Sodium Fluxes in Internally Dialyzed Squid Axons

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

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

ABSTRACT   The effects which alterations in the concentrations of internal sodium and high energy phosphate compounds had on the sodium influx and efflux of internally dialyzed squid axons were examined. Nine naturally occurring high energy phosphate compounds were ineffective in supporting significant sodium extrusion. These compounds were: AcP, PEP, G-3-P, ADP, AMP, GTP, CTP, PA, and UTP.[1] The compound d-ATP supported 25–50% of the normal sodium extrusion, while ATP supported 80–100%. The relation between internal ATP and sodium efflux was nonlinear, rising most steeply in the range 1 to 10 μM and more gradually in the range 10 to 10,000 μM. There was no evidence of saturation of efflux even at internal ATP concentrations of 10,000 μM. The relation between internal sodium and sodium efflux was linear in the range 2 to 240 mM. The presence of external strophanthidin (10 μM) changed the sodium efflux to about 8–12 pmoles/cm² sec regardless of the initial level of efflux; this changed level was not altered by subsequent dialysis with large concentrations of ATP. Sodium influx was reduced about 50% by removal of either ATP or Na and about 70% by removing both ATP and Na from inside the axon.

INTRODUCTION

Previous studies on squid giant axons subjected to internal solute control by dialysis have shown that the sodium fluxes across the membrane were approximately normal in magnitude and that the efflux of sodium depended upon internal ATP. The preparation also exhibited many of the other properties known to be characteristic of the sodium efflux in squid axons.

The experiments to be described were undertaken in order to characterize

---

[1] The following abbreviations are used in this paper: AcP, acetyl phosphate; ADP, adenosine-5'-diphosphate; AMP, adenosine-5'-monophosphoric acid; ATP, adenosine-5'-triphosphate; CTP, cytidine-5'-triphosphate; d-ATP, 2'-deoxyadenosine-5'-triphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-N,N'-tetraacetic acid; GTP, guanosine-5'-triphosphate; G-3-P, D-glyceraldehyde-3-phosphate; PA, phosphoarginine; PEP, phospho(enol) pyruvate; TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; UTP, uridine-5'-triphosphate.

181

the relation between internal ATP and sodium efflux, and to determine the extent to which some other high energy phosphate compounds of common biological occurrence can support sodium extrusion. Because strophanthidin reduces the Na efflux of squid axons (apparently by interfering with the ability of the membrane to hydrolyze ATP) measurements of Na efflux were made under conditions where $[ATP]_i$ was varied over wide limits, and the response of the efflux to this inhibitor noted.

TABLE I

COMPOSITION AND IDENTIFICATION OF SOLUTIONS USED*

Ion	ASW‡	Li-ASW	A'	E'	F	G	H	M	N	O	O'
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
Na^+	429		80	80		80		231		80	80
Li^+		429							80		
K^+	9	9	304	305	385	303	383	153	304	304	304
Mg^{++}	48	48	4	4	4	4	4	4	4	4	4
Ca^{++}	9	9									
Cl^-	496	496	88	88	88	88	88	88	88	88	88
Isethionate⁻			151	151	151	151	151	151	151	151	151
L-Aspartate⁻			151	151	151	151	151	151	151		
D-Aspartate⁻										151	151
CN^-			2	2	2						
SO_4^-	25	25									
EGTA⁼											0.1
EDTA⁼	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
TES⁻	4	4		1	1	1	1	2	2	2	2
Taurine			275	275	275	275	275	275	275	275	275

* pH of external solutions 7.5, of internal solutions 6.9-7.1.
‡ CN-ASW formulated by addition of 1-4 mM NaCN, buffered to pH 7.5.

A second phase of the work was concerned with measurements of sodium influx because recent experiments on erythrocytes (Garrahan and Glynn, 1967 a, b, c, d, e) indicate that under proper conditions, it is possible to demonstrate a component of the sodium influx that appears to be a one-to-one exchange of internal for external sodium ions. In the present work experiments were done to characterize the sodium influx in dialyzed squid axons and to determine the extent to which observed alterations in influx could be ascribed either to the presence of $[ATP]_i$ and/or $[Na]_i$.

METHODS

The experiments reported here were performed upon live specimens of Loligo pealei, during the period January, 1967 to January, 1968. Animals were obtained either at the Marine Biological Laboratory, Woods Hole, Massachusetts, or from Ocean City, Maryland. The apparatus and procedures used to dialyze isolated squid axons have been extensively described previously (Brinley and Mullins, 1967; Mullins and Brinley, 1967). Only the salient modifications will be mentioned here.

Dialysis Capillaries The porous glass capillaries used in the present studies had the same outside diameter as those used in earlier studies, but the wall thickness was about $\frac{1}{2}$ to $\frac{1}{3}$ as great. The porosity of these capillaries as supplied by the manufacturer (Corning Glass Co., Corning, N. Y.) was considerably greater than that of earlier lots and could be easily increased to levels required for the present purposes simply by soaking the capillaries in 70 mM Na-EDTA (pH 6.80) for 5–50 hr at room temperature. Capillaries thus treated proved to have a permeability to ATP, phenol red, and a test protein (hemoglobin) comparable to that of the earlier capillaries, but the water filtered at the standard flow rate (1λ/min) was about 10 % of that reported previously because of the much lower pressure drop in the porous region resulting from the larger lumen. The simplified preparative procedure yielded capillaries with greater mechanical strength which were less prone to break. Capillaries were stored in distilled water containing 20 % ethanol to retard bacterial growth on the porous surface.

Solutions The solutions used in this study are given in Table I. The external solutions are conventional. A large variety of internal solutions were used, reflecting a continuing search for a more suitable medium. The purpose for which each of the dialysis media was used is indicated below.

Strophanthidin seawater was prepared by dissolving an appropriate amount of strophanthidin in artificial seawater. No alcohol was used as a solvent.

Standard Media Solutions A', G, G', O, O' The rationale behind the inclusion of the major ingredients has been previously discussed (Brinley and Mullins, 1967). Minor modifications in the present series involved replacement of L-aspartate by its biologically inactive D-isomer, addition of EGTA or EDTA to chelate traces of Ca that might be present, and addition of TES as a buffering agent.

Influx Experiments, with Altered $[Na]_i \cdot E$, F, F'

Efflux Experiments with Altered $[Na]_i \cdot H,M,N'$ Solutions with sodium concentrations intermediate between those listed were prepared by mixing appropriate amounts of the stock solutions.

High Energy Phosphate Compounds The barium salt of phosphoarginine was obtained as a gift from Professor J. F. Morrison. All other compounds were obtained from Sigma Chemical Co. and stored at $-90°C$. Concentrated stock solutions were made up as either Na or K salts buffered to pH 7.0 and stored at $-90°C$. Deterioration of ATP and PA under these conditions was less than 2–3 % per month. ATP- and/or PA-containing solutions were made up by appropriate additions of the stock solutions to aliquots of dialysis media. The magnesium concentration of the dialysis media was always 4 mM in excess of the di- or triphosphonucleotide concentration.

Biochemical Procedures Acetyl phosphate was assayed by a chemical procedure as described by Lipmann and Tuttle (1945). (See also Stadtman, 1957.)

ATP analyses were done by the firefly flash method as previously modified and described by us (Mullins and Brinley, 1967). Although this method had the requisite sensitivity for analysis of picomole amounts of ATP in samples of dialysate, the presence of nonspecific transphosphorylases in the commercial luciferin-luciferase

extract made it impossible to assay for ATP in the presence of any large amount of other triphosphonucleotides. Accordingly the commercial extract was put through the purification procedure described by Strehler (see Bergmeyer, p. 572, Method b). This procedure enhanced the selectivity for ATP in comparison with d-ATP by a factor of 10–20. A representative analysis using the purified material is shown in the Results section where experiments involving d-ATP are presented.

TABLE II

ENERGY SOURCES FOR SODIUM EXTRUSION

Compound	No. of exper- iments	Physiological concentration	Test concentration	Na extrusion % of normal
		mM	mM	
ATP*	9	2–5	5	80–100
PA	3	3	10	<2
PEP	2		2.5	<2
AcP	3		2–3	<2
G-3-P	2		5	<5
ADP }*	2	0.05–0.1	0.05–1	<5–10
AMP }		<0.05	20	
UTP*	1		~2	<5
CTP*	1		~2	<5
GTP*	2		1.4	<5–10
d-ATP*	2		0.1–5	~25–50

* $[Mg]_i$ set at 4 mM in excess of ($\sim P_i$)

RESULTS

The Influence of High Energy Phosphates Other Than ATP on Sodium Extrusion

Previous studies (Mullins and Brinley, 1967) have shown that ATP alone was capable of serving as a substrate for the operation of the Na pump of the squid axons and that the efflux observed was comparable to values found in intact or injected axons. A further finding was that phosphoraginine (PA) did not energize more than a trivial amount of Na extrusion and that such extrusion as was observed was correlated with the appearance of an $[ATP]_i$ of the order of 10 μM in the dialysis effluent. Such a finding, while ruling out PA as a substrate, gives no information about the possibility that other compounds might be able to serve as substrates for Na extrusion. The experiments to be described below were done first, to examine the extent to which the structure of the ATP molecule itself can be modified and still retain substrate activity, and second to examine other high energy phosphate compounds not related to ATP which we had reason to think might have some ability to fuel Na extrusion.

