ATP]. These experiments were performed for the purpose of attempting to determine a threshold for the anomalous ATP effects previously described. In the experiment illustrated in Fig. 5, an axon was first

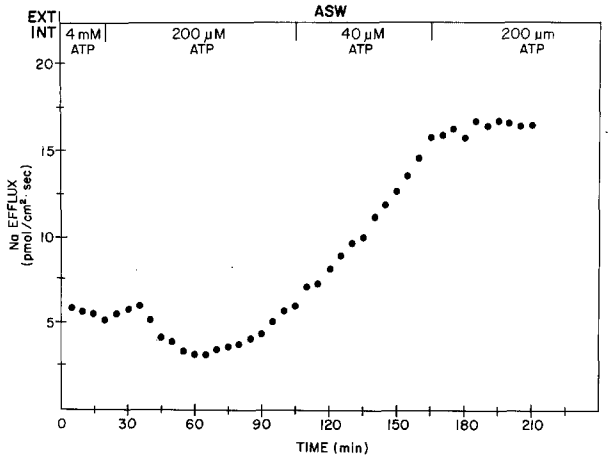


**Fig. 5.** Threshold effects of dialysis with low non-zero concentrations of ATP on Na efflux. External medium was ASW. [ATP] of internal dialysis fluid was initially 4 mM and then decreased to 200 $\mu$M and finally 40 $\mu$M; restoring [ATP] to 200 $\mu$M arrested the increase in Na efflux. The dialysis fluid, except for [ATP], was of the same composition as used in Fig. 2. Axon diameter was 830 $\mu$m

dialyzed with high Na dialysis fluid with [ATP] = 4 mM, and subsequently dialyzed with solutions with [ATP] = 200 $\mu$M and [ATP] = 40 $\mu$M, respectively. The rationale for these choices was the hope that a certain low value of $[ATP]_i$ might satisfy the ATP requirement for preventing the anomalous effect and yet permit at least some observation of the classically expected unfueling effect. The behavior of Na efflux during dialysis with 200 $\mu$M ATP differed from that occurring in both normal dialysis and dialysis with an ATP-free medium. After the usual delay to permit concentration changes at the membrane, Na efflux fell by almost 50% before beginning a steady rise.

The initial drop in Na efflux might be attributed to partial unfueling of the axon—this unfueling would take place at $[ATP]_i$ less than 1 mM but higher than 200 $\mu$M (there is no difference between effects at 4 mM and 1 mM ATP), since washout of ATP to its new level of 200 $\mu$M would take some time to complete. However, the subsequent, seemingly spontaneous rise in the Na efflux suggests that at some intermediate $[ATP]_i$ the anomalous effect is triggered (or significantly augmented) so that Na efflux rises instead of declines. Again, this occurs at some undetermined $[ATP]_i$ greater than 200 $\mu$M but less than 1 mM, but certainly less than the level at which unfueling is first observed in this preparation. When the initial Na efflux value was reached, the dialysis fluid level of [ATP] was changed to 40 $\mu$M in the hope that the nearly constant, positive slope of the Na efflux curve could be altered significantly. The results of Fig. 5 show that the rate of rise of the Na efflux continued unabated during dialysis with the 40 $\mu$M ATP solution, suggesting that the anomalous effect is a threshold phenomenon that is fully in action at an $[ATP]_i$ not less than 200 $\mu$M; during the 1-hr period of dialysis with this medium the $[ATP]_i$ level inside the axon could not have fallen below the 40 $\mu$M level. The conclusion reached is that essentially the same anomalous effect on Na efflux is observed when $[ATP]_i$ is lowered to values not less than 40 $\mu$M as is observed at near 200 $\mu$M. When experiments (*data not shown*) were performed with dialysis fluids containing ATP at values higher than 200 $\mu$M, results were much like those obtained during normal dialysis with $[ATP]_i$ = 1 mM. Therefore, the threshold for the anomalous effect may be said to be greater than but close to 200 $\mu$M ATP. Since this limiting concentration is some 40 times greater than the ATP concentration required to unfuel squid giant axons, it becomes clear why present and previous (Forbush, 1979) efforts have failed to demonstrate the unfueling effect of low $[ATP]_i$ in *Myxicola* giant axons. After 1 hr of dialysis with 40 $\mu$M ATP, the dialysis fluid was changed once again to one containing 200 $\mu$M ATP. Though the leveling off of Na efflux toward the end of the experiment (around $t$ = 165 min) does coincide with a change in [ATP] in the dialysis fluid from 40 to 200 $\mu$M, it cannot be stated that the change in [ATP] is the cause. Two reasons justify this view: (*i*) Actual changes in $[ATP]_i$ only gradually reflect changes in [ATP] of the dialysis fluid, and as can be seen in Fig. 5 the onset of the leveling off may actually precede the modification of the dialysis fluid; and (*ii*) If given enough time, preparations showing an anomalous increase in Na efflux will eventually show a leveling off in Na efflux (e.g., Figs. 4, 7, 9, and 10), even if no further modifications of internal or external fluids are made. Whether or not the initial decline in Na efflux observed after initiation of dialysis with 200 $\mu$M ATP represents the beginning time course of a true unfueling effect remains problematical. However, the results in Fig. 5 suggest the interesting possibility that a narrow range of $[ATP]_i$ exists within which unfueling can be discerned, perhaps because a threshold of $[ATP]_i$ value exists below which the anomalous rise in Na efflux is triggered (i.e., the Na efflux does not overtake the unfueling effect until $[ATP]_i$ reaches a low enough level), or possibly because the rise in Na efflux is activated to a lesser extent at certain $[ATP]_i$ than at lower concentrations (*see* Discussion).

