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THE EFFECTS OF INJECTING 'ENERGY-RICH' PHOSPHATE COMPOUNDS ON THE ACTIVE TRANSPORT OF IONS IN THE GIANT AXONS OF *LOLIGO*

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In the previous paper (Caldwell, 1960) it was shown that the phosphagen and adenosine triphosphate (ATP) of squid giant axons break down in the presence of fairly high concentrations of inhibitors such as cyanide, dinitrophenol or azide and that some recovery occurs when the inhibitors are removed. Since these agents also reduce the outflow of sodium ions from giant axons to a low value (Hodgkin & Keynes, 1955) it is natural to suppose that ATP or some other energy-rich phosphate compound may provide the energy for running the ionic transport system. This would fit with the generally accepted view as to the function of ATP in linking biochemical and physiological events in cells (Lipmann, 1941). The aim of the experiments described here was to test the assumption by seeing whether injected ATP or arginine phosphate could restore normal ion transport to a fibre poisoned with cyanide or dinitrophenol. The results show that both arginine phosphate and ATP have a restorative action, but that the former is a more effective substitute for normal metabolic activity. One of the properties of the ionic transport system is that the outflow of sodium ions from a normal fibre is reduced to about one quarter by removing potassium ions from the external medium (Hodgkin & Keynes, 1955). This may happen because metabolism drives a cycle in which an uptake of K^+ is coupled to an extrusion of Na^+. Whatever the mechanism, it was plainly important to determine whether the sodium efflux which resulted from the injection of a phosphate ester had the same potassium sensitivity as the normal efflux. It turns out that high concentrations of certain compounds, arginine phosphate and phosphoenolpyruvate, restore K sensitivity, but that others, ATP and ADP, give an outflow of sodium which is not reduced by removing K.

The experiments described here were carried out during the last four months of the years 1956–59. Preliminary accounts by the authors

concerned with different phases of the work were given at various meetings and symposia (Caldwell & Keynes, 1957; Caldwell, 1958; Keynes, 1958; Caldwell, Hodgkin & Shaw, 1959).

METHODS

Material. Giant axons from *Loligo forbesi* with diameters varying between 600 and 1000 μ were isolated and cleaned by the usual methods. Axons of over 700 μ are found in the large specimens of *L. forbesi* which are plentiful at Plymouth during the last 3 months of the year. These large squid are often damaged in the trawl or while being transported to the laboratory. A further hazard is rough weather which sometimes makes it difficult to bring any live squid to the laboratory. About half the work was done with live squid from the aquarium; these were normally used on the day that they were caught. The remaining experiments were

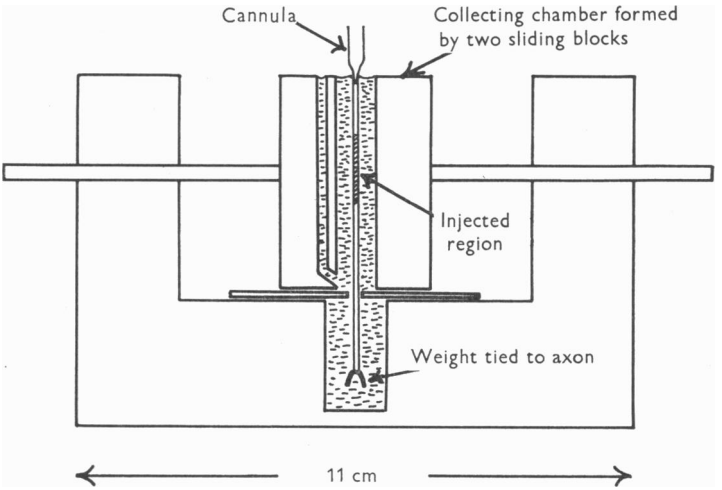

Fig. 1. Collecting cell for measuring the efflux of radioactive ions from squid axons. During the impalement of the axons with a micro-electrode or micro-syringe, the sliding blocks were pulled apart in order to allow more room for manoeuvre. They could be pushed together again with the microsyringe in position inside the axon, their sides being well greased with petroleum jelly so that they formed a watertight rectangular compartment whose volume (above the lower gate) was about 4 ml. Chlorided silver wires (not shown in the diagram) were used to apply shocks between the two compartments, thus stimulating the axon at the point where it passed through the gate. The drawing is roughly to scale, the over-all length of the cell being 11 cm, its height 7 cm, and its width 4 cm.

carried out with axons from refrigerated mantles. When the trawl was brought up, live squid were decapitated, and after removing the viscera the mantles were placed in large Thermos flasks filled with ice-cold sea water. Axons were dissected a few hours later, a bright light being used to illuminate the opaque mantle; they seemed to be in good condition since they gave spikes of 105–110 mV for many hours.

Procedure for injections and for measuring effluxes. The fibre was cannulated and mounted in the cell shown in Fig. 1. A long electrode consisting of a 100 μ capillary filled with isotonic KCl was inserted for a distance of 3–4 cm. At this stage the sliding blocks of Perspex were separated so as to allow the axon to be viewed from the front and side, a mirror being

employed in the usual way. The action potential was measured and the impalement was regarded as satisfactory if the spike amplitude was greater than 105 mV over a length of 2 cm; it was also desirable that the micro-electrode should lie fairly near the axis of the fibre. The micro-electrode was removed and a microsyringe of the type described by Hodgkin & Keynes (1956) was lowered into the fibre to a distance of about 35 mm from the tip of the cannula. The microsyringe had previously been filled with a solution containing ^{22}Na and this was injected uniformly over a distance of 10 or 12 mm. The microsyringe was then withdrawn, washed thoroughly with distilled water and reloaded with phosphate ester. The tip of the syringe was lowered to the end of the region injected with ^{22}Na, or, in the later experiments, to a point 3 mm beyond this region. Diffusion of phosphate ester from the tip of the syringe was prevented by a small air bubble in the tip. The movable blocks which had previously been greased with petroleum jelly were then pressed together so that the axon was enclosed in a volume of 4 ml. Samples were collected by sucking the cell dry with a syringe; this removed about 97 % of the fluid. If the composition of the solution had to be changed the cell was washed with the new solution before taking a sample; as a control the same procedure was often used when there was no change in composition. After collecting ^{22}Na from the normal fibre, cyanide or DNP was applied. After 90 min the Na efflux had always fallen to a low value and the time had come to observe the effect of the injection. Since the microsyringe was already in position, all that had to be done was to inject the column of phosphate ester and to continue collecting samples in the same way as before. The exact procedure varied slightly during the course of the work. In 1956 and 1957 the columns of ^{22}Na and phosphate ester were both 12 mm in length. Since about 2·5 hr elapsed between the two injections, the patch of ^{22}Na must have spread longitudinally by diffusion and some of the labelled ions would not have been reached by the phosphate ester; calculations in which a diffusion coefficient of 10^{-5} cm^2/sec was used indicated that, after 2·5 hr, 28 % of the total ^{22}Na (p. 569) would have been outside the column of phosphate ester. In 1958 the column of ^{22}Na was reduced to 10 mm and the column of phosphate ester increased to 16 mm, giving an overlap of 3 mm at either end. Under these conditions 12 % should have diffused beyond the column of phosphate ester in 2·5 hr. The corresponding figure for the 1959 experiments, in which the column of ^{22}Na was 12 mm and of $\sim$ P was 18 mm, is 10 % in 2·5 hr.

The action potential was checked from time to time and the experiment was discontinued if there was any sign of block or decrement in the injected region. This was important because control injections of salt solutions which have no effect on excitable axons may give an apparent rise in efflux in derelict axons. (These spurious effects may sometimes be due to fluid being squeezed out of the ends of branches.)

At the end of the experiment the fibre was removed and the total quantity of ^{22}Na was measured. In many experiments the concentrations of Na and K in extruded axoplasm were also determined by flame photometry.

External solutions. The artificial sea water normally used was the bicarbonate sea water described in the previous paper (Caldwell, 1960, p. 552); its pH was about 8. In some experiments with 0·2 mM DNP a phosphate sea water of a lower pH (*ca.* 6·7) was made up with the composition given by Hodgkin & Keynes (1955).

Phosphate compounds used for injection. The solutions used for injection were adjusted to a pH of about 7 with KOH or HCl and were stored at $-20°$ C. At the end of each group of experiments the solutions were tested chromatographically for impurities, the amounts of phosphorus present as the principal component and as the main impurities being determined by the method described previously (Caldwell, 1960). Table 1 gives information about the source and purity of the various solutions used; the figures for the impurities refer to the final composition of the solutions and not to the products supplied by the manufacturers.

Radioactive isotopes. ^{22}Na was obtained from the Radiochemical Centre, Amersham, as an aqueous solution of $^{22}NaCl$. After concentration by evaporation, the pH was adjusted

to 7, and any precipitate was removed. The amount of inactive Na in the sample varied from year to year; the calculated increase in the internal sodium concentration produced by the injection was usually between 5 and 30 mM.

Artificial sea water containing ^{24}Na or ^{42}K was made by treating $^{24}Na_2CO_3$, $^{42}K_2CO_3$ or $^{42}KHCO_3$, supplied by AERE Harwell, with a slight excess of HCl, heating to dryness and adding a solution containing the other constituents of artificial sea water.

Except in the 1959 experiments, in which γ rays from ^{22}Na were detected with a Labgear scintillation counter, the methods of counting liquid samples or the axon were essentially similar to those used by Hodgkin & Keynes (1955).

TABLE 1

Substance	Year used	Source	K or Na salt	Main impurities (as a % of the P in the principal constituent)
Arginine phosphate	1956*A*	*J*	K	2–8% as orthophosphate (P_i)
	1956*B**	*P*	K	0–6% as P_i
	1957*	*P*	K	Nil
	1958*	*P*	K	Nil
ATP	1956*A*†	*P*	K	28% as ADP, < 2% as P_i
	1956*B*†	*P*	K	< 18% as ADP, < 9% as P_i
	1957*A*†	*P*	K	18% as ADP, 9% as P_i
	1957*B*	*S*	K	12% as ADP, 4% as P_i
	1958	*S*	K	33% as ADP, 23% as P_i
	1959	*S*	Na, K	4% as ADP
ADP	1957‡	*S*	K	10% as ATP, 15% as P_i
	1958‡	*S*	K	33% as ATP, 15% as P_i
	1959	*S*	Na, K	< 5% as ATP
Phosphoenol-pyruvate	1958	*S*	Na	No P_i visible on chromatogram
	1959	*S*	Na	6% as P_i
Creatine phosphate	1958	*S*	Na, K	No P_i visible on chromatogram
GTP	1959	*S*	Na, K	4% as P_i, some GDP?
ITP	1959	*S*	Na, K	6% as P_i, some IDP?

