Some Factors Influencing Sodium Extrusion by Internally Dialyzed Squid Axons

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ABSTRACT Squid giant axons were internally dialyzed by a technique previously described. In an axon exposed to cyanide seawater for 1 hr and dialyzed with an ATP-free medium, the Na efflux had a mean value of 1.3 pmole/cm²sec when $[Na]_i$ was 88 mM, in quantitative agreement with flux ratio calculations for a purely passive Na movement. When ATP at a concentration of 5–10 mM was supplied to the axoplasm by dialysis, Na efflux rose almost 30-fold, while if phosphoarginine, 10 mM, was supplied instead of ATP, the Na efflux rose only about 15-fold. The substitution of Li for Na in the seawater outside did not affect the Na efflux from an axon supplied with ATP, while a change to K-free Na seawater reduced the Na efflux to about one-half. When special means were used to free an axon of virtually all ADP, the response of the Na efflux to dialysis with phosphoarginine (PA) at 10 mM was very small (an increment of ca. 3 pmole/cm²sec) and it can be concluded that more than 96% of the Na efflux from an axon is fueled by ATP rather than PA. Measurements of [ATP] in the fluid flowing out of the dialysis tube when the [ATP] supplied was 5 mM made it possible to have a continuous measurement of ATP consumption by the axon. This averaged 43 pmole/cm²sec. The ATP content of axons was also measured and averaged 4.4 mM. Estimates were made of the activities of the following enzymes in axoplasm: ATPase, adenylate kinase, and arginine phosphokinase. Values are scaled to 13°C.

INTRODUCTION

In a previous report (Brinley and Mullins, 1967) we have shown that squid axons subjected to internal solute control by dialysis retain their ability to generate a metabolically dependent efflux of sodium ions. The purpose of the present study was to examine this sodium efflux more closely both with respect to the kinds of substances that could energize the Na extrusion mechanism and with respect to the possible dependence of the Na efflux on the presence of both Na and K in the bathing seawater.

Metabolic inhibitors such as cyanide and dinitrophenol act in squid axons by preventing the generation of high energy phosphate bonds by oxidative

metabolism. The application of such substances to squid axons has been shown (Caldwell et al., 1960 *a*) to reduce Na efflux from normal values of about 60 pmole/cm² sec at 20°C to about 10 pmole/cm² sec with a concomitant reduction of [ATP] to about 10% of normal. This level of Na efflux during inhibition of metabolism is about seven times larger than that calculated for a purely passive outward leakage of Na. Such a result is understandable on two different bases; either the inhibitors do not reduce $[ATP]_i$ to zero or there are alternate sources of fuel or alternate pumps for Na extrusion. An effect of CN on purely passive Na permeability has been ruled out by the experiments of Caldwell et al. (1964). Internal dialysis experiments are capable of reducing $[ATP]_i$ to 1–10 μM and are therefore able to answer the question of whether a residual [ATP] in axons inhibited with CN is fueling extrusion.

Since it is clear that ATP is of major importance in energizing Na extrusion from axons, it seemed useful to have measurements of its consumption during Na extrusion. Such measurements, it is found, can only be interpreted if one has some idea of the axoplasmic concentrations of enzymes that transform ATP. Measurements of both ATP consumption and the activities of the enzymes ATPase, adenylate kinase, and arginine phosphokinase have been made during the course of this study.

The general conclusions from the studies summarized above are that dialyzed axons show an efflux of Na in the absence of fuel that can be calculated from a knowledge of Na influx, $[Na]_i$, and membrane potential by applying flux ratio considerations. Under conditions in which care has been taken to exclude substances such as ADP from the axoplasm, phosphoarginine at a concentration of 10 mM causes a negligible activation of the Na extrusion mechanism, while ATP is capable of fueling Na extrusion at rates comparable to those seen in injected axons. The K-free and Na-free responses of the Na efflux in dialyzed axons are similar to those seen in normal axons.

METHODS

Internal Dialysis The methods used to control internal solute concentrations have been previously described (Brinley and Mullins, 1967). For certain purposes, it was found desirable to free the axoplasm at both the left- and right-hand junctions of the nonporous/porous glass dialysis capillary of substances that might diffuse longitudinally from axoplasm in the nonporous region to axoplasm in the porous region, and there affect reactions under study. To perform such a washing of the axoplasm, the dialysis capillary was advanced along the axon until the porous region lay not in the center of the axon but about 10 mm to the left of center. This initial positioning is shown in Fig. 1 a. With the porous region in such a position, nonradioactive and fuel-free internal dialysis solution (composition given in Table I) was driven through the capillary at a rate of 0.5 μl/min. This effectively removed from the axoplasm solutes not supplied in the dialysis fluid and this action is shown in Fig. 1 a where a loss of stippling in the axoplasm corresponds to this removal of solutes.

The dialysis capillary was then slowly advanced to the right at a rate of about 1 mm/min until it reached the position shown in Fig. 1 b. The direction of travel was then reversed and the porous region of the capillary brought to the center of the axon. In general, washing operations with nonradioactive fluids were started about 10 mm to the left of the ultimate position of the left-hand border of the porous region and the right-hand border of the porous capillary was advanced about 5 mm beyond its ultimate position in the axon. With the porous region centered in the axon, the fluid

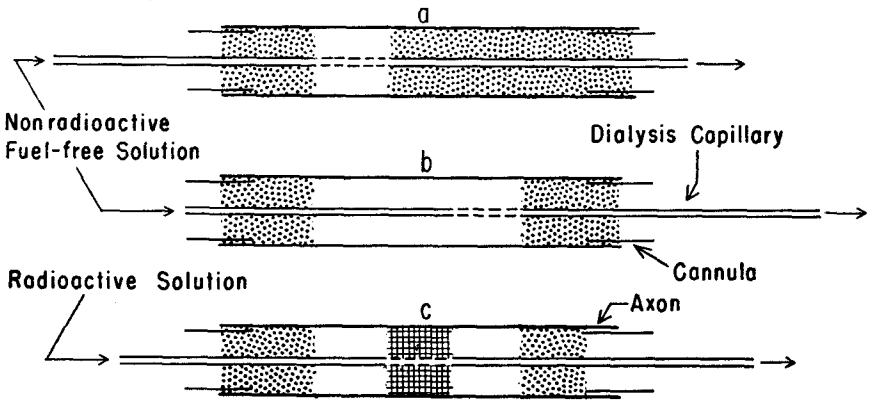

FIGURE 1. The method for removing substrates from the end regions of the axon is shown in this figure. The axon is cannulated and the porous capillary is inserted and brought to the position shown in *a*. The loss of stippling by the axoplasm indicates the removal of substances not contained in the nonradioactive dialysis fluid. The dialysis capillary is advanced toward the right at a rate of 1 mm/min until the porous region reaches the position shown in *b*. The result of this manipulation is to remove substrates from the central part of the axon. The direction of movement of the capillary is reversed and brought to the location shown in *c*; the fluid flowing through the dialysis capillary is changed to a medium containing ^{22}Na and this is shown as cross-hatching in the axoplasm. The region of flux measurement is thus bordered on each side by axoplasm that has been washed free of substrates such as ADP.

flowing through the capillary was changed to one containing a radioisotope such as ^{22}Na and the center of the axon equilibrated with tracer; this is shown in Fig. 1 c as a cross-hatched area. This method does not prevent longitudinal diffusion, but it does prevent the diffusion of substrates from the nondialyzed to the dialyzed region of the axon by removing these from the ends of the axon.

