Potassium Fluxes in Dialyzed Squid Axons

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

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

ABSTRACT Measurements have been made of K influx in squid giant axons under internal solute control by dialysis. With $[ATP]_i = 1$ μM, $[Na]_i = 0$, K influx was 6 ± 0.6 pmole/cm² sec; an increase to $[ATP]_i = 4$ mM gave an influx of 8 ± 0.5 pmole/cm² sec, while $[ATP]_i$ 4, $[Na]_i$ 80 gave a K influx of 19 ± 0.7 pmole/cm² sec (all measurements at ~16°C). Strophanthidin (10 μM) in seawater quantitatively abolished the ATP-dependent increase in K influx. The concentration dependence of ATP-dependent K influx on $[ATP]_i$, $[Na]_i$, and $[K]_o$ was measured; an $[ATP]_i$ of 30 μM gave a K influx about half that at physiological concentrations (2–3 mM). About 7 mM $[Na]_i$ yielded half the K influx found at 80 mM $[Na]_i$. The ATP-dependent K influx responded linearly to $[K]_o$ from 1–20 mM and was independent of whether Na, Li, or choline was the principal cation of seawater. Substances tested as possible energy sources for the K pump were acetyl phosphate, phosphoarginine, PEP, and *d*-ATP. None was effective except *d*-ATP and this substance gave 70% of the maximal flux only when phosphoarginine or PEP was also present.

INTRODUCTION

It has been known for a number of years (Hodgkin and Keynes, 1955 *a*) that the influx of potassium into cephalopod axons is significantly reduced by metabolic inhibitors. Since K influx, at least in isolated squid axons, is against a small but finite electrochemical gradient, the observed reduction in influx brought about by inhibitors such as cyanide presumably reflects the existence of a K pump dependent upon metabolic energy. The K influx in squid axons has never been systematically studied, largely, it would appear, because of the technical difficulty in making serial determinations of influx on the same axon. Experiments designed to measure K influx by the extrusion of axoplasm from paired axons, are not only tedious, but prone to some uncertainties because of the small size of the metabolically dependent component relative to the total K influx. The present experiments have made use of a porous glass capillary technique (Brinley and Mullins, 1967) which obviates this difficulty since sequential determinations of influx can be made over the

same region of an axon for several hours while maintaining a control of internal solutes in the axoplasm.

Since internal dialysis of an axon is capable of reducing $[ATP]_i$ to the order of 1 μM, it appeared useful to define the K pump of the squid axon as the increment in K influx that is observed when $[ATP]_i$ is increased from 1 μM to any higher value. The K pump defined in this way is identical to one defined as the decrement in K influx that is observed when strophanthidin is applied externally at a concentration of 10 μM. In spite of this fortunate agreement, some reservation must be made about the magnitude of the K pump at low $[ATP]_i$ until technical improvements in the dialysis technique allow one to reach much lower levels of $[ATP]_i$.

The main points examined in this study were: the dependence of the K pump on $[K]_o$, $[Na]_o$, $[Na]_i$, $[ATP]_i$, $[ADP]_i$, and on some other substrates that might serve as energy sources. The conclusion drawn from the experiments done is that the K pump is linearly dependent on $[K]_o$ and independent of $[Na]_o$, while both ATP and Na must be present inside the axon for maximal activation. Aside from ATP and *d*-ATP, no substrate had any significant action on the K pump, while ADP inside the axon was an inhibitor.

METHODS

The general methods used to study squid axons under conditions of internal solute control by dialysis have been described previously (Brinley and Mullins, 1967; Mullins and Brinley, 1967; Brinley and Mullins, 1968) and these were used in the present study. Only the salient modifications in the technique are described below.

Experimental Material The experiments reported here were performed upon live specimens of *Loligo pealei* during the months of May, 1967 and January–October, 1968. Experiments during the months of July and August were done at the Marine Biological Laboratory in Woods Hole, Massachusetts. The remainder of the experiments were done in Baltimore using squid obtained from Ocean City, Maryland.

Apparatus The axon chamber was essentially as described previously, but the micromanipulator system for introducing the porous capillary into the axon has been improved by the addition of a motordrive which advances or withdraws the capillary at a rate of 1 mm/min. This microdrive is used for the final positioning of the capillary in the axon. In some experiments it was noted that the axoplasm tended to adhere to the porous region of the capillary. Subsequent movement of the capillary would thus transmit a shear force via the axoplasm to the membrane. To minimize this adhesion, the microdrive motor was provided with a reversing switch and a timing cam which allowed the microdrive to oscillate a distance of 0.25–0.50 mm within a period of 30 sec. This dithering mode was used during the periods of end washing of axoplasm (see Mullins and Brinley, 1967) when the capillary was being neither advanced nor withdrawn. It was also used at any time before final positioning of the porous region.

The Diffusion of Material in Dialyzed Squid Axons One important technical limitation of the dialysis technique in measuring influx is that the response of the system to changes in membrane flux is limited by the fact that isotope crossing the membrane must diffuse across several hundred microns of axoplasm before it can be picked up by the capillary. Moreover, after isotope enters the capillary, there is a transit time of 1–3 min, depending upon flow rate, before the material can be collected at the tip of the porous capillary. We have determined the response time of the system empirically by assuming that the action of 10 μM strophanthidin on K influx is as rapid as its action on Na efflux, i.e., the effect is complete within 1 min (Baker, 1968). The slow reduction in K influx as observed in our experiments (see Fig. 2) is therefore presumably due to intrinsic diffusional delays in the axoplasm and porous capillary. In this manner, the half-time of response is estimated to be about 3–5 min. In contrast, the response of the system to abrupt changes in efflux is limited only by the washout time of the chamber slot which can be made less than 1 min.

A second major technical limitation of the method results from longitudinal diffusion of isotope in the axoplasm at the junction between the porous and nonporous areas of the capillary. In this region not only specific activity of isotope, but also the concentration of dialyzable material in the axoplasm is a complicated function of space and time. The chamber used in the present experiments was designed primarily for efflux experiments, and its performance in that mode of operation has already been described (Brinley and Mullins, 1967). For influx experiments the flow system was disconnected and radioactive seawater was added to the center slot. The capillary was positioned so that the porous/nonporous region lay nearly opposite the orifice of the guard outflow. Each guard syringe withdrew fluid at the rate of about 50 λ/min. In this way, isotope was continuously circulated from the center region through part of the guard chamber and out through the guard outflow (see Fig. 6, Brinley and Mullins, 1967). Since the end regions of the chamber were water-tight, this arrangement reduced, but by no means eliminated, exposure to isotope of those portions of the axon which lay beyond the porous region. A rough test of the adequacy of isotope exclusion was made by removing all isotope from the chamber at the end of a 2 hr influx dialysis and noting the final base line level of radioactivity collected at the outflow of the porous capillary. This residual radioactivity, which represented longitudinal diffusion of isotope from the end regions of the axon where the capillary is nonporous into the central region where the capillary is porous, amounted to about 10% of that present in influx samples taken during the experiment. Since this contribution of radioactivity comes from regions which are not under diffusional control, it will not respond to changes in the dialysis medium and it is not easy to decide how to correct the experimental records for this effect. It probably contributes to the base line influx which is not affected by metabolic changes and may be responsible for the fact that the "no fuel" base line influx after a long dialysis is slightly higher than the original base line.

Solutions The solutions used in this study are given in Table I. The external solutions are conventional. Strophanthidin seawater was prepared by dissolving an appropriate amount of strophanthidin in artificial seawater. Alcohol was never used as a solvent.

The rationale behind the formulation of the internal solutions has been described previously (Brinley and Mullins, 1967). Two significant modifications have been made in the present series of experiments. Most of the axons used for experiments in the later spring and summer were unusually small with many branches and did not survive well in the dialysis chamber. In an effort to maintain the membrane potential, the internal chloride was reduced from 80 mM to 8 mM. A second modification was to increase EGTA from 0.1 to 0.5 mM to increase the binding capacity of the dialysis solution for calcium.

TABLE I

COMPOSITION AND IDENTIFICATION OF SOLUTIONS USED

Ion	ASW	Li ASW	Choline ASW	1 K ASW	20 K ASW	E′	F′	O′	V	W	AB	AC	AD	AE	AF
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
K^+	9	9	9	1	20	304.4	382.4	304.4	304.4	384.4	309	309.5	389	309	389
Na^+	429	—	4	429	419	80	—	80	80	—	80	80	—	80	—
Li^+	—	429	—	—	—	—	—	—	—	—	—	—	—	—	—
$Choline^+$	—	—	425	—	—	—	—	—	—	—	—	—	—	—	—
Mg^{++}	48	48	48	48	48	4	4	4	4	4	4	4	4	4	4
Ca^{++}	9	9	9	9	9	—	—	—	—	—	—	—	—	—	—
Cl^-	496	496	496	48	48	88	88	88	8	8	8	8	8	88	88
$Isethionate^-$	—	—	—	—	—	151	151	151	151	151	151	151	151	151	151
L-$Aspartate^-$	—	—	—	—	—	151	151	—	—	—	—	—	—	—	—
D-$Aspartate^-$	—	—	—	—	—	—	—	151	231	231	231	231	231	151	151
CN^-	—	—	—	—	—	2	—	—	—	—	—	0.5	—	—	—
$SO_4^=$	25	25	25	25	25	—	—	—	—	—	—	—	—	—	—
$EGTA^=$	—	—	—	—	—	—	—	0.1	0.1	0.1	0.5	0.5	0.5	0.5	0.5
$EDTA^=$	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—	—	—	—	—	—	—	—
TES^-	4	4	4	4	4	—	—	2	2	2	5	5	5	5	5
Taurine	—	—	—	—	—	275	275	275	275	275	275	275	275	275	275

RESULTS

Potassium Fluxes in Intact Axons

In order to have data to compare with dialyzed axons, a few determinations of K influx were made in intact, lightly cleaned axons. The axons were held in ^{42}K seawater for various periods of time and the axoplasm was then extruded and counted. The results are presented in Table II; the K influx in these control axons averaged 20 pmoles/cm² sec (in subsequent measurements of flux this unit will be abbreviated as f for flux) at 16°C while in axons treated with 10 μM strophanthidin, the influx was reduced to 7 f. Comparable data on dialyzed axons give values of 19 f and 8 f, respectively. (It will be shown later that the difference between the normal and strophanthidin-poisoned K influx is a valid operational measure of the size of the ATP-

dependent component.) We conclude therefore that the procedure of dialysis has not altered the magnitude of either component of the K influx.

Potassium Influx in Dialyzed Axons

Some preliminary experiments were undertaken to determine whether the requirements for activating potassium uptake were similar to those for sodium extrusion, i.e. the presence inside an axon of both ATP and Na (Mullins and Brinley, 1967). Attention was therefore directed initially toward obtaining a stable base line of K influx in the absence of ATP followed

TABLE II

POTASSIUM INFLUXES IN INTACT AXONS (15.5°C)

Axon	Diameter	Soak time	Rinse time	Mass	Influx	
					Control	10 μM stroph
	μ	*min*	*min*	*mg*	*pmoles/ cm² sec*	
020168–1	500	37	4	2.8	16	
020168–3	475	42	6	3.5	23	
020168–4	500	44	7	4.6		6
020168–5	525	45	7	4.5	15	
020168–6	500	47	6	7.2		8
020168–7	450	33	6	3.1		8
020168–9	450	36	7	3.5		5
052268–3	475	37		5.0	24	
Mean					20	7
± SE					1.3	0.8
N =					4	4

by an experimental period in which ATP was increased to concentrations of the order of 2–4 mM. Such experiments were carried out at an $[Na]_i$ of 80 mM because most of our Na efflux data has been collected at this $[Na]_i$. The results obtained in these preliminary experiments showed that K influx in an axon with an $[ATP]_i$ of the order of 1 μM was about 10 f and there was about 9 f increase in flux when ATP was added to the dialysis fluid. It is not possible to reverse the effect of high concentrations of ATP when these have been applied for any length of time mainly, we believe, because some of the ATP diffuses longitudinally at the porous/nonporous junctions and this ATP becomes a reservoir that will supply the dialyzed region for long periods of time. Because the Na and K pumps are sensitive to [ATP] of the order of <10 μM, such an effect is understandable. It is possible, however, to test the condition of the axon near the end of a long period of dialysis either by (*a*) removing Na from the dialysis medium or (*b*) applying strophanthidin to the seawater bathing the axon. Both of these treatments reduce the ATP-dependent K influx to low levels. An experiment in which Na was removed

from an axon is shown in Fig. 1; it is clear that the removal of Na from the axoplasm reduces K influx to a level very close to that observed in the absence of ATP.