Notes: * Made by a method based on that of Ennor, Morrison & Rosenberg (1956). † Made from the Ba salt obtained as a by-product in the arginine phosphate preparation. This was dissolved in dil. HNO_3, the ATP precipitated as the Hg salt, brought into solution again with H_2S and reprecipitated as the oxine salt with acetone. The oxine salt was redissolved, the pH adjusted to 7 with KOH and after removal of the oxine with chloroform the ATP was precipitated as the K salt with acetone. ‡ Prepared from Ba salt supplied by Sigma Chemical Company. *J*, *Jasus* muscle, gift from Professor Ennor; *P*, *Palinurus* muscle; *S*, Sigma Chemical Co.; GTP, guanosine triphosphate; ITP, inosine triphosphate.

RESULTS

The effect of injections on Na efflux in poisoned fibres

Active and inactive substances. If a giant axon is immersed for 1½–2 hr in 2 mM-CN (pH 8) the arginine phosphate and ATP disappear from the axoplasm and the Na efflux is reduced to ¼–1/10 of its normal value. Under these conditions the Na efflux can be increased for a period of 30 min, or longer, by injecting arginine phosphate or ATP; similar solutions which had been hydrolysed by boiling to adenosine monophosphate (AMP) and inorganic phosphate, or to arginine and inorganic phosphate, were without effect when injected into poisoned fibres (Figs. 2 and 3). Control injections

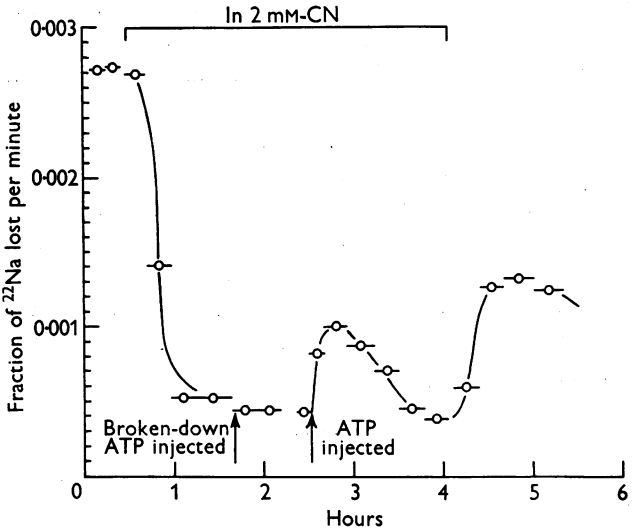

Fig. 2. 780 μ axon loaded with ^{22}Na over 12 mm. At second arrow, 16·4 nmole ATP was injected over the same 12 mm, giving a mean internal concentration of 2·9 mM (5·8 mM ~ P). At the first arrow an equal volume of the same ATP solution which had been hydrolysed by boiling was injected. At the end of the experiment the action potential was 99 mV, and the resting potential 56 mV. Fibre reference, 19D6 (19 December 1956). 1 nmole = 10^{-9} mole.

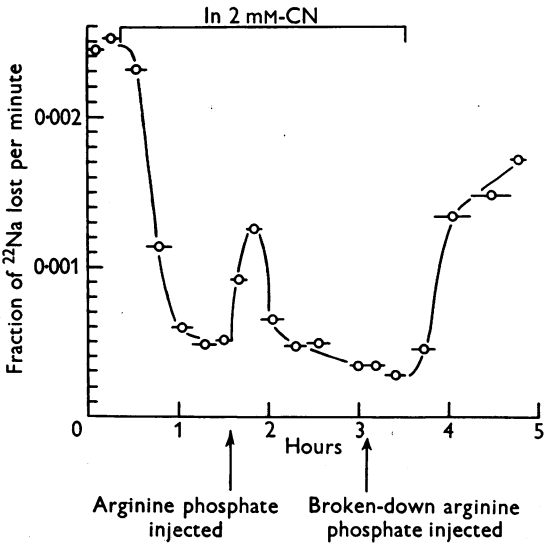

Fig. 3. 800 μ axon loaded with ^{22}Na over 12 mm. At first arrow 32·4 nmole arginine phosphate was injected over same 12 mm, making mean internal concentration 5·4 mM. At second arrow an equal volume of the same solution, hydrolysed by heating, was injected. Temperature 20° C. At end of experiment resting potential was 58 mV, action potential 99 mV. Fibre reference, 4O6.

of KCl, K_2SO_4, K isethionate or $MgCl_2$ also gave negative results. Arginine phosphate and ATP had the same restorative effect on fibres poisoned with 0·2 mM dinitrophenol (DNP) (pH 6·7) as on those poisoned with 2 mM-CN.

In all, seven compounds containing energy-rich phosphate bonds have been tested, namely, arginine phosphate, ATP, ADP, phosphoenolpyruvate, GTP, ITP and creatine phosphate. The first five gave substantial increases in Na efflux but ITP caused only a slight rise and creatine phosphate was without effect (Fig. 4). The difference between creatine phosphate and arginine phosphate is to be expected if the effects of the latter depend on catalysis by arginine phosphokinase (Ennor & Morrison, 1958) or by an enzyme of similar specificity.

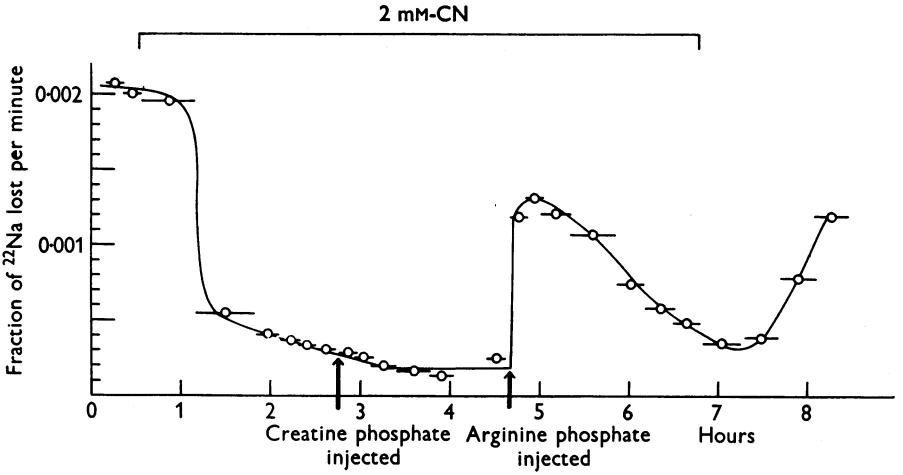

Fig. 4. The effect on the Na efflux from a CN-poisoned axon of injecting first creatine phosphate, and secondly arginine phosphate. The length injected with ^{22}Na was 10 mm, and with phosphate ester 16 mm. The mean concentrations in the axon immediately after the injections were 15·3 mM creatine phosphate and 15·8 mM arginine phosphate. Axon 10D8, diameter 766 μ. Temperature 19° C. At the end of the experiment the action potential was 90 mV.

External applications of ATP and arginine phosphate. Figure 5 shows that ATP had no effect on the sodium efflux of a poisoned fibre when applied externally at a concentration of 3·8 mM. Arginine phosphate at a concentration of 12 mM was also ineffective when applied externally.

The effect of increasing concentrations of ATP and arginine phosphate. The action of injected phosphate esters is transient, as one would expect if they are used up in driving ions against concentration differences, or destroyed in side-reactions. With quantities of arginine phosphate or ATP equivalent to about half the total energy-rich phosphorus in an

unpoisoned fibre the period of enhanced Na efflux lasted for about 30 min. With ten times the quantity, the Na efflux remained at a high level for several hours (e.g. Fig. 10).

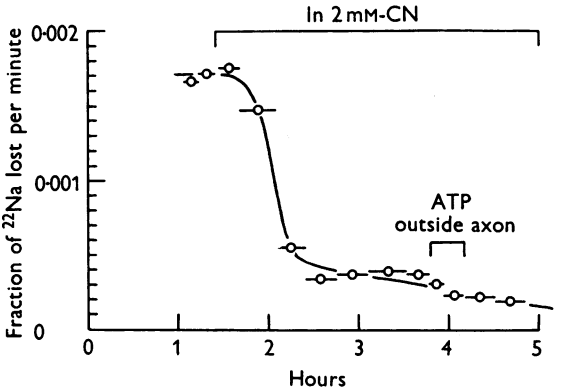

Fig. 5. The effect on the Na efflux from a CN-treated axon of applying ATP externally. This experiment was done with the collecting cell modified so that the external volume was 0·5 ml. During two collecting periods the CN sea water contained 3·8 mM ATP (7·6 mM ~ P). Axon 15N7, diameter 845 μ. Temperature 18° C. Final resting potential was 52 mV, action potential 103 mV.

Figure 6 illustrates the effect of injecting first a small and secondly a larger quantity of arginine phosphate into the same fibre. In the first case the quantity of arginine phosphate injected was sufficient to give a concentration in the axoplasm of 4·4 mM (as compared with 1·5–5 mM in unpoisoned fibres); in the second, the concentration was 3·5 times larger. The experiment shows that the extra Na efflux resulting from the second injection reached a higher peak and lasted longer than that from the first. A similar experiment with two different concentrations of ATP is given in Fig. 7. In both cases (Figs. 6 and 7) the percentages of labelled Na which emerged as a result of the injection were roughly proportional to the quantity of energy-rich phosphate injected (Table 2).

One way of expressing the quantitative effect of injecting a substance is to give the ratio of the number of internal sodium ions ejected to the number of energy-rich phosphate bonds injected. In order to calculate such an Na: ~ P ratio it is necessary to estimate the internal sodium concentration at the time of the injection. Column 7 in Table 2 gives a series of such estimates. The values marked with an asterisk were obtained by interpolating between an initial sodium concentration taken as about 55 mM and the final sodium concentration measured by flame photometry on extruded axoplasm at the end of the experiment. The exact value taken for the initial sodium concentration depended on the time from decapitation and was based on analyses of unpoisoned axons made at

various times after death. The average of the values marked with an asterisk is 101 mM and an internal sodium concentration of 100 mM has been assumed in other cases. As a check, three pairs of axons were dissected and one axon of each pair was used for an injection experiment (31O8, 12N8 and 17N8 in Table 2); both were put into cyanide at the same time and the control was removed for analysis soon after its companion had

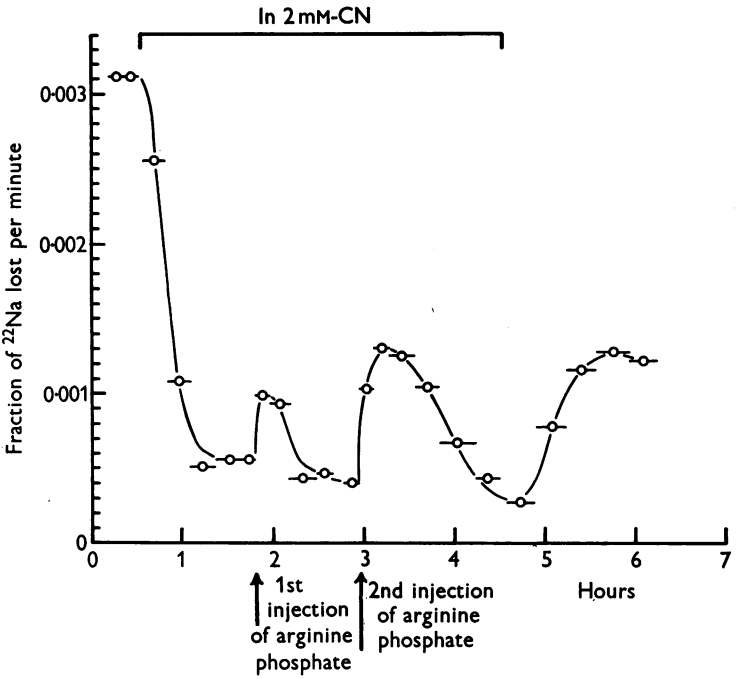

Fig. 6. The effect on Na efflux of injecting two different quantities of arginine phosphate. The axon was loaded with ^{22}Na over a distance of 12 mm, and at the first arrow sufficient arginine phosphate to give an internal concentration of 4·4 mM was injected over the same 12 mm. At the second arrow a larger amount of arginine phosphate was injected, giving a concentration of 15·4 mM. Axon 5O6, diameter 668 μ, temperature 17·5° C. At end of experiment resting potential was 59 mV, action potential 98 mV.

been injected. These axons had sodium concentrations of 94, 84 and 71 mM; to this should be added the quantity of Na injected with the ^{22}Na. In these experiments the Na injected was of the order of 25 mM so that the estimate of 100 mM would seem to be roughly right.

