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THE INCORPORATION OF INORGANIC PHOSPHATE INTO ADENOSINE TRIPHOSPHATE BY REVERSAL OF THE SODIUM PUMP

BY P. J. GARRAHAN* AND I. M. GLYNN
From the Physiological Laboratory, University of Cambridge

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SUMMARY

1. Resealed ghosts were prepared containing much potassium, very little sodium, and adenosine triphosphate (ATP), adenosine diphosphate (ADP) and ^{32}P-labelled orthophosphate (P_i) at concentrations such that the ratio [ATP]/([ADP].[P_i]) was low. Iodoacetate, iodoacetamide or both were also present. The ghosts were incubated in high-sodium, potassium-free media with and without ouabain, or in high-potassium media, and the incorporation of ^{32}P into ATP and ADP in 15 min was measured.

2. There was some incorporation of ^{32}P into the nucleotides whatever the medium, possibly because of the residual activity of glycolytic enzymes, but in every experiment there was extra incorporation when the ghosts were in a high-sodium, potassium-free medium. This extra incorporation was largely abolished by ouabain (5 experiments) and partly abolished by oligomycin (1 experiment).

3. It seems that if conditions are such that the over-all reaction associated with transport ATPase activity leads to an increase in free energy, the transport system will run backwards at a measurable rate and ATP will be synthesized at the expense of energy derived from ionic concentration gradients.

4. The nature of the transport system is discussed in the light of the findings of this paper and the four preceding papers.

INTRODUCTION

If the cation pump in the red cell membrane expels three sodium ions and takes up about two potassium ions for each molecule of ATP hydrolysed (Sen & Post, 1964; Gárdos, 1964; Whittam & Ager, 1965; Glynn 1962; Garrahan & Glynn, 1967*d*), the free energy avail-

* Present address: 2da Cátedra de Fisiologia y Centro de Investigaciones Cardiológicas, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

able to drive the reaction forwards must be quite small under physiological conditions. Calculations given in the Appendix suggest a figure of less than 4000 cal. By making the concentration gradients for sodium and potassium even more adverse than under physiological conditions, it should be thermodynamically possible to drive the pump backwards and synthesize ATP though, of course, theory cannot predict that the reaction would occur at a measurable rate. T. I. Shaw (personal communication) did not succeed in an ingenious attempt to demonstrate reversal of the sodium pump in squid axons, but his experiments left the possibility of reversal still an open question. Investigators who have looked for ATP–P_i exchange associated with transport ATPase activity have never found it (Skou, 1960; Fahn, Koval & Albers, 1966), but they have always looked in broken cell preparations, where ionic concentrations are inevitably the same on both sides of the membrane and where, in consequence, ΔG is likely to be heavily in favour of ATP break-down. The effects of inorganic phosphate on the behaviour of the transport system in resealed ghosts are more encouraging, since they suggest that the final step in the over-all ATPase reaction may be reversible (Garrahan & Glynn, 1967*c*).

In a preliminary attempt to reverse the pump, resealed red cell ghosts containing potassium in high concentration, adenosine diphosphate (ADP), adenosine monophosphate (AMP) and ^{32}P-labelled P_i, were incubated in media rich in sodium and containing no potassium. After 15 min, heavily labelled ATP and ADP could be detected, but as the labelling was not prevented by ouabain and occurred equally well when the external solution contained potassium rather than sodium, it could not be attributed to reversal of the pump and was, presumably, caused by glycolytic enzymes. Schrier & Doak (1963) have shown that triose phosphate dehydrogenase and, to a lesser extent, phosphoglycerate kinase are partly bound to the membrane in red cells, and glycolytic ATP–P_i exchange in red cell membranes has recently been reported by Ronquist & Ågren (1966). Furthermore, the lysing solution used in the experiment had a rather high osmolarity and there is reason to believe that the ghost preparation was heavily contaminated with intact cells, some of which might have been stretched sufficiently for inorganic phosphate to have entered (cf. Sen & Post, 1964). In subsequent experiments, particular care was taken to exclude intact cells from the ghost preparations and to inhibit any residual triose phosphate dehydrogenase, as far as possible, with sulphydryl inhibitors chosen for their relative ineffectiveness on the transport ATPase system.