The method for testing all the compounds was essentially the same and can

be summarized by the following. Squid axons were subjected to extensive "predialysis" of both the end regions and the center, using a dialysis fluid free of high energy phosphate compounds (Mullins and Brinley, 1967). This procedure suffices to remove substantially all ATP. Typical analyses of the dialysis effluent showed an [ATP] of 1–5 μM. The procedure also removes ADP and
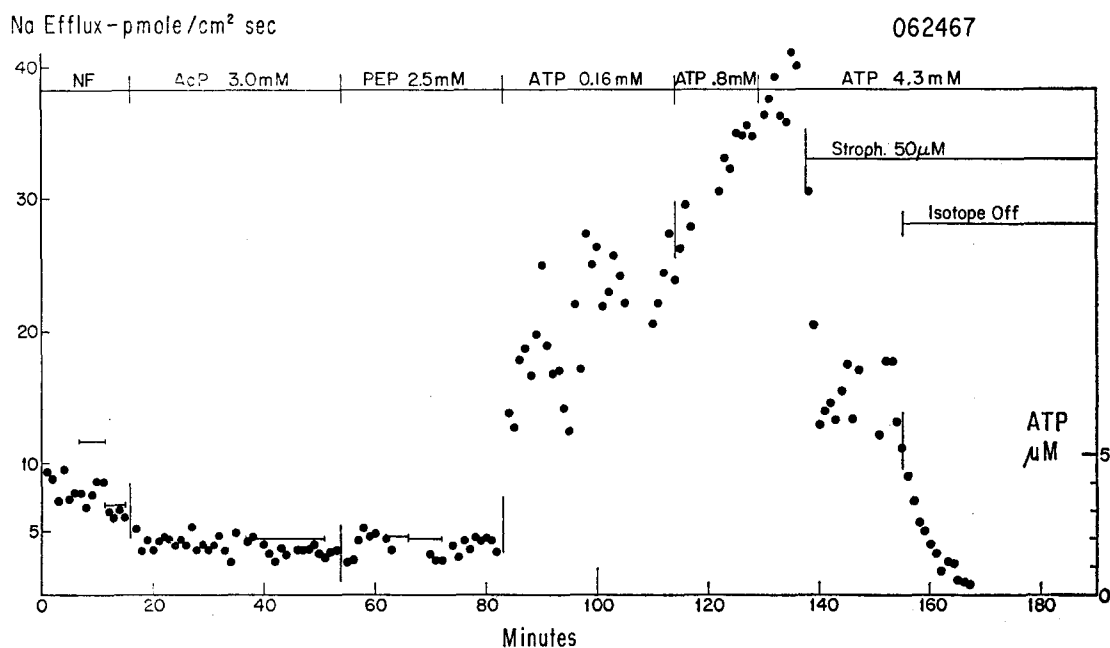
Na Efflux-pmole/cm^2 sec   062467

FIGURE 1. Na efflux as a function of time and showing the effects of AcP, PEP, ATP, and strophanthidin. The labels at the top of the drawing indicate concentrations of high energy phosphates supplied. The initial dialysis medium contained no fuel (NF) and the external medium was seawater. ATP concentrations in the effluent dialysate are indicated by the horizontal bars and refer to the right-hand ordinate. The predialysis with no fuel medium did not reduce the [ATP]$_i$ as much as usual. The initial efflux was therefore higher than usual but dropped as the ATP concentration fell. [Na] in dialysate was 80 mM.

other substances of molecular weight less than 1000 not present in the dialysis medium; there is some evidence that pretreatment of the axon with CN seawater is helpful in reducing [ATP]. Following the predialysis, the medium was switched to one containing ^{22}Na and a "no fuel" base line obtained. Finally, a ^{22}Na medium containing the desired concentration of test substrate was applied. The experiment was usually concluded with a brief application of ATP as a check on the performance of the Na pump. The results of experiments on eleven high energy phosphate compounds are summarized in Table II and described in detail in the following section.

ACETYL PHOSPHATE   Recent reports (Bond et al., 1966; Bader and Sen, 1966) have shown that AcP is capable of forming a phosphorylated intermediate with membrane ATPase, presumably in the same manner as ATP. It seemed important, therefore, to compare the action of AcP on Na efflux with that of ATP. Fig. 1 shows the result of an experiment in which the test dialysis

TABLE III

EFFECT OF EXTERNAL STROPHANTHIDIN

(10–15 μM) ON SODIUM EFFLUX

Axon reference	Axon diameter	Temperature	Dialysis solution	$[ATP]_i$*	$[PA]_i$‡	$[Na]_i$	m^o_{Na}	Strophanthidin insensitive	Inhibited / Control
	μ	°C		μM	μM	mM		p/cs	
120765	617	10	A'	4000	10,000	89	22	11	0.50
062467	625	15	G	4300‡	5000	80	38	15	0.39
071467-2	500	17	G	4300‡	5000	80	28	8	0.28
080467-2	550	~17	H,M,N	5000‡	5000	10	3	1	0.33
100567-1	500	16	O	50	5000	80	25	15	0.60
				5000‡				17	
101767-1	605	16	O'	25	500	80	17	10	0.60
101767-2	625	16	O'	500	500	80	21	12	0.60
110767-1	575	16	O'	28	5000	80	20	8	0.44
110767-2	500	16	O'	35	5000	80	10	10	1.0
110867 ·	475	15	O'	20	5000	80	13	8	0.61
				5000‡	5000			8	
121967-2	625	~16	O'	57	5000	80	27	16	0.59
				5000‡	5000			21	
122067-1	620	~17	O'	4	5000	80		~8	
				4000‡	5000			11	
122067-2	575	17	O'	22	5000	80	12	10	0.83
				4000				9	
122067-3	600	15	O'	3		80	3	11	3.9
				5000‡	5000			13	

* $[Mg]_i$ set at 4 mM in excess of $[ATP]_i$.

‡ Indicates a nominal concentration not verified by analysis of effluent dialysis fluids. All PA values are nominal.

medium contained 3 mM AcP. Additional details are given in Table III. The Na efflux during dialysis with this solution is actually lower than the base line efflux but this is probably the result of a declining [ATP] in the dialysis effluent as shown by the horizontal bars on the graph and the right-hand ordinate. The axon was capable of giving a normal response to fuel as demonstrated by the fact that a change to an internal dialysis medium of 160 μM ATP produced a large rise in Na efflux. Furthermore the Na efflux energized by ATP showed an appropriate sensitivity to externally applied strophanthidin. In all, three axons were tested for a response of the Na efflux to 2–3 mM AcP, but none was seen. Because of the low levels of radioactivity collected, an incre-

ment of efflux as large as about 2% of the normal efflux could have escaped attention.

Chemical assay (see Methods) of the material used showed that it was at least 85% AcP, and that 5 mM solutions of AcP (in standard dialysis media) showed a decomposition of less than 1%/hr when allowed to stand at room temperature for several hours (i.e. for periods far in excess of those of the experiments). Determination of the rate of hydrolysis of AcP in the presence of extruded axoplasm indicated that the rate was not significantly greater than the spontaneous breakdown. The negative results observed during dialysis therefore cannot be ascribed to very rapid hydrolysis of AcP during radial diffusion from the porous capillary to the axolemma. The AcP was a dilithium

102567-I

< No Fuel >| |< G-3-P >| |< d-ATP 0.32 mM

Na Efflux
pmole/cm² sec
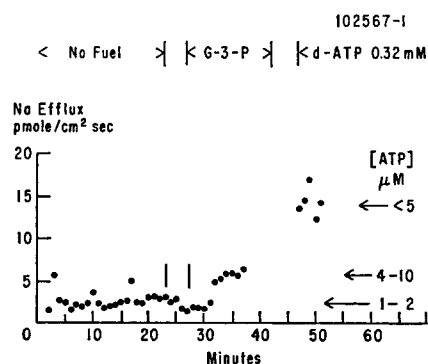
FIGURE 2. The effect of D-glyceraldehyde-3-phosphate (5 mM) and d-ATP on sodium efflux as a function of time. The horizontal arrows indicate the ATP concentrations measured in the effluent dialysate during perfusion with the various solutions.

salt; however, this small amount of lithium (4–6 mM) should not have affected the results, since in another experiment the extrusion process was not inhibited by the presence of far larger amounts of lithium (see below).

PHOSPHO(ENOL)PYRUVATE AND GLYCERALDEHYDE-3-PHOSPHATE These two intermediates of glycolysis might be presumed to occur normally, at least in small amounts, in intact squid axons and therefore might support some degree of Na extrusion. However, dialysis with millimolar concentrations failed to show any evidence of active Na transport (see Figs. 1 and 2). Actually the high energy compound, 1, 3-diphosphoglycerate (1, 3-DPG), is of greater interest than its precursor, glyceraldehyde-3-phosphate (G-3-P). This compound is not readily available commercially and therefore G-3-P + P_i was used in the dialysis medium instead. Hoskin (1966) has shown that isolated squid axoplasm can convert glucose to carbon dioxide via the glycolytic pathways, implying the existence of glycolytic enzymes, although glyceraldehyde-P-dehydrogenase was not specifically identified. Because the standard free energy of formation of 1, 3-DPG from G-3-P is + 1.5 kcal/mole (at pH 7.0), the amount of 1,3-DPG actually formed in the axoplasm under the conditions of the present experiments probably resulted in an intracellular

concentration of no more than a few micromolar at most. These results therefore do not provide direct information on the ability of 1,3-diphosphoglycerate to support sodium extrusion in the squid axon.

THE TRIPHOSPHATES OF URIDINE, CYTOSINE, AND GUANOSINE   Perhaps the most obvious modification of the ATP molecule is to replace the adenine ring with other purine or pyrimidine rings. One or two experiments were done with each of the compounds UTP, CTP, and GTP and the results are shown in Table II where the Na efflux that they produce may be compared with values
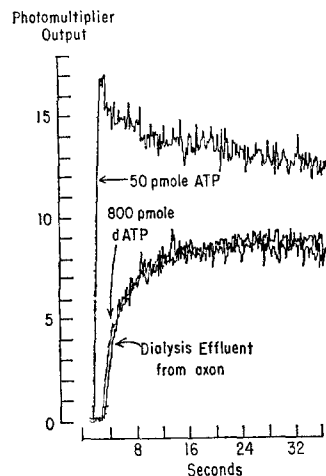
FIGURE 3. Recorder tracing showing photomultiplier output from the firefly flash reaction as a function of time for samples of dialysis media before and after passage through axon 102567-1 during dialysis with d-ATP (see Fig. 2). For comparison, the response of the system to about 6% as much ATP is also illustrated. The times of start of the firefly flash reaction have been slightly staggered to avoid complete superposition.

we have obtained with ATP. For each of these compounds there was at best a 5–10% increase in Na efflux during test dialysis. Even this small increase may be the result of ATP, however, since we could not analyze for [ATP] in the dialysis effluent. (The firefly flash assay for ATP in use at the time of these experiments could not discriminate between large excesses of XTP and ATP itself nor could we analyze the compounds used for traces of ATP present as a contaminant.) The conclusion from this study is that the Na pump has a specific requirement for an adenine ring.