The Influence of $[ATP]_i$ on K Influx

Previous experiments on squid axons (Caldwell, Hodgkin, Keynes, and Shaw, 1960 *b*; Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969) have shown that both cyanide and ouabain are able to reduce K influx. The inferences drawn from these experiments were that a part of the K influx

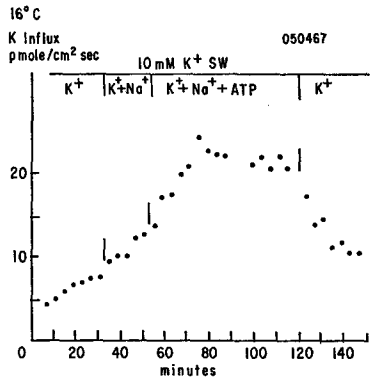

FIGURE 1. This shows the general response of K influx to changes in the internal dialysis medium. The addition of 80 mM Na produces an increase in K efflux while the subsequent addition of 2.5 mM ATP increased K influx greatly. This increased K influx could be decreased by removal of Na_i. The external solution was seawater throughout the experiment. Here and on other figures SW stands for seawater.

depends on $[ATP]_i$ either directly or via a coupling of K influx to the Na pump.

In our present experiments, therefore, it seemed worthwhile to determine the relation between K influx and $[ATP]_i$ over a wide range of concentrations. The general procedure was similar to that already described for determining the relation between Na efflux and $[ATP]_i$ (Brinley and Mullins, 1968). An axon was subjected to a prolonged "predialysis" with nonradioactive, fuel-free solutions in order to reduce the $[ATP]_i$ to 1–3 μM. The axon was then dialyzed with perfusion solutions containing successively increasing [ATP], and the response of K influx noted. In most of the present experiments, as in the previous ones, 0.5–4.0 mM phosphoarginine (PA) was included in the dialysis medium to maintain a low [ADP] throughout the axoplasm. The usual method employed in an experiment and the results obtained are illustrated in Fig. 2. This axon was given a preliminary immersion in artificial seawater containing 1 mM NaCN for 1 hr to reduce the ATP before the pre-

dialysis was begun. After the predialysis had reduced $[ATP]_i$ to 2 μM, isotope was added outside the axon and the no fuel[1] dialysis continued to obtain a base line. In this experiment, the $[ATP]_i$ was then increased to 75 μM. A prompt increase in K influx occurred. A further increase in [ATP] to 230 μM resulted in a further increase of influx; however, a final increase of [ATP] to 10,000 μM produced no further increase in influx.

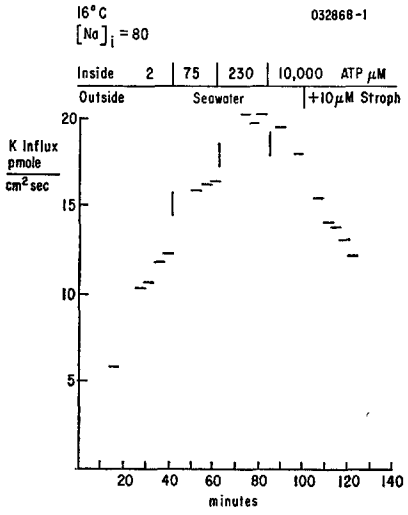

FIGURE 2

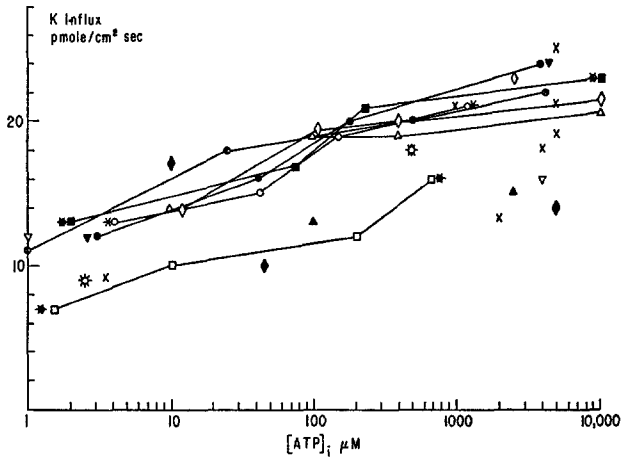

FIGURE 3

FIGURE 2. This shows an experiment in which $[ATP]_i$ was varied from its initial dialysis level of 2 μM to 10,000 μM. The K influx is shown on the ordinate. The outside solution was artificial seawater except for that period of time when 10 μM strophanthidin was added to the seawater.

FIGURE 3. This shows the results from 22 axons when the K influx was measured as a function of [ATP] in the dialysis solution. Single values for axons not measured in a systematic way are shown as points on the graph while the lines connect the range of $[ATP]_i$ measured with respect to K flux on a particular axon.

The results from this and 21 other axons in which the influx was measured at various $[ATP]_i$ are collected in Fig. 3. The graph illustrates several interesting features of the [ATP] dependence of the K influx.

1. An increase in $[ATP]_i$ from 1 to 10 μM produces about 4 f increase of influx, which is about one third of the total ATP-dependent influx observed in normally fueled fibers.

2. The relation between influx and $[ATP]_i$ may be less steep at [ATP] greater than physiological. This effect is apparent in three of the six experi-

[1] This term is used to denote dialysis solutions not containing either ATP or PA. The usual [ATP] in the dialysis effluent was 1–3 μM. The term "fuel" connotes the addition of from 0.5 to 5 mM PA to dialysis fluids containing ATP at various concentrations.

ments in which four or more concentrations were tested in the same axon. However, the effect is not marked and is not reflected in the collected data. It should not be ascribed to a marked increase in [ADP] since in the cases illustrated, PA was included in the dialysis media.

3. There is considerable variability in the absolute value of the influx at any given [ATP]. This variability seems to be the result of variability in the size of the 1 μM ATP component. The data in Fig. 3 have been replotted in Fig. 4 where the residual K influx present at 1 μM ATP has

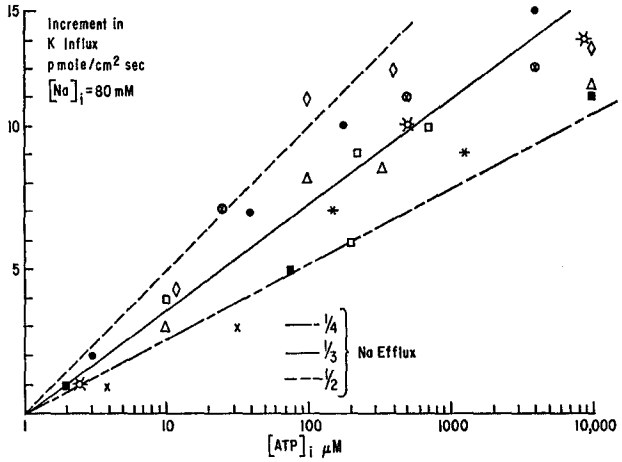

FIGURE 4. The data from Fig. 3 have been transcribed by measuring the increment in K influx from the value measured at or interpolated to 1 μM $[ATP]_i$. The lines drawn are derived from data of Brinley and Mullins (1968) and represent the [ATP] dependence of Na efflux divided by 4, 3, or 2, corresponding respectively to a test of coupling of K influx to Na efflux in ratios given by these numbers.

been subtracted. Fig. 4 shows that the increment in flux at any given [ATP] shows less variation than does the total flux.

Because of the rather steep relation between influx and [ATP] and because we have been unable to reduce the ambient [ATP] below 1 μM, it is not possible at present either to define or to characterize the 1 μM ATP influx accurately. It seems to be larger in smaller axons, those in poor condition, or those with more than the usual number of branches. In the present series of 47 axons, the size of 1 μM ATP influx has varied from 2 to 22 f without any obvious effect on the magnitude of the K pump or its strophanthidin sensitivity. All the measurements of the [ATP] dependence of the K influx are given in Table III.

The results obtained by Caldwell, Hodgkin, Keynes, and Shaw (1960 *a*) suggested that the properties of the K pump with respect to ATP might be different from those of the Na pump because the injection of ATP into squid

TABLE III

POTASSIUM FLUXES IN DIALYZED SQUID AXONS

Axon	Diameter	Duration of experiment	No fuel			Fuel					
			[ATP]	K	K + Na	[ATP]	K	K + Na	K + Na + stroph	K + Na + fuel − stroph	K + Na + fuel − no fuel
	μ	min	μM	f	f	μM	f	f	f	f	f
050467	400[c]	124[1]	—	7	13	2,500*	—	22	—	—	9
051767	650[b]	140[2]	—	—	—	2,500‖	—	13	2	11	—
051867-1	535[a]	113[2]	8	—	—	2,500‖	—	23	13	10	—
051867-2	501[a]	110[2]	—	—	—	4,900‖	—	12	4	8	—
013068-1	872[b]	175[3]	<1	6	12	4,000‖	—	16	11	5	4
013068-2	825[b]	162[3]	—	—	—	2,000‖	—	13	7	6	—
013168	600[b]	69[3]	—	—	—	4,000‖	—	18	—	—	—
022868	600[c]	171[4]	10	4	—	5,000‖	7	21	—	—	—
022968-1	625[b]	168[4]	—	4	—	5,000‖	9	19	—	—	—
022968-2	600[b]	150[4]	—	4	—	5,000‖	7	25	—	—	—
030168	600[b]	168[5]	3	—	12	4,000‖	—	24	—	—	12
032768	550[c]	118[5]	4	—	13	1,250*	—	21	—	—	8
032868-1	575[c]	124[5]	2	—	13	10,000‡	—	20	11	9	7
032868-2	475[c]	160[5]	1	—	7	700‡	—	16	—	—	9
032968-1	600[d]	106[5]	2	7	—	1,000‡	9	21	—	—	—
040368-1	750[c]	155[4]	5	7	—	3,600‖	9	—	—	—	—
040368-2	700[c]	113[5]	3	—	7	—	—	—	(8)	—	—
040468-1	650[c]	124[4]	—	—	—	3,500‡	7	23	—	—	—
040468-2	625[b]	132[4]	—	—	—	3,000‡	6	21	—	—	—
040468-3	625[c]	131[4]	3	8	—	—	—	—	—	—	—
040568	550[a]	112[4]	—	—	—	2,000‡	—	16	—	—	—
040968	575[b]	165[5]	12	—	14	10,000‡	—	21	—	—	7
041068-1	575[b]	102[4]	2	—	9	—	—	—	—	—	—
041068-2	600[c]	102[4]	—	—	—	4,200‡	—	22	11	11	—
041168	550[c]	147[5]	7	—	8	—	—	—	—	—	—
050768	625[a]	132[5]	1	—	8	—	—	—	—	—	—
050868-1	525[b]	50[5]	1	—	7	—	—	—	—	—	—
050868-3	650[b]	100[5]	1	—	2	2,000*	—	16	—	—	14
070968	575[c]	80[5]	—	—	—	2,000‖	—	23	13	10	—
071268-3	530[c]	57[5]	—	—	—	2,000*	—	(25)	(18)	7	—
072668-1	500[d]	128[6]	10	(36)	—	5,000‖	—	(51)	(36)	15	—
072668-2	475[c]	121[6]	10	—	—	5,000‖	—	(42)	(32)	10	—
072768	550[c]	109[7]	12	—	(29)	—	—	—	—	—	—
073068	500[c]	113[7]	9	—	12	—	—	—	—	—	—
073168-1	550[c]	180[7]	4	—	(17)	—	—	—	—	—	—
073168-2	510[c]	107[8]	1	—	(22)	—	—	—	—	—	—
080168	550[d]	138[7]	7	—	(17)	6,400‖	—	(36)	(19)	17	19
080268	475[c]	78[8]	4	—	(18)	—	—	—	—	—	—
080668	550[c]	120[7]	3	—	(15)	—	—	—	—	—	—
101068-1	660[d]	86[9]	—	—	—	3,600‖	—	15	—	—	—
101068-2	500[d]	108[9]	—	—	—	3,600‖	—	—	11	—	—
101168-1	500[d]	100[9]	—	—	—	3,600‖	—	17	12	5	—
101168-2	510[d]	95[9]	—	—	—	3,600‖	—	38	21	19	—
101568-1	495[d]	126[9]	—	—	—	4,000‖	—	(35)	(19)	16	—
101568-2	500[d]	131[9]	—	—	—	4,000‖	—	(33)	(23)	10	—
101568-3	525[d]	116[9]	—	—	—	4,000‖	8	22	11	11	—
101668-1	575[d]	136[9]	4	—	7	4,000‖	—	15	6	9	8
102468-1	550[c]	150[9]	1	—	11	4,000‖	11	21 (26)	(18)	8	10
Mean				6	10		8	19	10	10	10
± SE				0.6	0.8		0.5	0.7	1.0	1.0	1.0
N =				8	16		9	27	13	19	9

* PA = 0.