This paper reports the results of six consecutive attempts to demonstrate reversal of the pump. For reasons that will be discussed later, the conditions differed slightly in the different experiments, but all six

showed extra incorporation of labelled P_i into ATP under conditions favourable for reversal, and in all six this extra incorporation was greatly reduced by an inhibitor thought to be specific for the pump system.

A preliminary account of these experiments has been published (Garrahan & Glynn, 1966).

METHODS

Procedure for the reversal experiments

The preparation of resealed ghosts. Cells from 40 ml. of fresh citrated blood were washed 4 times at 37° C with a solution containing (mM): K 150; Mg 1; Tris 1·4; Cl 147; phosphate 2·5; iodoacetate 2; pH 7·4 at 37° C. In some of the experiments 5 mM iodoacetamide was also included. The cells were allowed to stand for 2 min between each wash to allow time for the exit of glucose. The washed cells were resuspended in more of the wash solution and were incubated for 1 hr at 37° C to allow time for the inhibitors to act and to decrease, to some extent, the concentrations of glycolytic intermediates. The cells were then washed 4 times at room temperature with a wash solution containing (mM): K 105; Mg 30; Tris (pH 7·4 at 20° C) 13; Cl 173; iodoacetate 2; iodoacetamide 5. In the first two experiments the inhibitors were omitted from this wash solution. The cells were packed at 1500 *g* for 7 min at room temperature and were squirted into 100 volumes of a suitable lysing solution at 5° C. The composition of the lysing solution differed slightly in the different experiments. Its osmolarity was never more than 60 ideal m-osmole/l. It always contained (mM): ATP 1; ^{32}P-labelled P_i (pH 7·4) 5; and iodoacetic acid (adjusted to pH 7·4 with Tris base) 2. The sodium concentration was zero in the fourth experiment of Table 1, 0·2 mM in the experiment of Table 2, 2·0 mM in the first and third experiments of Table 1 and in the experiment of Table 3, and 4·8 mM in the second experiment of Table 1. In the experiments of Table 1, ADP was present in 2 mM concentration in the second, reduced nicotinamide-adenine dinucleotide (NADH) in 1 mM concentration in the third, and iodoacetamide in 5 mM concentration in the third and fourth. The Mg concentration was always 1 mM greater than the sum of the concentrations of ATP, ADP and P_i.

Isotonicity was restored by the addition of a suitable amount of 3 M-KCl, and the suspension was centrifuged at 16,000 *g* for 15 min at 5° C. All but 30 ml. of the supernatant was discarded. The ghosts were resuspended in the remaining supernatant and incubated for 30 min at 37° C. After incubation, the suspension was centrifuged at 1500 *g* for 1 min to bring down any unlysed cells. The supernatant suspension was transferred to a polythene tube and centrifuged at 20,000 *g* for 5 min at 5° C. The clear supernatant was discarded and the ghosts were washed 4–5 times at 5° C with a wash solution containing (mM): Na 140; Tris (pH 8·3 at 5° C; 7·4 at 37° C) 6·4; Mg 7; Cl 157; iodoacetate 2.

In the second experiment of Table 1 the lysing solution contained extra Mg to cover the ADP, and extra Mg was therefore also added to the wash solution. The composition of the wash solution in this experiment was therefore (mM): Na 137; Tris 6·4; Mg 9; Cl 158; iodoacetate 2. The washed ghosts were suspended in more of the same wash solution at a haematocrit of about 15 % (based on the original cell volume).