Another possibility that was considered was that a less specific nucleotide requirement for prevention of the anomalous rise of Na efflux existed

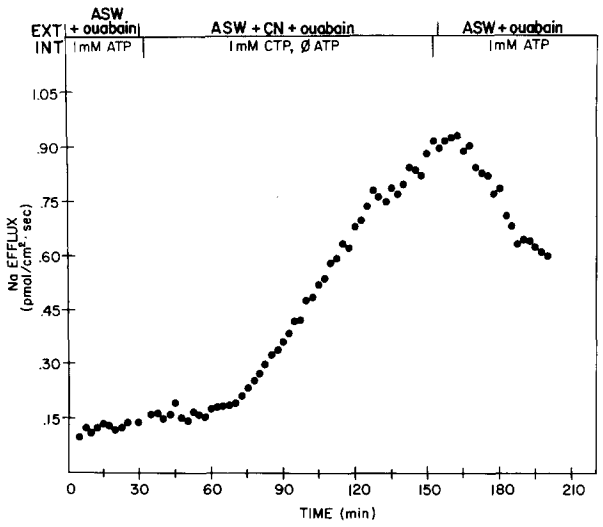


**Fig. 6.** CTP does not prevent the rise in Na efflux caused by removing ATP from the dialysis fluid. Dialysis fluid initially was low Na (2.15 mM Na) 1 mM ATP solution; the ATP was then substituted with 1 mM CTP for a period of about 1½ hr, and the ATP subsequently restored. External solution was ASW, $10^{-4}$ mM ouabain, and 0 or 2 mM CN (as KCN). Axon diameter was 396 $\mu$m