‡ PA = 500–1000 μM.

‖ PA = 1500–5000 μM.

[a] Temperature = 12.0–13.5°C.

[b] Temperature = 13.6–15.0°C.

[c] Temperature = 15.1–16.5°C.

[d] Temperature = 16.6–18.0°C.

[1] Perfusion solution = E′F′.

[2] Perfusion solution = E′.

[3] Perfusion solution = O′.

[4] Perfusion solution = V, W.

[5] Perfusion solution = V.

[6] Perfusion solution = AC, AD.

[7] Perfusion solution = AB, AC.

[8] Perfusion solution = AC.

[9] Perfusion solution = AE, AF.

Parentheses indicate values not included in the mean.

axons restored Na efflux but not the K sensitivity. It seemed likely that it was the products of ATP hydrolysis, i.e. ADP, AMP, or P_i, that brought about this change since Garrahan and Glynn (1967 *b*) have shown for red cells that high internal phosphate changes Na/K exchange to Na/Na exchange. It seemed worthwhile, therefore, to measure K influx in the presence and absence of ADP. An experiment was done in which the axon was dialyzed with equimolar concentrations of ATP and ADP. The concentration was deliberately set rather high (2 mM) so that axoplasmic enzymes would be unable to

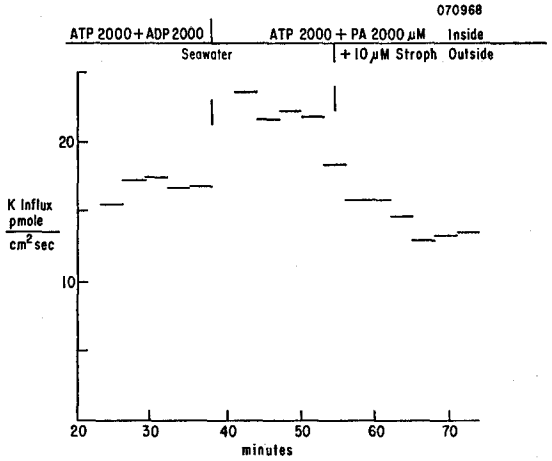

FIGURE 5. K influx is plotted against dialysis time when the internal dialysis solution contained ATP + ADP followed by ATP + PA. Concentrations are given in the legend at the top of the figure. The axon was in artificial seawater throughout except for the period at the end of the dialysis when 10 μM strophanthidin was added to the seawater.

appreciably alter the concentrations supplied. The results are shown in Fig. 5. After the (ATP + ADP) base line had been obtained, the internal medium was changed to (ATP + PA); the first measurement taken after this solution change is somewhat higher than subsequent samples and may be the result of a transiently very high [ATP] from the transphosphorylation reaction ADP + PA → ATP + A. The subsequent base line was quite stable and a change to 10 μM strophanthidin seawater outside the axon allowed one to calculate that the K pump with $[Na]_i$ = 80 mM and $[ATP]_i$ = 2 mM gave a flux of 10 f, while in the presence of 2 mM ADP, the K pump gave a flux of only 5 f. The experiment makes it clear that ADP reduces the amount of K pumping although there is still an appreciable K pumping when $[ADP]_i = [ATP]_i$.

The Influence of Compounds Other than ATP on K Influx

Previous studies (Brinley and Mullins, 1968) have shown that ATP was the only commonly occurring high-energy substrate that would support Na

extrusion in dialyzed squid axons; it seemed important to obtain similar information on the K influx. The method for testing all the compounds was essentially the same and can be summarized as follows. Squid axons were extensively "predialyzed" to remove the naturally occurring high-energy phosphates, specifically, PA, ADP, AMP, and ATP. The predialysis was continued until on-line ATP analysis indicated that the [ATP] in the dialysis effluent was 1–5 μM. The test compound was then added to the perfusion

TABLE IV

EFFECT OF SELECTED HIGH-ENERGY PHOSPHATE COMPOUNDS ON K INFLUX IN DIALYZED SQUID AXONS

Axon	Diameter	[ATP]	[~P]	K influx	Extra K influx	Estimated K flux from ATP	Flux due to ~P	Fueled K influx
	μ	μM	μM	f	f	f	f	f
				Phosphoarginine				
050768	625*	1		8				
		4	2000	12	4	3	1	—
050868-3	650‡	1		2				
		2	2000	3	1	<1	<1	
		7	2000	6	4	3	1	15
072768	550§	12		29				
		15	2000	31	2	1	1	16
				Phospho(enol)pyruvate				
072768	550§	12		29				
		14	2000	31	2	1	1	—
073068	500§	9		12				
		—	4000	15	3	—	3	—
073168-1	500§	4		17				
		8	4000	17	0	—	0	—
				Acetyl phosphate				
050768	625*	1		8				
		3	2000	9	1	0	1	—
073068	500§	9		12				
		6	4000	14	2	0	2	—
				2'-Deoxyadenosine-5'-triphosphate				
073068	500‖	1		12				
		—	2000	20	8	—	8	
073168-1	550§	8		17				
		—	2000	23	6	—	6	
080168	550¶	7		17				
		—	2000	18	1	—	1	19
080668	550‖	3		14				
		—	2000	15	1	—	1	
		—	2000	21	7	—	6	

* = 13°C.
‡ = 15°C.
§ = 16°C.
‖ = 16.5°C.
¶ = 17.5°C.

fluid, and its effect on both the K influx and effluent [ATP] noted. The results of experiments on four high-energy compounds are summarized in Table IV and described in detail below.

PHOSPHOARGININE This compound was studied in the greatest detail because it is the only high-energy compound besides ATP which is present in axoplasm in millimolar concentrations, and because it has been thought to be involved in the relationship between Na extrusion and K uptake in squid axons (Caldwell et al., 1960 *a*). The results of the three experiments

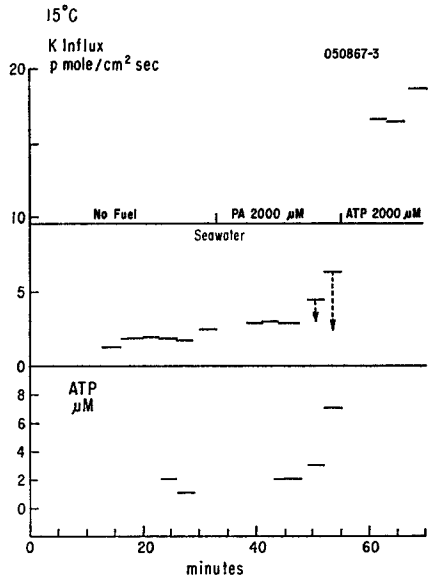

FIGURE 6. This shows the response of K influx to 2 mM phosphoarginine as well as changes in [ATP] in the dialysis effluent. The increment in K influx observed with PA also led to an [ATP] of about 7 μM. The dashed vertical line and arrows indicate a correction for K influx based on the measured sensitivity of K influx to $[ATP]_i$. The final internal solution change was to 2 mM ATP, and this led to a K influx of about 20 f.

are listed in Table IV, and an experiment is illustrated in Fig. 6. Here the addition of a physiological concentration (2 mM) of PA caused an increase of at most 2–4 f of K influx in a well-dialyzed axon. This increase was accompanied by a slight but definite increase in [ATP] from 3 to 6 μM. This increase in ambient [ATP] was usually seen when physiologically occurring phosphorylating agents were dialyzed into the axoplasm and is probably the result of the phosphorylation of small amounts of residual ADP which had not been removed from the axoplasm. In view of the demonstrated sensitivity of the K influx to micromolar concentrations of ATP, it seems justified to subtract from the PA-induced influx increment, the flux increment that can reasonably be thought to result from the ATP generated. This correction is indicated by the dashed vertical lines in the figure and also given in Table IV. When this is done, the increment in K influx attributable solely to PA is at most 1 f. In this axon, the ATP increment, taken as a measure of the magnitude of the K pump, was about 15 f. We conclude therefore that in this

experiment the PA-induced K influx was only about 5 % of that induced by a physiological concentration of ATP. Two additional experiments showed a similar lack of effect of PA and we conclude that PA is not a significant fuel source for K uptake.

PHOSPHO(ENOL)PYRUVATE Three experiments were done to test the ability of millimolar concentrations of phospho(enol)pyruvate (PEP) to support K uptake. The mean increment in influx, above that attributable to ATP, was only 1 f. Since the physiological concentration of PEP is probably in the range of 50 μM, or about 2 % of the test concentration, this compound seems clearly to be excluded as a physiological substrate.

ACETYL PHOSPHATE This compound was tested because of reports (Bond, Bader, and Post, 1966; Bader and Sen, 1966) that acetyl phosphate (AcP) is capable of forming a phosphorylated intermediate with membrane ATPase, and it seemed possible that it might also induce K uptake. However, in two experiments conducted in the same manner as the preceding two series, at most 1–2 f could be attributed to the presence of large concentrations of AcP in the dialysis medium.

2′-DEOXYADENOSINE-5′-TRIPHOSPHATE This substance was tested because of a recent report (Whittam and Wiley, 1967) that deoxyadenosine could support active K transport in intact red cells. The first experiments designed to test the efficacy of this material showed a puzzling variability. Two technically satisfactory experiments indicated that 2′-deoxyadenosine-5′-triphosphate (*d*-ATP) produced about one-half of the increment in K influx that was given by ATP. However, an equally satisfactory experiment, 080168, gave at most an increment of 1 f although the K influx was clearly capable of responding to ATP, and this response was strophanthidin-sensitive. In the first two experiments the preparation was dialyzed first with PEP and then with *d*-ATP; some PEP, of course, remained in the axoplasm during dialysis with *d*-ATP. In the third experiment, however, *d*-ATP was the first compound tested after the no fuel base line had been obtained. The discrepancy between the two types of experiments was resolved by a fourth experiment (080668) in which, after the no fuel base line, the axon was first perfused with *d*-ATP and then with *d*-ATP in the presence of 2 mM PA. This is shown in Fig. 7 from which it is clear that *d*-ATP produced an increment in K influx only when the dialysis was accompanied by PA. Since an increment in K influx was observed when the axoplasm contained *d*-ATP and either PEP or PA, but not otherwise, we conclude that the presence of a transphosphorylating agent is necessary for the effect. This increase of the K influx cannot be ascribed to a nonspecific effect of the compound since it was promptly and completely abolished by 10 μM strophanthidin in all experiments, suggesting,

but by no means proving, that *d*-ATP acts upon the K influx in the same manner as does ATP.

The Effect of Strophanthidin on K Influx

It was noticed rather early in the experimental program that 10 μM strophanthidin seawater reduced K influx in fully fueled axons to the no fuel level. This statement is supported most clearly in the mean values for K influx shown in Table III. Both the no fuel and fuel + strophanthidin values are 10 f.