Incubation procedure. A series of flasks were prepared containing 30 ml. portions of suitable incubation media at 37° C. Portions of 1·5 ml. of ghost suspension were added and the flasks were shaken gently in the water-bath. After 15 min the flasks were removed to an ice-bath, and after a further 7 min the contents were transferred to polythene tubes and centrifuged at 20,000 *g* for 3 min at 5° C. The supernatants were sucked off, and the ghosts were lysed in a few ml. of water containing about 3 μmoles of unlabelled ATP, 3 μmoles of unlabelled ADP and 2·5 μmoles of unlabelled P_i to act as carriers for the labelled material. The haemolysates were transferred completely to small graduated tubes, and water was added to give final volumes of 5 ml. The graduated tubes were placed in an ice-bath and

deproteinized with trichloracetic acid (final concentration 5 g/100 ml.). The trichloracetic acid was removed by four extractions with equal volumes of ether, and the ether was blown off with a gentle stream of washed air for 5 min. The solutions were then ready for analysis.

Modified incubation procedure for the experiment of Table 2. This experiment was designed to permit determinations of the specific activities of ATP and ADP after the incubation. It was therefore necessary to carry out the incubation on a much larger scale, as carrier quantities of nucleotides could not be added later. Ghosts were prepared as described above but were finally suspended in the wash solution to give a 50% haematocrit (based on the original volume of the cells). Since resealed ghosts occupy less than half of the original cell volume the suspension was not unduly viscous. A series of flasks were prepared containing 54 ml. portions of suitable incubation media, and 6 ml. portions of ghost suspension were added. Subsequently the procedure was the same as in the other experiments except that carrier ATP, ADP and P_i were not added.

Incubation media. In each experiment the basic incubation medium was identical with the solution used to wash the cells after pre-incubation. When potassium was required, KCl was substituted for an equivalent amount of NaCl. Ouabain was added dissolved in a small amount of the incubation medium, except in the experiment of Table 3 when it was added in ethanol. Oligomycin was added as an ethanolic solution containing 5 mg/ml.

Separation of labelled products. Two alternative methods were used; an extraction method that was simple but merely separated inorganic from esterified phosphate, and a chromatographic method that was time-consuming but gave a good separation of ATP, ADP and P_i.

Extraction method. P_i in the solutions for analysis was converted into phosphomolybdate and extracted into isobutanol as described by Weil-Malherbe & Green (1951). To make sure no traces of P_i remained in the aqueous layer, about 2 μmole of unlabelled P_i was added and a further isobutanol extraction was carried out. This procedure was repeated once and then a fourth isobutanol extraction was carried out without preliminary addition of phosphate. The quantity of radioactivity left in the aqueous layer expressed as a fraction of the total radioactivity in the original solution showed how much of the original phosphate had been esterified. A comparison of the results of this method with the results of the chromatographic method, in an experiment in which both were used, showed that the esterified phosphate was mainly ATP with some ADP.

Chromatographic method. Separations were carried out on small columns of Dowex 1 (Cl) resin (200–400 mesh, × 8 cross-linked) 3 cm long and 0·4 cm in diameter. Before use, the columns were washed with 1 N-HCl until the effluents had an extinction at 259 mμ of less than 0·03, and then with water until the pH of the effluents was greater than 4·0. The solutions for analysis were diluted × $\frac{1}{2}$ with water (× $\frac{1}{4}$ in the experiment of Table 2); small portions were set aside for the determination of total radioactivity, and the remainder was run onto the columns at a flow rate of 0·2 ml./min. The columns were washed successively with: (i) 10 ml. of water, (ii) 20–25 ml. of 0·01 N-HCl, (iii) 15 ml. of 20 mM-NH_4Cl in 0·02 N-HCl, (iv) 15 ml. of 0·25 N-HCl. The effluents were collected in 5 ml. portions, and the radioactivity of each portion was determined by liquid scintillation counting using Bray's (1960) solution. Figure 1, from a preliminary experiment, shows the degree of separation of P_i, AMP, ADP and ATP obtained. In the experiment of Table 2, in which the specific activities of ADP and ATP were to be determined, the 259 mμ absorptions of relevant samples of effluent were also measured. Since iodoacetate has a strong absorption at 259 mμ, measurements were also made at 290 mμ. For adenine the ratio a_{290}/a_{259} is 0·002; iodoacetate gives a ratio of 0·64. Comparison of the measurements at the two wave-lengths showed that none of the observed absorption was caused by iodoacetate. With the eluting solutions used, the only phosphate ester likely to be confused with ATP is 2,3-diphosphoglycerate (Bartlett, 1959), and this would have been present only in trace amounts. In a simple trial to check that the phosphate ester eluted by the 0·25 N-HCl really was 'acid labile', 96·3% of the counts were found to be extractable as orthophosphate after 10 min treatment at 100° C with 1 N-HCl.