From the estimates of $(Na)_I$, the fibre diameter and the percentage of internal sodium leaving the fibre as a result of the injection, it is simple to calculate the quantity of Na ejected and hence to obtain Na: $\sim$ P. The values which are given in Column 9 of Table 2 are probably too small, because some ^{22}Na would have diffused beyond the column $\sim$ P. The

fraction (F) of the ^{22}Na which should have been outside the $\sim$ P column can be calculated by the methods of Carslaw & Jaeger (1947) as

$$F = \frac{\sqrt{Dt}}{h}\left(\text{ierfc } l/2\sqrt{Dt} - \text{ierfc } \frac{2h+l}{2\sqrt{Dt}}\right), \quad (1)$$

Where D is the diffusion coefficient of Na in axoplasm, taken, by analogy

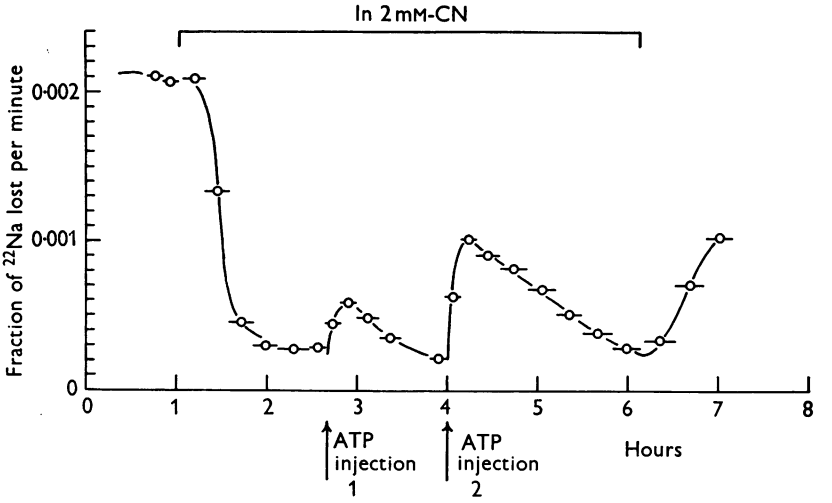

Fig. 7. Injection of two different amounts of ATP into the same axon. Axon loaded with ^{22}Na over 12 mm. Injection 1 was 5·9 nmole ATP over same 12 mm, making mean internal (ATP) 1·2 mM. Injection 2 was 31 nmole ATP, making mean internal (ATP) 6·2 mM. Axon 18D6, diameter 725 μ. Temperature 19·5° C. At end of experiment resting potential was 47 mV, action potential 71 mV.

with K which has nearly the same mobility in axoplasm as in aqueous solution (Hodgkin & Keynes, 1953), as 10^{-5} cm^2/sec;

$2h$ is the total length of the column of ^{22}Na;

$2h+2l$ is the total length of the column of $\sim$ P;

t is the time between the two injections; and

ierfc has its usual meaning (for definition and tables see Carslaw & Jaeger (1947), pp. 371–373). With the range of values involved, the second term in eqn. (1) is very small and can be neglected.

The corrected results, which are listed in Column 10 of Table 2, give mean values ($\pm$ S.E. of mean) of Na: $\sim$ P of $0{\cdot}73 \pm 0{\cdot}06$ for ATP, $0{\cdot}40 \pm 0{\cdot}05$ for ADP and $0{\cdot}64 \pm 0{\cdot}06$ for arginine phosphate. These values correspond to 1·46 Na per molecule of ATP, 0·40 Na per molecule of ADP and 0·64 Na per molecule of arginine phosphate.

The Na: $\sim$ P ratios obtained for the other compounds were 0·7 and 0·8 for phosphoenolpyruvate, 0·27 and 0·47 for GTP and 0·03 for ITP.

There was no detectable ATP in the sample of ITP, but it is difficult to be sure that the slight effect obtained with ITP was not due to the presence of some other nucleotide.

TABLE 2. Relation between quantity ~ P injected and quantity Na extruded

1	2	3	4	5	6	7	8	9	10	11
Fibre reference	Temperature (°C)	Axon diameter (μ)	Initial concentration (mM ~ P)	Quantity ~ P injected (nmole/cm)	Na emerging (%)	Estimated $[Na]_i$ (mM)	Quantity Na ejected (nmole/cm)	Na: ~ P Uncorrected	Na: ~ P Corrected	Notes
					ATP					
18 D 6	20	725	2·4	9·8	1·1	100	4·6	0·47	0·63	
18 D 6	20	725	12·4	51	4·8	100	19·8	0·39	0·58	
20 N 7	19	890	2·8	17	1·5	103*	9·6	0·56	0·79	
19 D 6	19	780	5·8	27	2·7	100	12·9	0·47	0·66	
19 D 7	18	911	7·4	49	4·2	98*	27	0·55	0·78	
22 N 7	19	867	8·2	49	5·9	98*	34	0·70	1·02	1
24 O 7	18	890	8·2	51	5·6	92*	32	0·62	0·83	2
27 S 7	18	850	9·0	51	4·2	100*	24	0·47	0·66	3
30 O 7	18	842	9·2	51	4·1	119*	27	0·53	0·75	4
10 N 9	19	776	9·5	45	6·1	100	29	0·65	0·74	
4 O 7	17	830	9·6	51	6·5	124*	44	0·85	1·30	5
11 D 6	18	581	12·6	33·6	4·3	100	11·4	0·34	0·46	
7 N 8	17	805	14·3	73	9·3	100	44	0·61	0·73	
12 S 7	19	578	19·6	51	4·9	102*	13·1	0·26	0·34	
Mean ± S.E.			9·4						0·73 ± 0·06	
					ADP					
11 N 9	17	806	2·1	11·5	0·6	100	3·0	0·26	0·30	
22 O 7	17	826	16·8	90	4·7	89*	22·4	0·25	0·34	
7 N 7	18	796	18·0	90	6·1	85*	25·8	0·29	0·42	
16 D 8	17	606	23·1	67	> 9·6	100	> 28	*ca.* 0·45	*ca.* 0·55	
Mean ± S.E.			15						0·40 ± 0·05	
					Arginine phosphate					
5 O 6	18	668	4·4	15·3	1·1	100	3·8	0·25	0·33	
5 O 6	18	668	14·7	51·5	4·6	100	12·5	0·24	0·35	
4 O 6	20	800	5·4	27·0	1·5	100	7·5	0·28	0·37	
7 O 8	20	898	7·4	47	4·3	100	27	0·57	0·70	
21 N 8	16	844	8·5	47	2·0	100	11·3	0·24	0·28	
26 S 6	20	706	9·1	35·5	2·9	100	11·3	0·32	0·45	
20 S 6	21	664	10·3	35·5	3·4	100	11·7	0·33	0·43	
17 N 8	17	725	11·5	47	9·5	100	39	0·82	1·0	
13 D 7	18	690	12·1	45	10·6	100	39·6	0·88	1·23	2
24 S 6	22	578	13·5	35·5	5·0	100	13·1	0·37	0·51	
10 D 8	19	766	15·8	71	9·5	100	44	0·61	0·80	
31 O 8	17	810	18	90	9·8	100	50	0·56	0·83	6
28 O 8	19	840	22	122	12·5	100	68	0·56	0·63	
27 O 8	19	814	23·5	122	13·7	100	72	0·59	0·67	
5 N 8	17	834	24	133	16·8	100	92	0·69	0·75	6
21 O 8	17	761	27	122	12·7	100	58	0·47	0·57	
23 O 8	18	686	33	122	17·4	100	64	0·52	0·60	
12 N 8	16	800	37	188	31·8	100	160	0·85	1·02	
Mean ± S.E.			17						0·64 ± 0·06	

Columns 6 and 8 give the quantities of Na emerging as a result of the injection; the residual efflux in cyanide has been subtracted. Na: ~ P ratios in column 10 have been corrected for diffusion of ^{22}Na beyond the phosphate column by using a diffusion coefficient of 10^{-5} cm^2.sec. Concentrations (mM) are in m-mole/l. axoplasm. Since ATP contains 2 ~ P per molecule the concentration in mM ~ P is twice the concentration in mM; for ADP and arginine phosphate, which contain 1 ~ P per molecule, the two units are identical. Unless otherwise stated, 2 mM-CN and 10 mM-K were used. 1 nmole=10^{-9} mole.

Notes: * Estimated from analysis of axoplasm at the end of the experiment; in other cases a value of 100 mM has been assumed. 1. Preceded by injection of 0·3 M-$MgCl_2$. 2. 0·2 mM DNP at pH 6·8, 10 mM-K. 3. Preceded by injection of 0·5 M-$MgCl_2$. 4. 0·2 mM DNP at pH 6·8, K-free. 5. 2 mM-CN, K-free. 6. 2 mM-CN, 20 mM-K.