Analytical Methods In the course of the present series of experiments it was found necessary to make analyses for ATP, ADP, AMP, and occasionally for total adenine and for phosphoarginine. Three different methods for ATP analysis were used, involving the following reactions:

(*a*) glucose + ATP → glucosephosphate

glucosephosphate + TPN → phosphogluconate + TPNH

(*b*) ATP + phosphoglycerate → diphosphoglycerate + ADP
diphosphoglycerate + DPNH → phosphoglyceraldehyde + DPN + P_i

(*c*) ATP + luciferin + O_2 → $h\nu$ + adenyl-oxyluciferin + pyrophosphate

The TPNH formed in reaction (*a*) was measured spectrophotometrically at 340 nm. The reagents for such a reaction were contained in a capsule (ATP Calsul- Calbiochem, Los Angeles, Calif.) that could be dissolved in water in a spectrophotometer cuvette and the ATP sample for analysis added. The spectrophotometer used had a stability of 0.001 absorbance/hr and a sensitivity of 0.001 absorbance. Such a sensitivity

TABLE I
COMPOSITION OF SOLUTIONS

Substance	Seawater			Dialysis fluid*
	Normal	K-free	Li	
	mM	mM	mM	mM
K^+	9	0	9	304
Na^+	425	425	2	75–85
Li^+	0	0	423	
Mg^{++}	48	48	48	0.1–4‡
Ca^{++}	9	9	9	
Cl^-	496	496	496	83–93
CN^-	2	2	2	2
$SO_4^=$	25	25	25	
Isethionate$^-$				151
Aspartate$^-$				151
Taurine				275
EDTA				0.1
Glucose	2	2	2	

* Described in text as "no fuel" (NF); additions made were ATP 5–14 mM (described as "ATP"), phosphoarginine 10 mM (described as "PA"), or [(ATP 5–14 mM) + (PA 10 mM)] (described as "fuel" or "F").
‡ Early experiments had [Mg] of 0.1–4 mM; in later experiments [Mg] was 4 mM in addition to [ATP].

corresponded to the detection of about 10^{-10} mole ATP in a 0.5 ml sample with a light path of 1 cm. This method was used to check ATP stock solutions from time to time and was used to measure 5 μl samples of effluent from the dialysis tube when concentrations were expected to be in the range of 1–10 mM ATP, corresponding to 5–50 nmole ATP. A second method of ATP analysis (*b*) was used for axoplasm samples. The reagents were supplied in the form of a test kit (ATP test, Boehringer Co., Mannheim, Germany) and the reaction was again run in a cuvette volume of 0.5 ml; the sensitivity for ATP was, as above, 0.1 nmole. Under some experimental conditions it was necessary to make analyses for ATP on 2 μl samples containing ATP at 1–100 μM, or 2–200 pmole per sample. Since the enzymatic methods described above were insufficiently sensitive, these analyses were carried out using desiccated firefly tails (Sigma) (using reaction *c*). The firefly tail extract was held in a cuvette

positioned in front of a photomultiplier and the light emission was recorded as a function of time. Peak light emission was measured from records and calibrated with ATP standards. The reagent blank in these measurements was less than 10^{-14} mole ATP, and 10^{-12} mole could be measured with an analytical accuracy of 5%.

The measurement of both ADP and AMP was carried out with reagents supplied as a test kit (ADP/AMP test, Boehringer, Mannheim, Germany) and involved the following enzyme-catalyzed reactions.

$$\text{ADP} + \text{PEP} \rightleftarrows \text{ATP} + \text{pyruvate}$$
$$\text{pyruvate} + \text{DPNH} \rightleftarrows \text{lactate} + \text{DPN}$$

The decrease in absorbance resulting from the conversion of DPNH to DPN was measured and could be related to the ADP present. The subsequent addition of myokinase to the above reaction mixture allowed the following reaction to proceed:

$$\text{AMP} + \text{ATP} \rightleftarrows 2\ \text{ADP}$$

and the reaction of the ADP formed with the test reaction above allowed a quantitative estimate of the AMP present. All the above analytical methods are fully described in Bergmeyer (1965).

Axoplasm was analyzed for ATP by extruding about 5 mg onto a small piece of plastic film and weighing this rapidly on an electrobalance (Cahn Instrument Co., Paramount, Calif.). The sample was then placed in 0.1 ml of ice-cold 6% perchloric acid and homogenized with a glass rod and a few grains of sand. The tube was centrifuged and an aliquot of the supernatant neutralized by triethanolamine buffer was taken for ATP analysis by the DPN method.

Stock solutions of ATP were usually made up at 0.5 M and were stored at -90°C. They were analyzed periodically for ADP and AMP in order to have a sensitive measure of decomposition of the solution. Because of possible variations in the hydration of ATP salts, it was necessary to analyze stock solutions for [ATP] by the TPN method as well as to have ADP/AMP measurements as a check on decomposition. Occasional checks were made on the total adenine nucleotide content of such solutions by measuring absorbance at 259 nm. ATP solutions ordinarily used had at least 98 mole % ATP.

Effluent Samples from Dialysis Tube Measurements of ATP consumption by axoplasm and ATP concentrations in the effluent during dialysis with both fuel-free solutions and PA were made by a calibrated glass capillary tube that was positioned over the end of the dialysis capillary with a micromanipulator. Fluid samples so collected had volumes of from 1–5 μl, corresponding to from 2–10 min of dialysis time. Upon collection, these calibrated capillary tubes were placed inside a stoppered plastic test tube and transferred at once to a −20°C deep freeze. At the end of the experiment, the samples were either analyzed immediately or transferred to storage at −90°C.

RESULTS

Analysis of Axoplasm for ATP Although analyses of the ATP concentration in axoplasm of *L. forbesi* have been published (Caldwell et al., 1960 *a*), it

seemed important to have values for *L. pealei*. Axoplasm (or whole axon) was treated with perchloric acid as described in Methods and subjected to enzymatic analysis for ATP. Two axons were also analyzed for ADP and gave values of 0.2 and 0.6 mM. ATP was present in easily detectable quantities and the measurements obtained are given in Table II. The mean [ATP] was 4.4 mM.