ADP AND AMP   A second modification of the structure of ATP is to remove a phosphate group, thereby transforming the molecule into ADP. The interpretation of experiments using ADP as a substrate is complicated by the presence of adenylate kinase in axoplasm. This enzyme will carry out the following reaction: 2 ADP = ATP + AMP. The adenylate kinase reaction has been shown to occur in axoplasm (Caldwell et al., 1964; Mullins and Brinley, 1967); therefore appreciable amounts of ATP could be expected to be formed in axoplasm undergoing dialysis with ADP. Fortunately, the equilibrium constant for the reaction is about 0.5 (Eggleston and Hems, 1952) so that the

formation of ATP can be considerably reduced by incorporating in the dialy-
sis fluid rather large concentrations of AMP (20 mM) and correspondingly
smaller concentrations of ADP (0.05–1.0 mM). When equilibrium conditions
prevailed in axoplasm, the calculated [ATP] agreed rather well with re-
sults obtained by an analysis of the dialysis effluent. The observed increment
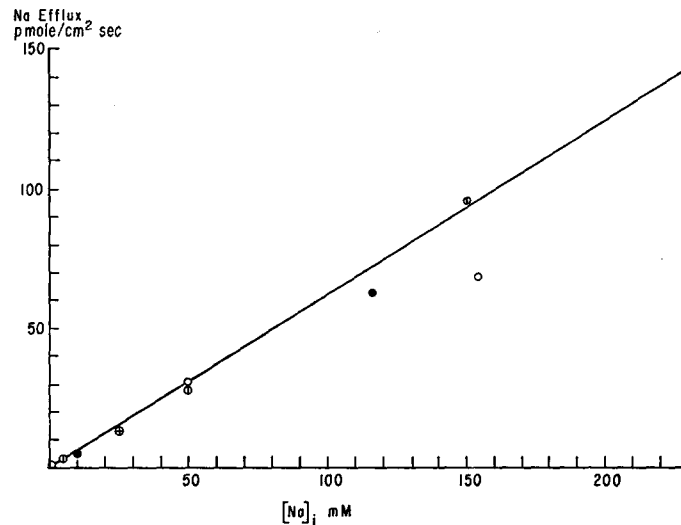in Na efflux also agreed well with that expected on the basis of the observed

FIGURE 4. Collected data from four experiments showing the relation between Na
efflux and [Na]$_i$. The [Na] in the effluent dialysate was not analyzed, but effluent anal-
ysis in similar situations indicated that at [Na]$_i$ less than 20 mM, the actual concentration
might exceed the nominal one by 1–2 mM. All experiments were done with a sequence of
increasing [Na]$_i$. At least 30 min of dialysis with low concentrations (less than 50 mM
[Na]$_i$) preceded the actual measurement. Dialysis solutions contained 5 mM ATP + 5
mM PA.

[ATP] but the scatter in the data could conceal a flux contribution from
(ADP + AMP) of as much as 5 pmoles/cm^2 sec or 10% of the total pump
efflux.

d-ATP   Another modification of the ATP molecule is possible by chang-
ing the sugar connecting the adenine ring with the triphosphate moiety. We
have used a deoxyribose (d-ATP) substitution because a report by Whittam
and Wiley (1967) suggested that the use of deoxyadenosine in intact red blood
cells allowed active K transport to proceed.

An experiment involving dialysis with d-ATP is shown in Fig. 2, where it
may be seen that a substantial increase in Na efflux occurs when this com-
pound is supplied. The possibility that the sample of commercially prepared
d-ATP was contaminated seemed unlikely but could not be dismissed; nor

could we exclude the possibility that the axoplasm was capable of converting some d-ATP into ATP. With a suitable purification of the firefly extract used for ATP analysis (see Methods) it was possible to analyze for 10–100 pmoles of ATP in the presence of a 10- to 100-fold excess of d-ATP. The result of a typical analysis upon the effluent dialysate obtained in the experiment of Fig. 2 is shown in Fig. 3. These records allow the comparison of a flash emitted by the entering perfusion solution with a comparable sample of the same perfusion solution after it had passed through the axon. A third trace shows the response of the analytical system when a sample containing about 5% as much pure ATP is injected. A generous estimate for the maximum possible ATP
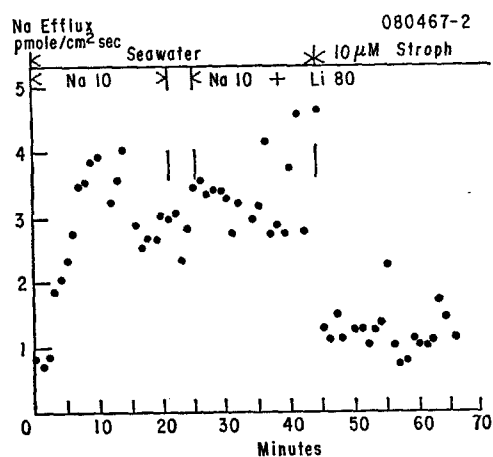
FIGURE 5. This experiment shows the effects of internal lithium on the Na efflux from an axon dialyzed with 10 mM [Na]$_i$. The Na efflux from the axon is also shown when seawater + 10 μM strophanthidin was applied. The legend at the top of the figure indicates the sequence of internal and external solution changes. The fuel in the dialysis medium was 5 mM ATP + 5 mM PA.

present in the effluent dialysate based on these data would be 1–2% of the d-ATP concentration, or 3–6 μM. Since the observed increment in Na efflux is far larger than could be attributed to an [ATP]$_i$ of this magnitude, the conclusion is that d-ATP itself was capable of operating the Na pump about 25–50% as well as ATP.

The Effect of [Na]$_i$ on Sodium Efflux

The dialysis technique affords a convenient way of adjusting the [Na]$_i$ to almost any desired value and thus allows one to study the dependence of Na efflux on [Na]$_i$.

The axons used for this study were fueled either with 1 mM each ATP + PA or with 5 mM each ATP + PA: the sodium concentration in the dialysis medium was adjusted to desired values by substituting Na for K in the solution. The largest value of [Na]$_i$ was 220 mM and this required a reduction in the [K]$_i$ in the medium from 300 to 160 mM. Alterations in the axoplasmic [K] of this magnitude do not have a large effect on membrane potential (Baker et al., 1962 a, b) and the axons recovered well after dialysis with 220 mM [Na]$_i$

as judged by the stability of efflux at lower $[Na]_i$ and by their bioelectric behavior. The results of four experiments are illustrated in Fig. 4, which shows that the Na efflux appears linear over a concentration range of 220 to 1 mM. No analyses of the effluent dialysate were done during this series of experiments so no definite statement can be made about the adequacy of the control of $[Na]_i$ at the very low levels. However, an analysis during an influx experi-
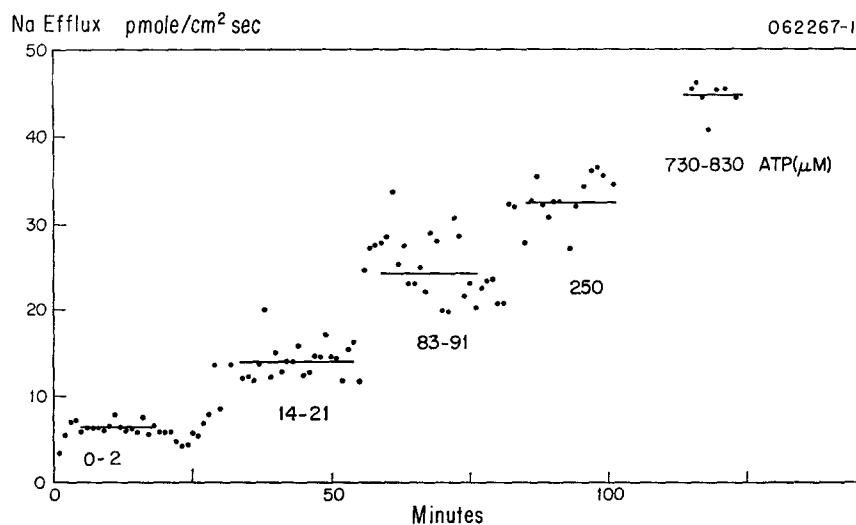

FIGURE 6. The effect of step increases in [ATP] on Na efflux in a dialyzed squid axon. Where a range of ATP values is given, the first value refers to the [ATP] in the fluid entering the dialysis capillary and the second to the [ATP] in the effluent dialysate. The 2 μM concentration was measured on the effluent dialysate during dialysis with fuel-free solution. The 250 μM concentration was determined on inflowing solution only. Sodium concentration in dialysate was 80 mM.

ment (see below) with nominally Na-free medium indicated an actual Na level in axoplasm of less than 1 mM. We infer therefore that a similar degree of control was effected during this series of experiments.

To determine whether $[Li]_i$ would affect Na efflux, an axon was first dialyzed with $[Na]_i = 10$ mM, and then a change was made to a medium of $[Na]_i = 10$ mM $+ [Li]_i = 80$ mM (by substituting Li for K in the internal dialysis fluid). Fig. 5 shows the results of this experiment. It is apparent that the Na efflux is unaltered in magnitude in the presence of eight times as much Li as Na, suggesting that $[Li]_i$ does not inhibit the Na extrusion mechanism.