Since the estimates of Na: ~ P are based on measurements of sodium efflux, rather than on net movements of sodium, they cannot be used to calculate the efficiency with which phosphate bond energy is transformed into secretory work. For example, if ATP increases Na influx as well as Na efflux, a calculation of efficiency based on efflux measurements would be erroneously large. An error in the opposite direction would occur if ATP and arginine phosphate are broken down in side reactions which have nothing to do with ion transport. In spite of these complications it is still interesting to see whether the observed Na: ~ P ratio can be checked by other methods. One method is to assume that cyanide blocks production of ATP rather promptly and that the lag in the fall of Na efflux depends on the energy-rich phosphate bonds stored in arginine phosphate and ATP. The fraction of internal sodium which leaves the fibre under the influence of the natural store of ~ P can be worked out from the area under the curve for the decline in the Na rate constant after applying cyanide; in measuring the area the basal efflux should, as usual, be subtracted from the efflux at any moment. The conclusion from analysing 29 experiments in Table 2 is that $5{\cdot}6 \pm 0{\cdot}3\%$ (mean and S.E.) of the labelled sodium moved out of the fibre during the initial period in cyanide. With an internal sodium concentration of 60–80 mM this is equivalent to a decrease in sodium concentration of 3·4–4·5 mM. From Caldwell (1960), the concentration of arginine phosphate is about 3·3 mM and of ATP about 1·3 mM (2·6 mM ~ P) so that Na: ~ P = 0·6 – 0·8. A similar figure is obtained by taking the approximate figure of 4Na per O_2, calculated (Hodgkin & Keynes, 1954) for *Sepia* axons and frog muscle; with a ~ P:O_2 ratio of 3 this gives Na: ~ P = 2:3. Since the three estimates all give roughly the same Na: ~ P ratio the tentative conclusion is that injected phosphate compounds may be used in the same way as those supplied by the normal metabolic cycle.

The Na: ~ P ratios described here are smaller than those calculated from the oxygen consumption and sodium transport of short-circuited frog skin (Zerahn 1956; Leaf & Renshaw, 1957). However, in the frog skin experiments, which gave Na: ~ P ratios of 3 if the basal oxygen consumption was subtracted, sodium ions were probably being transported against a lower electrochemical potential difference than that in nerve or muscle.

The extent to which the Na efflux is restored by injections

Arginine phosphate. The relation between the initial concentration of arginine phosphate in the axoplasm and the absolute magnitude of the sodium efflux is illustrated by Fig. 8, curve *A*. In order to reduce scatter, the observations have been collected in groups according to the concentration

of ~ P. The efflux reached a saturating value of about 50 pmole/cm^2.sec at concentrations of 7–15 mM ~ P, and then remained constant. The values plotted were not corrected for longitudinal diffusion of ^{22}Na,

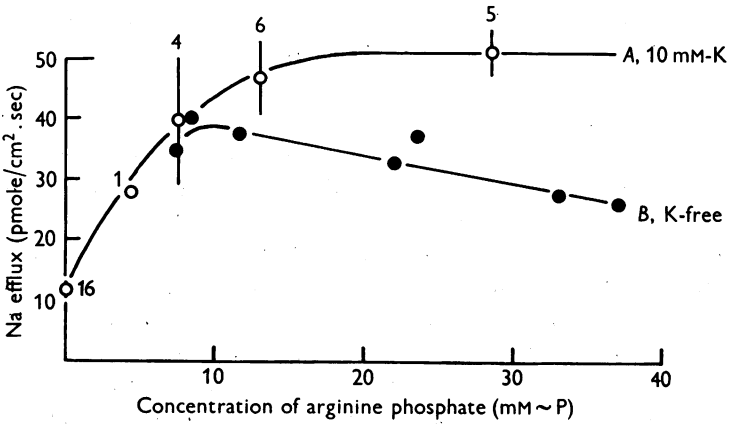

Fig. 8. Relation between initial concentration of injected arginine phosphate and peak Na efflux, ○, from observations in 10 mM-K; ●, from observations in K-free solution. The results of individual experiments in 10 mM-K have been collected in the following groups: < 5, 5–10, 10–20, 20–40 mM ~ P. ○ are the average of each group, the numbers in each group are shown against the points and ± 2 × S.E. of mean by the vertical lines. Values in K-free solutions (●) are individual results normalized by multiplying the ordinate of curve *A* by the figure for K sensitivity (Column 5 in Table 5).

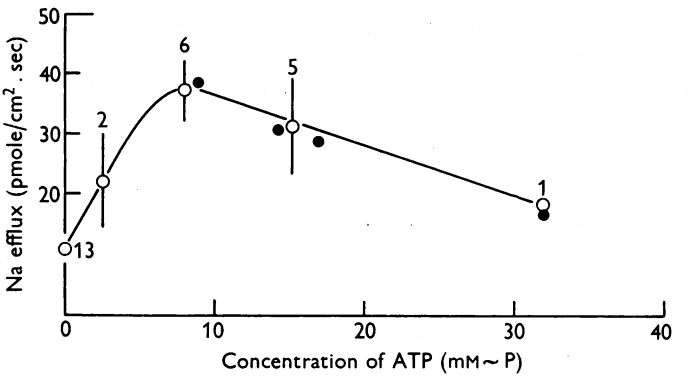

Fig. 9. Relation between initial concentration of ATP (mM ~ P) and peak sodium efflux. ○, from observations in 10 mM-K, averages after grouping as in Fig. 8; ●, from observations in K-free solution, obtained in the same way as in Fig. 8.

the reason being that since the efflux reaches saturation, longitudinal diffusion of arginine phosphate, which was neglected in deriving eqn. (1), would reduce the correction factor to an indeterminate extent. In con-

sidering the extent of restoration we wished to avoid any procedure which might exaggerate the effect of the injection. Further evidence of saturation was provided by the observation that with large doses of arginine phosphate the efflux often remained approximately constant for 1–3 hr and then declined relatively quickly (Fig. 10).

In order to decide whether arginine phosphate is fully restoring the sodium efflux, the best criterion is the rate constant for the loss of internal

TABLE 3. Rate constants and peak sodium fluxes after injection, arginine phosphate and phosphoenolpyruvate

1	2	3	4	5	6	7	8	9	10	11	12
				Rate constant (min^{-1}/1000)				Extent of restoration			
Fibre reference	Temperature (°C)	Axon diameter (μ)	Initial concentration (mM ~ P)	Normal, k_1	Poisoned, k_2	Injected, k_3	Normal, k_4	$\frac{k_2}{k_1}$	$\frac{2k_3}{k_1+k_4}$	Peak Na efflux after injection (pmole/cm².sec)	Calculated correction factor
				Arginine phosphate							
5 O 6	18	668	4·4	3·1	0·50	0·99		0·32	0·45	28	1·32
5 O 6	18	668	14·7		0·35	1·28	1·3	0·41	0·58	36	1·45
4 O 6	20	800	5·4	2·5	0·48	1·26	1·7	0·52	0·60	42	1·32
7 O 8	20	898	7·4	1·65	0·26	1·40	1·76	0·85	0·82	52	1·24
21 N 8	16	844	8·5	1·14	0·29	0·74	—	0·65	—	26	1·17
26 S 6	20	706	9·1	3·5	0·32	1·40	1·5	0·40	0·56	41	1·41
20 S 6	21	664	10·3	3·6	0·61	1·93	2·1	0·54	0·68	53	1·30
17 N 8	17	725	11·5	1·65	0·36	1·42	—	0·86	—	43	1·22
13 D 7	18	690	12·1	3·5	0·53	1·98	—	0·57	—	57	1·41
24 S 6	22	578	13·5	3·35	0·58	2·06	2·7	0·62	0·68	50	1·39
10 D 8	19	766	15·8	2·0	0·18	1·32	1·2	0·66	—	42	1·31
31 O 8	17	810	18	2·9*	0·44*	1·92*	1·2*	0·66*	0·94*	65*	1·48
28 O 8	19	840	22	2·1	0·35	1·62	1·5	0·77	0·90	57	1·12
27 O 8	19	814	23·5	2·1	0·38	1·54	1·45	0·73	0·87	52	1·14
5 N 8	17	834	24	3·0*	0·35*	1·94	1·5*	0·65	0·86	67*	1·09
21 O 8	17	761	27	2·1	0·30	1·60	1·4	0·76	0·91	51	1·22
23 O 8	18	686	33	2·2	0·37	1·85	1·6	0·84	0·97	53	1·15
12 N 8	16	800	37	1·3	0·35	1·35	1·1	1·05	1·12	45	1·20
				Phosphoenolpyruvate							
24 N 9	16	804	17	1·52	0·23	1·00	.	0·66	—	47	1·14
26 N 8	15	860	20	1·51	0·27	1·14	—	0·76	—	57	1·22
21 N 8	16	844	21	1·14	0·34	0·83	—	0·73	—	41	1·36

* In 20 mM-K, 13D7 was in 0·2 mM DNP at pH 6·8, the remainder in 2 mM-CN.

Fluxes are calculated with $[Na]_i$ as in Table 1. k_1 is the rate constant in artificial sea water at the beginning of the experiment; k_2 in CN or DNP just before injecting; k_3 was measured at the peak of the response to injection, usually 15 min after injection; k_4 after recovery in artificial sea water. The correction factor (which has not been applied) gives the calculated ratio of the total ^{22}Na to the quantity which had not diffused beyond the column of ~ P.

sodium. Rate constants after injection, which do not depend on estimates of the internal sodium concentration, are given in Table 3 (Column 7). From Column 9 it will be seen that with high concentrations of arginine phosphate (> 20 mM) the rate constant after injection into a poisoned fibre is about 0·8 of that in the unpoisoned fibre at the beginning of the experiment. However, since the rate constant at the end of the experiment was less than that at the beginning, this comparison is weighted against the injection. Column 10, which gives the ratio of the rate constant after injection to the mean of the initial and final values, probably affords a

fairer basis for comparison; from this it will be seen that with high concentrations of arginine phosphate restoration is about 90% complete. Bearing in mind that the correction for longitudinal diffusion of ^{22}Na has not been introduced, the conclusion is that high concentrations of arginine phosphate produce a complete, or very nearly complete, restoration of the sodium efflux. However, the concentrations required to do this are larger than those in the normal fibre; this aspect is considered further on p. 27.