TABLE II

[ATP] IN AXOPLASM (20–22°C)

Axon reference	Axoplasm sample	Time after death of squid	[ATP]
	mg	*hr*	*mM*
071565	8.9	3.0	5.7
071665	6.0	3.0	3.0
072065-1	7.6	2.5	3.5
072065-2	6.5	3.1	3.5
072065-3	4.3	1.3	4.3
072065-4	5.6	4.0	4.8
072765-1	5.2	4.0	4.9
072765-2	3.5	4.5	5.8
Mean			4.4±0.4
n =			8

Enzymes Concerned with Phosphate Transfer It is generally recognized that there are three enzymes in squid tissue mainly concerned with intracellular transformations of adenosine phosphates; these are:

ATPase	$ATP \rightarrow ADP + P_i$	(1)
Adenylate kinase	$2ADP \rightleftarrows ATP + AMP$	(2)
Arginine phosphokinase	$PA + ADP \rightleftarrows ATP + A$	(3)

The relative importance of these different reactions might be expected to be connected with the concentrations of these enzymes in axoplasm; hence measurements were made to have some idea of the activity of each enzyme.

The activities of the enzymes adenylate kinase and arginine phosphokinase could be measured on the same sample of axoplasm inasmuch as both involve the phosphorylation of ADP to ATP. The method of performing the analysis was as follows: to an "ATP Calsul" sufficient ADP was added to make the solution in the cuvette 5 mM with respect to this substance. The result obtained is shown in the lower curve of Fig. 2 where the addition of ADP results in a prompt increase in absorbance as the ATP present in the ADP as an impurity undergoes reaction. When this reaction had stopped, as judged by a constant

absorbance with time, a small amount of axoplasm was added to the cuvette and dispersed in the reaction mixture. The result of this addition was an increase in absorbance that was linear with time and presumably a measure of the extent to which reaction (2) was taking place. As the reaction mixture contains so much ADP that its concentration does not change, and because the ATP formed is removed by the analytical reaction in the test kit, the

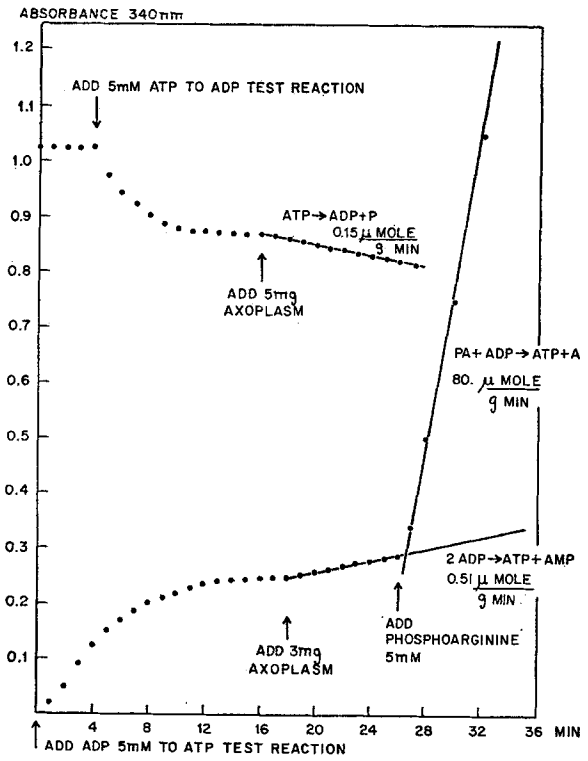

FIGURE 2. The absorbance at 340 nm of either TPNH (lower curve) or DPNH (upper curve) is plotted as a function of time. The appearance of 1 mole of TPNH corresponds to the consumption of 1 mole of ATP by the test reaction. Similarly, the consumption of 1 mole of DPNH (in the upper curve) corresponds to the consumption of 1 mole of ADP by the test reaction.

reaction rate should measure the concentration of that component of the reaction mixture which is limiting; thus presumably is the enzyme adenylate kinase.

With the addition of sufficient phosphoarginine to the mixture in the cuvette to make its concentration 5 mM, the rate of increase of absorbance with time changed to a much larger value, as shown in Fig. 2. This new rate is presumably the sum of reactions (2) and (3) proceeding simultaneously. From the known extinction coefficient of TPNH, the cuvette volume, and the mass of

axoplasm added, the enzyme activity could be calculated in terms of moles of substrate converted per unit time and per unit of axoplasm mass. Values for both enzymes are given in Table III. While adenylate kinase activity appeared to be relatively independent of whether the axoplasm was fresh or had been stored for some time, arginine phosphokinase activity was much more labile in that it decreased greatly if axoplasm or axons were stored overnight at 4°C or for a few hours at room temperature (see also Morrison et al., 1957).

The ATPase activity of axoplasm could be conveniently followed by meas-

TABLE III

SUMMARY OF AXOPLASMIC ENZYME DATA (25°C)

Axon reference	Axon diameter	Axoplasm sample	Enzyme activity*			Remarks
			Adenylate kinase	ATPase	Arginine phospho-transferase	
	μ	mg	μmole/g min	μmole/g min	μmole/g min	
080266-1	400	4.1	0.11		33	Axon partially dried
080266-2	385	6.1	0.51	0.15	80	Fresh axon
080466	384	6.4	0.67	0.30	45	Stored overnight, 4°C
080966		5.5	0.21			Mitochondria-free axoplasm
081066		3.0	0.35			Assay in 2 mM CN + 500 mM K isethionate
081666		1.5	0.53			Assay in 2 mM CN + 500 mM K isethionate Stored overnight, 4°C
081666		12		0.26		Mitochondria-free axoplasm
Mean			0.40	0.24	53	
n =			6	3	3	

* ATP or PA 5 mM.

uring the ADP produced (see reaction 1) from ATP using a Boehringer ADP/AMP test kit. In this assay, the [ATP] was made 5 mM in the cuvette and the initial decrease in absorbance measured in the top curve of Fig. 2 was the reaction of ADP present as an impurity in the ATP. After the absorbance had reached a steady value with time, axoplasm was added to the reaction cuvette and thoroughly dispersed. Upon this addition, the reaction started and could be followed as a linear decrease in absorbance with time. ATPase activity could be calculated from this slope and a knowledge of the DPNH extinction coefficient, cuvette volume, and mass of axoplasm added.

In some of the ATPase assays, the ADP test kit reagents were made up in 0.5 M K isethionate rather than distilled water. Under these conditions, it was conceivable that the mitochondria of the axoplasm might remain intact and functional to some extent although no substrate was supplied. To pre-

vent any possible loss of ADP by phosphorylation in the mitochondria, CN was added to the reaction cuvette at a concentration of 2 mM. These assays for ATPase were the same as those carried out in distilled water without CN.