The Effect of $[ATP]_i$ on Na Efflux

One useful piece of information that was not difficult to obtain by the internal dialysis technique was the relationship between internal [ATP] and Na efflux. Preliminary measurements had indicated that it would be necessary, in work-

ing out this relationship, to use [ATP] as low as 10 μM and it was also clear from axoplasmic consumption measurements that given the flow rates we used (about 1 λ/min) it would not be possible to supply sufficient ATP to the axoplasm under these conditions. A convenient way around this difficulty was to include PA in the dialysis medium. Other experiments have shown that this substance does not fuel Na extrusion but it can be expected to maintain the [ATP] at values only slightly different from those fixed by the experimenter.
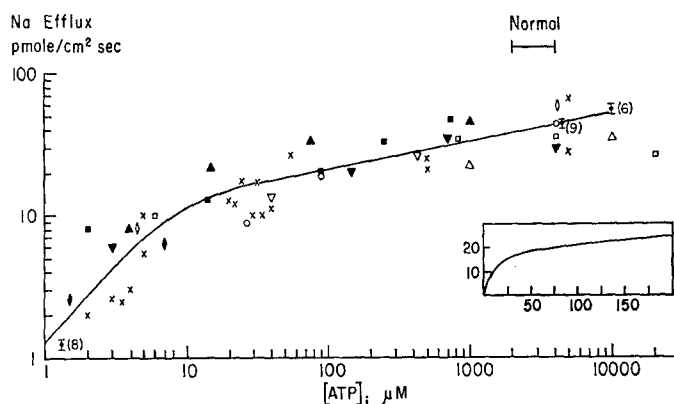

FIGURE 7. The relationship between [ATP]$_i$ and Na efflux in 47 dialyzed squid axons is shown on a log-log plot. The three points with vertical bars and numbers in parentheses represent previously published mean $\pm$ SD values from 20 axons. Crosses represent 18 single values from individual axons, while the other symbols refer to 9 axons in which more than one [ATP] was tested during the dialysis. A horizontal bar indicates the range of [ATP] in axoplasm extruded from freshly dissected axons taken from living squid. Insert shows data for the range from 0 to 200 μM ATP plotted on a linear scale. Data normalized to 15°C.

Our internal dialysis fluids contained, therefore, either 0.5 or 5.0 mM PA in addition to any ATP. The usual experiment used a well-predialyzed axon on which a fuel-free base line was obtained. A change to an ATP-containing fluid was then made and another base line obtained. In this manner, by a stepwise increase of [ATP] in the dialysis fluid, several values of Na efflux at different [ATP]$_i$ were obtained. The [ATP] in the dialysis fluid both entering and leaving the axon was analyzed to obtain the effective concentration, which was taken as the mean between these two values; in most cases the numbers agreed to within 25%. An experiment of this sort is shown in Fig. 6. The collected results from a number of axons are shown in Fig. 7. This curve has some unusual features: the Na efflux does not appear to saturate with increase in [ATP] although the slope is greatest over the range from 1 to 100 μM ATP, a range very much lower than physiological concentrations. A reservation about the efflux observed at high [ATP] is that because we used

the salt K_2MgATP, large concentrations involved the addition of substantial extra K^+ and $[K]_i$ was elevated 10–20% at the highest [ATP].

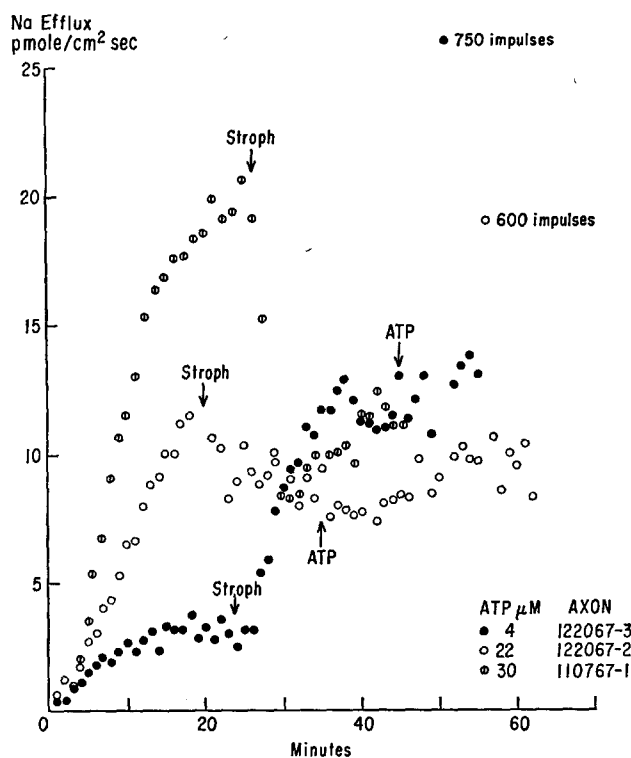In a few cases, at the end of the experiment the external seawater was re-

FIGURE 8. The effect of 10 μM strophanthidin seawater on the Na efflux from three dialyzed squid axons. Axons were dialyzed with fluids of varying [ATP] and the values given in the lower right of the figure were obtained by analysis of the dialysis effluent. The external solution was changed from artificial seawater to artificial seawater + strophanthidin at the times indicated by the arrows marked "stroph". In the experiments at 4 and 22 μM [ATP]$_i$, the perfusion solution was later changed to one containing 4000 μM ATP at the times indicated by the arrows marked "ATP". Near the end of these two experiments, the axons were stimulated at 25/sec to give the total number of impulses as indicated. The response to external strophanthidin of squid axons dialyzed with high concentrations of ATP is shown in Figs. 1 and 5.

placed with either lithium-containing or potassium-free seawater. Lithium seawater produced a mean reduction of efflux of about 10% in three dialyzed axons with internal ATP of 19–44 μM, which is only slightly larger than the average reduction of 5% seen in fully fueled axons. However, the reduction in sodium efflux resulting from removal of external potassium appeared to be less marked; only about 10–20% of the efflux was inhibited compared to about 50% in fully fueled axons. There was some doubt about the condition of the

axons when they were tested, since the tests were made at the end of a sequence of solution changes when the axons may have begun to deteriorate. The tentative conclusion drawn from these observations is that the efflux observed during dialysis with low [ATP]$_i$ is not simply Na:Na exchange, although it may not exhibit as much potassium sensitivity as efflux from fully fueled axons.
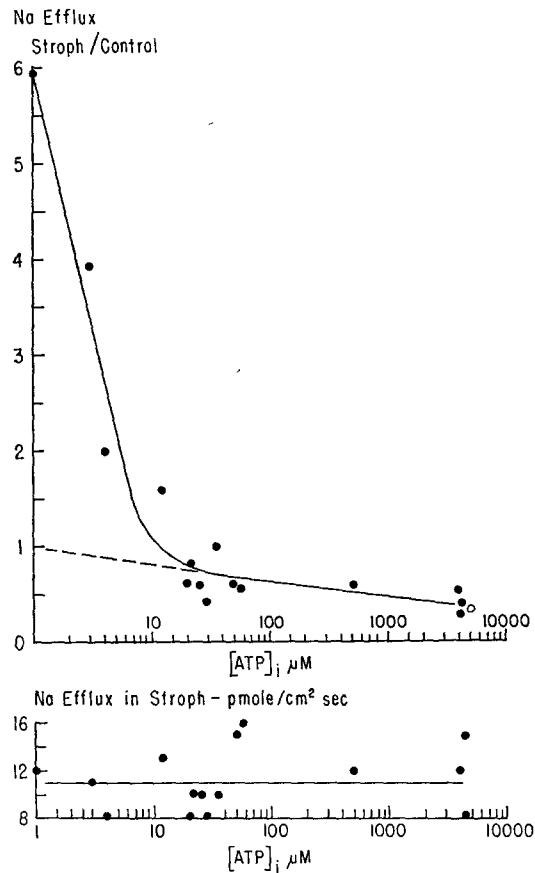

FIGURE 9. Effect of 10 μM strophanthidin seawater on sodium efflux from dialyzed squid axons as a function of [ATP]$_i$. The upper curve shows the ratio of Na efflux in the presence of strophanthidin to control efflux. The lower curve shows the absolute value of efflux in the presence of strophanthidin. Solid circles, [Na]$_i$ equal 80 or 89 mM. Open circle, [Na]$_i$ equals 10 mM.

The Effects of Strophanthidin and [ATP]$_i$ on Na Efflux

The cardiac glycoside, ouabain, and its aglycone, strophanthidin, have been extensively used as inhibitors both of Na extrusion in cells and of ATP hydrolysis by membrane ATPase preparations. Indeed, such inhibitors have been used to define active transport as that part of the Na efflux which is inhibited. As noted previously, about 10 pmoles/cm²sec of Na efflux appeared to remain in dialyzed or injected squid axons (Caldwell and Keynes, 1959; Brinley and Mullins, 1967) after treatment with cardiac glycoside in high concentration, while a removal of ATP from the axoplasm by dialysis resulted in virtually

complete inhibition of Na pumping (Mullins and Brinley, 1967). The nature of this glycoside-insensitive Na efflux was obscure and experiments were designed to study it further.

Since, as was shown above, Na efflux from squid axons is linearly related to $[Na]_i$, it was of interest to see whether the fraction of the Na efflux which is sensitive to strophanthidin would change if $[Na]_i$ were reduced. An experiment of this sort was made with an $[Na]_i$ in the dialysis fluid of 10 mM; the axon was fueled with 5 mM ATP + 5 mM PA. Fig. 5 shows the result of this experiment. The Na efflux was appropriate to this level of internal Na and upon the application of strophanthidin 10 μM, the efflux was reduced to one-third of
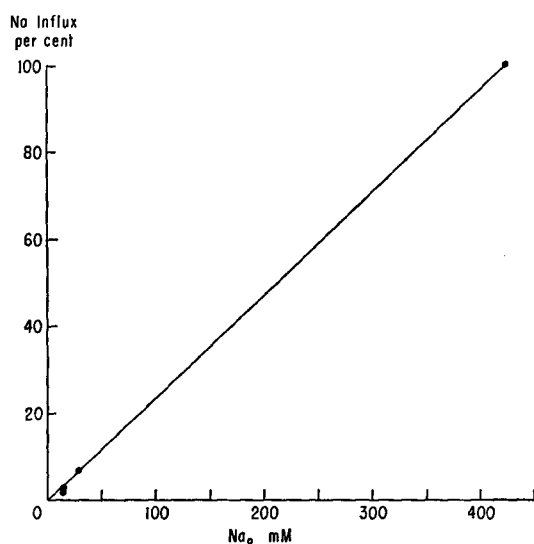
No Influx
per cent

Figure 10. Relation between Na influx and $[Na]_o$ in squid axons dialyzed with millimolar concentrations of ATP and PA. Na influx is plotted relative to influx in 430 mM Na artificial seawater. Collected results from three experiments.

its previous value. This is quite similar to the response of an axon fully fueled and with an internal Na of 80 mM (see the last part of Fig. 1). The conclusion from this experiment is, therefore, that strophanthidin is capable of making a large reduction in Na efflux even when $[Na]_i$ (and therefore Na efflux) is quite low.