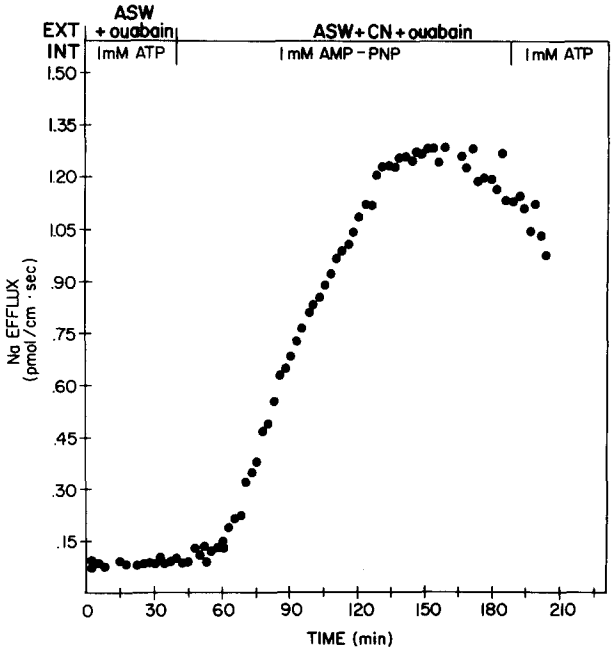


**Fig. 7.** AMP-PNP does not prevent the rise in Na efflux caused by removing ATP from the dialysis fluid. As in Fig. 6, the dialysis fluid was initially 1 mM ATP; the ATP was then replaced with 1 mM AMP-PNP for a little more than 2 hr, and the ATP subsequently restored. Internal dialysis fluid was 2 mM Na (low Na) and 4 mM Li. The Li was introduced because the AMP-PNP was in the form of the tetraLi salt. External solution was ASW with the inhibitors indicated in the graph ($10^{-4}$ mM ouabain and 2 mM CN as KCN). Axon diameter was 495 $\mu$m

that could be satisfied by other nucleotides. In this case, axons could be unfueled by lowering of ATP levels and the anomalous rise of Na efflux prevented by use of an appropriate nucleotide substitute. In this event, the effect of unfueling axons of ATP should be observed as a lowering of Na efflux. The nucleotide CTP and the nonhydrolyzable analog of ATP, AMP-PNP, were used. The results are shown in Figs. 6 and 7 and indicate that neither CTP nor AMP-PNP had any detectable ATP-like effect. The Na efflux increased during dialysis with an ATP-free medium in the presence of each of the ATP substitutes, just as in the previously described experiments in which no other nucleotides were present. When ATP was resupplied to the axon interior late in the ATP-free dialysis period, prompt reversal of Na efflux towards normal values ensued in each case. This is consistent with the nucleotide requirement being most likely specific for ATP, the normal hydrolyzable substrate. It can be noted in Fig. 7 that a maximum Na efflux was reached during dialysis with the ATP-free medium before readmission of ATP to the dialysis medium—the Na efflux had increased about 15-fold before the leveling off occurred. There is also some evidence for a decline in the rate of rise of Na efflux seen in Fig. 6 to a rate below the apparently linear rate of rise measured earlier in the dialysis with the ATP-free medium. Such tendencies of Na efflux to level off were seen only after the Na efflux had increased several-fold; the significance of this is unknown but a suggested possibility is that the presumably enzymatic Na transport process unleashed by low $[ATP]_i$ levels has a definite $V_{max}$ (*see* Discussion).

In the course of the experiments, $[Na^+]$ in the dialysis fluid was varied by design to determine the influence of $[Na^+]_i$ on the results. Experiments were initially with $[Na^+]$ in the dialysis fluid set to a value of 20 mM, near the normal physiological value for *Myxicola* giant axons (Abercrombie & Sjodin, 1977). Subsequently, $[Na^+]_i$ was varied between 85 $\mu$M and 200 mM (data for some of the values not shown). In this entire range of $[Na^+]_i$, the graphs of experimental data looked qualitatively the same and differed only in the absolute magnitude of the Na efflux, which appeared to be approximately linearly related to $[Na^+]_i$. The main features of this work, i.e., increases in Na efflux during dialysis with ATP-free and low-ATP media, and recovery toward normal Na efflux values upon readmission of ATP to dialysis media, were unchanged by changing the $[Na^+]_i$ level. In Figs. 2–7, the particular value of $[Na^+]$ used in each case is stated in the legends.