Because of the peculiar effect that strophanthidin had on Na efflux in increasing its no fuel value from about 1.5 f to 12 f (Brinley and Mullins, 1968), it appeared necessary to see whether this inhibitor had any such

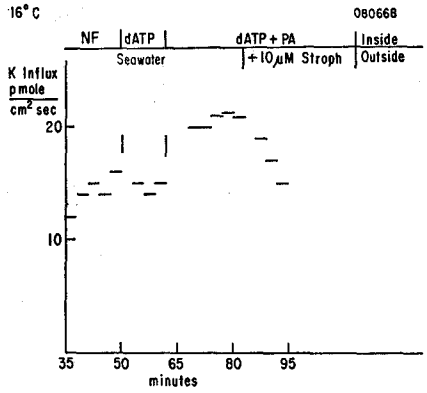

FIGURE 7. This shows an experiment in which *d*-ATP was used as a fuel source for internal dialysis. K influx is shown for NF (no fuel) followed by dialysis with *d*-ATP. The internal dialysis solution was changed to *d*-ATP + PA and the artificial seawater outside the axon was replaced with seawater containing 10 μM strophanthidin.

similar action with respect to K influx which might not be apparent from the mean values given in Table III. The experiment was done by extensively predialyzing an axon so that its $[ATP]_i$ was 3 μM; isotope was then added to the seawater outside the axon and K influx measurements were begun. The seawater was then changed to one containing 10 μM strophanthidin and K influx was followed; a final change was to add fuel to the dialysis fluid (ATP 4 mM + PA 4 mM) and measurements were continued. The experiment is shown in Fig. 8 and it is clear that neither the addition of strophanthidin nor the subsequent addition of fuel had any effect on K influx. Such an experiment, in addition to the many done in connection with other sorts of measurements, makes it quite apparent that strophanthidin does not affect the passive K influx but does totally inhibit (within the accuracy of measurement) the ATP-dependent K influx.

K Influx from Na-Free Seawater

The effect that the removal of Na from seawater might have on K influx was investigated because of recent suggestions (Sjodin and Beaugé, 1968 *a*) that

some substitutes for Na^+ such as Li^+ might compete with K^+ for influx via the K pump; it has also been suggested (Baker et al., 1969) that Na^+ may have direct inhibitory effects on the K pump. An experiment to test these possibilities is shown in Fig. 9. An axon predialyzed with an ATP- and Na-free dialysate was treated with ^{42}K seawater and its K influx measured. The internal solution was then changed to ATP 5 mM, PA 1 mM and influx measurements continued. A further change was to add ATP + PA + Na 80 mM to the internal dialysis fluid and again estimate K influx in Na and Li

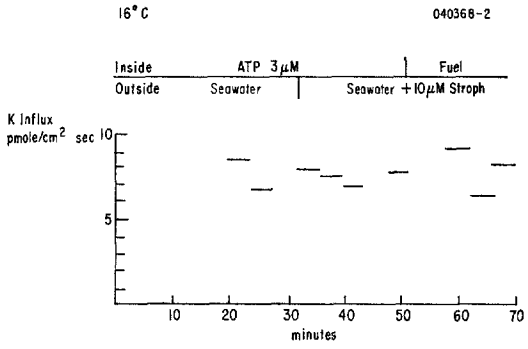

FIGURE 8

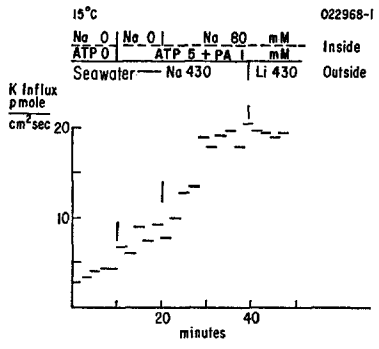

FIGURE 9

FIGURE 8. This shows K influx as a function of time when the axon had been well predialyzed and had an initially low [ATP]. Strophanthidin artificial seawater was applied and no change in K influx was noted. A further internal fluid change was to a fuel of ATP 4 mM + PA 0.4 mM; this treatment was without effect on the K influx.

FIGURE 9. This shows a change in K influx when $[ATP]_i$ is increased from 0 to 5 mM while the $[Na]_i$ is kept nominally at 0. This change was followed by an increase of [Na] to 80 mM and the seawater outside the axon was changed from Na to Li seawater. This change produced no variation in K influx.

seawater containing 10 mM K. It is clear that there is no change in K influx when going from Na to Li seawater. A second similar experiment was made in which choline$^+$ was substituted for Na^+ in seawater but again there was no discernible change in K influx when external Na was removed and replaced by this presumably inert cation. We conclude, therefore, that although both Na and K influx show an ATP dependence (Brinley and Mullins, 1968 and present results), the presence or absence of $[Na]_o$ does not influence the active K influx.

The Effect of $[K]_o$ on K Influx

A knowledge of the way in which K influx responds to $[K]_o$ in seawater is helpful in arriving at ideas about the mechanism of pumping. If K influx were closely coupled to Na efflux, then (at constant $[Na]_i$) the K pump ought

to be half-maximal at about 10 mM $[K]_o$ because Na efflux responds to $[K]_o$ in this manner. A complication with changes in [K] of seawater is that small changes in membrane potential are to be expected when going, for example, from 1 to 20 mM $[K]_o$. In a study of *Sepia* axons, however, Hodgkin and Keynes (1955 *b*) noted that K influx was relatively insensitive to membrane potential; we have also noted that hyperpolarizing current densities of the

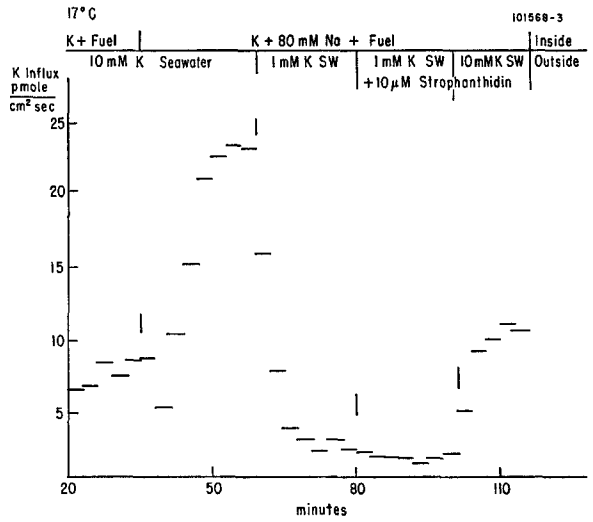

FIGURE 10. This figure shows an experiment in which the K influx was evaluated a two different [K] (10 mM and 1 mM) in seawater. The first step was to measure influx from 10 mM K seawater when the internal medium was K + fuel. The internal solution was then changed to K + 80 mM Na + fuel and a new base line obtained; the next change was to reduce the K in seawater to 1 mM and obtain a fueled K influx at this level. This was followed by the addition of strophanthidin to 1 mM K seawater and a base line for K influx in the presence of strophanthidin was obtained; the final maneuver was to increase [K] in seawater again to 10 mM and evaluate a new strophanthidin base line.

order of 25 μamp/cm^2 produce only a 10–15 % decrease in the K influx into unfueled axons. The membrane potential changes to be expected in going from 1 to 20 mM $[K]_o$ are far less than that produced by the hyperpolarization cited above so that an error in the estimate of passive K influx appears unlikely. In any case, the K influx of no fuel axons was measured at each of four experimental concentrations of K in seawater, namely 1, 3, 10, and 20 mM. Experiments were usually done by measuring influx in a fuel-free axon at two of the experimental concentrations given above; the internal dialysis medium was then changed to one containing fuel and measurements at the two concentrations were repeated. An experiment was usually concluded by

adding 10 μM strophanthidin to the seawater as a check on the stability of the passive influx. Measurements of the sort described above are shown in Fig. 10, while collected data on the axons used are given in Table V and Fig. 11. This curve was constructed by subtracting the K influx at 1 μM ATP from that measured at a particular $[K]_o$ when the dialysis fluid contained $[ATP]_i$ 4 mM, PA 0.4–4.0 mM; $[Na]_i$ was 80 mM in all the measurements made. This ATP-dependent K influx is expressed relative to the influx at 10 mM $[K]_o$. One point emerges from the measurements made; K influx is approximately linear with $[K]_o$ over the range from 1 to 20 mM.

TABLE V

EFFECT OF $[K]_o$ ON K INFLUX

Axon	ASW (1 K) Fuel	ASW (1 K) NF* or S‡	ASW (3 K) Fuel	ASW (3 K) NF or S‡	ASW (10 K) Fuel	ASW (10 K) NF or S‡	ASW (20 K) Fuel	ASW (20 K) NF or S‡	K pump 1 K	K pump 3 K	K pump 10 K	K pump 20 K
					pmoles/cm²sec							
072668-1§	—	—	—	—	51	36	102	70	—	—	15	32
072668-2§	—	—	—	—	42	32‡	93	64	—	—	10	29
101568-1‖	5.6	3.2‡	—	—	35	19‡	—	—	2.4	—	16	—
101568-2‖	4.2	3.2‡	—	—	33	23‡	—	—	1.0	—	10	—
101568-3‖	2.7	1.8‡	—	—	22	11‡	—	—	0.9	—	11	—
040869‖	—	—	—	—	30	17‡	60	39‡	—	—	13	21
040969-1‖	—	—	—	—	20	8‡	42	17‡	—	—	12	25
040969-2‖	—	—	10	7‡	36	24‡	—	—	—	3	12	—
041069‖	—	—	6	2	22	8	—	—	—	4	14	—
Mean									1.4	3.5	13	27
± SE									0.5	—	1	3
N									(3)	(2)	(9)	(4)

* No fuel $[ATP]_i$ 1–4 μM.
‡ 10 μM strophanthidin seawater.
§ Dialysis solution AC,AD.
‖ Dialysis solution AE.

Potassium Influx As a Function of $[Na]_i$

Measurements of the dependence of K influx on $[ATP]_i$ were obtained at a fixed $[Na]_i$ of 80 mM. It has been observed that K influx is increased in axons in which $[Na]_i$ has been increased (Sjodin and Beaugé, 1968 *a*), so it appeared important to examine the dependence of the ATP-sensitive K influx on $[Na]_i$.

An experiment is shown in Fig. 12 in which an axon was first predialyzed with a medium nominally free of both Na and ATP. Isotope was then added to the seawater outside the axon and the Na- and ATP-free base line of about 6 f obtained. Next, ATP and PA were added to the Na-free dialysate and the

influx rose promptly to about 8 f. Subsequent addition of 8 mM Na resulted in a K influx of about 12 f. A final experimental maneuver, that of increasing the Na to 20 mM, gave an influx of about 17 f.

The results of four experiments of this type are collected in Fig. 13. Also included in the figure are the mean values for a number of experiments with either 0 or 80 mM $[Na]_i$. The data suggest that K influx increased more than

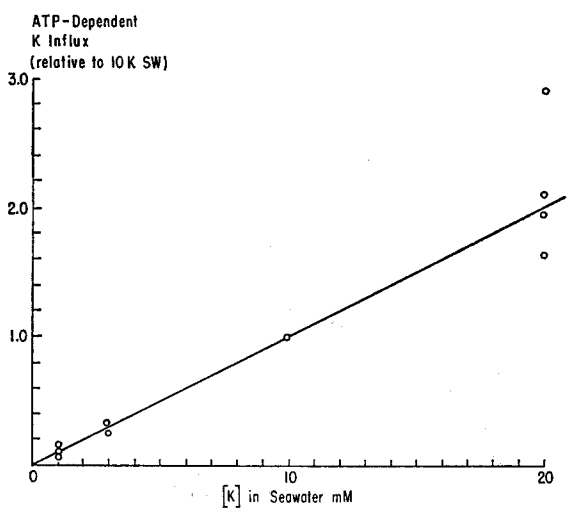

FIGURE 11

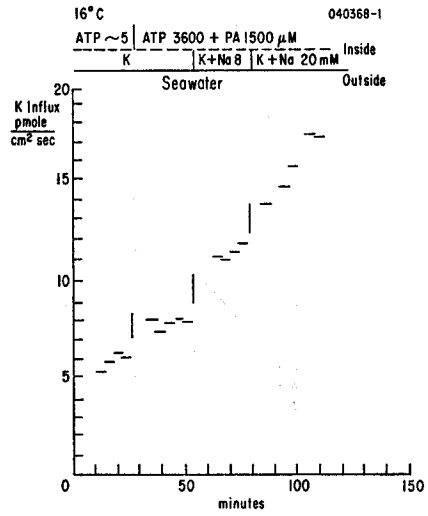

FIGURE 12

FIGURE 11. This curve shows the collected data on K influx *vs.* $[K]_o$ in seawater. The ordinate is the ATP-dependent K influx plotted relative to 10 mM K artificial seawater and the points show the measurements that have been made at 1, 3, and 20 mM [K] relative to 10 mM K. Each point is a comparison between 10 mM K and the test [K]. The point at 10 mM K represents nine separate influx measurements. The data for the calculation of the K pump flux are given in Table V.