A second variation of the experimental conditions is to keep the internal Na concentration at 80 mM and to lower $[ATP]_i$. An $[ATP]_i$ was selected that was much lower than physiological concentrations but nonetheless yielded a rather large efflux. A value of 30 μM ATP was chosen and the top curve in Fig. 8 shows the response of the Na efflux to strophanthidin under these conditions. This is the usual decline in Na efflux to a value of about 0.5 that of the control efflux, or a response similar to that seen in Fig. 5. A value for ATP of 22 μM was selected on the basis that it ought to produce a quite low Na efflux but one easily distinguished from the leakage efflux. An experiment with this concen-

tration of ATP is also shown in Fig. 8. Here, the result is quite different from that obtained with low internal Na in that the application of strophanthidin produced virtually no change in Na efflux, nor did a subsequent change in [ATP]$_i$ to 5 mM result in any increase in Na efflux. If instead of supplying an internal ATP concentration of 22 μM, the axon was well-washed and had a very low ATP concentration (as measured in the dialysis effluent) of 2–3 μM, then the efflux of Na which was initially about 2 pmoles/cm²sec, changed upon the application of strophanthidin to a value of about 10 pmoles/cm²sec. A convenient method of summarizing the effects of strophanthidin on Na
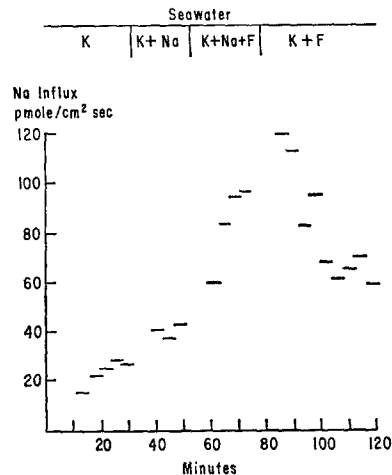
FIGURE 11. Effect on sodium influx of adding ATP or internal sodium to the dialysis medium. Axon was in artificial seawater throughout the experiment. "F" refers to "fuel" which was ATP 7 mM + PA 5 mM.

efflux is to note that it changes the Na efflux to a value of roughly 10 pmoles/cm²sec, no matter what the initial value of Na efflux might have been. A summary of the results with strophanthidin is given in Table III. The ratio: efflux in strophanthidin/control efflux has been plotted in Fig. 9 as a function of internal ATP.

The Effect of [Na]$_o$ on Sodium Influx

Previous work with dialyzed axons (Brinley and Mullins, 1967) suggested that the Na influx was about 1.5 times higher than the usual values given in the literature for injected axons, and was presumably an unavoidable concomitant of the technique itself. Since rather drastic alterations in Na efflux were made in the course of the experiments described above, it was clearly important to discover whether there were comparable alterations in Na influx.

Previous experiments (Mullins and Brinley, 1967) have shown that the replacement of Na in seawater by Li led to no change in Na efflux in fully fueled axons, suggesting that Na:Na exchange did not occur under these conditions.

One would conclude, therefore, that Na influx was purely passive and that the influx should be directly proportional to external sodium. Experiments to measure Na influx were made by dialyzing axons with a fluid containing 80 mM Na and 5 mM each of ATP and PA. The seawater outside the axon contained ^{24}Na and samples of the internal dialysis fluid were collected to measure the counts appearing in axoplasm. The seawater used had 430, 30, or 15 mM, Na and influx was evaluated at the highest $[Na]_i$ and at one lower $[Na]_i$. The collected results on Na influx as a function of $[Na]_o$ in 3 fueled axons are shown in Fig. 10; the linear relationship between Na influx and $[Na]_o$ is apparent. This implies a constant P_{Na} in a fueled axon independent of both $[Na]_o$ and $[Li]_o$. Intermediate $[Na]_o$ were not tested so it is possible that the apparent linearity of Na influx with $[Na]_o$ is fortuitous.

The Effect of $[Na]_i$ and $[ATP]_i$ on Sodium Influx

Since experiments with axons poisoned with cyanide and then injected with large amounts of ATP have shown (Caldwell et al., 1960) that Na influx is increased about 20% under these conditions, the proposition that metabolism may affect P_{Na} as well as the efflux of Na seemed an important one to examine.

Axons predialyzed with a medium free of both fuel and Na had a low Na influx from seawater containing a normal $[Na]_o$, as is shown in Fig. 11. A subsequent change of the internal dialysis fluid to one containing 80 mM Na led to a rise in Na influx, while the further addition of ATP 5 mM and PA 5 mM increased the Na influx still more. The effect of $[Na]_i$ is quite reversible, and the Na influx fell when internal Na was removed from the dialysis medium. A summary of the experiments done is given in Table IV from which it can be seen that internal ATP and internal Na act more or less independently of each other in increasing Na influx. Individual axons are likely to have considerable variation in their measured Na influx values so that for purposes of analysis it was better to compute for a particular axon the ratio of the change, and take the mean of these values. This has been done in columns 9–13 of Table IV and from the means it can be seen that the addition of Na to the internal dialysis medium in the absence of ATP increases Na influx to 1.33 that of the control (column 9). Column 12 gives the value for an equivalent change when ATP is present in the axon and here the Na influx increases to 1.5 its previous value so that it would appear that the inclusion of Na in the dialysis fluid has about the same effect whether or not ATP is present. If ATP alone is added (column 10), without any Na inside, Na influx increases to 1.7 times the control value, while if ATP is added in the presence of $[Na]_i$ the value is 2.0. Considering the relative inaccuracy of comparisons between influxes in different axons, the values 1.7 and 2.0 show a satisfactory agreement. The experimental findings can be summarized by saying that the addition of both ATP and Na to the inside of an axon increases the Na influx about 140% above the influx into

TABLE IV

INFLUENCE OF [Na]$_i$ AND [ATP]$_i$ ON SODIUM INFLUX*

Axon reference (1)	Axon diameter (2)	Temperature (3)	Dialysis solution (4)	Influx				Ratios				
				K‡ (5)	K+Na§ (6)	K+~P‖ (7)	K+Na+~P (8)	$\frac{K+Na}{K}$ (9)	$\frac{K+\sim P}{K}$ (10)	$\frac{K+Na+\sim P}{K}$ (11)	$\frac{K+Na+\sim P}{K+\sim P}$ (12)	$\frac{K+Na+\sim P}{K+Na}$ (13)
	μ	°C		*p-mole/cm² sec*								
080566-3	400	10				29	45				1.55	
020167-1	801	13	F′	24								
020167-2	718	~13	E′		37							
020167-3	701	~13	E′		37							
020267	567	11	E′		56							
032167-1	551	13	E′,F	30	40	65	110	1.33	2.15	3.51	1.63	2.64
032267	568	14	E′,F	33		42	56		1.27	1.70	1.34	1.27
042667	584	16	E′,F	21	33		42	1.57		2.00		
042767	685	15	E′,F	55	65			1.18				
051067	680	17	E′		40		83					2.08
					2.5¶		5.5¶					2.18¶
051167-1	835	15	E′		44		83					1.89
051167-2	768	15	E′		31							
					1.0**							
051167-3	780	16	E′				56					
					1.0**		1.4**					1.4**
Mean				33	43	45	71	1.33	1.71	2.40	1.50	1.97
±SD				±6	±3	±12	±12	±0.1		±0.5	±0.2	±0.2
No. of measurements				(5)	(9)	(3)	(7)	(3)	(2)	(3)	(3)	(4)

* [Mg]$_i$ set at 4 mM in excess of [ATP]$_i$.
‡ [K]$_i$ = 550 mM for 080566-3, 400 mM for all others.
§ [K]$_i$ = 440 mM, [Na]$_i$ = 110 mM for 080566-3; [K]$_i$ = 320 mM, [Na]$_i$ = 80 mM for all others.
‖ ~P = 10 mM ATP for 080566-3, 7 mM ATP + 5 mM PA for all others.
¶ [Na]$_o$ = 30 mM, [Li]$_o$ = 450 mM, omitted from mean.
** [Na]$_o$ = 15 mM, [Li]$_o$ = 450 mM, omitted from mean.

a dialysate containing only K. This change is made up of a 50% increase produced by Na alone and a 90% increase produced by ATP alone.

A puzzling result was the effect of ATP alone since one might imagine that it could not affect the membrane without the activating effect of some $[Na]_i$. The possibility existed that the large $[Na]_o$ of normal seawater supplied some Na to a region of the membrane where Na was required for activating Na pumping. One might expect therefore that the "ATP effect" would be re-
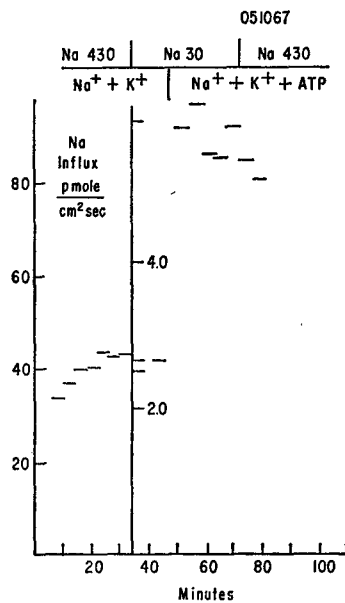

FIGURE 12. The effects of change in $[Na]_o$ and $[ATP]_i$ on Na influx in dialyzed squid axons. The legend at the top of the figure indicates (above the line) changes in external solutions and (below the line) changes in internal solutions. The first ordinate gives Na influx in seawater; the second ordinate refers to influx in the low Na seawater.

duced or absent at low $[Na]_o$. An experiment of this sort is shown in Fig. 12 where Na influx was measured in seawater with $[Na]_o = 430$ mM; the internal dialysis solution was fuel-free with Na 80 mM + K. When a base-line of normal influx had been established at about 40 pmoles/cm²sec, the seawater was changed to $[Na]_o = 30$ mM, $[Li]_o = 400$ mM, and the second ordinate scale on the graph is drawn to reflect the change in efflux to be expected on the basis (see above) that Na influx is linearly related to $[Na]_o$. Next a change to ATP 5 mM was made and the Na influx increased to 2.1 times its former value. This ratio remained unchanged when $[Na]_o$ was increased to 430 mM again so that the conclusion from this experiment is that $[Na]_o$ does not affect the change in Na influx brought about by ATP.