It seemed useful to know whether the ATPase activity observed in axoplasm was the result of mitochondria activity or whether the enzyme involved was dispersed in the nonparticulate fraction of the axoplasm. To make such measurements, axoplasm was extruded into capillary tubes with an inside diameter of 0.5 mm. These tubes were mounted in close-fitting Lucite blocks machined to fit into an ultracentrifuge rotor. The extruded axoplasm was subjected to centrifugation at 50,000 *g* for 4 hr at 5°C and the supernatant axoplasm was analyzed for ATPase activity. Electron microscopy of the supernatant and sediment confirmed that the experimental treatment was adequate to remove virtually all particulate matter from the axoplasm supernatant, while enzymatic analysis showed the supernatant to have largely the same ATPase activity as whole axoplasm (see Doane, 1967). The data on ATPase activity are shown in Table III.

ATP Consumption by Dialyzed Axons By measuring the [ATP] in the effluent from the dialysis tube, and knowing the flow rate and [ATP] in the dialysis fluid before it entered the axon, it is possible to calculate the ATP consumption of an axon. Such measurements were made on a number of axons and the results are shown in Table IV. The mean value for ATP consumption normalized to an [ATP] of 4 mM is 43 pmole/cm² sec and is similar to the mean Na efflux (51 pmole/cm² sec) measured on the same axons. It agrees rather well with the in vitro measurement of ATPase activity reported, 0.24 μmole/g min = 60 pmole/cm² sec at an [ATP] = 5 mM or 48 pmole/cm² sec when normalized to 4 mM.

Na Efflux in the Absence of ATP In order to measure the efflux of ^{22}Na from squid axons in the absence of high energy phosphate compounds, a number of axons were pretreated with 2 mM CN sea water for 1–3 hr before beginning an experiment. As shown by Caldwell et al. (1964), this procedure serves to reduce the [ATP] of axoplasm to about 120 μM. Our subsequent dialysis of the axoplasm with fuel-free fluid yielded a concentration of ATP in the dialysis effluent of about 2 μM. The Na efflux from such axons, though readily measurable, was extremely small and for eight axons the mean was 1.3 pmole/cm² sec at 14°C (see Table V).

Perfusion with media free of high energy phosphate compounds did not seem to affect the transport mechanism adversely, for it was found that the Na efflux rose promptly to values characteristic of fueled axons when the internal dialysis medium was changed to one containing ATP and PA. This prompt rise is illustrated in Fig. 3, where the flux begins to rise 2–3 min after dialysis with fuel begins. Actually the delay is considerably less than this

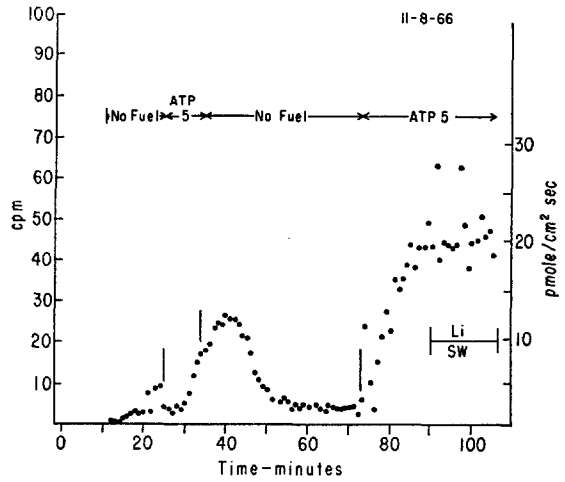

FIGURE 3. The efflux of Na is plotted as a function of time; the internal dialysis started at 12 min and was a no-fuel medium. The efflux under these conditions was about 1.8 pmole/cm²sec. A 10 min pulse of ATP at a concentration of 5 mM was applied and followed by no fuel and the Na efflux then fell essentially to its initial level. At 73 min, ATP 5 was applied and the Na efflux rose to 20 pmole/cm²sec. A change to Li seawater during the plateau had no effect on the magnitude of the Na efflux.

since about 1–2 min are required to flush out the dead space in the lumen of the capillary between the porous region and the capillary holder.

It might be argued that the observed flux increase is not due to ATP or PA, but to some toxic impurity in the fuel perfusion media which increases the membrane permeability to sodium or in some other way causes a large increase in efflux. The flux increase resulting from ATP perfusion, however, for short periods (about 10 min) is readily reversible as can be seen from Fig. 3, where the ^{22}Na efflux begins to fall within 5 min after a change to no-fuel solution. The flux rise and fall is nearly symmetrical and the nonfueled flux reaches almost the same level as before the ATP pulse. Four experiments were sufficiently complete so that the ratio of fueled to nonfueled flux could be calculated on the same axon, as shown in Table V. This ratio was 22, or somewhat smaller than the ratio calculated from the pooled data for all axons on which a fueled or nonfueled flux was determined. This ratio was 40/1.3 = 31 and forms the basis for an experimental test of the flux ratio equation which will be presented in the Discussion.

Na Efflux during Dialysis with Phosphoarginine Experiments similar to those described above for ATP were performed using PA in order to have measurements of the extent to which this compound could serve to energize the Na extrusion mechanism. These experiments were usually done by comparing the Na efflux produced with PA in the dialysis fluid with that pro-

duced when both ATP and PA were present. An experiment of this sort is shown in Fig. 4 where an initial dialysis with no fuel produced the low level of Na efflux that is ordinarily observed. A change in dialysis fluid to one containing PA at 10 mM resulted in a prompt rise in Na efflux to a level of roughly 20 pmole/cm² sec and a subsequent change to fuel led to an efflux of Na that was initially 40 pmole/cm² sec and a final efflux, just before a change to no fuel, of about 33 pmole/cm² sec. The axon showed a small decrease in Na efflux in Li seawater which was unusual. The [Mg] in the dialysis fluid was

TABLE IV

ATP CONSUMPTION BY DIALYZED AXONS DURING Na EXTRUSION (13–15°C)

				ATP consumption*				
Axon reference	Axon diameter μ	$[Na]_i$ mM	[ATP]‡ mM	μmole g min	μmole g min [ATP]	pmole cm² sec	pmole / cm² sec if [ATP] = 4 mM	Na efflux pmole/cm² sec
121565	517	90	4	0.16§	0.040	33	33	23
121665	580	90	4	0.08	0.020	20	20	23
062366-2	585	89	4	0.19	0.048	52	52	~70
070666	468	89	10	0.65	0.065	137	55	65
070766	468	89	10	0.77	0.077	163	65	75
071666	534	105	6	0.22	0.037	52	35	
				0.34¶	[0.057]	89	[59]	
			29	0.55	[0.019]	133	[18]	
072166	400	105	16	0.42	[0.026]	75	[19]	
			29	0.37	[0.012]	66	[29]	
Mean					0.048		43	51
n =					6		6	5

Brackets indicate values not included in means.

* Total Mg = 4 mM.

‡ Nominal ATP concentration. An indeterminate amount of hydrolysis occurred during storage estimated to be as much as 25% in some cases.

§ 19°C.