FIGURE 12. This shows a dialysis which evaluated the effect of 8 and 20 mM $[Na]_i$ on an axon. The exact sequence of solution changes and the reasons therefore are given in the text.

linearly with $[Na]_i$, but there is insufficient information to indicate the exact form of the relation. Of greater relevance is the dependence of the metabolic component of the influx upon $[Na]_i$. This component could be calculated by subtracting the 1 μM ATP K influx from the total influx. However, in view of the substantial variation of the ATP-independent component at low $[Na]_i$, the result would be of dubious significance. A second series of experiments was therefore undertaken in which the ATP-dependent component was determined directly at two different $[Na]_i$ (20 and 80 mM) in the same axon. The $[K]_o$ was 10 mM in all cases. The experiments were begun by comparing fuel-free K influx at one $[Na]_i$ with that at another. Fuel was then added to

the dialysis fluid and measurements again made at the same two values of $[Na]_i$. The experiment was terminated by adding strophanthidin to the seawater as a final check on the fuel-free base line. The results of these measurements are shown in the insert to Fig. 13 and are collected in Table VI.

One conclusion from experiments done as outlined above is that a $[Na]_i$ of 20 mM produces 70 % of the ATP-dependent K influx that is given by $[Na]_i$ = 80 mM. If the increment in K influx were proportional to $[Na]_i$, the value would be only 25 %. Even if one excludes the Na-independent, ATP-depend-

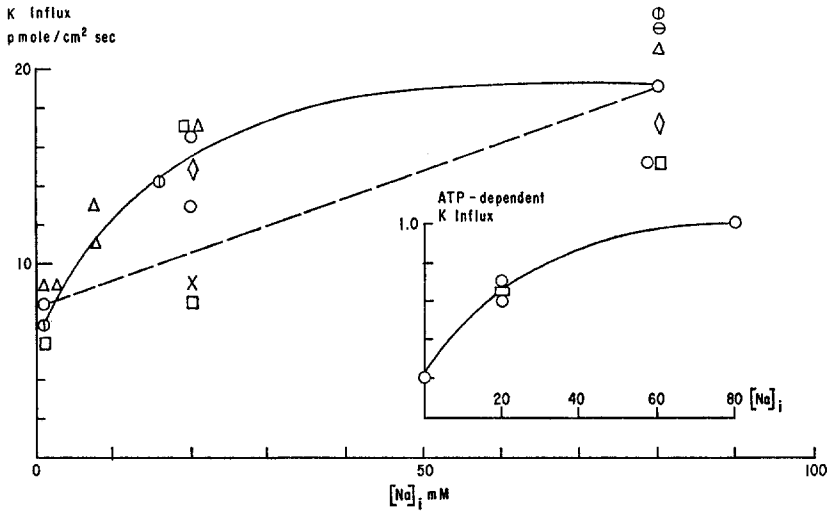

FIGURE 13. Collected data on the effect of $[Na]_i$ on K influx are given in this graph. The ordinate of the large graph is the measured K influx and the abscissa $[Na]_i$. Na artificial seawater was the external medium in all the experiments shown. The insert shows the ATP-dependent influx relative to $[Na]_i$ = 80 mM. The results included are from the three experiments that were especially done in order to evaluate both the passive and the active components of the K influx at two particular $[Na]_i$.

ent influx, the percentage increase predicted on the basis of linearity would be only 45 %. Thus, this second set of experiments confirms the collected data shown in Fig. 13; K influx is not a linear function of $[Na]_i$. The significance of this result with respect to Na/K coupling will be examined in the Discussion.

The Effect of ATP on K Efflux

Some experiments were done in which K efflux was measured under no fuel conditions and then the internal dialysis solution was changed to $[ATP]_i$ 2.5–4.0 mM and efflux measurements continued. An experiment of this sort is shown in Fig. 14 where it is clear that there is a decrease in K efflux of about 20 f when a physiological concentration of ATP is applied. Four such experiments were done and the mean decrease in efflux was 15 f. In some of these

experiments, 10 μM strophanthidin was added to the seawater outside the axon at the end of the experiment when $[ATP]_i$ was high; other experiments were done by adding strophanthidin to an axon dialyzed with high $[ATP]_i$ from the start; four such experiments gave a mean increase of K efflux of 15

TABLE VI
EFFECT OF $[Na]_i$ ON POTASSIUM INFLUX
(pmoles/cm²sec)

	$[Na]_i$ = 20 mM		$[Na]_i$ = 80 mM		K pump		Column 6
Axon	F*	NF or S‡	F*	NF or S‡	$[Na]_i$ = 20 mM	$[Na]_i$ = 80 mM	Column 7
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
101068-1	8	—	15	—	—	(<7)	—
101068-2	17	10‡	—	11‡	7	—	—
101168-1	15	—	17	12‡	3	5	0.6
101668-1	13	7	15	7‡	6	8	0.8
102468-1	17	11	(21)§		6	—	—
			26§	18	—	9	0.7
Mean					5.5	7	0.7

* Fuel ATP 4 mM, PA 4 mM.
‡ 10 μM strophanthidin seawater.
§ Axon showed base line drift during experiment. Influx = 21 pmoles/cm²sec immediately after dialysis with solution AE + fuel. Influx = 26 pmoles/cm²sec immediately before poisoning with strophathidin.

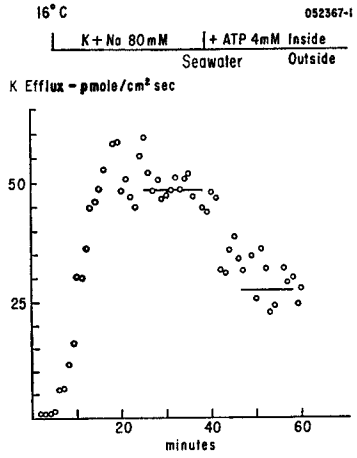

FIGURE 14. K efflux was evaluated in this axon after predialysis had reduced $[ATP]_i$ to 9 μM. The curve shows the response of K efflux to the subsequent addition of 4 mM ATP to the dialysis fluid.

f. It would appear, therefore, that there is an ATP-dependent K efflux in squid axons.

An increase in K efflux with strophanthidin is seen in sartorius muscle (Harris, 1957; Sjodin and Beaugé, 1968 *b*) and in barnacle muscle fibers

(Brinley, 1968), while an increase with low ATP has been noted in *Sepia* axons (Hodgkin and Keynes, 1955 *a*) and in squid axons during hypoxia (Shanes and Berman, 1955). Some caution must be exercised in interpreting these results as a demonstration of carrier-mediated K efflux because Hodgkin and Keynes (1955 *b*) have shown that K efflux in *Sepia* axons is very sensitive to membrane potential and K efflux varies *e*-fold for an 8 mv change in E_m. It is possible, therefore, that the changes observed in K efflux in the presence

TABLE VII

EFFECT OF 10 μM STROPHANTHIDIN ON Na INFLUX IN INTACT SQUID AXONS

Axon	Diameter	Temperature	Uptake time	Rinse time	Axoplasm mass	Preincubation in glycoside	Na influx	
							Stroph 10 μM	Control
	μ	*°C*	*min*	*min*	*mg*	*min*	*pmoles/ cm² sec*	
062768-2	430	5-7	28	8	4.3	8	22	—
062768-3	495	5-7	22	9	4.6	—	—	34
062868-1	500	23	22	9	3.3	—	—	40
062868-2	462	23	23	13	3.1	11	39	—
062868-4	580	23	28	9	5.7	—	—	30
062868-5	600	23	28	8	4.3	10	44	—
062868-6	561	23	28	11	4.8	—	—	38
071368-1	450	25	25	6	3.3	—	—	29
071368-3	450	25	34	6	3.8	—	—	37
071368-4	450	25	28	8	3.6	0	24	—
071768-2	460	25	25	7	2.0	—	—	42
071768-3	440	25	24	6	1.8	7	37	—
071868-1	490	26	27	7	2.3	—	—	30
071868-2	460	26	26	7	3.3	10	36	—
071868-3	460	26	25	7	3.5	—	—	42
Mean							34	36
± SE							4	2
N							(6)	(9)

of ATP and strophanthidin are reflections of small changes in E_m induced by the Na extrusion which is a concomitant of increasing $[ATP]_i$. The results we have must thus be regarded as provisional, pending a further study in which membrane potential is controlled.

The Effect of Strophanthidin on Na Influxes in Intact Axons

For reasons which will be considered in the Discussion, it became desirable to determine whether Na influx was affected by strophanthidin. Accordingly a number of influx measurements using ^{24}Na and the extrusion technique were made on intact axons which had been sparingly cleaned except at the

big end. The experiments were performed on five different batches of squid and the results are shown in Table VII; these show that 10 μM strophanthidin was without effect on Na influx, although this concentration is far in excess of that producing maximal action on both Na efflux and K influx (Baker et al., 1969). All except one of the test axons were given a preliminary soak in nonradioactive artificial seawater with strophanthidin in case there was any delay in the onset of inhibition. Most of the experiments were done at 23–26°C which is considerably above our usual experimental temperature of 13–17°C. This was done deliberately on the assumption that the hypothetical strophanthidin-sensitive Na influx represented some sort of metabolic process and hence might have a large temperature coefficient. Most of the determinations were made using paired axons, although in our experience the major cause of variability in Na influx is the trauma of cleaning which varies greatly from axon to axon.

DISCUSSION

Potassium Flux Balance in Dialyzed Squid Axons The axons dialyzed during July and August, 1968 had, with one or two exceptions, ATP-independent components of the K influxes which were two to five times larger than the comparable fluxes of axons taken from winter or spring squid, although the size of the ATP-dependent component was the same. A similar variation exists in the experiments performed in October, 1968, in which three of the nine axons had rather large influxes. The axons used during the July-October series of experiments consistently had far more than the usual number of small branches and it seems not unreasonable to attribute the higher fluxes to leakage of K into or out of the branches. Such a "leakage" flux might be expected to occur independently of any metabolic component and to be essentially an artifact of the preparation. It did not seem necessary to eliminate these axons entirely from Table III because the size of the metabolic component and its response to strophanthidin (e.g. experiment 080168) agreed well with the data obtained on the other axons. We have not, however, averaged any ATP- or strophanthidin-independent components which were more than 15 f. The following discussion applies only to the selected data unless otherwise noted since these results are derived from axons which we believe are more nearly comparable to our previously published data on Na fluxes.

Mean values for K influx in intact axons have been collected from published sources and are listed in Table VIII along with mean values for the winter-spring series of dialyzed axons. Potassium influx in dialyzed axons is very close to that in intact axons, and more importantly, the metabolically dependent component is, with one exception, within 20 % of the ATP-sensitive component in dialyzed axons.