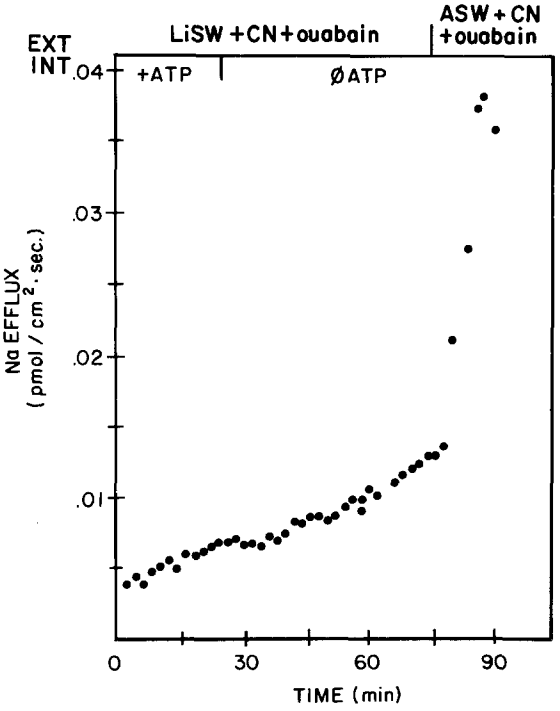


**Fig. 8.** Na efflux increases in external Na-free medium substituted with Li if ATP is removed from dialysis fluid. ATP was removed from dialysis fluid for a period of 45 min in LiSW containing ouabain ($10^{-4}$ M); the axon was then placed in ASW, 2 mM KCN. Dialysis medium was very low Na (85 $\mu$M), demonstrating that low $[Na]_i$ also does not appreciably affect the occurrence of the anomalous effect. Axon diameter was 650 $\mu$m

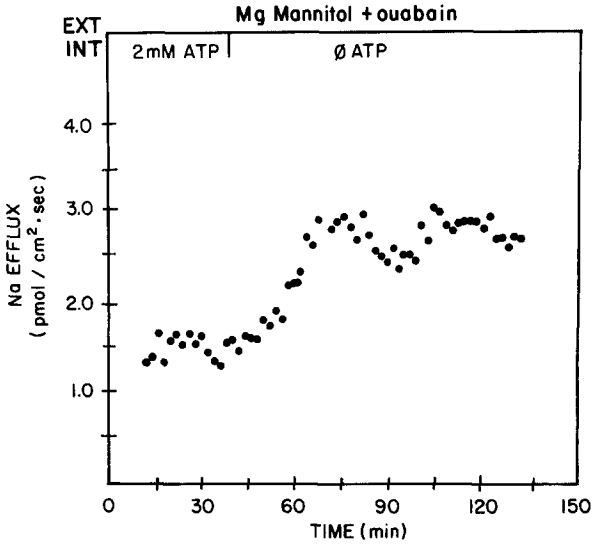


**Fig. 9.** Na efflux increases if external Na is replaced with Mg mannitol and ATP is removed from dialysis fluid. ATP initially at 2 mM was removed from the dialysis fluid when Mg mannitol was substituted for Na in the external medium (0 Ca, Mg mannitol). Dialysis fluid was normal Na (20 mM Na) and contained KCN (2 mM). Axon diameter was 675 $\mu$m

Some experiments were also performed using K-free external solutions.

Two other possibilities were examined, namely, that the increased Na efflux occurring at lowered $[ATP]_i$ values represents an increased rate of Na/Na exchange or an increased rate of Na/Ca exchange. Experiments similar to those already reported were performed with $[Na]_o$ replaced by $[Li]_o$ (*see* Figs. 3 and 8) and in the absence of external Ca ions (*data not shown*). The results obtained in Li solutions were qualitatively similar to those in Na media in that increased Na efflux still occurred at lowered values of $[ATP]_i$. In one experiment, however, going from LiSW to Na seawater (ASW) gave a prompt and large increase in Na efflux after 1 hr of dialysis with an ATP-free solution (Fig. 8). Removing Ca ions from the external solutions did not alter the outcome of the experiments. There is, thus, some evidence that the major external monovalent cation is of importance in the anomalous, low-ATP effect. To further investigate this hypothesis, some experiments were performed in Mg mannitol solutions containing neither K, Na, nor Ca ions (*see* Abercrombie & Sjodin, 1977; Sjodin & Abercrombie, 1978). The results are shown in Figs. 9 and 10. Dialysis with an ATP-free solution led to about a 70% increase in the Na efflux even in a solution nominally free of any monovalent cation. Unlike in the previous experiments, resupplying ATP by dialysis failed to restore the Na efflux to initial values (*data not shown*). However, replacing the Mg mannitol external medium with a buffered and osmotically matched NaCl solution brought about a large (approximately threefold) increase in Na efflux. Returning to the Mg mannitol solution again lowered the Na efflux. It seems clear that, though some elevation of Na efflux at lowered $[ATP]_i$ still occurs in Li and in Mg mannitol solutions, the increase is considerably higher if external Na ions are present. It seems safe to conclude that, whatever is the fundamental basis for the anomalous ATP effect in *Myxicola* giant axons, the effect predominates to a greater extent if external Na ions are present. Though this suggests at least the partial involvement of a Na/Na exchange in the anomalous ATP effect, further investigation would be required to prove this.