¶ 24°C.

only 4 mM in this experiment and the extrusion mechanism may well have been deficient in Mg. This notion is to some extent borne out by the response of the Na efflux to a change to no fuel at 115 min. The efflux appears to rise before undergoing the usual fall toward levels of Na passive efflux. The effluent from the dialysis tube was analyzed for ATP 16 min after the change to no fuel and the insert in Fig. 4 shows the [ATP] in mM in 2 min samples of the effluent. The last sample had a value of 2 mM and the efflux of Na was still at normal levels. An important observation from this experiment was that the level of Na efflux produced by dialysis with PA was about half that produced by ATP + PA. In four experiments of this sort, the mean Na

efflux during PA dialysis was 44 % of that during dialysis with PA + ATP; the results are shown in Table 5, column 5. In one of these experiments, 111566-1, the concentration of ATP present in the PA dialysis fluid which had passed through the axon was analyzed and found to be 0.6 mM. Analysis of the stock PA solution which had been used to prepare the PA dialysis

TABLE V

CONDITIONS AFFECTING SODIUM EFFLUX IN DIALYZED SQUID AXONS

Axon reference	Na efflux pmole/cm²sec		Ratio of sodium efflux							
			SW			K-free SW / SW		Li SW / SW		
	F	NF*	NF/F	PA/F	ATP 5/F	F	ATP 5	F	PA 10	ATP 5
1	2	3	4	5	6	7	8	9	10	11
100566	70							1.3		
101266	30							1.0		
								0.8		
101866	38	2.0	0.05	0.50				0.7		
								0.9		
102566	27	0.9	0.03	0.20					1.0	
102666		1.7								
110866-1	41			0.44						
110866-2	46	0.9	0.02							1.0
111066-1		1.3								
111066-2	57				0.67			1.2		0.7
111566-1	26	2.0	0.08	0.62						
111566-2	23				1.0	0.60	0.68			
111666	25					0.31				
111666-1	40									1.0
112266-1	41						0.79			0.9
012567-1		0.3								
012567-2		1.5								
012667	52									
Mean	40	1.3	0.05	0.44	0.84	0.46	0.74	0.9	1.0	0.9
n =	13	8	4	4	2	2	2	6	1	4

* Fluxes corrected for extra efflux during stimulation (1 imp/10 sec). Fuel is "F" and no-fuel is "NF".

fluid indicated that ATP present as an impurity in PA would have contributed no more than 2.5 μM to the effluent ATP concentration. The conclusion seemed inescapable, therefore, that PA was phosphorylating some substrate in the axon. A consideration of the experimental arrangement which we used led to the hypothesis that ADP diffusing longitudinally from the non-dialyzed region of the axon into axoplasm outside the junction of porous/

nonporous glass might be phosphorylated by the enzyme arginine phosphokinase. The situation is further complicated by the fact that ^{22}Na and PA will diffuse longitudinally in a direction opposite to that of ADP, so that in the region of the junction between porous and nonporous glass, the specific activity of tracer as well as the amount of ATP formed by a reaction of PA with ADP, will be indeterminate. Although the guard system serves to exclude from the collecting system any ^{22}Na crossing the membrane in the end regions, there is no control to prevent ADP in the end regions from diffusing

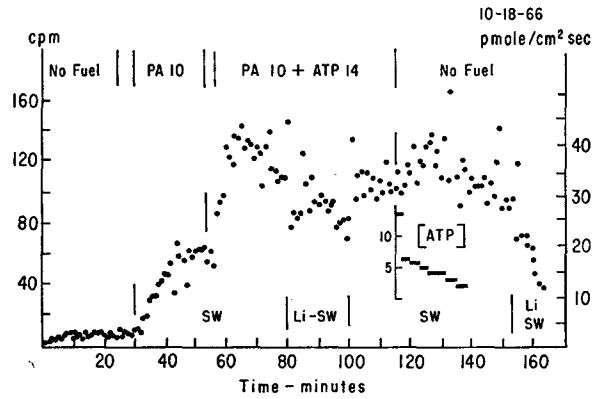

FIGURE 4. Na efflux is plotted as a function of time for the various internal and external changes in solutions made. The initial dialysis was with no fuel and the Na efflux was about 2.5 pmole/cm²sec. A change to phosphoarginine 10 mM led to a rise in efflux to about 20 pmole/cm²sec and a further change to PA 10 + ATP 14 mM resulted in an efflux of Na of about 40 pmole/cm²sec. A change to Li seawater outside appeared to reduce the Na efflux somewhat while a change to no fuel seemed initially to allow an increase in Na efflux followed by a decline of the efflux toward fuel-free values. The insert shows the [ATP] in mmoles/liter found by analysis of the dialysate for 16 min following the change to no fuel internal dialysis solution. The final value for [ATP] was about 2 mM and there was, at this time, no obvious effect on Na efflux.

across the wall of the porous capillary, being carried downstream by the flow of fluid, and redistributed along the length of the dialyzed region. Diffusion of ADP at the downstream junction will also occur, but in this case would not reach the porous region because it would be carried away by the dialysis fluid flow into the nonporous glass. A theoretical analysis of the situation seemed unpromising because the relation between ATP concentration and sodium efflux was not known. Therefore the problem was approached experimentally as follows.

The end regions of an axon were dialyzed as described in Methods. This should have removed ADP and other solutes from regions of the axon adjacent to the porous length of the capillary, although no tests were made. An

experiment is shown in Fig. 5, where Na efflux is given on the left-hand ordinate and [ATP] in the dialysis effluent in μM is indicated on the right-hand ordinate. The initial dialysis was with no fuel and yielded a Na efflux of 0.3 pmole/cm^2 sec while [ATP] was about 1.5 μM. The internal dialysis was then changed to PA at 10 mM and the efflux of Na rose to about 3.3 pmole/cm^2 sec but the [ATP] also rose about 10-fold. The results of four such experiments are shown in Table VI where the first entry, experiment 111567-1 shows the results obtained when there was no preliminary wash of the end

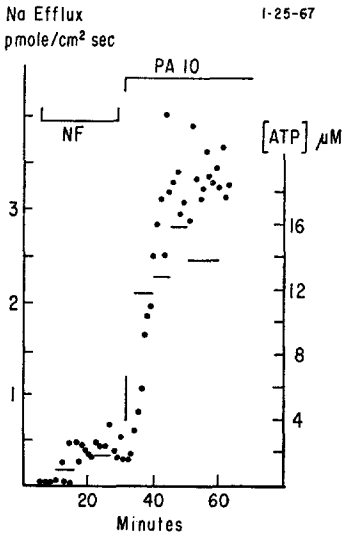

FIGURE 5. Na efflux is plotted as a function of time for an axon that was subjected to a predialysis of the axoplasm on each side of the region where efflux was measured. The left-hand ordinate is Na efflux and the right-hand ordinate is [ATP] in the dialysis fluid coming from the axon in μmole/liter. The solid circles are Na efflux while the horizontal bars are [ATP] in a 4 min sample of dialysis fluid. The initial dialysis was with no fuel and Na efflux was 0.3 pmole/cm^{2}sec, while [ATP] was 1.5 μM. A change to PA 10 in the internal dialysis fluid led to an Na efflux of 3.3 pmole/cm^{2}sec and an [ATP] of about 15 μM. Note that the highest value of Na efflux is only 1/15th of normal values while [ATP] is less than 1/300th of normal.