TABLE 4. Rate constants and peak sodium fluxes after injection, nucleotides

1	2	3	4	5	6	7	8	9	10	11	12
				Rate constant (min^{-1}/1000)				Extent of restoration			
Fibre reference	Temperature (°C)	Axon diameter (μ)	Initial concentration (mM ~ P)	Normal, k_1	Poisoned, k_2	Injected, k_3	Normal, k_4	$\frac{k_3}{k_1}$	$\frac{2k_3}{k_1+k_4}$	Peak Na efflux after injection (pmole/cm².sec)	Calculated correction factor
						ATP					
18 D 6	20	725	2·4	2·1	0·29	0·59		0·28	0·38	18	1·35
18 D 6	20	725	12·4		0·22	1·00	1·0	0·48	0·65	30	1·49
20 N 7	19	890	2·8	1·4	0·26	0·69	—	0·49	—	26	1·41
19 D 6	19	780	5·8	2·7	0·43	1·00	1·3	0·37	0·50	32	1·41
19 D 7	18	911	7·4	2·2	0·41	1·27	—	0·58	—	47	1·41
22 N 7	19	867	8·2	1·95	0·28	1·12	—	0·57	—	40	1·45
24 O 7	18	890	8·2	2·3	0·40	1·18	—	0·51	—	40	1·33
27 S 7	18	850	9·0	2·2	0·12	0·95	—	0·43	—	34	1·43
30 O 7	18	842	9·2	1·8	0·34*	0·97*	—	—	—	46*	1·41
10 N 9	19	776	9·5	1·5	0·21	0·97	1·0	0·66	0·79	31	1·13
4 O 7	17	830	9·6	2·2	0·26*	0·92*	—	—	—	43*	1·54
11 D 6	18	581	12·6	3·8	0·56	1·56	1·9	0·41	0·55	38	1·39
7 N 8	17	805	14·3	1·5	0·35	0·90	1·0	0·60	0·72	32	1·19
19 N 8	17	746	17·0	1·0	0·24	0·55	—	0·55	—	17	1·25
12 S 7	19	578	19·6	4·2	0·59	1·60	1·6	0·38	0·5	39	1·33
24 N 8	16	720	32	1·9	0·30	0·60	—	0·32	—	18	1·22
						ADP					
11 N 9	17	806	2·1	1·5	0·28	0·54	—	0·36	—	18	1·14
22 O 7	17	826	16·8	2·2	0·28	0·73	—	0·33	—	23	1·35
7 N 7	18	796	18·0	3·0	0·25	0·96	—	0·32	—	29	1·45
16 D 8	17	606	23·1	2·2	0·38	1·2	—	0·55	—	30	1·22
						GTP					
18 N 9	16	786	10·4	1·28	0·18	0·75	0·74	0·59	0·74	25	1·23
12 N 9	19	774	10·7	2·00	0·22	0·85	0·68	0·43	0·64	27	1·11
						ITP					
13 N 9	18	744	11·2	1·81	0·29	0·40	1·0	0·22	0·28	12	1·14

* In K-free solution. 24 O 7 and 30 O 7 were in 0·2 mM DNP at pH 6·8, the remainder in 2 mM-CN. Other details as in Table 3.

Adenosine triphosphate. Figure 9 gives the relation between the initial concentration of ATP and the estimated sodium efflux. The efflux appears to rise to a maximum of about 40 pmole/cm².sec at 10 mM ~ P and then to decline at higher concentrations (see Table 4). On p. 581 it is shown that high concentrations of ATP, ADP and pyrophosphate depress the sodium efflux of unpoisoned fibres. This probably accounts for the decline in Fig. 9, but makes it difficult to assess the restorative effect of ATP.

The K sensitivity of the Na efflux

Arginine phosphate. In an unpoisoned squid axon removal of external K causes a sudden and reversible decrease in the efflux of sodium. The ratio (Na efflux) $_{\text{K-free}}$:(Na efflux)$_{10\text{mM-K}}$ varied between 0·2 in some fresh fibres to 0·5 or more in axons which had been isolated for long periods; for example, 0·7 in an axon used 24 hr after death of the squid. In the experiments considered here, which were all carried out on axons from refrigerated mantles (p. 562), the average value of the ratio was 0·35; a mean value of 0·30 was obtained on *Sepia* axons (Hodgkin & Keynes, 1955).

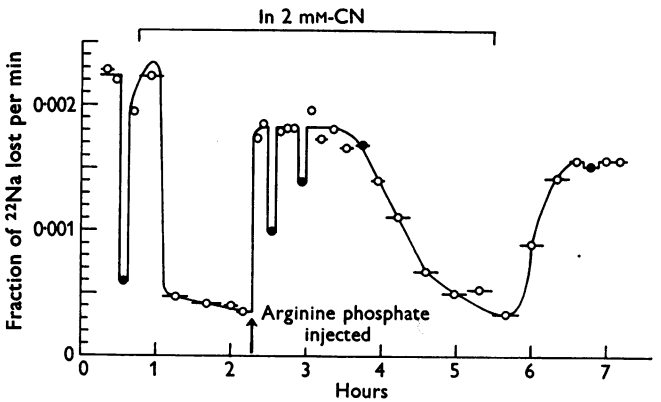

Fig. 10. Effect of injecting a large quantity of arginine phosphate into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 10 mm and with arginine phosphate, 15 mm. Immediately after the injection the mean concentration of arginine phosphate in the fibre was 33 mM. Axon 2308, diameter 686 μ, 18° C. At the end of the experiment the action potential was 101 mV.

Since the Na efflux is normally K-sensitive, it was important to find out whether the Na efflux resulting from injections had the usual response to removal of K. An additional reason for carrying out such experiments is that strong inhibitors such as 2 mM-CN or 0·2 mM DNP at pH 6·7 cause a transient rise in the Na efflux into a K-free solution at a time when the Na efflux into 10 mM-K is still near its normal value. This and related effects which will be described in a later paper may be summarized by saying that the K sensitivity of the Na efflux is more susceptible to interference with metabolism than is the absolute level of the Na efflux into normal sea water (Caldwell, Hodgkin, Keynes & Shaw, 1960).

Figure 10 illustrates the effect of injecting a high concentration of arginine phosphate into a fibre poisoned with cyanide on the sodium efflux in 10 mM-K (○) and in a K-free solution (●). In the unpoisoned

fibre the rate constant in 10 mM-K was $2{\cdot}2 \times 10^{-3}$ min^{-1} and removal of K reduced this to $0{\cdot}6 \times 10^{-3}$ min^{-1}. After the fibre had been in cyanide for 90 min the rate constant in 10 mM-K was $0{\cdot}4 \times 10^{-3}$ min^{-1}; on injecting arginine phosphate (33 mM ~ P) the rate constant in 10 mM-K rose to $1{\cdot}85 \times 10^{-3}$ min^{-1} and remained at this level for an hour. The efflux was initially cut to about a half by removing external K but the K sensitivity decreased fairly rapidly and had disappeared by the end of the plateau. The loss of K sensitivity may be due partly to a loss of arginine phosphate and partly to an accumulation of arginine (see p. 562); an accumulation of arginine might also explain the fact that although the sodium efflux rose

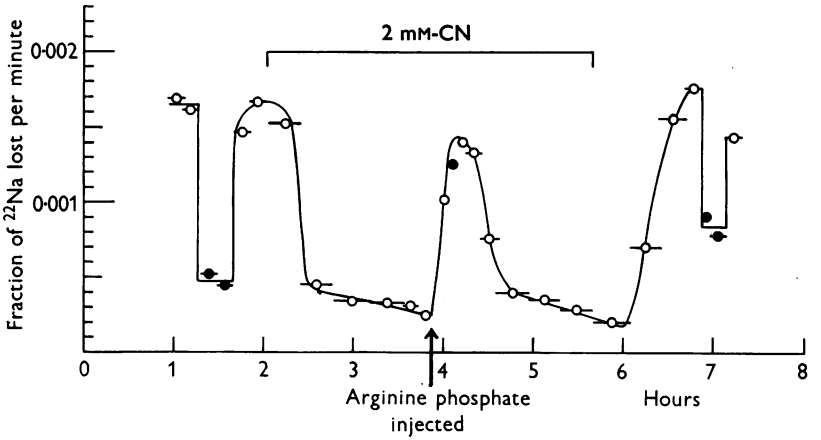

Fig. 11. Similar to Fig. 10 but injecting a smaller quantity of arginine phosphate. The length injected with ^{22}Na was 10 mm, and with arginine phosphate 15 mm. Immediately after the injection the mean concentration of arginine phosphate in the fibre was 7·4 mM. Axon 708, 898 μ, 20° C; final action potential, 99 mV.

to a fairly high level when cyanide was removed there was only a very slight recovery of K sensitivity.

Figure 11 illustrates the action of a dose of arginine phosphate which was about one quarter of that considered previously. In this case the period of enhanced sodium efflux lasted for a relatively short time and there was no plateau. A single observation made near the peak of the curve indicated that the efflux was little affected by removing K. From Table 5 it can be seen that the Na efflux became K-sensitive when the injected arginine phosphate exceeded a concentration in the axon of about 8 mM.

The relation between the concentration of arginine phosphate and the Na efflux into a K-free solution is illustrated by curve *B* in Fig. 8. In contrast to the efflux into 10 mM-K, which remained approximately constant over the range 10–30 mM ~ P, the efflux into a K-free solution probably reached a maximum at about 10 mM ~ P and then declined

slowly as the concentration was increased to 35 mM ~ P. The two curves in Fig. 8 account satisfactorily for the way in which the Na efflux into 10 mM-K and K-free solution vary with time after injection of a large quantity of arginine phosphate. Suppose that, as in the experiment of Fig. 10, the initial concentration of arginine phosphate is 33 mM. As the concentration

TABLE 5. Effect of injections on K sensitivity of sodium efflux

1	2	3	4	5	6	7
		(Na efflux in K-free solution):(Na efflux in 10 mM-K)				
Fibre reference	Initial concentration (mM ~ P)	Normal	In CN	In CN soon after injection	In CN long after injection	Normal after recovery
			Arginine phosphate			
7O8	7·4	0·30	—	0·9–1·0	—	0·5
21N8	8·5	0·41	—	1·0	—	—
17N8	11·5	0·43	1·2	0·83	1·3	
(17N8	45·6		1·3	0·68	—	—)
31O8†	18	0·18†	1·1†	0·49†	—	0·85†
28O8	22	0·29	1·0	0·64	—	0·78
27O8*	23·5	0·34	1·4*	0·73*	1·1	1·00*
5N8†	24	0·18†	1·5†	0·51†	1·2†	0·60†
23O8	33	0·27	—	0·54	1·0	0·98
12N8	37	0·35	1·2	0·46	—	0·86
(19N8	43	0·31	1·4	0·51	—	—)
			Phosphoenolpyruvate			
24N9	17	0·42	1·1	0·65	1·23	
26N8	20	0·30	1·4	0·68	—	—
(21N8	21	0·41	—	0·81	—	—)
			ATP			
10N9	9·5	0·31	1·2	1·03	—	0·69
7N8	14·3	0·41	1·4	0·93	—	0·9
19N8	17·0	0·31	1·4	0·94	—	—
24N8	32	0·29	1·4	0·91	—	—
			ADP			
16D8	16	0·45	1·4	1·02	—	—
			GTP			
18N9	10·4	0·46	1·3	1·1	1·5	0·85
12N9	10·7	—	—	1·3	—	—

* Axon in K-free solution for most of experiment, 10 mM-K for short periods only. † 20 mM-K instead of 10 mM-K throughout. Results in brackets are second injections of phosphate ester done at a later stage of the experiment. In Columns 5 and 6, 'soon' and 'long' mean about 15 and 120 min, respectively.

falls from 33 to 10 mM the Na efflux into 10 mM-K should remain constant but the efflux into K-free solution should rise steadily. K sensitivity ought to disappear at the end of the plateau. These predictions were borne out by the experiment of Fig. 10 and by other experiments of a similar type.