Measurement of the inhibitory effect of iodoacetamide on the transport ATPase of red cell membranes

Cells from 60 ml. of week-old blood from a blood bank were washed 4 times in a solution containing (mM): Na 152; Tris 5; Cl 153; ethylenediaminetetra-acetate (EDTA) 1; pH 7·7 at 20° C. The washed cells were packed at 1500 *g* for 7 min at 20° C and were lysed in 10 volumes of a solution containing (mM): Na 2; Tris 2; Cl 1·2; EDTA 1; pH 7·7 at 20° C. The ghosts were spun down at 16,000 *g* for 15 min at 5° C and were washed 4 times with 2–3 volumes of a solution identical with that used for the initial lysis. After each wash the ghosts were centrifuged at 20,000 *g* for 3 min at 5° C. The washed ghosts were resuspended in a solution containing (mM): Na 90; K 30; Tris 10; Cl 127; EDTA 0·1; pH 7·7 at 20° C (7·4 at 37° C), to give a haematocrit of about 10% (based on the original cell volume).

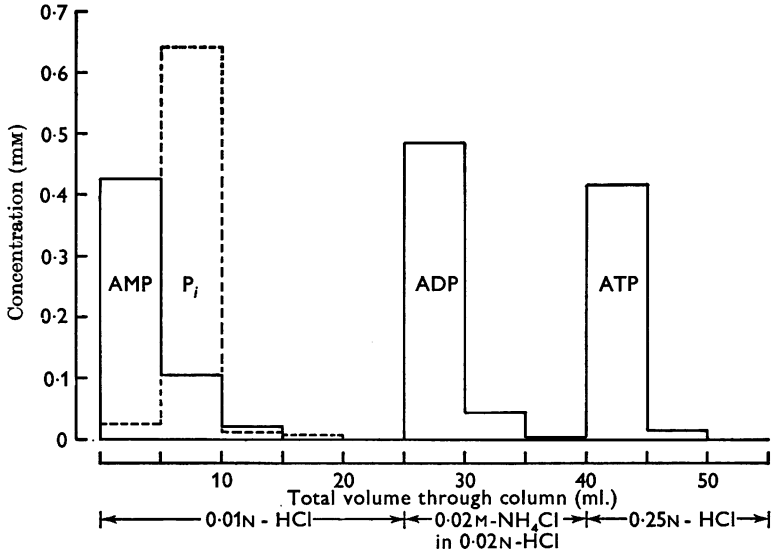

Fig. 1. Elution chart, from a preliminary experiment, showing the separation of P_i, AMP, ADP and ATP on a Dowex 1 column.