regions. The final four entries in the table show that removal of ADP from the end regions by predialysis results in a marked reduction of the sodium efflux and a concomitant reduction of the concentration of ATP appearing in the perfusate. The data indicate that 10 mM phosphoarginine can produce at most about 5–15% of the normal sodium efflux. However, we doubt that this small increase in efflux seen during PA perfusion should be ascribed to phosphoarginine since it correlates well with the appearance of a large increment in the concentration of ATP in the dialysate. The increment in ATP is consistent in each of the three experiments for which data are complete,

and is the result of multiple analyses of dialysis effluent before and during PA dialysis.

The single experiment, 120866-2, provides qualitative support for the notion that most of the ADP phosphorylated by PA comes from the upstream side of the axon. In this experiment only the left-hand (upstream) portion of the axon was predialyzed; however, the ATP concentration appearing during subsequent PA dialysis has been reduced practically to the level observed when both end regions were predialyzed.

Effect of K-Free Seawater on Na Efflux An intact squid axon injected with radioactive Na shows a large decrease in Na efflux when the seawater

TABLE VI

RELATION BETWEEN Na EFFLUX AND [ATP] IN DIALYSIS FLUID

Axon reference	Dialysis with NF		Dialysis with PA 10		Efflux of Na pump	
	[ATP]	Na efflux	[ATP]*	Na efflux		
	μM	pmole/cm²sec	μM	pmole/cm²sec	pmole/cm²sec	Δ[ATP] μM
111566-1		2.2	[600]‡	22	20	600
120866-2			[55]§			
010467-1	0.5	3.7	23	14.4	11	23
010467-2			11			
012567-1	1	0.3	12	3.4	3	11
012567-2	3	1.5	12	4.1	3	9
Mean	1.5	1.9	15			
n =	3	4	4			

Brackets indicate data excluded from the mean.

* Corrected for ATP in PA (2.5 μM).

‡ No predialysis of end regions.

§ Predialysis of left end region of axon only.

surrounding the axon is made K-free (Hodgkin and Keynes, 1955). The magnitude of this effect appears to be greatest in fresh axons and declines in axons that have been isolated for many hours. The nature of the change responsible for the loss of a K-free effect is unknown. Since we were interested in the extent to which dialyzed axons resemble normal axons with respect to their Na efflux, it seemed important to discover whether such axons did show a K-free effect. An experiment to test for a K-free effect in dialyzed axons is shown in Fig. 6. The axon was held in CN seawater for 2 hr prior to internal dialysis; it was then equilibrated with fuel and Na efflux measurements were begun. At 25 min, the seawater surrounding the axon was changed to K-free and the Na efflux promptly fell to 0.30 of its former value. Because the Na efflux was still rising at the time when the change to K-free seawater was made, it continued to rise in K-free seawater and when a change to seawater

was made, the Na efflux increased from 0.40 to 1.0. Four axons were tested for K-free effects and the results are summarized in Table V (columns 7 and 8). Although these results indicate that the K-free effect is greater in axons dialyzed with fuel than with ATP alone, the variability of the effect among axons and the fact that rather few experiments were done make it impossible to be certain that the difference is real. In the experiment shown in Table V (axon 111566-2) the same axon was dialyzed with fuel and then with ATP; there was no difference in the K-free effect with these two substrates. The

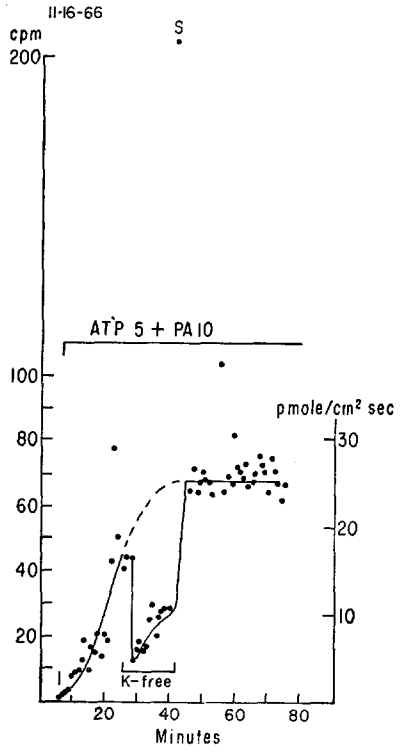

FIGURE 6. Na efflux is plotted as a function of time for an axon dialyzed with fuel (ATP 5 mM + PA 10 mM). The dialysis was started at 6 min and at 25 min the seawater surrounding the axon was changed to K-free seawater. The result was clearly a prompt drop in Na efflux. The change to K-free seawater was made when the Na efflux was only 65% of its ultimate value so the efflux in K-free seawater rises with time. A change to seawater at 42 min led to a prompt rise of Na efflux to its plateau. The dashed line shows the time course of Na efflux that might be expected if K-free conditions were not imposed.

average of four determinations of the K-free effect in three axons showed that Na efflux was reduced to 0.56 of control values, indicating that internal dialysis did not abolish the K-free effect, although it is probably smaller in magnitude than in injected axons tested early in an experiment.

The Effect of Na-Free Seawater on Na Efflux Except for Na-loaded axons (Mullins and Frumento, 1964), conditions have not been described in which the intact squid axons undergoes a Na-Na exchange but it was plainly important to discover whether dialyzed axons retained a Na efflux that was independent of the presence of Na^+ in the bathing seawater. Such experiments as we made were usually done by applying seawater in which all the

Na except 2 mM was replaced by Li and the response of the efflux of ^{22}Na noted. Measurements on axons that remained in satisfactory condition throughout the experiment showed that the substitution of Li for Na in seawater had no influence on Na efflux as shown by Fig. 3 and the data in Table V. Some axons (such as that shown earlier as 101866) showed a 10–30% decrease in Na efflux when Li was applied but were in good condition as judged both by their bioelectric behavior and by a demonstration that Na efflux could be reduced to low levels when no-fuel conditions were imposed on the inside of the axon.