One point not illustrated by Fig. 10 is that in cyanide either before or long after the injection there is a small effect of K on sodium efflux which is in the opposite direction to that in the normal fibre. In the poisoned fibre removal of K usually increases the residual efflux by 10–40% instead of decreasing the efflux by 60–80% as in a normal fibre. The effect of a maximal injection of arginine phosphate is thus to alter the ratio (Na efflux)$_{K\text{-free}}$:(Na efflux)$_{10\ mM\text{-}K}$ from 1·1–1·4 in cyanide to about 0·5 soon after the injection.

The general conclusion from the experiments with arginine phosphate is that this compound is capable of restoring a K-sensitive efflux of sodium but only when applied at concentrations which are much higher than those in a normal fibre.

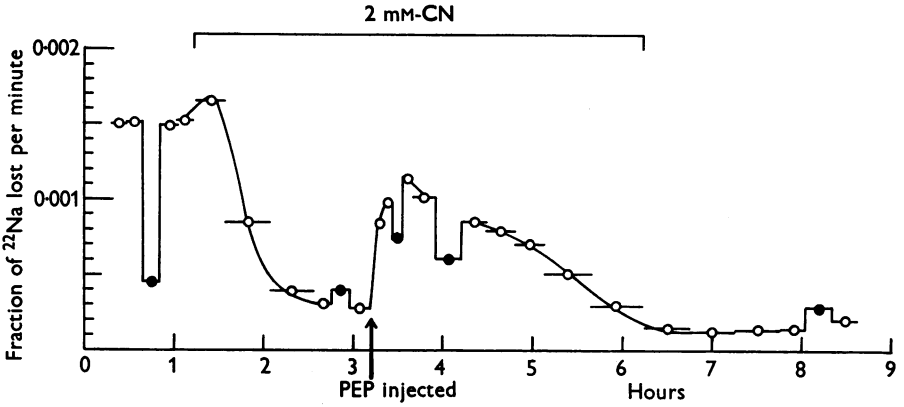

Fig. 12. Effect of injecting phosphoenolpyruvate (PEP) into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 10 mm, and with PEP 16 mm. Immediately after the injection the mean concentration of PEP in the fibre was 20·5 mM. Axon 26N8, 860 μ, 17° C; final action potential, 87 mV.

Phosphoenolpyruvate. Figure 12 gives the result of injecting phosphoenolpyruvate (PEP) into an axon poisoned with cyanide. The recovery of K sensitivity is definite, but not as marked as with arginine phosphate; the reason may be that the PEP was injected as a sodium salt, for there were indications that the K effect was small when the internal sodium was high.

An unexpected conclusion about the action of PEP, which depends on a single experiment, is that after the response to the injection had worn off there was no final recovery on removing cyanide. This result is illustrated in Fig. 12; the other experiments with PEP, which were terminated in cyanide, provided no evidence about recovery. Since there was always a substantial recovery on removing cyanide after injections of ATP or arginine phosphate, it seems that PEP may give rise to some compound with a blocking action which outlasts removal of cyanide.

Adenosine triphosphate. The effect of injecting a large concentration of ATP is illustrated by Fig. 13. The sodium efflux did not rise to such a high value as that obtained after injections of arginine phosphate and it

was not appreciably reduced by removing external K; similar results were obtained in all the other experiments with ATP (Table 5). In one case an injection of arginine phosphate made the Na efflux sensitive to external potassium after a previous injection of ATP had failed to do so.

Although the results of injecting ATP were clearly different from those of injecting arginine phosphate, it is not right to conclude that ATP had no effect at all on K sensitivity. When external K was removed the Na efflux of a fibre in cyanide normally increased, instead of decreasing as in an unpoisoned fibre. After an ATP injection removal of K no longer increased the Na efflux, so that in this sense the ATP did have a small effect on K sensitivity.

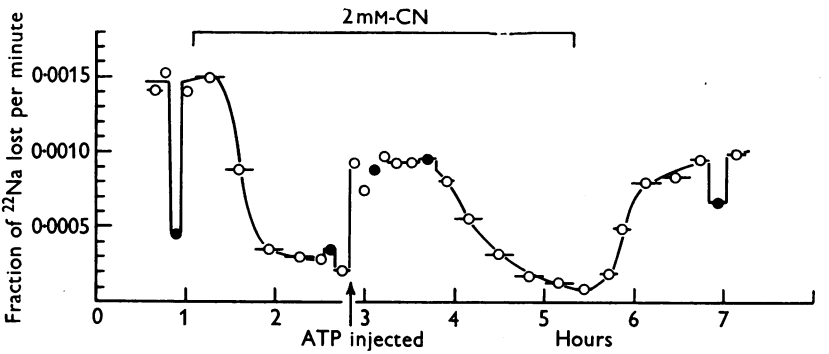

Fig. 13. Effect of injecting ATP into a CN-poisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 12 mm and with ATP, 18 mm. Immediately after injection the mean concentration of ATP in the fibre was 4·8 mM (9·5 mM ~ P). Axon 10N9, 776 μ, 19° C; final action potential, 92 mV. The ATP contained about 4 % of the phosphorus as ADP.

Adenosine diphosphate, guanosine triphosphate and inosine triphosphate. These compounds increased the Na efflux of a cyanide poisoned fibre but did not restore the normal K sensitivity (Table 5). The Na efflux after a GTP injection resembled the residual efflux in cyanide in being increased by removing K instead of being reduced as in the normal fibre.

The effects of injections on Na efflux in unpoisoned fibres

Arginine phosphate. Injections of arginine phosphate into normal fibres had little effect on Na efflux or on its K sensitivity. Tests were made with external potassium concentrations of 0, 10, and 20 mM; in no case was there any appreciable change in Na efflux after the injection of arginine phosphate. These results suggest that the normal fibre contains enough arginine phosphate to saturate the transport mechanism.

Since the K sensitivity of the sodium efflux declined when fibres were left for 12–24 hr in sea water, it seemed possible that arginine phosphate might have a beneficial effect when injected into an old fibre. This point was tested on one fibre which was used 15 hr after the death of the squid.

Figure 14 shows that injection of arginine phosphate affected neither the K sensitivity nor the absolute level of the sodium efflux. The conclusion is that the low K sensitivity of old fibres does not depend on a low concentration of arginine phosphate but on some other change.

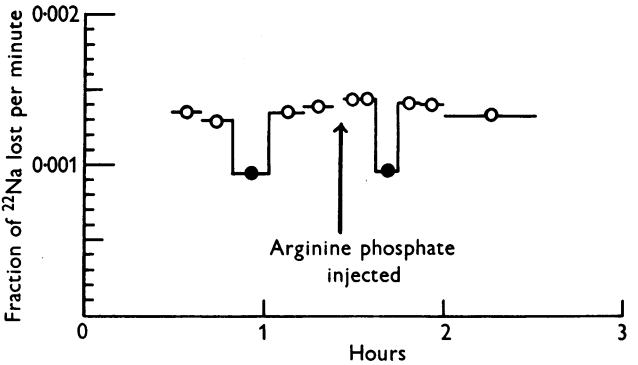

Fig. 14. Effect of injecting arginine phosphate into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 10 mm and with arginine phosphate, 16 mm. Immediately after injection the mean concentration of the injected arginine phosphate in the fibre was 37 mM. Axon 14N 8, 805 μ, *ca.* 18° C. This axon, which was taken from a squid which had just died, was kept for 15 hr, mainly at 0° C, before use. The action potential was 104 mV at the beginning of the experiment and 102 mV at the end.

Adenosine triphosphate. When injected in fairly high concentrations (> 3 mM), ATP depressed the efflux of sodium from an unpoisoned fibre. Figure 15*B* illustrates the decrease in efflux resulting from an injection which gave an initial rise of 8 mM (16 mM ~ P). The effects of high concentrations of pyrophosphate were similar to those of ATP (Fig. 15*A*) A single experiment showed that ATP at a concentration in the axon of 5 mM depressed the efflux into K-free solution in about the same proportion as that into 10 mM-K. High concentrations of ATP seem to depress the absolute level of the Na efflux but not to alter its K sensitivity. As the depressant action of ATP was reminiscent of the inhibitory effect of an excess of ATP on the adenosine triphosphatase of myofibrils (Perry & Grey, 1956), the effects of Mg were investigated. Introduction of Mg (as $MgCl_2$) in an amount equivalent to the ATP appeared to have a protective effect, neither the Mg nor the subsequent dose of ATP causing any marked change.

Adenosine diphosphate. Figure 16 shows the result of injecting sufficient ADP to raise the concentration in the axon by 2·6 mM. The effect of ADP was to raise the Na efflux into K-free sea water and to cause a slight reduction in the efflux into 10 mM-K. In contrast to ATP, ADP reduces K sensitivity.

With a much larger quantity of ADP (13 mM in the axon) the injection left the Na efflux into K-free sea water unaltered and reduced the efflux into 10 mM-K to the K-free level. Apparently a high concentration of ADP has an inhibitory effect which is similar to that of excess ATP.

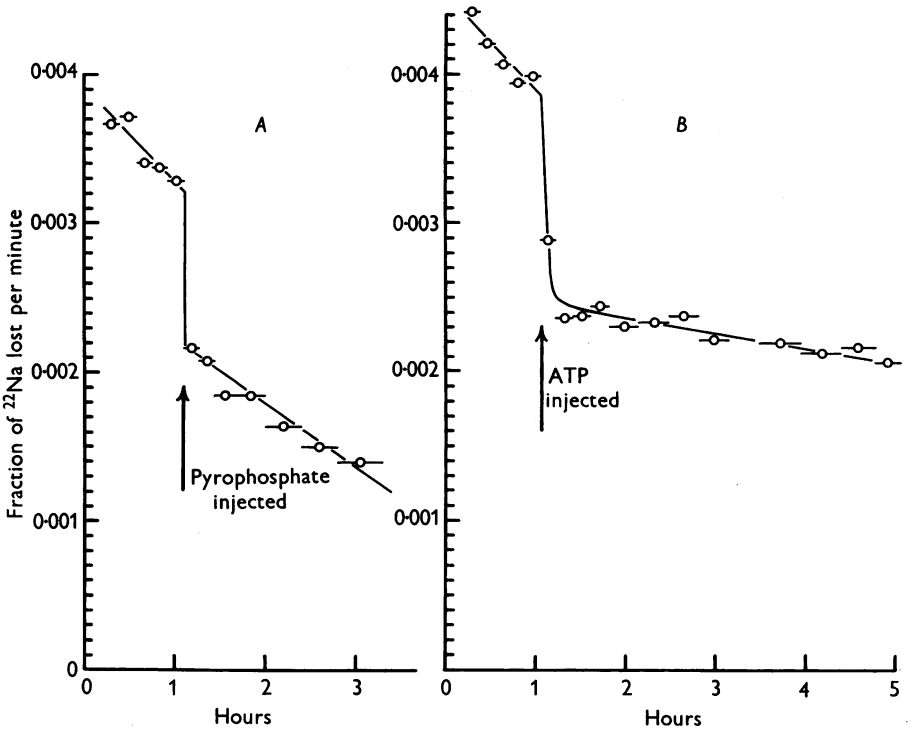

Fig. 15. The effect on Na efflux from unpoisoned axons of injecting pyrophosphate and ATP. In both experiments the axons were loaded with a 12 mm column of ^{22}Na, and the phosphate compounds were injected over the same 12 mm. *A*: axon 17S7, diameter 658 μ, temperature 18° C, concentration of potassium pyrophosphate inside axon after injection was 14·4 mM. *B*: axon 16S7, diameter 640 μ, temperature 18·5° C, concentration of K salt of ATP inside axon after injection was 8·0 mM (16 mM ~ P).