As external K ions are normal activators of the Na pump and the affinity of pump sites for external K is a function of internal ATP levels (Beauge & DiPolo, 1981), it seemed important to determine any influence of $[K]_o$ on the effects being reported. The anomalous increase in Na efflux in ATP-depleted axons was observed in K-free media and increasing the value of $[K]_o$ had no apparent effect on the results.

From the nature of the results presented, it does not seem likely that the increase in Na efflux occurring at low $[ATP]_i$ levels represents an effect on

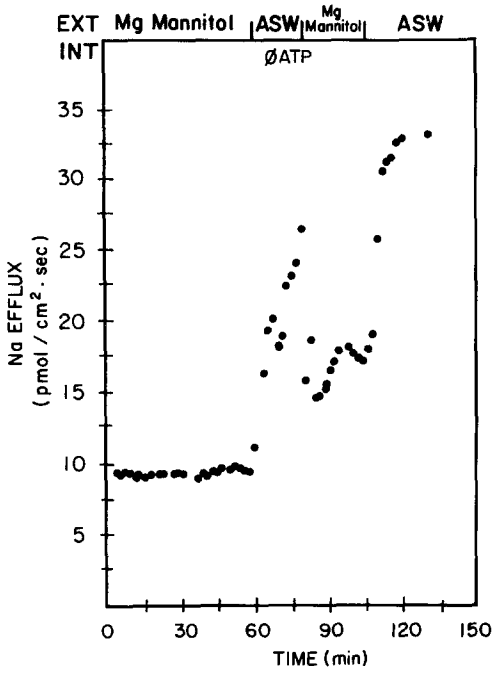


**Fig. 10.** The anomalous efflux is reversibly increased by external Na. Axons in Mg mannitol ($10^{-4}$ ouabain) and dialyzed with fluid containing no ATP (showing a modest "anomalous" increase in Na efflux) were made to show a large increase in Na efflux upon immersion in ASW ($10^{-4}$ ouabain); this increase could be reversed to a large extent by reimmersion in Mg mannitol. Subsequent return to the ASW once again caused a large increase in the anomalous Na efflux. Dialysis solution was normal Na (20 mM) throughout. Axon diameter was 550 $\mu$m

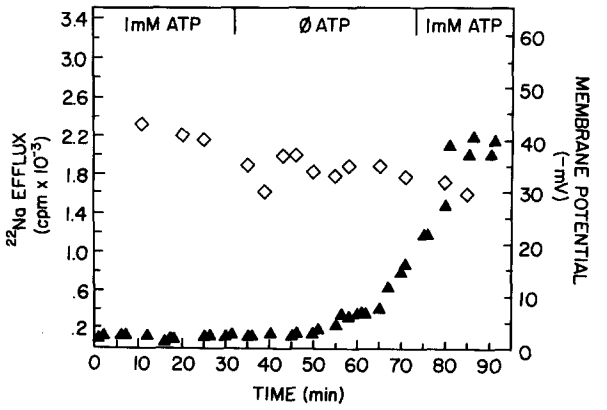


**Fig. 11.** Axon membrane potential is largely independent of the anomalous Na efflux. The membrane potential was monitored together with $^{22}$Na efflux during an experiment to determine if the anomalous Na efflux was accompanied by commensurate large changes in the membrane potential. The membrane potential declined steadily with time, as is usually observed in dialyzed axons, but did not show any perturbations that could be ascribed to the presence or absence of ATP in the dialysis fluid. Na efflux increased anomalously as expected with respect to [ATP]. Calcium was alternately added and removed from the external solution at intervals of 7–12 min throughout the experimental period starting at $t$ = 11 min and ending at $t$ = 85 min, without any demonstrable effect on either Na efflux or the membrane potential. Experiment was performed in LiSW. Axon diameter was 575 $\mu$m