DISCUSSION

The dependence of the normal sodium efflux in squid axons upon metabolic energy has been well-established by two general approaches. Either the production of energy was inhibited by inhibitors of aerobic metabolism such as cyanide or the utilization of energy for sodium transport was prevented by one of the cardiac glycosides. Such procedures usually reduced the sodium efflux to 10–20 pmole/cm^2 sec, and led to the conclusion that ATP is the major, but perhaps not the only, source of energy for sodium extrusion. The residual efflux of sodium was still several times greater than that predicted by the Ussing flux ratio equation. Possible explanations for the discrepancy are: incomplete inhibition of ATP production, other energy sources, or exchange diffusion. The dialysis technique afforded a way of discriminating between these explanations since substrates could be supplied separately to the axon. When axons were perfused with ATP-free media, the efflux was 1.3 pmole/cm^2 sec. Since the influx in a similar series of perfused axons was 57 pmole/cm^2 sec, the ratio of efflux/influx is 0.023. This agrees closely with the flux ratio calculation of 0.017, assuming the membrane potential of our axons was —60 mv. The ratio of no fuel/fuel sodium efflux in dialyzed axons can also be calculated as an independent experimental test of the flux ratio equation, on the assumption that the observed sodium efflux was equal, or nearly so, to the influx in these dialyzed axons. We have, of course, no evidence that the sodium extrusion process was operating maximally during the experiments in which we measured efflux; the results, however, 0.045 (ratio calculated from data on the same axon) and 0.033 (ratio calculated from pooled efflux data) are in close agreement with the theoretical value.

As can be seen from Table VI, we have been unable to reduce the ATP concentration in the effluent dialysis media from no-fuel axons below about 1.5 μM. Although this value is only 0.034% of the normal ATP concentration in our axons (4.4 mM), it might explain the fact that the observed ratios are all slightly larger than the theoretical. In considering this explanation, it may be noted that the absolute value of the sodium efflux calculated from the flux ratio equation, 1.0 pmole/cm^2 sec, is only 0.3 pmole/cm^2 sec less than

the observed fuel-free efflux. The actual amount of sodium efflux required to produce the discrepancy in the flux ratios is therefore extremely small. The conclusion drawn from these experiments is that in the absence of ATP, the observed sodium efflux agrees quantitatively with the flux ratio prediction. There is no evidence of exchange diffusion under these circumstances.

Although the experiments described in this study indicated that ATP was sufficient to produce a large sodium efflux, they did not exclude the possibility that phosphoarginine may normally provide energy for sodium transport. A critical test of the effects of PA was not possible until ATP and ADP were removed from the axoplasm. This is true because, in the case of ATP, a rather large sodium efflux can result from a small [ATP]. The removal of ADP is also a requirement because the high activity of arginine phosphokinase in axoplasm will lead to ATP formation by the reaction of PA with ADP. The procedures which we used to remove ADP from the axoplasm have already been described in the Methods section. Although we did not directly determine ADP removal from the axoplasm during the "predialysis," we infer that any free ADP was in fact removed; our control experiments (Brinley and Mullins, 1967) indicate that the porous capillary removed substances up to at least 1000 molecular weight with a half-time of 2–5 min. The results of these experiments clearly indicate that dialysis with 10 mM PA (which is about twice the normal axoplasm concentration) under conditions where phosphorylation of ADP is minimal, produces only about 3–4 pmole/cm^2 sec of sodium efflux, and even this is associated with the appearance of ATP at a concentration of about 12 μM in the effluent dialysis fluid. We conclude therefore that PA is not a direct energy source for the sodium extrusion.

Fresh, intact, isolated cephalopod axons show characteristic changes in sodium efflux when exposed to sodium-free solutions. The sodium efflux is increased about 20–50% in *Sepia* (Hodgkin and Keynes, 1955). In squid axons there may be some species variation. Caldwell and Keynes, using *Loligo forbesi* (Keynes, 1965, Fig. 9), found an increase of efflux in sodium-free solutions which was about the same as that found in *Sepia*. On the other hand, Sjodin and Beauge (1967), using *Lologio pealei*, found essentially no effect of Na-free solutions on sodium efflux. Despite the quantitative differences found by different laboratories, what is clear from published data is that lithium solutions do not reduce the sodium efflux from normal squid axons.

Whatever the ultimate explanation for the sodium-free effect in intact axons, it appears that squid axons dialyzed with high energy phosphate media resemble intact axons in the response to lithium seawater.

This seemed a point worth establishing since Caldwell et al. (1960 *b*) have shown that microinjection of ATP into axons previously poisoned with cya-

nide produced a sodium efflux nearly normal in magnitude, but which was reduced substantially when lithium was substituted for sodium in the external solution.

Potassium-free external solutions reversibly reduce the sodium efflux from cephalopod axons. The ratio $(\text{Na efflux})_{\text{K-free}}/(\text{Na efflux})_{\text{SW}}$ is about 0.30 for *Sepia* (Hodgkin and Keynes, 1955) and about 0.35 for *Loligo forbesi* (Caldwell et al., 1960 *a*) and 0.41 for *Loligo pealei* (Sjodin and Beauge, 1967). The effect is apparently rather labile since it tends to become less marked after axons have been isolated for many hours, although the size of the sodium efflux is little changed. It is barely detectable in cyanide-poisoned axons whose Na efflux has been restored by large injections of ATP.

The present results indicate that at least some potassium sensitivity persists during dialysis since mean ratio of efflux in K-free to efflux in normal seawater was 0.56 in four determinations. In the injected axons studied by Caldwell et al. (1960 *a*) some potassium-sensitive sodium efflux was induced by injections of PA, although the concentrations of PA required to do so were between two and eight times the amount of PA present in unpoisoned fibers. These authors considered two possible explanations for this induced sensitivity: a direct effect of phosphoarginine on the transport mechanism, or the production of a high ATP/ADP ratio in the axoplasm resulting from the rapid resynthesis of ATP from ADP. The second explanation was favored, but the first could not be excluded since the microinjection experiments afforded no convenient way of controlling the ATP/ADP ratio without the simultaneous presence of a phosphorylating agent such as PA or PEP. The dialysis experiments, which afford a means of controlling the ATP/ADP ratio without using PA, suggest that PA is not absolutely essential. In two experiments, 111566-2 and 112266-1, a significant, though small, K-free effect was seen during perfusion with 5 mM ATP plus 0.5 mM ADP. Theoretical calculations based on the diffusion of ATP from the capillary and its consumption in the axoplasm (Brinley and Mullins, 1967) indicate that the ATP/ADP ratio at the axolemma was between 4 and 10, with an absolute ATP concentration of about 4.0–4.5 mM during these experiments.