Arginine. In fibres poisoned with cyanide a high concentration of arginine phosphate increases the K sensitivity of the sodium efflux. One might therefore expect that a high concentration of arginine would reduce the K sensitivity of the normal efflux. Figure 17 shows that the initial effect of injecting arginine into an unpoisoned fibre was to increase the Na efflux into K-free solution. Before injecting arginine the ratio $(\text{Na efflux})_{\text{K-free}} : (\text{Na-efflux})_{10\,\text{mM-K}}$ was 0·23; 5 min after the injection it was 0·8. On leaving the fibre there was a slow recovery in K sensitivity and a final injection of arginine phosphate caused only a slight improvement.

The slow recovery is not understood, since there was not enough phosphate in the axon to convert much of the arginine into arginine phosphate.

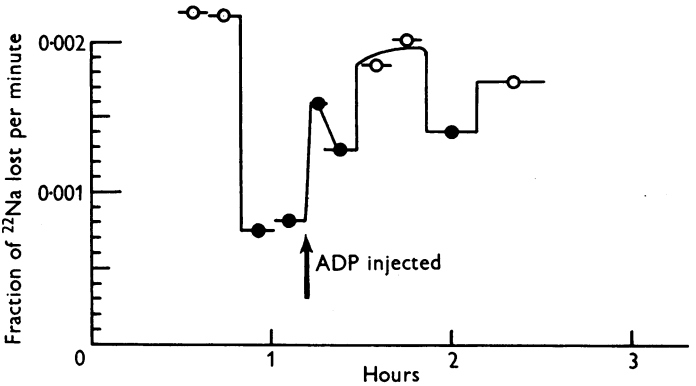

Fig. 16. Effect of injecting ADP into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 10 mm and with ADP, 16 mm. Immediately after injection, the mean concentration of ADP in the fibre was 2·6 mM. Axon 27N8, 860 μ, 19° C; final action potential, 80 mV.

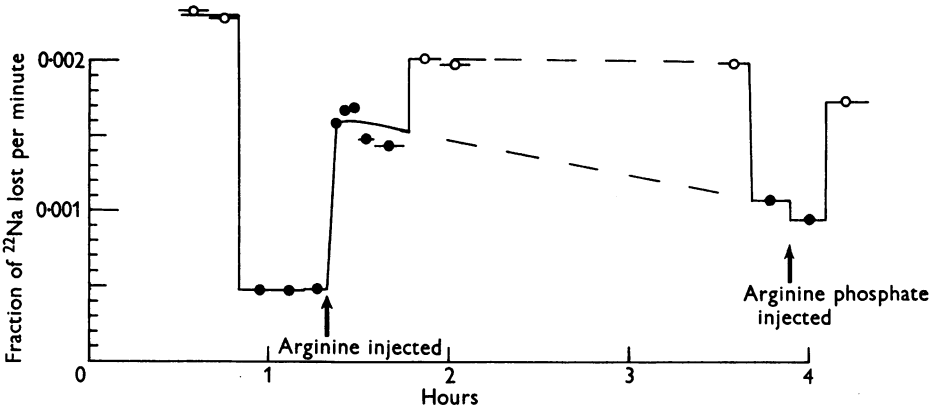

Fig. 17. Effect of injecting arginine HCl into an unpoisoned fibre on Na efflux into K-free solution (●) and 10 mM-K (○). The length injected with ^{22}Na was 10 mm and with arginine HCl 16 mm. Immediately after injection the mean concentration of arginine in the fibre was 62 mM. Axon 9D8, 788 μ, 18° C, final action potential 92 mV.

The effect of injections on the other Na and K fluxes in poisoned fibres

Na influx: the effect of CN and of ATP injections. The experiments on Na influx were done before the difference between the effects of ATP and of arginine phosphate on the Na efflux had been appreciated, and were confined to the action of injected ATP. The Na influx was determined by exposing the axon to a solution containing ^{24}Na for 10 min, and then taking

counts for about an hour with a stream of inactive sea water flowing past the axon. The removal of adherent small fibres and connective tissue from the giant axon was done as completely as possible, but even so about 40 min elapsed before all the superficial ^{24}Na was washed away. However, after this period the radioactivity declined with a low rate constant of the same order (0·01–0·02 hr^{-1}) as that found for CN-treated axons injected

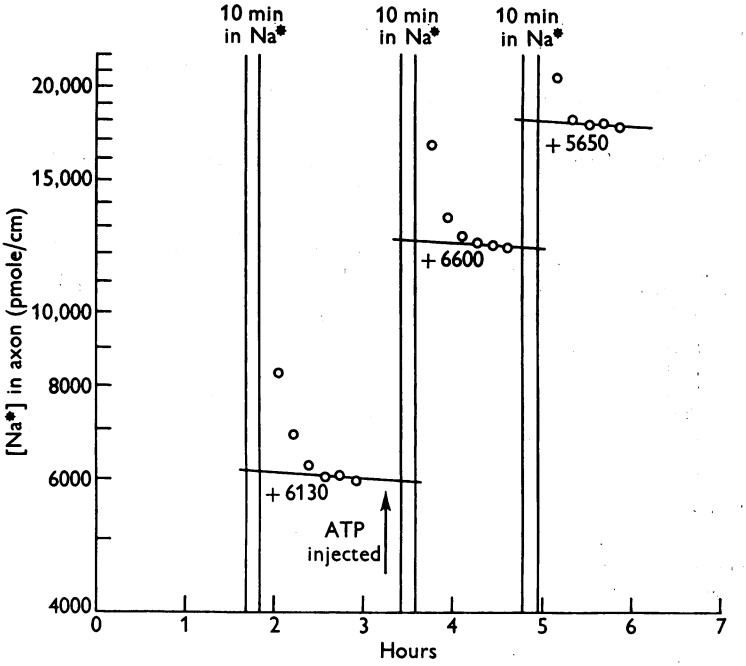

Fig. 18. The effect of injecting ATP on the influx of labelled sodium. Both the ^{24}Na solution and the inactive solution in which counts were taken contained 2 mM-CN, and the axon had been in the CN-sea water for 2·2 hr before the first period in ^{24}Na. Sufficient ATP was injected at the arrow to give a mean concentration of 5·3 mM in the axoplasm, over a length of 20 mm. Axon 9D7, diameter 768 μ, temperature 17° C. At the end of experiment the resting potential was 58 mV, action potential 97 mV.

with ^{22}Na, and could therefore be taken to represent labelled Na which had penetrated into the axoplasm. In earlier work with a similar technique (see Hodgkin & Keynes, 1955) it was easier to distinguish between extracellular and intracellular radioactivity, since the ^{24}Na was introduced by stimulation, and relatively more entered the interior of the axon. Clearly, it would be desirable to make more measurements with extruded axoplasm in order to avoid this difficulty.

One experiment is illustrated in Fig. 18 and the results of this and four others are listed in Table 6. It appears that, as in DNP-treated *Sepia*

axons (Hodgkin & Keynes, 1955), the Na influx was little changed by poisoning with 2 mM-CN (8 unpublished experiments with smaller axons which were done in 1952 gave an average resting Na influx into unpoisoned squid axons of 49 pmole/cm².sec; Shanes & Berman (1955) give 62 pmole/cm².sec for the axons of *L. pealii*). For what it is worth, the table suggests that there may be a slight increase in Na influx immediately after the injection of ATP, which would be qualitatively consistent with the idea that ATP promotes an exchange diffusion of Na across the membrane. But the action of ATP on the influx seems to be definitely less than its stimulating effect on the efflux.

TABLE 6. The effect of injecting ATP on the influx of labelled sodium into CN-poisoned axons

Axon diameter (μ)	Influx before injection (pmole/cm².sec)	Influx 15 min after injection (pmole/cm².sec)	Influx 1·5 hr after injection (pmole/cm².sec)	[ATP] after injection (mM ~ P)
850	35	21	—	8·6
880	33	71	38	8·0
768	42	45	39	10·6
856	48	50	54	8·6
935	53	72	37	7·2
Mean	42	52	42	8·6

All the axons had been treated with 2 mM-CN for at least 1·2 hr before making the first influx measurement. In the first experiment the Na influx before poisoning was determined as 32 pmole/cm².sec; in the other experiments the unpoisoned influx was not measured. Temperature 17–19° C.

K efflux: the effect of CN and of ATP injection. Figure 19 shows that neither cyanide nor a subsequent injection of ATP had any marked effect on the efflux of labelled potassium. A second experiment on similar lines gave the same result. This emphasizes the difference in nature between the passive K efflux, effectively independent of metabolism, and the metabolically-driven Na efflux.

K influx: effects of cyanide and of injecting ATP or arginine phosphate. Determinations of K influx by counting intact squid axons proved to be complicated by the presence of substantial amounts of K in a superficial region of the fibre which exchanged much more rapidly than K introduced into the axon by micro-injection. It was therefore necessary to rely on measurements with extruded axoplasm, pairs of axons being used wherever possible. These results are given in Table 7. Tentative conclusions are (1) cyanide reduces K influx to about 25–50%; (2) after cyanide poisoning, injections of ATP, in concentrations giving about the maximum Na efflux, may give an increase in K influx—however, this increase was

small and was not statistically significant; (3) after cyanide poisoning, injections of arginine phosphate increase K influx about threefold.