electrodiffusional or passive Na efflux. For example, even if low ATP levels led to severe depolarization of the fibers and passive Na efflux increased several-fold, the results presented would not be expected because the electrodiffusional component of Na efflux is normally such a low fraction of the total Na efflux. Membrane depolarization, however, could lead to a lower energy barrier for Na extrusion so that Na efflux could become increased. It seemed important, therefore, to examine the behavior of the membrane potential during dialysis with ATP-free media. The results of a typical experiment are shown in Fig. 11. The resting membrane potential shows the usual decline in negativity that occurs during prolonged dialysis. The point that Fig. 11 demonstrates, however, is that during the time interval in which Na efflux is experiencing a large increase, a fourfold increase in the interval from 50 to 70 min, the membrane potential curve is fairly flat and cannot explain the large increase in Na efflux that occurs.

## Discussion

It was previously noted that the sensitivity of Na efflux in *Myxicola* giant axons to reduced internal levels of ATP is quite unlike that observed in squid giant axons (Forbush, 1979). In the cited previous work, it was not possible to demonstrate a dependence of active or pumped Na efflux on $[ATP]_i$ in the sense expected if ATP were the immediate energy source for the pump. In the present work it was likewise impossible to demonstrate that ATP is the major energy source of the Na pump in *Myxicola* giant axons. During dialysis with an ATP-free medium, at about the time that a decline in Na efflux due to the beginning of unfueling could be expected, the Na efflux increased. If it were simply a matter of nothing happening to Na efflux during dialysis with an ATP-free medium, one could advance the hypothesis that [ATP] at the plasma membrane in *Myxicola* giant axons does not follow closely changes in [ATP] in the bulk of the axoplasm. Forbush (1979) has propounded effective arguments opposing this hypothesis. In our work, the Na efflux responds to a change in [ATP] in dialysis fluid at just about the time one would expect the change to be felt at the plasma membrane, arguing that the [ATP] level at the membrane really does respond to changes in [ATP] in the dialysis medium. The effects are specific for ATP as shown in experiments with either CTP or AMP-PNP as substitutes for ATP. One then has to consider possible explanations for the anomalous direction of the change in Na efflux.

One possibility that has to be examined is that internal stores of ATP, or internal sources of ATP production, are concentrated at the inner plasma membrane surface in *Myxicola* giant axons and that these stores or sources liberate ATP near the Na pump sites in response to a drop in ambient [ATP]. Two factors argue against this hypothesis. One argument is that the anomalous effect occurred after prolonged exposure to metabolic poisons that should have inactivated any such putative processes. Thus, this would appear an effective argument, unless considerable amounts of ATP were stored in a bound form from which free ATP could be liberated at a high rate for quite some time whenever a deficit of free ATP occurs in the cell, or if ATP could be regenerated biosynthetically by a process intractable to the metabolic poisons that were used; alternatively, the argument would not hold if a significant quantity of ATP were sequestered in compartments that were in effect physically or biochemically isolated from the dialysis fluid and its constituents, including metabolic inhibitors. A stronger argument comes from the observation that increasing $[ATP]_i$ above the normal value of about 1 mM does not increase the Na efflux above the normal value seen in microinjected axons (Abercrombie & Sjodin, 1977). However, even this argument can be countered if one assumes that the putative, ATP storage compartments are tightly annexed to the membrane sites of the Na pump, so that the compartments themselves prevent direct access of ATP in the dialysis fluid to at least some of the pump sites (*see* similar arguments made by Forbush, 1979).

An alternative explanation is that it is the passive or electrodiffusional Na efflux that increases in response to reduced $[ATP]_i$. For example, low values of $[ATP]_i$ could lead to increases in $[Ca^{2+}]_i$, which in turn could activate channels that conduct Na ions. The problem with this explanation is that large increases in Na influx would also be expected to occur were this the case, and such increases in Na influx have not been observed (Forbush, 1979). A variant of this hypothesis is that even though new Na channels are not generated, membrane depolarization leads to a large increase in the passive Na efflux through normally existing channels. This hypothesis was rejected earlier. The normal passive Na efflux is too low to produce the observed effects, even with a large increase in its value due to depolarization, and, moreover, the needed depolarization simply does not occur.