Na Influx As Affected by Na:Na Exchange

If the internal dialysis medium contains only K or (K + ATP), there is no possibility of a one-for-one exchange of Na across the membrane since no Na is

included inside (analysis of a dialysate sample showed $[Na]_i < 1$ mM); if the internal dialysis solution contains $(Na + K)$ but no fuel, then the Na efflux is so small (of the order of 2–3 pmoles/cm²sec) that it cannot account for the increment in Na influx (of the order of 10 pmoles/cm²sec). These conclusions, while quite straightforward, did not rule out the possibility of an unfamiliar artifact; thus it appeared useful to measure Na influx and Na efflux on the same axon. Fig. 13 shows the results of this experiment; dialysis was initially
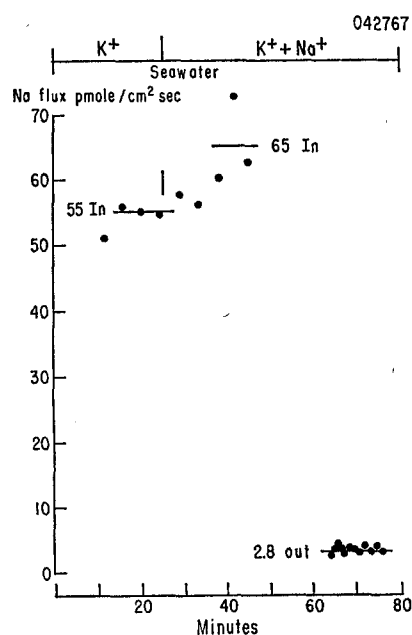

Figure 13. An influx-efflux experiment on a squid axon dialyzed with fuel-free medium is shown. The axon was in standard artificial seawater throughout the experiment and influx measured with ^{24}Na. At the time indicated the internal solution was changed from one that was Na-free to one containing 80 mM Na. External ^{24}Na was then removed, ^{22}Na added to the internal dialysis solution, and Na efflux measured.

with K only followed by $(Na + K)$. After the influx had been determined with ^{24}Na, the seawater was removed, the chamber thoroughly flushed, and then perfusion with the same internal dialysis medium (containing only Na + K) plus ^{22}Na was begun. The efflux samples collected were stored until the ^{24}Na had decayed and then the ^{22}Na was counted and Na efflux calculated. The increment in influx following the addition of $[Na]_i$ was 10 pmoles/cm²sec while the total efflux determined under the same perfusion conditions some 30 min later was only 2.8 pmoles/cm²sec. This latter value exceeded the passive efflux calculated from the flux ratio equation by only about 1 pmole/cm²sec. About half of this excess could be attributed to the residual ATP of 1–5 μM which normally is present in exhaustively dialyzed axons. The amount of efflux unaccounted for and available to balance the assumed influx due to Na:Na exchange was therefore about 5% of the amount required. One con-

cludes that a simple one-for-one Na:Na exchange cannot explain the observed findings in ATP-deficient axons.

Inasmuch as the methods for measuring influx in dialyzed squid axons are admittedly imprecise, the arguments advanced in the preceding paragraphs cannot exclude the presence of some Na:Na exchange under the conditions of these experiments, but the amount must be rather small. Alterations of sodium influx as small as 10 pmoles/cm²sec could readily be detected in the present series, and definitely excluded from a Na:Na exchange mechanism. The mean control sodium influx in this series was 71 pmoles/cm²sec; we estimate therefore that an undetected exchange mechanism could account for no more than about $10/71 = 14\%$ of the control influx.

DISCUSSION

Flux Balance in Dialyzed Axons A previous report indicated that the Na influx in dialyzed axons was about 50% above that in intact or injected axons (Brinlev and Mullins, 1967). The fact that the influx in the present series averages 71 pmoles/cm²sec as against 57 pmoles/cm²sec for the earlier experiments with dialyzed axons can probably be ascribed to the additional trauma attendant upon the various external and internal solution changes required for these more recent experiments. A slight correction should probably be made to the Na influx because the internal perfusion solution contained 80 mM Na in most experiments, and $[Na]_i$ in fresh axons (with which the comparisons are made) is more nearly 50 mM. On the assumption that the increment in Na influx is linearly related to $[Na]_i$, the influx in axons dialyzed with an internal sodium similar to that found in fresh axons would be $47 + 50/80 \times (71\text{-}47) = 62$ pmoles/cm²sec (see Table IV), a value still about 25–50% above that found for fresh axons.

Although the magnitude of the influx, and hence the sodium permeability, is higher than in normal axons, the response of the influx to ATP seems to be about the same. In the present series the ratio of unfueled/fueled influx lies in the range 0.5–0.6, whereas the ratio in axons first treated with cyanide and then injected with ATP was about 0.8 (Table 6, Caldwell et al., 1960). Qualitative agreement is about all that can be expected because the range of internal ATP concentrations and ATP/ADP ratios differed widely in the two series.

The values for Na efflux obtained in the present study (40–50 pmoles/cm²sec) at $Na_i = 80$, $ATP_i = 4$, and $T = 15°C$) seem to agree rather well with other data when appropriately scaled for temperature, internal sodium, and ATP: 41 (Caldwell et al., 1960), 58 (Sjodin and Beaugé, 1967), 40 (Canessa et al., 1968), 45 (DeWeer, unpublished data). Since virtually all of the Na efflux can be attributed to some sort of ATP-dependent pump or pumps, we infer that the extrusion process is working relatively normally, and

that the excess of influx over efflux is probably due to abnormal sodium permeability rather than a deficient operation of the extrusion process.

The net gain of sodium (71 − 48 = 23 pmoles/cm²sec) observed in the present experiments is balanced rather well by a net loss of potassium from dialyzed fibers which we estimate (Mullins and Brinley, unpublished data) to be 15–20 pmoles/cm²sec. It appears therefore that there is no uncompensated cation transfer across the axolemma of the dialyzed axon. A net gain of sodium in isolated cephalopod axons has also been noted by some others (e.g., Steinbach and Spiegelman, 1943; Shanes and Berman, 1955; Keynes and Lewis, 1951).

Energy Sources for Na Extrusion   For some time there has been strong evidence that the substrate for the Na pump in nerve is either ATP or some substance readily formed from ATP. Such a conclusion was suggested by the effects on Na efflux of (*a*) inhibitors of oxidative phosphorylation such as CN, (*b*) uncouplers of phosphorylation such as dinitrophenol, and (*c*) inhibitors of membrane transport such as ouabain. The preparation of an ATPase from nerve membrane fragments followed by the demonstration that this enzyme was activated by Na and K and inhibited by glycosides (Skou, 1957, 1960) lent further support to the role of ATP as the source of energy for Na extrusion.

Because of the rapidity with which PA and ATP can be interconverted, inhibitor studies on cells undergoing Na transport are incapable of showing whether a substance such as PA is the actual substrate for Na extrusion. By using an experimental arrangement which removed intermediates necessary for oxidative phosphorylation, glycolysis, or transphosphorylation it was possible to assay the activity of selected compounds in energizing Na transport.

Previous studies with ATP as a substrate demonstrated that this substance was capable of fueling Na extrusion at virtually normal rates. The present studies have confirmed this finding and extended the range of these observations to other substrates. Of the 11 compounds tested, the only other substance capable of giving an appreciable Na extrusion was deoxyribose ATP. Our measurements show that d-ATP yields a Na extrusion that is of the order of 25–50% of that produced by an equivalent concentration of ATP but too few measurements have been made to allow a really quantitative comparison between the two substrates.

The triphosphates UTP, CTP, and GTP were essentially without effect on Na extrusion; the small effect on efflux that was actually observed could well be the result of a small ATP contamination of the compounds used or of some transphosphorylation taking place in the axoplasm. The inactivity of these compounds implies a specificity requirement for an adenine ring in any compound that is to serve as a substrate. Dialysis with ADP + AMP resulted in an increment of Na efflux that could be reasonably ascribed to ATP formation,

suggesting that these substances per se cannot support Na extrusion. The inactivity of the intermediates of glycolysis, PEP and 3-phosphoglycerate, further reinforces the notion of a high degree of substrate specificity on the part of the Na pump.

An interesting finding in connection with these substrate studies was the lack of effect of acetyl phosphate on Na extrusion. It has been shown (Bond et al., 1966; Bader and Sen, 1966) that this substance reacts with membrane ATPase fragments to form a phosphorylated intermediate that is similar to the intermediate formed between the enzyme and a terminal phosphate of ATP. Evidence that such an intermediate is related to Na pumping is given by the finding that Na^+ is required for the formation of the intermediate and that K^+ promotes the decomposition of the intermediate. The decomposition with the release of phosphate is inhibited by ouabain. In the case of acetyl phosphate, the intermediate forms without the presence of Na, suggesting that this substrate has modified the ion specificity requirements of the enzyme. Therefore, the expectation would be that in the presence of AcP the Na pump might transport any cation, including Na; however, the results clearly show a failure of this compound to affect Na transport in squid axons. The conclusion appears inescapable that either dialysis removes an unidentified cofactor necessary in the AcP reaction, or that the formation of a phosphorylated intermediate, as shown by enzyme studies may not be directly related to Na transport in squid axons.

Considered together, these results indicate that ATP is the only high energy phosphate likely to be present in the axon at reasonable concentrations which will support a normal amount of sodium extrusion, although several of the substances tested are probably present in small amounts in normal axoplasm. We infer that if there is more than one Na extrusion mechanism, then, under the conditions of the present experiments, ATP is the only fuel source.

Dependence of Efflux on $[ATP]_i$ *and* $[Na]_i$ The experiments reported have shown that both $[Na]_i$ and $[ATP]_i$ have important effects on both Na efflux and Na influx. A general statement about these effects is that increases in either $[Na]_i$ or $[ATP]_i$ increase both Na efflux and Na influx. The details of these changes are discussed below.