These results are consistent with the idea that injections of arginine phosphate are more effective than ATP in restoring the coupling between K entry and Na exit. However, it now seems unlikely that there can be a

TABLE 7. K influxes in paired axons

I. Effect of CN (uncleaned axons)

Diameter (μ)		K influx (pmole/cm^2.sec)		
		2 mM-CN	Unpoisoned	Difference
A	*B*	*A*	*B*	*B*−*A*
638	638	5·8	20·7	14·9
712	690	13·6	14·8	1·2
622	645	8·7	21·6	12·9
Mean ± S.E.		9·3 ± 2·3	19·0 ± 2·2	9·7 ± 4·1

II. Effect of ATP after CN (cleaned axons)

Diameter (μ)			2 mM-CN, injected		Concentration of ATP in axon
A	*B*	2 mM-CN	with ATP	Difference	(mM ~ P)
955	986	6·6	9·1	2·5	6·8
655	684	3·6	4·4	0·8	14
850	924	2·6*	8·0*	5·4*	7·2
Mean ± S.E.		4·3 ± 1·2	7·2 ± 1·4	2·9 ± 1·3	

III. Effect of arginine phosphate after CN (cleaned axons)

Diameter (μ)			2 mM-CN, injected with arginine		Concn. of arginine phosphate in axon
A	*B*	2 mM-CN	phosphate	Difference	(mM)
681	709	5·0	14·3	9·3	48
761	756	4·3	12·9	8·6	42
841	810	4·4	12·5	8·1	37
806	821	3·2	14·1	10·9	35
844	831	4·6	14·6	10·0	35
Mean ± S.E.		4·3 ± 0·3	13·7 ± 0·4	9·4 ± 0·5	

* These axons were stimulated at 156/sec for 20 min before being placed in cyanide.

ATP or arginine phosphate was injected over a length of 20 mm about 10 min before a 10 min exposure to ^{42}K. After removal from ^{42}K, axons were soaked 10 min in inactive sea water before extruding axoplasm; in the case of injected axons, axoplasm was taken from 15 mm in the injected region. Control and injected axons were kept for the same period in CN (1½–2 hr). Temperature, 17–19° C. The mean resting influx in unpoisoned cleaned axons was 16·3 ± 2·2 pmole/cm^2.sec (5 experiments).

one-to-one coupling between K entry and Na exit. From Table 7 the Na efflux resulting from an injection of arginine phosphate is probably about 50 pmole/cm^2.sec. Since the Na efflux is reduced to about 25 pmole/cm^2.sec on removing K one might expect a K influx of 25 pmole/cm^2.sec if the K effect depends on a component of the Na efflux which is coupled in a one-to-one fashion with K influx. In practice, the extra

K influx resulting from an injection of arginine phosphate was about 10 pmole/cm^2.sec. Both this result and those obtained with normal fibres are better fitted by a theory in which an entry of one K^+ ion promotes the exit of 2 or 3 Na^+ ions.

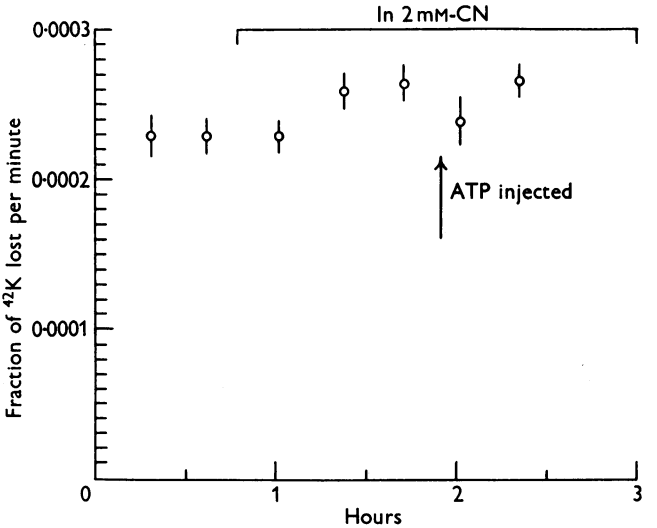

Fig. 19. The effect of CN and injection of ATP on the potassium efflux from a squid axon. The axon was loaded with ^{42}K by the injection of a 13 mm column of 0·57 M-K*Cl at zero time. At the arrow, sufficient ATP was injected to give a mean concentration of 8 mM ~ P over the same 13 mm. The lengths of the vertical lines indicate $\pm 2 \times$ S.E. of the counts. Axon 11N7, diameter 912 μ, temperature 17° C. At end of experiment resting potential was 59 mV, action potential 108 mV.

DISCUSSION

The object of this investigation was to see how far the effects of metabolism on the movements of Na and K could be imitated by injecting substances like arginine phosphate and ATP. The general answer is that both compounds increase the Na efflux of a fibre poisoned with cyanide or DNP, but that injected arginine phosphate is a more effective substitute for metabolism than injected ATP. Thus, arginine phosphate restores the normal coupling between Na efflux and K influx, whereas injected ATP seems to have little effect on K influx and gives a Na efflux which is not K sensitive. Of the other compounds containing ~ P which were tested, only phosphoenolpyruvate gave a Na efflux which was reduced by removing external K; creatine phosphate caused no increase and ITP a very slight increase in Na efflux; ADP and GTP caused a substantial increase in efflux which was not reduced by removing external K.

The compounds which increase Na efflux might all do so by providing a supply of ATP at the membrane. For ATP itself no intermediate reaction is required; for the other three compounds in which we were principally interested the reactions and enzymes might be as follows:

I. ADP + arginine P = ATP + arginine (Arginine phosphokinase),

II. ADP + pyruvate P = ATP + pyruvate (Pyruvate phosphokinase),

III. 2 ADP = ATP + AMP (Adenylate kinase).

In this hypothesis it is assumed that the axoplasm contains the necessary enzymes and that a small amount of ADP is left after cyanide poisoning. Evidence for these assumptions is provided by recent unpublished observations which show that injection of arginine phosphate into fibres poisoned with cyanide does regenerate ATP from AMP and that injection of phosphopyruvate under similar conditions regenerates both ATP and arginine phosphate. Some further assumption is needed to explain the effects of arginine phosphate and phosphoenolpyruvate in making the sodium efflux sensitive to removal of potassium. One possibility is that the transport system requires for its normal operation not only a supply of ATP but also that there should be a high ATP:ADP ratio near the membrane. On this view, the effects of arginine phosphate or phosphopyruvate on the coupling between K and Na movements arise from their action in keeping ADP at a low level by reactions I and II; ATP does not restore K sensitivity because it rapidly produces ADP by reaction III. The results of injecting ADP or arginine into unpoisoned fibres were consistent with this idea, since both substances increased the Na efflux into a K-free solution without having much effect on the efflux into a solution containing 10 mM-K. However, such experiments do not prove that the conversion of ATP to ADP is the reaction immediately concerned with ionic transport. It could equally well be argued that the transporting mechanism requires a high ratio of arginine phosphate to arginine, and that ADP affects the coupling between K and Na movements by converting arginine phosphate to arginine. Indeed, if the various forms of high-energy phosphate are in rapid equilibrium, it may be very difficult to decide which phosphate compound is most directly concerned in driving ion transport.

A quantitative difficulty raised by the experiments is that although injected arginine phosphate gave a complete, or nearly complete, restoration of the normal sodium efflux, the concentration required was about twice that in a normal fibre. Still higher concentrations were needed to make the Na efflux K sensitive. Two possible causes of the discrepancy will be considered. In the first place, the figure for the concentration of arginine phosphate was obtained by dividing the quantity injected per centimetre

by the cross-sectional area of the axon. The peak sodium efflux was reached 10–15 min after the injection and not instantaneously as would be expected if radial diffusion of arginine phosphate were so rapid that it could be neglected. By the time the Na efflux reaches its peak some arginine phosphate has probably broken down, so the total quantity in the fibre should be less than the amount injected. Furthermore, the concentration at the surface of the axon should be less than the mean concentration, since there must be a diffusion gradient in the axoplasm. Calculations with an equation in Carslaw & Jaeger (1947, p. 308, eqn. 7) suggest that with a diffusion coefficient of $0{\cdot}4 \times 10^{-6}$ cm^2/sec the surface concentration of arginine phosphate would reach a maximum in about 15 min and that the maximum surface concentration would be only one third of the initial mean concentration. A similar argument should hold if the diffusion coefficient of arginine phosphate in axoplasm is not as low as $0{\cdot}4 \times 10^{-6}$ and the delay in the rise of Na efflux is due to an intermediate chemical reaction.

Another explanation of the quantitative discrepancy is that the poisoned fibre probably contains an abnormal amount of break-down products such as arginine, AMP or inorganic phosphate. In the presence of these compounds it may require an unusually large concentration of arginine phosphate to restore a situation approaching that in the normal fibre.

The same arguments might also explain why very high concentrations of arginine phosphate are needed to restore the K sensitivity of the Na efflux. It seems likely that the loss of K sensitivity depends on the accumulation of a break-down product which may perhaps be identified with ADP. Possibly only a very slight rise in concentration, say from 0·01 to 0·1 mM, may be enough to abolish K sensitivity. In that case significant amounts of ADP might accumulate near the membrane unless the mitochondria in the immediate vicinity were metabolizing normally. Very large concentrations of arginine phosphate might then have to be injected into poisoned fibres in order to reduce the ADP at the surface to the normal level.

SUMMARY

1. Injections of adenosine triphosphate or diphosphate, guanosine triphosphate, arginine phosphate and phosphoenolpyruvate (PEP) caused a transient increase in the efflux of labelled sodium from squid giant axons which had been poisoned with 2 mM cyanide. Injections of creatine phosphate, adenosine monophosphate, arginine, inorganic phosphate, pyrophosphate and various salt solutions had no restorative action on fibres poisoned with cyanide; inosine triphosphate had only a very slight effect.

2. Injections of ATP and arginine phosphate also had a restorative effect on fibres poisoned with 0·2 mM DNP at pH 6·7; the other compounds were not tested.

3. ATP and arginine phosphate had no effect when applied externally to fibres poisoned with cyanide.

4. The duration of the response to ATP or arginine phosphate varied between about half an hour with a small quantity to several hours when injecting 5–10 times the quantity in an unpoisoned fibre.

5. The number of Na ions extruded was roughly proportional to the number of molecules of phosphate ester injected. Na: $\sim$ P ratios were about 0·7 for ATP, arginine phosphate and PEP, about 0·4 for ADP and GTP and about 0·03 for ITP.

6. The Na effluxes obtained by injecting high concentrations of arginine phosphate into poisoned fibres were intermediate between the levels in unpoisoned fibres at the beginning and end of the experiment.

7. The Na effluxes resulting from injections of ATP, ADP or GTP differed from the normal efflux in that they were not reduced by removing external potassium. On the other hand, the Na effluxes resulting from injections of arginine phosphate or PEP resembled the normal efflux in that initially they were substantially reduced by removing external potassium.

8. Injections of arginine phosphate increased the K influx of cyanide-poisoned fibres about threefold; ATP injections had relatively little effect.

9. The downhill fluxes (Na influx and K efflux), which were little altered by cyanide, were not much changed by injecting ATP into fibres poisoned with cyanide.

10. The Na efflux of unpoisoned axons was not affected by injections of arginine phosphate and was depressed by high concentrations of ATP, ADP or pyrophosphate. Injections of ADP or arginine made the Na efflux of the normal fibre insensitive to removal of external potassium.

11. The experiments provide further support for the view that metabolism drives the cation transport system by generating high-energy phosphate bonds which are carried to the membrane by substances like arginine phosphate and ATP. They also suggest that the presence of arginine phosphate as well as ATP may be required for the normal operation of the active transport mechanism.

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