Values for K efflux obtained in the winter-spring series of dialyses (34 f) also seem to agree rather well with values obtained on injected axons (Caldwell and Keynes, 1960, 38 f; Brinley and Mullins, 1964, 42 f, unpublished). The present values are about one-third less than the previously published mean value of 59 f for dialyzed axons (Brinley and Mullins, 1967). Although the earlier series of experiments did have some ATP included in the dial-

TABLE VIII
LITERATURE VALUES FOR K INFLUX

Reference	Temperature	K influx					Pump flux	
		Control	CN	DNP	Glycoside	NF*	T°C	17°C‡
	°C	pmoles/cm² sec						
Caldwell et al. (1960 *b*)§, ‖	18	24		11			13	12
Caldwell et al. (1969 *a*)§, ‖	17–19	19	9				10	9
Caldwell and Keynes (1960)§, ‖	11–18	16						
Tasaki (1963)¶, **	20					2–5		
Brinley and Mullins (1965)‖, ¶	5–10	15						
Sjodin and Beaugé (1967)‖, ¶	17.5	29	5				24	24
Baker et al. (1969)§, ‖	18–20	34			16		18	14
This paper‖, ¶	15.5	19			7		12	14
¶, ‡‡	13–17	19			9	9	10	12
Mean								14
± SE								2
N								(6)

* No fuel.
‡ Corrected to 17°C using $Q_{10} = 3$.
§ *Loligo forbesi*.
‖ Extrusion.
¶ *Loligo pealei*.
** Perfusion.
‡‡ Dialysis.

ysis medium, excess magnesium was not added. The efflux measurements are therefore more comparable to the present no fuel series which does in fact average 49 f.

Not only the absolute size of the efflux but also the response of the K efflux in dialyzed squid axons to ATP is in qualitative agreement with other data on cephalopod axons.

In dialyzed squid axons the ratio fueled/unfueled efflux is about 0.65 whereas in *Sepia* axons, the ratio of [DNP-poisoned efflux]/[control efflux] is about 0.6 (Hodgkin and Keynes, 1955 *a*), and in one squid axon the ratio of control/cyanide flux was about 0.9 (Caldwell et al., 1960 *b*, Fig. 19). Qualitative

agreement is about all that can be expected because the range of ATP and ADP concentrations probably differed widely in the various experiments.

A flux balance based on previous data on the Na fluxes (Brinley and Mullins, 1968) together with the results obtained for the K fluxes is shown below.

Flux	$[ATP]_i$, μM	
	~1	~4000
Na (in)	43	71
K (in)	10	20
Sum (Na + K)	53	91
Na (out)	1	48
K (out)	49	34
Sum (Na + K)	50	82

It appears that there is no large uncompensated net charge transfer across the preparation either with or without ATP and that the dialyzed squid axon resembles intact and injected squid axon in slowly gaining Na and losing K (Steinbach and Spiegelman, 1943; Shanes and Berman, 1955; Keynes and Lewis, 1951). These conclusions accord with what is known concerning the net fluxes of other ionic species across the membrane of isolated squid axons.

There is an interesting point with respect to the K fluxes which appear in the tabulation above. Under no fuel conditions, the ratio: K efflux/K influx is 5, a value similar to that found by Hodgkin and Keynes (1955 *b*) in their study of the "long pore effect." If we are correct in assuming that part of this K efflux is carrier-mediated, then the correct value for the flux ratio is obtained by reading K influx at 1 μM $[ATP]_i$ and K efflux at 4000 μM $[ATP]_i$; in this case the ratio is 3, a value very close to that calculated by assuming $E_m = -60$ mv, and calculating E_K for our experimental conditions as -90 mv.

It is not obvious whether the inequality in K fluxes is due to a deficient operation of the ATP-sensitive component of the influx or to an increase in permeability of the membrane to K presumably caused by the unavoidable trauma of dissection and cleaning. The present experiments argue against inadequate fueling of the ATP-sensitive component as an explanation for the imbalance because even in the presence of 10 mM ATP (plus phosphoarginine), which is at least two to three times the normal concentration in axoplasm, the ATP-sensitive component was no more than 20 f, whereas about 30 f is required to bring the fluxes into balance. A comparison of the K fluxes in the July-October series with those in the winter-spring series shows that the 1 μM ATP portion of the influx may vary over a factor as great as 5 while the ATP-dependent component remains very nearly the same. This observation would be easily explained if the K influx had a relatively constant metabolic com-

ponent of about 10–15 f in parallel with a passive leak resulting from a highly variable K permeability.

One way of explaining the net loss of K and gain of Na in dialyzed axons is to suppose that the K transport system has become less than normally selective and carries in about twice as much Na as K (i.e., 20 f of Na and 10 f of K). Under this hypothesis, the K influx with ideal physiological conditions would be about 20 + 10 = 30 f and the Na influx would be about 73 − 20 = 53 f. Not only would this hypothesis bring both the Na and K fluxes into approximate balance, but also it would provide a simple explanation for the ATP-dependent Na influx which is about 30 f in dialyzed squid axons (Brinley and Mullins, 1968), and which could be regarded as Na being carried inward on the sites which are normally exclusively K carriers.

There are, however, several pieces of evidence against this argument:

1. Potassium influx is independent of external sodium. As has already been shown (see Fig. 9) K influx is not changed by the replacement of external Na by either Li or choline. If the deficient K influx results from Na being transported on the K carriers, one would expect that removing the competing ions, i.e., Na, would facilitate K transport.

2. Potassium influx in intact isolated axons and dialyzed axons is the same. Inasmuch as the strophanthidin-sensitive component of the K influx is the same size in intact axons as in dialyzed fibers, the postulated deficient K transport cannot be ascribed to the dialysis procedure per se.

3. Sodium influx is insensitive to strophanthidin. Results have already been presented (see Table VII) to indicate that the Na influx in intact isolated squid axons is unaffected by external strophanthidin, although the hypothesis outlined above would require that the Na influx contain a component about 20 f in magnitude carried inward on the K carriers which are known to be strophanthidin-sensitive.

None of these three arguments is crucial evidence against the perverted carrier hypothesis, but as our knowledge of the factors responsible for the ion selectivity of the Na and K pumps is fragmentary at best, it seems safest to consider that the ATP-sensitive Na and K influxes take place via separate mechanisms.

The Dependence of K influx on Substrate Two experimental findings argue very strongly for the view that ATP is the normal energy source for the K pump in squid axons. First, no other substance tested (with the exception of *d*-ATP) showed any real ability to energize K uptake and second, the concentration dependence of K uptake on ATP showed a shape very similar to that for the effect of ATP on Na efflux. Taken together, these findings suggest that ATP is the fuel source for both the Na and K pumps; were it not for other results, they would provide strong support for the idea that the Na and K pumps are coupled at a constant ratio of 3 Na/1 K.

Figs. 3 and 4 make it clear that very low $[ATP]_i$ of the order of 30 μM is sufficient to yield half of the ATP-dependent K influx found at physiological [ATP] of 2–4 mM and that detectable increases in this flux can be noticed at far lower [ATP]. The figures also emphasize that while the presumably passive K influx may fluctuate considerably, the ATP-dependent increment in K influx is much more constant.

The substrate requirements of the Na and K pumps are different in the following respects. Experiments with injected axons (Caldwell et al., 1960 *b*) showed that a large Na efflux resulted from the introduction of ATP into axons that had been pretreated with CN^-, but that such axons did not show a decrease in Na efflux when the bathing seawater was made K-free. The injection of PA, on the other hand, also led to a large Na efflux but this efflux did show a decrease when K-free seawater was applied. In dialyzed axons (Brinley and Mullins, 1968) we have shown that ADP + AMP yields a small increase in Na efflux which can be accounted for by the measured $[ATP]_i$ which is generated from these substances by the adenylate kinase reaction. The experiments described in the present paper have shown that when $[ADP]_i = [ATP]_i$ there is a large inhibition of K influx. The experimental results cited above can be summarized by saying that the magnitude of the Na efflux is dependent on $[ATP]_i$ and largely independent of the breakdown products of ATP hydrolysis such as ADP (De Weer, unpublished data). Potassium influx, on the other hand, appears to be inhibited by ADP.

There is a small but easily measurable increase in K influx with an increase in $[ATP]_i$ when $[Na]_i$ is nominally zero. This component, with a magnitude of 2–3 f, can be considered within three different frameworks. First, it would be the result of an appreciable $[Na]_i$ that has not been allowed for in the measurements made. Such measurements as we have made during dialysis have shown that nominally Na-free solutions might actually have had a [Na] of about 1 mM when they passed through the internal dialysis system. Since our data show that an $[Na]_i$ of 20 mM gives about 70 % of the pumped K influx that is found at $[Na]_i = 80$ mM, it might be reasonable to suggest that the curve relating K influx to $[Na]_i$ is exceedingly steep and 1 mM $[Na]_i$ gives 20–30 % of the activation that is observed at 80 mM $[Na]_i$. There is a second possibility that Na diffusing from the outside of the membrane increases the local [Na] in a region inside that is concerned with activating the K influx; in this case the actual [Na] could be much greater than 1 mM.[2] A third possibility is that the K pump

[2] A somewhat analogous explanation has been offered for the residual Na efflux seen in K-free solutions. In this situation it is thought that K^+ accumulate in the Schwann cell cleft and thus prevent the attainment of truly K-free conditions outside the axolemma. However, there is no obvious structural element on the inside of the axolemma to act as a diffusion barrier. Furthermore the rapidity with which radioactive Na appears on the outside of squid axons following either injection or dialysis argues against any significant diffusion barrier on the inside of the axon.

has a basal rate of operation under $[Na]_i$-free conditions that is 20–30 % of its maximal rate. This would be similar to the situation for the Na pump in which a Na flux that is about 30 % of its maximal rate at $[K]_o$ = 10 mM is generated under K-free conditions. We might then describe this phenomenon as an Na_i-free effect, complementary to the better known K_o-free effect.

The effect of *d*-ATP (in the presence of PA) is that of yielding a K influx 70 % of that given by an equal concentration of ATP itself. A similar finding with respect to Na efflux has been made (Brinley and Mullins, 1968). A marked difference between *d*-ATP and ATP is that *d*-ATP is apparently much more sensitive to the presence of small concentrations of ADP in axoplasm than ATP is. This is inferred from the finding that while ATP alone is capable of supporting a normal magnitude of K flux via the K pump, *d*-ATP yields a somewhat smaller pump flux but only if a compound such as PA is present to phosphorylate any residual ADP.

There are suggestions in the literature which indicate that cation transport in other tissues may have components that do not require ATP. In human red cells, Garrahan and Glynn (1967 *a*,*b*) have demonstrated that about one-third of the total Na efflux is insensitive to ouabain and also to the absence of ATP since the ouabain-insensitive component persists in resealed red cell ghosts which have been washed free of ATP. Since the flux ratio calculation of the electrochemical gradient for Na indicates that no more than a few per cent of the Na efflux can be passive, one is forced to conclude that almost half of the normal Na efflux in red cells is fueled by an energy source other than ATP, or some other readily dialyzable high-energy compound.

Hoffman and Kregenow (1966), using a combination of ouabain and ethacrynic acid to inhibit Na efflux in human red cells, have also concluded that about 20–25 % of the Na pump is unlikely to be driven by ATP.

Strophanthidin Effects on Na and K Fluxes The results obtained in this study probably support equally well two rather different assumptions regarding Na/K transport. In the first, Na efflux is coupled to K influx with a variable coupling ratio, while in the second, two entirely separate ion pumps operate from a common energy source (ATP).

The results obtained by studying the dependence of the K influx on $[ATP]_i$ suggested a very close relationship between Na and K pumping, while the inclusion of ADP in the internal dialysis fluid showed this substance to have a differential effect largely on the K pump. In an attempt to explore further the similarities and differences between the ATP-dependent K influx and Na efflux, strophanthidin was used and its effects on K influx were examined.

The action of strophanthidin on the ATP-dependent Na efflux and K influx is the same in the sense that these fluxes are both abolished[8] by the inhibitor.

[8] Since we have defined the Na pump in terms of the increment in Na efflux produced by a change in $[ATP]_i$ from 1 to 4000 μM, it is clear that strophanthidin abolishes the pump defined in this manner although it does not abolish Na efflux.

In the case of Na efflux, however, a new Na efflux, insensitive to [ATP], is induced by strophanthidin (Brinley and Mullins, 1968) while K influx is unaffected. The measurements of Na influx in intact axons that we have made show that Na influx is unaffected by strophanthidin; this finding rules out a change in P_{Na} as an explanation for the increased Na efflux observed in ATP-free axons when strophanthidin is applied. The provisional measurements we have made of the effect of strophanthidin on K efflux suggest that it increases K efflux in fueled axons by about 15 f. This conclusion is rather tentative because, as explained in the Results section, it is not clear whether this result is to be ascribed to a small membrane potential change or to a carrier-mediated component of K efflux.