Sodium efflux appears to be a linear function of $[Na]_i$ over a concentration range of 2–240 mM, although some reservation must be made about this statement because at $[Na]_i = 11$ mM much of the Na efflux was cut off in Li seawater while this effect does not occur at $[Na]_i = 80$ mM. The linear relation between efflux and $[Na]_i$ in squid axons has been reported by several groups using varying concentration ranges (Hodgkin and Keynes, 1956; Sjodin and Beaugé, 1967); and represents a distinct difference from muscle where a highly nonlinear relation has been found (Keynes and Swan, 1959; Mullins

and Frumento, 1963; Brinley, 1968). It might be noted that when $[Na]_i$ = 1 mM and $[Na]_o$ = 430 mM, then E_{Na} = +150 mv and the difference $(E_{Na} - E_m)$ is 210 mv (assuming the resting potential remains at −60 mv). This large electrochemical potential difference would make it thermodynamically impossible for 3 Na to be extruded per ATP molecule split (Baker and Shaw, 1965) and would require exceedingly high efficiencies even if the stoichiometry were as low as 2 Na/ATP. We have made no systematic measurements of Na efflux into Na-free solutions at low $[Na]_i$ but the one value cited above suggests that Na:Na exchange becomes prominent when $[Na]_i$ is reduced, although it is absent at a concentration of 80 mM.

The fact that Na efflux is linearly related to $[Na]_i$ but that at low $[Na]_i$ the Na efflux is dependent on $[Na]_o$ gives support to the notion that a single mechanism is engaged in either Na:Na or Na:K exchange. This is inferred from a consideration of how a two-mechanism scheme might work: a slowing down of Na:K exchange would not necessarily lead to a starting up of Na:Na exchange. Presumably a Na:Na exchange mechanism requires less energy than the Na:K mechanism which seems to operate at higher $[Na]_i$. It should be noted, however, that the efflux of Na at low $[Na]_i$ retains the same fractional strophanthidin sensitivity as the efflux at higher $[Na]_i$; i.e., about ⅔. This result does not conflict qualitatively with the notion that the efflux is partly Na:Na coupled at low $[Na]_i$ since Garrahan and Glynn (1967 a) have shown that the Na:Na exchange in red cells is ouabain-sensitive.

The absence of an effect of $[Li]_i$ on Na efflux can be interpreted as an indication that this ion is inert insofar as it might affect the Na pump. However, because of the linear relationship between Na efflux and $[Na]_i$, a large $[Li]_i$ could affect the Na transport mechanism in a noncompetitive manner so that a metabolically dependent Li efflux might result without any affect on Na efflux. We have no experimental information on this point.

The sensitivity of Na efflux to $[ATP]_i$ is clearly different from its sensitivity to $[Na]_i$ in two respects. In the first, tests have shown that when $[ATP]_i$ is low, Na efflux is uninfluenced by Na-free conditions, implying that Na:Na exchange is not a prominent process. Second, the relationship between Na efflux and $[ATP]_i$ is linear over the concentration range of 1 to 10 μM, where efflux undergoes a change from about 1 to 10 pmoles/cm²sec. The relationship is much less steep between 100–1000 μM but shows no evidence of saturating even at the highest values of $[ATP]_i$.

Effects of Strophanthidin   The effect of strophanthidin and related substances has been generally recognized as one of reducing Na efflux in squid axons (Caldwell and Keynes, 1959), in muscle (Horowicz and Gerber, 1965), and in red blood cells (Glynn, 1957). In none of these preparations has the Na efflux been reduced to a level compatible with that of the passive Na efflux

as calculated from flux ratio considerations. The situation is quite similar in membrane ATPase preparations in which, typically, 10–20% of the enzyme activity cannot be inhibited.

Suggestions as to the nature of the strophanthidin-insensitive Na efflux have inclined toward another, glycoside-insensitive, Na pump (Hoffman and Kregenow, 1966). In considering how this suggestion applies to squid axons, it might be noted that when axons are poisoned with CN, the reduction in Na efflux is about the same as when they are treated with strophanthidin. The explanation for the CN-insensitive efflux is the existence of residual $[ATP]_i$ rather than a second nonATP fueled pump. Since very low internal ATP concentrations (of the order of 20 μM) suffice to give a Na efflux similar in magnitude to that found in CN- or strophanthidin-poisoned fibers, it is reasonable to expect that the application of a second inhibitor which further reduces $[ATP]_i$, possibly by interfering with ATP produced by glycolysis, would produce an additional fall in Na efflux. A possible, though erroneous, interpretation of such an experiment might be that the second inhibitor interfered with a second pump for Na. Because of the strong dependence of sodium extrusion on $[ATP]_i$ in squid axons, the existence of another, nonATP fueled, pump cannot be demonstrated until the axoplasmic ATP has been reduced to the order of 1 μM. Our results do not support the idea of a second pump.

Our experimental findings with strophanthidin suggest that the effect of this substance is to change Na efflux from an initial [ATP]-dependent value to a value of about 10 pmoles/cm²sec. These data could be easily understood if there were a component of sodium efflux insensitive to external strophanthidin. However, the existence of such a component cannot explain the effect of strophanthidin on the sodium efflux from axons dialyzed with extremely low $[ATP]_i$ of the order of 1–10 μM. In this case the Na efflux which is initially only about 2–5 pmoles/cm²sec rises to a value of about 10 pmoles/cm²sec when strophanthidin is applied. Explanations for this effect can be considered by assuming that strophanthidin: (*a*) increases P_{Na} of the membrane, or (*b*) initiates a Na:Na exchange, or (*c*) increases Na pumping.

The first explanation would require an almost fourfold increase in Na influx; it should be noted, however, that when ATP is absent from inside the axons, Na influx is about half its normal value so that only an over-all twofold increase in Na influx would result if this influx were compared with that in normal, fully fueled axons.

The second suggestion, that of a Na:Na exchange, is contradicted by a single experiment in which the strophanthidin-induced increase in ATP-independent sodium efflux was not reduced by substitution of lithium for sodium in the external seawater.

The difficulty with the third explanation, i.e. strophanthidin-induced sodium extrusion, is that this suggestion would require an energy source and

no high energy phosphate was supplied in the experiment described. One rather complicated explanation is that strophanthidin in the course of making most of the pump mechanism unable to utilize ATP, transforms some fraction of the pump mechanism in a manner such that it is able to utilize ATP at a concentration of 2–3 μM. One other energy source known to be available is the Na concentration gradient across the membrane. If the energy of this dissipative movement of Na^+ could be somehow coupled to the Na pump, some Na extrusion would be possible. Such a scheme does not rule out running the Na pump backward to make an energy source, but neither is it limited to such a scheme. Under no fuel conditions, Na influx is about 40 pmoles/cm²sec, and Na efflux is about 10 pmoles/cm²sec in the presence of strophanthidin; this would allow the entry of 4 Na to promote the extrusion of 1 Na, assuming that Na influx was uninfluenced by strophanthidin.

Sodium Influx The Na influx experiments were done to examine the extent to which variations in the internal concentrations of Na and ATP might influence Na movement. The results are best discussed by reference to the table below:

Internal solution Column No.	K 1	(K+Na) 2	(K+~P) 3	(K+Na+~P) 4	(2-1)	(3-1)	(4-1)
Mean Na influx	33	43	47	71	10	14	38
Na efflux (under comparable conditions)	0	2	0	48*			
Na net influx	33	41	47	23			

* Uninfluenced by Li seawater.

The first point to be examined is the nature of the change in Na influx when the internal dialysis solution is changed from one containing only K to one with Na + K. The mean increment in influx is 10 pmoles/cm²sec while the mean increment in efflux can be expected to be of the order of 2 pmoles/cm²sec a value close to that expected for a purely passive Na movement from the axon. Thus, there is very little Na efflux which can be attributed to a metabolic process or to Na:Na exchange diffusion so that the increment in influx could be described formally as a change in P_{Na} .

If instead of adding Na to the internal dialysis medium one adds (ATP + PA), there is an increment of influx of 14 pmoles/cm²sec (column 3–column 1). Although the addition of fuel does clearly increase Na influx, the absence of $[Na]_i$ precludes there being any Na efflux for coupling. This increment in Na influx could also be considered as resulting from an increment in P_{Na} of the membrane.

An interesting consequence of the dependence of Na influx on $[Na]_i$ is that net influx of Na is changed by only half the value of the Na efflux generated by pumping. This is seen by comparing the net flux of column 3 with that of column 4. The net influx is 23 pmoles/cm²sec in the presence of Na; this is just half the net influx in the absence of Na. As Na efflux is linearly dependent on $[Na]_i$, if P_{Na} also has a linear dependence, then the net flux will be only half the change in efflux for all concentrations.

It is interesting to compare the foregoing analysis of the effects of changes in $[Na]_i$ and $[ATP]_i$ with the conventional way in which data from inhibitor studies are treated. Since there is a large increase in Na influx when ATP is present inside the axon, it might be argued that this increment in influx resulting from the addition of substrate is a pumped ion movement. The fact that this ion movement is linear with $[Na]_o$ cannot be used as evidence against this notion because Na efflux, which is clearly a pumped ion movement, responds linearly to $[Na]_i$. These observations underline the difficulty in discriminating between pumped and passive ion movements when the ion flux in question does not involve movement against an electrochemical gradient.

By way of summarizing the influx data, it would appear that concomitant with turning on Na extrusion there is a substantial increase of Na influx. This change of flux cannot be largely a Na:Na exchange and can therefore be formally described as a changed Na permeability. It seems, however, that the presently accepted theoretical framework is inadequate to explain the observations properly since the changes observed are undoubtedly closely connected with Na pumping.

Energy Expenditure for Sodium Extrusion   Finally, it seems useful to compare the energy expenditure for ion pumping in some tissues in which reasonably accurate values for the ion fluxes and the electrochemical gradient are available. These are shown in Table V; from them, the minimum power input to the sodium and potassium pumps has been calculated. The following points can be noted.