Portions of ghost suspension (4·2 ml.) were added to a series of stoppered glass tubes immersed in an ice-bath, and ouabain and iodoacetamide were added as required in 0·2 ml. of distilled water. Similar volumes of water were added to the control tubes. All the tubes were incubated for 1 hr at 37° C, to allow time for the iodoacetamide to act, and were then returned to the ice-bath. ATP was added in 0·2 ml. of a solution containing (mM): ATP 75; Mg 77·5; Na 150; Tris 165; Cl 155; pH 7·4 (at 37° C) and the tubes were incubated for 1 hr at 37° C. After incubation, the tubes were returned to the ice-bath and deproteinized with trichloroacetic acid (final concentration 5 g/100 ml.). Phosphate was estimated in the supernatants by the method of Fiske & Subbarow (1925). Suitable enzyme and reagent blanks were prepared.

Testing for an inhibitory effect of iodoacetate on the efflux of sodium from resealed ghosts

Ghosts were prepared as in the second experiment of Table 1 except that ^{24}Na was added to the haemolysate before isotonicity was restored, so that the resealed ghosts contained ^{24}Na. The ghosts were incubated in a series of tubes following the usual procedure for efflux

experiments described by Garrahan & Glynn (1967*c*), and using an incubation medium containing 3 mM-K. Tubes were withdrawn from the warm bath after 5 and after 30 min, cooled in an ice-bath and centrifuged. ^{24}Na was estimated in the supernatants and also in the original labelled ghost suspensions.

Sources of materials

Iodoacetamide and iodoacetic acid were obtained from British Drug Houses Ltd. Solutions were prepared on the day of the experiment. Iodoacetic acid was neutralized with Tris base before use.

The sources of other materials are given in previous papers (Garrahan & Glynn, 1967*a*, *c*, *d*).

RESULTS

Preliminary experiments to test the effects of iodoacetate and of iodoacetamide on the transport ATPase activity of fragmented ghosts

In experiments on electric organ preparations, *p*-chlormercuribenzoate and *o*-iodosobenzoate were found to inhibit the transport ATPase strongly whereas iodoacetamide inhibited rather weakly (Glynn, 1963). Iodoacetamide therefore seemed likely to meet the need for a substance that

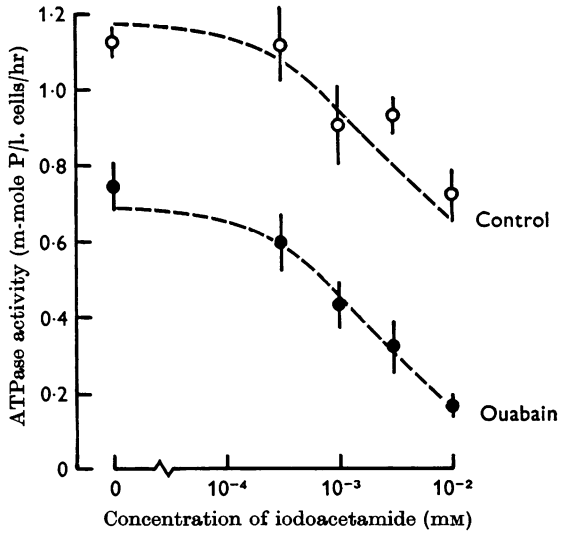

Fig. 2. The ATPase activity of a preparation of fragmented ghosts in the presence and absence of ouabain over a range of iodoacetamide concentrations. The inhibitors were allowed to act for 1 hr at 37° C before ATP was added; incubation was then continued for a further hour The final incubation medium contained (mM): ATP 3·3; Mg 3·4; Na 89; K 27; Tris 16; Cl 123; EDTA 0·1; pH (37° C) 7·4. The ouabain concentration was 5×10^{-5} g/ml.

would inhibit glycolysis strongly without too much affecting the cation transport system. Figure 2 shows the results of an experiment to check this point. The ATPase activity of a preparation of fragmented ghosts was measured in the presence and absence of ouabain over a range of iodo-

acetamide concentrations. It is clear that the ouabain-resistant ATPase was fairly strongly inhibited, whereas the ouabain-sensitive ATPase was not significantly affected even by 10^{-2} M iodoacetamide. A similar but less thorough experiment with iodoacetic acid over the same range of concentrations showed a similar lack of effect on the ouabain-sensitive ATPase.