The results with strophanthidin have some bearing on the stability of the ATP-dependent K influx in that this substance was usually applied at the end of a dialysis that might have lasted 150 min or more. The stability of the fueled K influx is considerable and in no experiment was there a suggestion that it might decline with time. The application of strophanthidin permitted one to evaluate passive influx at the end of a long dialysis and so to make certain that the apparent stability was not a fortuitous increase of leakage and a compensating decrease of the metabolic component. Out of 44 axons studied, all but 4 gave the usual response to dialysis with ATP. In two of these four cases there were reasons to believe that the axons had inadvertently come in contact with strophanthidin.

The results obtained also argue against the need for any dialyzable cofactor in the operation of the K pump. The sole internal requirement for obtaining some K pumping is the presence of MgATP, while optimal K pumping is obtained by the further addition of Na. If there were a further requirement for a cofactor, this ought to have suffered a large reduction in internal concentration during the dialysis—no effect on K influx was observed, however. In this respect K influx behaves differently from the K-free effect on Na efflux, which is invariably smaller at the end than at the start of an experiment.

The Effect of $[K]_o$ *on K Influx* The concentration dependence of ATP-sensitive K influx appears to be linearly related to $[K]_o$ (1–20 mM) when $[ATP]_i$ and $[Na]_i$ are high. In this respect K influx resembles Na efflux which also shows a linear concentration dependence on $[Na]_i$. On the other hand, there is evidence that Na efflux is strongly dependent on $[K]_o$ and that K influx is highly dependent on $[Na]_i$. The main point worth some discussion is the relationship between $[K]_o$ and active K and Na transport. If the dependence of Na efflux on $[K]_o$ is a reflection of a constant coupling between Na and K transport, a different concentration dependence for the two processes is impossible. A further point is that the promotion of Na efflux by $[K]_o$ is maximal at lower $[K]_o$ in various Na-free media (Baker, 1968) so that for values of $[K]_o$

less than saturating, the expected effect of Na-free external solutions would be to increase Na efflux. Constant coupling would then require an increase in K influx. Our experimental findings are, however, that choline seawater does not increase K influx as compared with Na seawater. Thus both the effects of varying $[K]_o$ and of Na-free solutions are different when Na efflux and K influx are considered. These results suggest that $[K]_o$ may exert a dual action on Na/K transport which might be described as: (*a*) K^+ are transported inward by a carrier-mediated mechanism that is far from saturated even at $[K]_o$ = 20 mM and (*b*) K^+ exert a catalytic action in controlling the rate of Na transport.

Baker et al. (1969) have measured K influx vs. $[K]_o$ on axons by extrusion of axoplasm. These results agree with ours for the range of $[K]_o$ = 1 to 10 mM. At 20 mM $[K]_o$ we find on the basis of four experiments that the ATP-dependent K influx rises proportionally to concentration while Baker et al. find at 30 mM $[K]_o$ a less than proportional increase in K influx.

TABLE IX

A COMPARISON OF THE Na^+ and K^+ PUMPS IN SQUID AXONS AT 16°C

Property	K^+ pump flux	Na^+ pump flux
$[K]_o$ 1–20 mM	Linear	S-shaped curve with half-maximum ∽7 mM
$[Na]_o$ 0–430 mM	No effect (Li^+, choline$^+$)	Increase (choline$^+$)
$[Na]_i$ 10–80 mM	Rises more steeply than linear	Linear
$[ATP]_i$ 1–4000 μM	Half-maximal ∽30 μM	Half-maximal ∽30 μM
$[ADP]_i$ = $[ATP]_i$	Strongly inhibited	No effect
Strophanthidin 10 μM	∽0	12 f
Magnitude	10 f	40 f

$[ATP]_i$ = 4, $[Na]_i$ = 80, $[K]_i$ = 355, $[Na]_o$ = 430, $[K]_o$ = 10 mM. Dependence of the pump flux on variations of each of these parameters individually is given above.

The Effect of $[Na]_i$ on K Influx Some considerable effort was devoted to working out the relationship between K influx and $[Na]_i$ at high $[ATP]_i$ because data for Na efflux vs. $[Na]_i$ have already been obtained (Brinley and Mullins, 1968) and the way in which K influx depended on $[Na]_i$ might afford some clue as to the mechanism of ion pumping. The notion that there is a fixed coupling between Na efflux and K influx would require that K influx show the same concentration dependence on $[Na]_i$ as does Na efflux but our results show that this clearly is not the case. As an example, K influx at $[Na]_i$ = 20 mM has been shown to be 70 % of that when $[Na]_i$ = 80 mM or about 7 f. Na efflux at 20 mM $[Na]_i$ is 10 f so that the two fluxes are roughly comparable, while at 80 mM $[Na]_i$, about 3–4 Na are extruded per K taken up. In addition, some of the Na efflux at $[Na]_i$ = 20 mM may not be Na/K exchange since we have noticed that Na efflux at low $[Na]_i$ is reduced when Li seawater replaces

Na seawater; this reduction in efflux is not observed when $[Na]_i$ = 80 mM. If some of the Na efflux at $[Na]_i$ = 20 mM were Na/Na exchange, the Na/K coupling ratio would then be less than unity. Baker et al. (1969) have suggested that Na/K coupling is 2/1 at normal $[Na]_i$; they used, however, the ouabain-sensitive Na efflux as a measure of the Na pump while our results show that this flux is only (40-12)/40 = 70 % of that of the Na pump. When this correction is made to their data, the observed coupling ratio is raised to 3/1. The differences between the Na and K pumps in squid axons are summarized in Table IX. A point which this table emphasizes is that the pump fluxes are linear with either Na or K concentration under our experimental conditions; the activating effects of $[Na]_i$ on the K pump and of $[K]_o$ on the Na pump are nonlinear curves that show saturation and both are half-maximal at about 7–10 mM. The shape of the curve relating $[K]_o$ to Na efflux is dependent upon the nature of the cations in the seawater outside the axon, and it has been reported (Baker, 1968) that in Na seawater this curve is S-shaped, while in choline seawater the curve is hyperbolic. It has usually been assumed that the explanation for the K-free effect (that is the reduction in Na efflux when the external solution is made K-free) is that because the movements of Na out of and K into the fiber are coupled, an absence of external K will not allow outward movement of Na. The measurements we have made, however, show that there are a number of differences between the K-free effect and the K pump and these are shown in Table X. The conditions shown below the dotted line have to do with fuel supply to the ion transport mechanism and may involve only slight differences. There is the possibility that when $[ADP]_i = [ATP]_i$ the K-free effect is totally abolished. (This is inferred from the data of Caldwell et al., 1960 *b*, and of De Weer, unpublished data.) However, our measurements show that there is an easily

TABLE X

A COMPARISON OF THE K PUMP AND THE K-FREE EFFECT IN SQUID AXONS

Condition	K pump flux	K-free effect on Na efflux
Na-free seawater	No effect	Markedly reduces $[K]_o$ necessary for full activation (choline)
Concentration dependence on $[K]_o$	Linear	S-shaped curve in Na seawater; hyperbolic in choline seawater
Stability with time	Stable	Tends to decrease markedly during experiment
Magnitude	Uniform (10 f ± 1)	Variable (0.25–1.0)
Strophanthidin	Abolishes	Decreases slope of curve relating $[K]_o$ to K-free effect
$[ADP]_i$	Decreases	Decreases

measurable K pump flux but data on the K-free effect are not extensive enough to establish this point. Baker et al. (1969) have shown that when $[K]_o$ is increased there is some increase in Na efflux in the presence of strophanthidin. The main points where there is quantitative information regarding the differences between K pump and K-free effect are in the action of Na-free seawater (Li or choline) and in the concentration dependence of the K-free effect on $[K]_o$.

Taken as a whole, the data suggest that the K and Na pumps, in *L. pealei*, are operating far from saturation and so show a linear dependence on the concentration of their respective ions. In addition, $[K]_o$ would appear to activate Na efflux, while $[Na]_i$ activates K influx. Such a modulation makes it possible to vary the apparent coupling of Na efflux to K influx over wide limits.

A number of investigators have reported that Na efflux is a linear function of $[Na]_i$ (Hodgkin and Keynes, 1956; Sjodin and Beaugé, 1967; Brinley and Mullins, 1968; Baker et al., 1969). The present finding that K influx is a nonlinear function of $[Na]_i$ therefore requires that the coupling ratio between K influx and Na efflux vary with internal sodium. This relation is shown in Fig. 15, where the coupling ratio is plotted for the $[Na]_i$ at which we have experimental data as well as for various $[ATP]_i$. To a good approximation, coupling is constant with $[ATP]_i$ except at very low concentrations where control over $[ADP]_i$ may be inadequate. The lower curve for coupling vs. $[Na]_i$ has been calculated by assuming that the entire ATP-dependent K influx is coupled to the ATP-dependent Na efflux, while the upper curve represents ratios calculated on the assumption that 2–3 f of ATP-dependent K influx observed at a very low $[Na]_i$ is in fact not associated with Na efflux. No great accuracy can be claimed for the calculated ratios because of the difficulties of making accurate influx measurements at low $[Na]_i$. The coupling ratio shown for $[ATP]_i$ = 4–6 mM and $[Na]_i$ = 80 mM in the figure (3.8), does not quite agree with the ratio plotted against $[ATP]_i$ for the same parameters (3.0) because the K influx in the two series varied by about 4 f, probably due to slight variations in temperature. Nevertheless, the inference appears inescapable that the coupling ratio is reduced from about 3:1 at a $[Na]_i$ of 80 mM to nearly 1:1 at low $[Na]_i$. The coupling ratio at a $[Na]_i$ of about 35–50 mM, which is about that encountered in fresh axons, would appear to be of the order of 2:1. Sjodin and Beaugé (1968 *a*) have also concluded that the Na/K coupling varies in intact and injected axons as a function of $[Na]_i$.

The conclusion that the coupling ratio is reduced at low $[Na]_i$ may only reflect the fact that the electrochemical gradient for Na efflux has been increased, and that fewer Na^+ can be extruded per cycle of the transport mechanism regardless of the absolute rate of operation. However, this explanation requires that a fixed number of ATP molecules be consumed per cycle, that

the K influx be an adequate measure of the cycling rate, and that the rate of ATP consumption vary with $[Na]_i$ in the same way as the K influx. If these three assumptions are correct, then the relative constancy of the regression of coupling ratio upon $[ATP]_i$ reflects a slower rate of cycling, but no change in the mode of operation. On the other hand, the regression of coupling ratio on $[Na]_i$ indicates a change in mode of operation as well as rate.

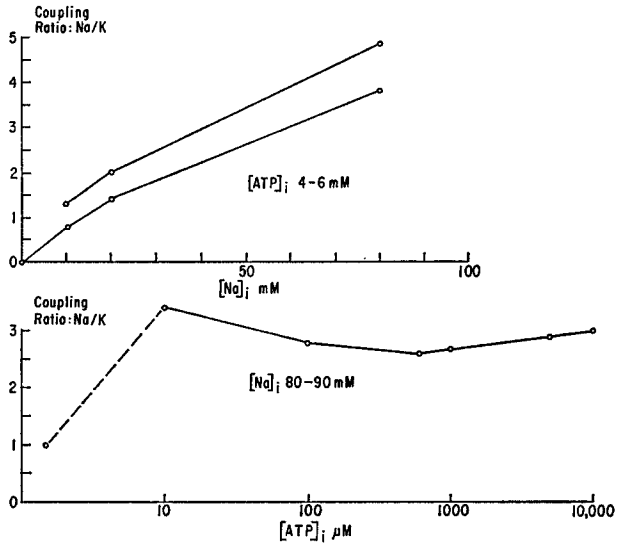

FIGURE 15. The upper graph shows the dependence of the apparent coupling ratio Na/K on $[Na]_i$ for (upper curve) an assumption that K influx under Na-free conditions is a base line from which coupling is to be computed. The lower curve is the apparent coupling between the ATP-dependent K influx and Na efflux. The lower graph shows Na/K coupling as a function of $[ATP]_i$. The value below 10 μM ATP is prone to large errors in flux estimate and is shown by a dotted line.