ATP Consumption and Na Extrusion

It should be noted that the method used to supply ATP to the Na extrusion mechanism in these experiments differs greatly from the manner by which the major fraction of the ATP is supplied physiologically. In the intact axon, ATP is presumably generated by mitochondria located close to the source of utilization of ATP; if we consider consumption of ATP at the axon membrane, then it is mitochondria located in the periphery of the axoplasm which take up the ADP produced by the hydrolysis of ATP by the membrane enzyme and regenerate it to ATP. The advantages of this local generation and

consumption system are that the [ADP] is kept low and the consumption of ATP is minimal. When ATP is supplied from a dialysis tube in the center of the axon, it must diffuse 150 μ to reach the membrane. In the course of this diffusion, ATP is subject to many side reactions unrelated to its action at the membrane. This axoplasmic ATP consumption reduces the [ATP] ultimately reaching the membrane and because it is necessary to poison the mitochondria with CN in order to control ATP generation, the result is that the [ADP] and [PO_4] concentrations may be expected to be higher than in the physiological situation. Since the free energy available from the reaction, ATP $\rightleftarrows$ ADP + P_i, is decreased as the concentrations of the products are increased, the method we have used to fuel Na extrusion cannot be expected to be as efficient as when mitochondria generate ATP. The use of phosphagen, in the form of phosphoarginine, enables one to overcome part of the difficulty to be expected when substrates are furnished by dialysis. This is so because PA is not subject to side reactions of the sort that affect ATP. Instead, it reacts with ADP, and effectively removes this product. The phosphate produced by ATP hydrolysis, however, is not removed by the action of PA on ADP so that the free energy available is not as great as it would be if there were a system to consume the phosphate produced.

These considerations are relevant to measurements of ATP consumption by squid axons because it is not clear that ATP consumption in axons supplied with ATP by dialysis can be expected to be the same as that in the normal axon in which most of the ATP is supplied to the membrane by mitochondrial oxidative phosphorylation. In the calculations that follow, all parameters are scaled to a temperature of 14°C using a temperature coefficient (Q_{10}) of 2.2. It is further assumed that the axoplasmic ATPase has an activity (μmole/g min) that is linearly dependent on [ATP]. If we examine the case of the intact axons first, Connelly (1952) and Connelly and Cranefield (1953) have measured a mean O_2 consumption for intact squid axons of 70 μl/g hr at 16°C. This value can be converted to ATP production, if a P/O_2 ratio of 6 is assumed; this is 0.31 μmole/g min at 16°C or 0.26 at our standard temperature of 14°C. Our mean activity of axoplasmic ATPase (Table III) is 0.24 μmole/g min at 25°C and 5 mM [ATP] while the mean axoplasmic [ATP] is 4.4 so that rescaling to 14°C and 4.4 mM yields an ATPase activity of 0.09 μmole/g min. The net ATP available for consumption by the membrane is, therefore, the difference between ATP production (0.26) and axoplasmic hydrolysis (0.09) or 0.17 μmole/g min.

For the dialyzed axon, our mean ATP consumption measured while Na extrusion was taking place (Table IV) is 0.048 μmole/g min per mM [ATP] at 14°C. Diffusion calculations indicate that for a substrate that is consumed (such as ATP), the steady-state concentration in axoplasm will be (at 14°C) 0.77 that of the supplied concentration so that to scale our consumption to

an [ATP] of 4.4 mM in the dialysis fluid involves an axoplasmic concentration of (0.77 × 4.4) or 3.4 mM. We take, therefore, 0.048 (Table IV) μmole ATP consumed/g min [ATP] and multiply by 3.4 mM and obtain 0.16 μmole/g min for ATP consumption by axons under internal dialysis. For axoplasmic ATP consumption, we take the value obtained in the paragraph above (0.09 μmole/g min at 14°C and 4.4 mM) and rescale to the concentration given above by multiplying by 0.77 or a value for ATPase of 0.07 μmole/g min. The difference between the total ATP consumption measured in vivo and the axoplasmic ATP consumption is (0.16–0.07) or 0.09 μmole/g min.

The values obtained from the calculations given in the two previous paragraphs are listed below:

	μmole/g min
a. ATP generated in intact axons (from O_2 consumption measurements)	0.26
b. ATP consumed by axoplasmic ATPase (from in vitro measurements)	0.09
c. ATP available to membrane (*a* − *b*)	0.17
d. ATP consumption measured in dialyzed axons extruding Na	0.16
e. ATP consumed by axoplasmic ATPase (from in vitro measurements)	0.07
f. ATP available to membrane (*d* − *e*)	0.09

The value listed in line *f* can be transformed to a surface consumption in a 500 μ axon and is then 17 pmole ATP/cm² sec. This can be compared with the simultaneously measured Na efflux (Table IV, column 9) of 51 pmole/cm² sec and gives a Na/~P of 3. For intact axons, the value listed in line *c* similarly can be transformed to a surface ATP consumption of 32 pmole/cm² sec. The foregoing calculation is admittedly arbitrary; values as low as 1.5 for a Na/~P ratio can be calculated using somewhat different methods of treating the data.

Baker and Shaw (1965) have measured the rate of release of P_i after the onset of CN treatment in whole axons and in axoplasm and have obtained the following values (scaled to 14°C): P_i release by whole axon 0.25 μmole/g min and P_i release by axoplasm 0.17 μmole/g min. If P_i release is related directly to ATP consumption, the difference between these values (0.08 μmole/g min) is very similar to the one we obtained with dialyzed axons (0.09), but the individual values are rather different. Their value for ATP consumption by whole axon is in excellent agreement with the results from O_2 consumption (line *a*) but the value for ATP hydrolysis by axoplasm is much higher than that given in line *b*. The general conclusion of Baker and Shaw is the same as ours with respect to there being a Na/~P ratio of about 2–3; however, it should be noted that the method used by Baker and Shaw

to estimate ATP consumption in the axon periphery inevitably resulted in including ATP hydrolysis by the Schwann cells (two membranes) in addition to the axon membrane. There is no information on which to base an estimate of the relative contributions of these three membranes; on the assumption that they all share equally in ATP hydrolysis, Na/~P ratios as high as 9 could be calculated.

The estimation of ATP consumption by the axoplasm is a critical measurement for the calculation of ATP utilization in Na extrusion. We have considered the following possible sources of error in our estimates of axoplasmic ATPase: that the ionic strength, pH, or activating ion requirements of the enzyme were not met in the assay system that we used. The nonspecific ATPase does not have an activating ion requirement (Bergmeyer, 1965) and the pH for the assay was 7.5 which, while less than the pH optimum for the enzyme, is reasonably close to the pH of axoplasm. The assay was usually made with a total salt (as KCl) concentration of about 150 mM; however, in one measurement, 500 mM K isethionate was used without any great difference in activity. Mitochondria in the presence of CN may have an ATPase activity, but we did not observe a difference between whole axoplasm and mitochondria-free axoplasm.

While Na/~P ratios may be of interest in connection with hypotheses about pump stoichiometry, the actual free energy available from ATP hydrolysis and its relationship to Na extruded is probably a more important consideration at present. Unfortunately, the evaluation of this free energy depends upon having reliable estimates of the concentrations of the following substances at the inside of the membrane: ATP, ADP, and HPO_4. Because it is almost certain that substances such as ADP are not uniformly distributed between the particulate matter and the homogeneous phase of the axoplasm, analyses of whole axoplasm for ADP are unlikely to yield satisfactory data for estimates of the free energy available for pumping.

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