These experiments suggested that either iodoacetamide or iodoacetic acid would be suitable. Iodoacetamide has the advantage that being uncharged it is likely to be able to penetrate the cell membrane readily; iodoacetate, on the other hand, is a more powerful inhibitor of glycolysis (Racker, 1965). In practice, iodoacetate was always included in the solutions for the preliminary incubation of the intact cells, in the lysing solutions and in the final incubation media. In some experiments iodoacetamide was also present during the preliminary incubation and in the lysing solution.

Reversal of the transport ATPase

In the preliminary attempt at reversal mentioned in the Introduction, AMP was included in the ghosts in an attempt to keep ATP at a low level through residual adenylate kinase activity. This may not have been wise. The fact that sodium: sodium exchange by the transport system requires ATP though there is little or no associated hydrolysis (Garrahan & Glynn, 1967*d*), suggests that ATP may be required to 'prime' the system. For this reason, in most of the later experiments the cells were lysed in solutions containing ATP as the only nucleotide. With 1 mM ATP in the lysing solution, about one-third of the ATP trapped in the ghosts must have been split during the pre-incubation necessary for resealing; for the experiment proper the ratios P_i:ADP:ATP were therefore something like 15:1:2.

The results of four experiments are shown in Table 1. In these experiments the ghosts were prepared with the compositions shown in the first column, and after resealing were incubated in sodium media, with and without ouabain, and in potassium media. After 15 min all enzymic activity was stopped with trichloracetic acid. Unlabelled ATP, ADP and P_i were added to act as carriers, and the nucleotides and phosphate were separated by chromatography, or by an extraction procedure, as described in the Methods section. The amount of radioactivity found in each fraction was expressed as a percentage of the total radioactivity in the ghosts.

In all four experiments more P_i was incorporated into ATP when the ghosts were in the sodium medium, and this extra incorporation was greatly diminished in the presence of ouabain.

The first experiment in the Table was the first to be performed, and the slight differences in the composition of the ghosts in the subsequent experi-

ments were made in the hope of decreasing the 'basal' incorporation or increasing the 'extra' incorporation. ADP was included in the second experiment in case the rate at which the pump ran backwards was limited by the concentration of ADP. In fact the ratio of extra incorporation to basal incorporation was lower than in the first experiment, perhaps because raising the level of ADP stimulated the phosphoglycerate kinase activity.

TABLE 1. The incorporation of ^{32}P-labelled P_i into 'energy-rich' phosphate through the reversed action of the sodium pump

Expt.	Composition of ghosts before sealing (mM)	Fraction of total counts incorporated into 'energy-rich' phosphate in 15 min (%)		
		'Na medium'	'Na medium' + ouabain	'K medium'
1	ATP 1; P_i 5; Mg 7; Na 2; K 140; Tris 5; Cl 144; iodoacetate 2	0·663	0·413	0·391
2	ATP 1; ADP 2; P_i 5; Mg 9; Na 4·8; K 134; Tris 9·4; Cl 143; iodoacetate 2	0·697	0·571	0·538
3	ATP 1; P_i 5; Mg 7; Na 2; K 147; Tris 5; Cl 150; NADH 1; iodoacetate 2; iodoacetamide 5	0·344	0·261	0·228
4	ATP 1; P_i 5; Mg 7; Na 0; K 147; Tris 5; Cl 150; iodoacetate 2; iodoacetamide 5	0·302	0·235	0·206