Active vs. Passive Ion Fluxes The division of an experimentally measured ion flux into two or more components (such as passive ion movement and active transport) requires certain assumptions regarding the components. The experiments reported in this study, taken together with those previously published (Brinley and Mullins, 1968), make it clear that increasing $[ATP]_i$ of an axon from about 1 μM to any higher value increases the inward fluxes of Na and K, the outward flux of Na, and probably decreases the K efflux as well. We have employed a uniform definition for this change in flux observed when $[ATP]_i$ is increased from 1 μM, and have called this a pumped flux.[4]

The definition given above is rather different from those conventionally

[4] A more suitable definition probably ought to be based on ATP consumption rather than $[ATP]_i$ as this would allow one, in principle, to discriminate between ion pumping which requires work as compared with ion movement which does not.

employed and it is instructive to examine the differences involved. One method that has been employed extensively to separate the ion fluxes of nerve fibers into passive and active components has been that of pretreatment with cyanide. The results obtained by Doane (1967) on squid axons show that maximal conversion of DPN to DPNH is produced by rather low concentrations of CN (100 μM) so that oxidative phosphorylation can be expected to be totally blocked at concentrations of ~2 mM. In turn, this should lead to a low but indeterminate $[ATP]_i$ because (*a*) the extent to which glycolysis can supply ATP in squid axons is unknown, and (*b*) some recycling of ADP through the adenylate kinase reaction will also lead to ATP production. It is not surprising, therefore, that Na efflux from a CN-poisoned axon is considerably greater than that calculated from flux ratio considerations, for a purely passive Na efflux.

We have shown (Fig. 5) that ADP is an inhibitor of K influx. In a CN-treated axon it is to be expected that ADP is present in concentrations far greater than those for ATP, and that therefore K influx is inhibited far more than it might otherwise be solely on the basis of the [ATP] present. Thus, while the general conditions imposed on an axon by pretreatment with CN (transformation of ATP to ADP, AMP, and P_i and of phosphoarginine to arginine and P_i) can be specified, the actual concentration of [ATP] realized in an experiment, and the extent to which the breakdown products of ATP are present may be rather variable.

The cardiac glycosides have been used as specific inhibitors of membrane transport and their action on ion fluxes has been used as a means of defining active transport by equating glycoside-sensitive fluxes with active transport (Glynn, 1957). In the case of K influx in squid axons, it appears that the definition of the K pump based on glycoside inhibition is identical with that based on the increase in K influx as $[ATP]_i$ is increased from 1 μM to physiological concentrations. For the Na efflux, however, it is clear that the glycoside-sensitive Na efflux and the ATP-dependent Na efflux are different. The former is about 30 f while the latter is about 40 f. As the total Na efflux is about 40 f, there is more than just a quantitative difference between the two definitions as the following makes clear. In the presence of ouabain there is a residual Na efflux of about 10 f and it is logical to call this the "ouabain-insensitive component of the Na efflux." The existence of such a component suggests that the Na pump may be a dual mechanism either operating on two different fuels or exchanging two different kinds of ions. Our results on the effects of ATP and strophanthidin on Na efflux (Brinley and Mullins, 1968) have shown, however, that Na efflux reaches values close to those calculated from flux ratio considerations when $[ATP]_i$ is 1 μM, and that this flux is increased from about 1 to 10 f when strophanthidin is added. In the presence of strophanthidin, the Na efflux is insensitive to $[ATP]_i$; it is apparent then that

the magnitude of the Na pump is 40 f and that the Na efflux in the presence of glycoside is another mode of operation of the Na transport system but it is not a component of the normal Na efflux.

The problem of obtaining a consistent definition of active transport using inhibitors can be further illustrated by considering the effect of glycosides on Na and K transport in axons containing about 30 μM ATP. The ATP-sensitive K influx in such axons is about 4 f (see Fig. 4) and is also ouabain-sensitive. However, the glycoside-sensitive Na efflux under these conditions is zero (Brinley and Mullins, 1968, Figs. 7 and 9). The most straightforward interpretation of these results would be simply that under these circumstances some glycoside-sensitive K transport exists in the absence of glycoside-sensitive Na transport. However, this explanation contradicts the major assumption underlying the use of glycosides, which is that they inhibit only coupled ion pumps. This apparently uncoupled glycoside-sensitive K influx cannot be dismissed simply as a side effect of the inhibitor because the size of this flux (about 4 f) is one-third of the total active K pump.

The considerations discussed above suggest that control of [ATP] and its products of hydrolysis is essential if the active transport components of a measured ion flux are to be specified and they also argue against the division of a flux into inhibitor sensitive and insensitive components. As an extension of this idea, one might also argue that while the "K-free Na efflux" and "Na-free Na efflux" are clearly operational terms, they suggest that what is being measured is the extent to which K or Na influx is coupled to Na efflux. Arguments have been advanced earlier in this discussion against the idea that the K-free effect necessarily is closely related to K pumping, while the fact that muscle fibers show a Na-free effect when they have been treated with glycoside (Horowicz, 1965; Keynes, 1966; Sjodin and Beaugé, 1968 *b*) makes it clear that there are difficulties with the idea that Na-free external solutions always measure the extent to which Na:Na exchange takes place.

The definition of active transport flux based on the assumption that only passive ion transport takes place when $[ATP]_i$ is 1 μM is entirely arbitrary and needs careful examination. The reason for selecting this concentration is purely experimental—it has not been possible to reduce internal [ATP] below this level, probably because ATP is continuously being supplied to the axoplasm from some internal compartment, quite possibly the mitochondria. We infer that $[ADP]_i$ must be considerably greater than 1 μM, possibly 5 μM, under conditions where $(ATP]_i = 1$ μM, because this is the concentration of ATP that appears in the dialysis effluent when PA is added to the dialysis fluid. One might then inquire whether this relatively large [ADP] rather than the low [ATP] might be inhibiting Na efflux. Two pieces of information argue against this view: the slope of the curve of Na efflux vs. $[ATP]_i$ is linear in the range of 1–10 μM, although the ratio [ATP]:[ADP] would vary from 1/5 to

2/1. The second point is that in a CN-treated axon which would be expected to have an [ADP] of the order of 1 mM, the injection of an equivalent concentration of ATP gives a near normal Na efflux (De Weer, unpublished data).

Very low values of Na efflux cannot be measured with any great precision, but the fact that at $[ATP]_i = 10$ μM, Na efflux is 10 f suggests that for $[ATP]_i = 1$ μM, Na pump should be about 1 f. The observed Na efflux is about 1.3 f so that perhaps 1 f out of 40 f is actively transported at 1 μM ATP. This is a small error in terms of the maximum Na efflux but it may have an important bearing on the correct value for passive Na efflux. If active transport of Na at 1 μM ATP is 1 f, then with a total flux of 1.3 f, there is a passive Na efflux of 0.3 f. With $E_m - E_{Na}$ of 100 mv, a flux ratio of 56 is to be expected so that Na influx ought to be 17 f. Such a value was in fact found in lightly cleaned, intact squid axons (Brinley and Mullins, 1965) but in dialyzed squid axons at $[ATP]_i = 1$ μM, the mean value is 43 f. The latter value for influx can be understood as an unavoidable concomitant of the internal dialysis procedure that makes the membrane leaky to Na, and to a passive efflux of Na that is closer to 0.8 f rather than 0.3 f as was assumed, because efflux measurements are not accurate enough to make certain of this value unless $[ATP]_i$ can be reduced to values much smaller than 1 μM. On the other hand the discrepancy may be due to a carrier-mediated component of Na influx even when $[ATP]_i$ is 1 μM, and which might be induced by the experimental manipulation necessary to effect internal dialysis.

Measurements of the ATP-dependent component of K influx present some slightly different problems. Instead of having to measure flux at a level of about 1 f, it appears that passive K influx is of the order of 10 f. Given the base line stability of approximately 10 % this means that accuracy of flux measurement will be of the order of ± 1 f. The K pump at $[ATP]_i$ of 10 μM is of the order of 3 f so that on a linear basis, the K pump influx at 1 μM would be 0.3 f or much less than base line flux stability. We have shown that when $[ATP]_i = [ADP]_i$ there is about a 50 % inhibition of K influx so that given the tentative value of $[ADP]_i$ of 5 μM, then at $[ATP]_i = 5$ μM K influx which might be expected to be 1.5 f, would be reduced by ADP inhibition to 0.75 f. This value, too, is below the precision of measurement so that we conclude that corrections for the ATP-dependent K influx at $[ATP]_i = 1$ μM appear unnecessary. Some inhibition of K influx by the ambient $[ADP]_i$ would appear to exist but is unlikely to be measurable given the limitations on the precision with which influx can be estimated.

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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. 1968. Recent experiments on the properties of the Na efflux from squid axons. *J. Gen. Physiol.* **51** (5, Pt. 2):172.

BAKER, P. F., M. P. BLAUSTEIN, R. D. KEYNES, J. MANIL, T. I. SHAW, and R. A. STEINHARDT. 1969. The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol. (London).* **200**:459.

BOND, G. H., H. BADER, and R. L. POST. 1966. Acetyl phosphate as substrate for (Na + K) ATPase. *Fed. Proc.* **25**:567.

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. 1965. Ion fluxes and transference number in squid axons. *J. Neurophysiol.* **28**:526.

BRINLEY, F. J., JR., and L. J. MULLINS. 1967. Sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* **50**:2303.

BRINLEY, F. J., JR., and L. J. MULLINS. 1968. Sodium fluxes in internally dialyzed squid axons. *J. Gen. Physiol.* **52**:181.

CALDWELL, P. C., A. L. HODGKIN, R. D. KEYNES, and T. I. SHAW. 1960 *a*. The effects of injecting "energy-rich" phosphate compounds on the active transport of ions in the giant axons of *Loligo*. *J. Physiol. (London).* **152**:561.

CALDWELL, P. C., A. L. HODGKIN, R. D. KEYNES, and T. I. SHAW. 1960 *b*. Partial inhibition of the active transport of cations in the giant axons of *Loligo*. *J. Physiol. (London).* **152**:591.

CALDWELL, P. C., and R. D. KEYNES. 1960. The permeability of the squid giant axon to radioactive potassium and chloride ions. *J. Physiol. (London).* **154**:177.

DOANE, M. G. 1967. Fluorometric measurement of pyridine nucleotide reduction in the giant axon of the squid. *J. Gen. Physiol.* **50**:2603.

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 stoichiometry of the sodium pump. *J. Physiol. (London).* **192**:217.

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

HARRIS, E. J. 1957. Permeation and diffusion of K ions in frog muscle. *J. Gen. Physiol.* **41**:169.

HODGKIN, A. L., and R. D. KEYNES. 1955 *a*. Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol. (London).* **128**:28.

HODGKIN, A. L., and R. D. KEYNES. 1955 *b*. The potassium permeability of a giant nerve fibre. *J. Physiol. (London).* **128**:61.

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 characteristics of new energy dependent cation transport processes in red blood cells. *Ann. N. Y. Acad. Sci.* **137**:566.

HOROWICZ, P. 1965. Sodium movements in frog's sartorius muscle. *Acta Physiol. Acad. Sci. Hung. Suppl.* **26**:14.

KEYNES, R. D. 1966. Exchange diffusion of sodium in frog muscle. *J. Physiol. (London).* **184**:31P.

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

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

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. *Curr. Mod. Biol.* **1**:105.

SJODIN, R. A., and L. A. BEAUGÉ. 1968 *a*. Coupling and selectivity of sodium and potassium transport in squid giant axons. *J. Gen. Physiol.* **51** (5, Pt. 2):152.

SJODIN, R. A., and L. A. BEAUGÉ. 1968 *b*. Strophanthidin-sensitive components of potassium and sodium movements in skeletal muscle as influenced by the internal sodium concentration. *J. Gen. Physiol.* **52**:389.

STEINBACH, H. B., and S. SPIEGELMAN. 1943. The sodium and potassium balance in squid nerve axoplasm. *J. Cell. Comp. Physiol.* **17**:187.

TASAKI, I. 1963. Permeability of squid axon membrane to various ions. *J. Gen. Physiol.* **46**:755.

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