Another possible explanatory argument is that the rise in Na efflux is caused by a rise in the extracellular potassium ion concentration due to periaxonal K accumulation. Two facts argue against this possibility. The anomalous effect on Na efflux was not influenced by K-free conditions in the external solution and a considerable anomalous effect was observed in the presence of ouabain, which inhibits activation of the Na pump by external K.

Ruling out these explanations leaves possible only one general conclusion: the aberrant behavior observed at low $[ATP]_i$ must represent an activation of a Na transport process, possibly even an activation of the Na pump, making it operate in a highly abnormal mode. Some hint that this may be the case can be seen in Fig. 2. Here a ouabain-sensitive Na efflux was demonstrable during dialysis with an ATP-free medium and during a period of rapid rise of the Na efflux. Cardiac steroid sensitivity of Na efflux after prolonged dialysis with an ATP-free medium (containing CN) was also observed by Forbush (1979). It can be seen in Fig. 2, that after ATP has been removed from the dialysis fluid and ouabain added to the external medium, Na efflux decreases steadily and then resumes its rise again at a rate very close to that seen just before the ouabain was added. If one assumes that two separate and independent mechanisms are concomitantly in operation in this behavior of the Na efflux, that is, the Na efflux is made to rise through one process that depends on low ATP levels inside the cells, while Na efflux is made to decrease by the inhibition of a separate mechanism, namely the Na pump, then the relevant region of the curve in Fig. 2 being discussed can be viewed as the algebraic summation of these two concurrent processes, with the ouabain-sensitive component being gradually reduced until 30 min have elapsed and no further reduction is possible. Such an algebraic approach (using graph distances from Fig. 2) leads to the conclusion that the ouabain-sensitive Na efflux is greater in magnitude than the entire normal Na efflux, which is presumably almost entirely via the Na pump in the first place. One would have to conclude on this basis, that the ouabain-sensitive pump rate had to have increased over its normal value, along with the rest of Na efflux, in response to the lowered value of $[ATP]_i$. But if this were the case, the steep, positive slope for Na efflux seen in Fig. 2 after ATP depletion, but before the addition of ouabain, would have to be much steeper than that which is seen after the resumption of the increase in Na efflux that occurs about 30 min after the addition of ouabain. As the two slopes in question are about equal in magnitude, one must conclude that the anomalous process giving rise to increased Na efflux under these conditions is not independent of the Na pump itself, i.e., that the two countervailing processes overlap at least in part. On the basis of these considerations, it would seem that the action of low $[ATP]_i$ values would have to be, at least partially, on the Na pump itself.

Beyond what has already been said, it is difficult to explain the anomalous effect of low $[ATP]_i$ levels on Na efflux in *Myxicola* giant axons. The effect seems likely not to be on Na channels, but at least in part on the Na pump itself. Evidence has been presented here that Na/Na exchange, which is not very significant in normal axons (Abercrombie & Sjodin, 1977), may form a significant part of the increased Na efflux observed at lowered $[ATP]_i$. The anomalous effect may be regulatory in nature. When the value of $[ATP]_i$ declines at the membrane, mechanisms to compensate for the anticipated unfueling may be set into play to maintain Na pumping. The putative regulatory mechanisms may involve one or more of the following: (*i*) increased turnover rate of the pump, (*ii*) release of ATP from stored forms, and (*iii*) shifting of some of the Na efflux to Na/Na exchange to conserve energy. Of these mechanisms, only the second, notwithstanding the aforementioned difficulties, would account for the fact that Na efflux cannot be shut off by prolonged dialysis with ATP-free media when $[ATP]_i$ in the bulk of the axoplasm can be expected to have fallen to values of 1 $\mu$M or below. Whatever the mechanism, it merits further study, since it may represent a unique, heretofore unknown, adaptive response to losses of cellular energy. Unfortunately, the question of alternate energy sources for the Na pump in *Myxicola* giant axons remains unresolved as does the question of compartmentalization of intrafibrillar ATP near the membrane in this preparation. Further work is required to provide firm answers to these questions.

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