1. The thermodynamic requirement for energy expenditure to effect inward potassium ion movement for the tissues listed in Table V, except for the erythrocyte and isolated squid axon, is nearly zero. This conclusion does not depend upon the exact value chosen for the active potassium influx, because the electrochemical gradient for entries other than the erythrocyte and isolated squid axon is not significantly different from zero. Even though the absolute power input to the erythrocyte potassium pump is very small, it is still finite because the electrochemical gradient is definitely different from zero. In the case of the isolated squid axon, an energy requirement appears to stem from the fact that such axons are depolarized by about 17 mv compared with axons *in situ* (Moore and Cole, 1960) but even under these

TABLE V

MINIMUM POWER INPUT FOR SODIUM AND
POTASSIUM PUMPS IN SELECTED TISSUES

Tissue	Active fluxes		$[K]_o$	$[K]_i$	$[Na]_o$	$[Na]_i$	$-E_m$	$-E_K$	E_{Na}	$\|E_K-E_m\|$	$E_{Na}-E_m$	Power input		Total energy input	% used by Na pump
	m_{Na}^o	m_K^i										Na	K		
	$\frac{pmole}{cm^2 sec}$	$\frac{pmole}{cm^2 sec}$	$m M$	$m M$	$m M$	$m M$	mv	mv	mv	mv	mv	$\frac{nwatt}{cm^2}$	$\frac{nwatt}{cm^2}$	$\frac{nwatt}{cm^2}$	
Squid axon															
in vitro	40*	10‡	10	350	425	80	60	86	41	26	101	400	26	2300§	17
in situ	17||	10||	20	350	350	50	77	76	47	~0	124	210	~0		
Lobster axon															
in vitro¶	5	12	10	233	465	37	75	76	61	~0	136	68	~0		
Frog sartorius muscle	4**	~0‡‡	2.5	120	120	10	93	93	60	~0	153	60	~0	330§§	18
Barnacle depressor muscle||||	4	~1	10	160	465	21	67	67	75	~0	142	57	~0		
Erythrocyte (human)	0.04¶¶	0.05¶¶	4	120	140	10	7	86	66	79	73	0.3	0.4	2***	23
						40			23		30	0.1			

* Mullins and Brinley (1967).
‡ Caldwell et al. (1960).
§ Oxygen consumption (Connelly and Cranefield, 1953).
\|\| Estimated from fluxes in fresh axons.
¶ Brinley (1965).
** Mullins and Noda (1963).
‡‡ Sjodin and Beaugé (1968).
§§ Estimated from oxygen consumptions (Hegnauer et al., 1932).
\|\|\|\| Brinley (1968).
¶¶ Hoffman and Kregenow (1966).
*** Estimated from lactate production (Whittam and Wiley, 1967).

conditions the power requirement for potassium pumping is still only about 5% of the requirement for the sodium pump.

2. The energy expenditure for an isolated squid axon pumping Na is rather large compared with the other nerve or muscle preparations listed in Table V, but there is reason to suppose that such a preparation has been made highly leaky to Na by the procedures necessary to isolate it. An intact squid axon has a power expenditure for Na pumping much more comparable with that for muscle fibers or lobster nerves. These tissues do not have cut branches and the conditions in the isolated preparation probably resembles more closely those found in the in vivo tissue.

3. The energy requirement for sodium pumping in the nerve and muscle preparations is about 60–200 nwatts/cm² whereas the power input for the erythrocyte is about 0.5% of this value.

4. The power inputs to the erythrocyte sodium and potassium pumps are approximately the same, whereas in all other tissues the relative energy expenditure for the potassium pump is very much less if not actually zero.

5. The minimum energy for sodium extrusion is about 20% of the total energy produced by the cell.

REFERENCES

Bader, H., and A. K. Sen. 1966. K-dependent acyl phosphatase as part of the (Na + K) dependent ATPase of cell membranes. *Biochim. Biophys. Acta.* 118:116.

Baker, P. F., A. L. Hodgkin, and T. I. Shaw. 1962 a. Replacement of the axoplasm of giant nerve fibers with artificial solutions. *J. Physiol. (London).* 164:330.

Baker, P. F., A. L. Hodgkin, and T. I. Shaw. 1962 b. The effects of changes in internal ionic concentrations on the electrical properties of perfused giant axons. *J. Physiol. (London).* 164:355.

Baker, P. F., and T. I. Shaw. 1965. A comparison of the phosphorous metabolism of intact squid nerve with that of the isolated axoplasm sheath. *J. Physiol. (London).* 180:424.

Bergmeyer, H. U., editor. 1965. Methods of Enzymatic Analysis. Academic Press, Inc., New York.

Bond, G. H., H. Bader, and R. L. Post. 1966. Acetyl phosphate as substrate for (Na + K) ATPase. *Federation Proc.* 25:567.

Brinley, F. J., Jr. 1965. Sodium, potassium, and choride concentrations and fluxes in the isolated giant axon of *Homarus. J. Neurophysiol.* 28:742.

Brinley, F. J., Jr. 1968. Sodium and potassium fluxes in isolated barnacle muscle fibers. *J. Gen. Physiol.* 51:445.

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

Caldwell, P. C., A. L. Hodgkin, R. D. Keynes, and T. I. Shaw. 1960. The effects of injecting "energy-rich" phosphate compounds on the active transport of ions in the giant axons of Loligo. *J. Physiol. (London).* 152:590.

CALDWELL, P. C., A. L. HODGKIN, R. D. KEYNES, and T. I. SHAW. 1964. The rate of formation and turnover of phosphorus compounds in squid giant axons. J. Physiol. (London). 171:119.

CALDWELL, P. C., and R. D. KEYNES. 1959. The effect of ouabain on the efflux of sodium from a squid giant axon. J. Physiol. (London). 148:8.

CANESSA, M., F. ZAMBRANO, and E. ROJAS. 1968. The loss and recovery of the sodium pump in perfused giant axons. J. Gen. Physiol. 50(5, Pt. 2):162.

CONNELLY, C. M., and P. F. CRANEFIELD. 1953. The oxygen consumption of the stellate nerve of the squid. Proc. 19th Intern. Congr. Physiol. Soc., Montreal. 276.

EGGLESTON, L. V., and R. HEMS. 1952. Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. Biochem. J. 52:156.

GARRAHAN, P. J., and I. M. GLYNN. 1967 a. The behaviour of the sodium pump in red cells in the absence of external potassium. J. Physiol. (London). 192:159.

GARRAHAN, P. J., and I. M. GLYNN. 1967. b The sensitivity of the sodium pump to external sodium. J. Physiol. (London). 192:175.

GARRAHAN, P. J., and J. M. GLYNN. 1967 c. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalyzed by the sodium pump. J. Physiol. (London). 192:189.

GARRAHAN, P. J., and I. M. GLYNN. 1967 d. The stoichiometry of the sodium pump. J. Physiol. (London). 192:217.

GARRAHAN, P. J., and I. M. GLYNN. 1967 e. The incorporation of inorganic phosphate into adenosine triphosphate by reversal of the sodium pump. J. Physiol. (London). 192:237.

GLYNN, I. M. 1957. The action of cardiac glycosides on sodium and potassium movements in human red cells. J. Physiol. (London). 135:148.

HODGKIN, A. L., and R. D. KEYNES. 1956. Experiments on the injection of substances into squid giant axons by means of a microsyringe. J. Physiol. (London). 131:592.

HOFFMAN, J. F., and F. M. KREGENOW. 1966. The characterization of new energy dependent cation transport processes in red blood cells. Ann. N. Y. Acad. Sci. 137:566.

HOROWICZ, P., and C. L. GERBER. 1965. Effects of external potassium and strophanthidin on sodium fluxes in frog muscle. J. Gen. Physiol. 48:489.

HOSKIN, F. C. G. 1966. Anaerobic glycolysis in parts of the giant axon of squid. Nature. 210:856.

KEYNES, R. D., and P. R. LEWIS. 1951. The sodium and potassium content of cephalopod nerve fibers. J. Physiol. (London). 114:151.

KEYNES, R. D., and R. C. SWAN. 1959. The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle. J. Physiol. (London). 147:591.

LIPMANN, F., and L. C. TUTTLE. 1945. A specific micromethod for the determination of acetyl phosphates. J. Biol. Chem. 159:21.

MOORE, J. W., and K. S. COLE. 1960. Resting and action potentials of the squid giant axon in vivo. J. Gen. Physiol. 43:961.

MULLINS, L. J., and F. J. BRINLEY, JR. 1967. Some factors influencing sodium extrusion by internally dialyzed squid axons. J. Gen. Physiol. 50:2333.

MULLINS, L. J., and A. S. FRUMENTO. 1963. The concentration dependence of sodium efflux from muscle. J. Gen. Physiol. 46:629.

MULLINS, L. J., and K. NODA. 1963. The influence of sodium-free solutions on the membrane potential of frog muscle fibers. J. Gen. Physiol. 47:117.

SHANES, A. J., and M. D. BERMAN. 1955. Kinetics of ion movement in the squid giant axon. J. Gen. Physiol. 39:279.

SJODIN, R. A., and L. A. BEAUGÉ. 1967. The ion selectivity and concentration dependence of cation coupled active sodium transport in squid giant axons. Currents Mod. Biol. (Holland). 1:105.

SJODIN, R. A., and L. A. BEAUGÉ. 1968. Strophanthidin-sensitive components of potassium and sodium movements in skeletal muscle as influenced by the internal sodium concentration. J. Gen. Physiol. 52: In press.

Skou, J. C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta.* **23**:394.

Skou, J. C. 1960 Further investigations on Mg + Na activated adenosine-triphosphatase, possibly related to the active linked transport of Na and K across the nerve membrane. *Biochim. Biophys. Acta.* **42**:6.

Stadtman, E. R. 1957. Preparation and assay of acetyl phosphate. *In* Methods in Enzymology. S. P. Colowick and N. O. Kaplan, editors. Academic Press, Inc., New York. **3**:931.

Steinbach, H. B., and S. Spiegelman. 1943. The sodium and potassium balance in squid nerve axoplasm. *J. Cellular Comp. Phyisol.* **17**:187.

Whittam, R., and J. S. Wiley. 1967. Potassium transport and nucleoside metabolism. *J. Physiol. (London).* **191**:633.