In the second, third and fourth experiments the P_i and nucleotides were separated by chromatography; in the second experiment similar results were also obtained by the extraction procedure; in the first experiment the extraction procedure alone was used. In the three experiments for which figures are available, the ratio (ATP counts)/(ADP counts) was about 3:1, but as the ADP was presumably labelled from ATP by adenylate kinase activity it is convenient to consider merely 'energy-rich' phosphate. It is possible that, through an error, the estimate of total counts in the first experiment was too low so that the figures for incorporation in that experiment may be too high, though their relative magnitudes will be correct. The 'Na medium' used in the first, third and fourth experiments contained (mM): Na 140; Mg 7; Tris (pH 7·4 at 37° C) 7·8; Cl 157; iodoacetate 2. The 'Na medium' used in the second experiment was similar but contained 9 mM-Mg. The 'K media' were identical with the 'Na media' except that K replaced 95% of the Na. The ouabain concentration was 5×10^{-5} g/ml.

Reduced nicotinamide-adenine dinucleotide (NADH) was included in the third experiment in the hope that it would inhibit oxidative phosphorylation by triose phosphate dehydrogenase. It had little effect. In the fourth experiment the ghosts were prepared free of sodium so that the force driving the pump backwards would be greater. Sodium had not been omitted in the earlier experiments in case some sodium was necessary to get the system into a state in which it could be made to run backwards. In the event, the ghosts lacking sodium behaved much like the ghosts in the earlier experiments.

Before concluding that the extra incorporation in the sodium medium was brought about by reversal of the transport ATPase, we must exclude certain alternative explanations:

(1) The figures in Table 1 show amounts of activity expressed as fractions of the total counts present in the ghosts, and this could be misleading

TABLE 2. The incorporation of ^{32}P-labelled P_i into ATP and ADP: direct measurements of relative specific activities

Incubation medium	Relative specific activities: counts/mole ATP × 100 / counts/mole P_i	Relative specific activities: counts/mole ADP × 100 / counts/mole P_i
'Na medium'	1·89	0·89
'Na medium' + oligomycin (10 μg/ml.)	1·57	0·83
'K medium'	1·37	0·72

The ghosts were sealed in a medium containing (mM): ATP 1; P_i 5; Mg 7; Na 0·2; K 151; Tris 5; Cl 154; iodoacetate 2. The 'Na medium' was the same as that used for most of the experiments of Table 1. The 'K medium' was identical except that K replaced 90% of the Na.

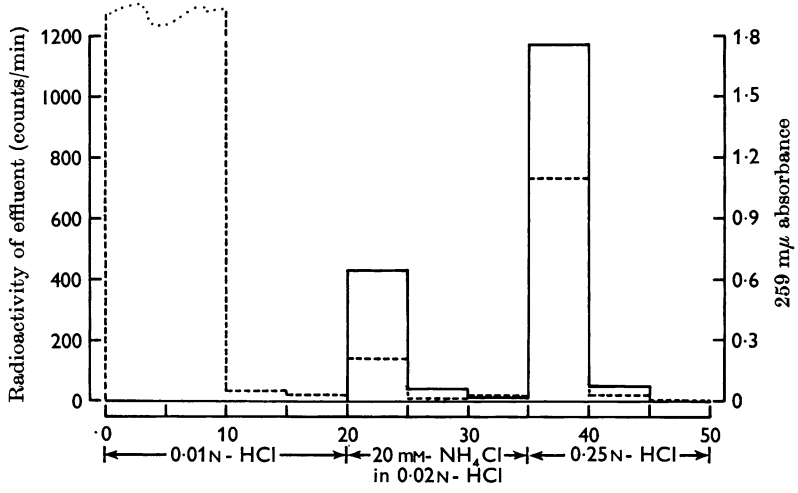

Fig. 3. Elution chart showing the separation of P_i, ADP and ATP from the flask containing extracellular potassium in the experiment of Table 2. The labelling of ADP was presumably the result of adenylate kinase activity. This activity would also have caused double labelling of the ATP, which may account for the fact that the specific activity of the ATP was about double that of the ADP. The major part of the P_i had passed through the column before the elution was started. The 259 mμ absorption of the first 20 ml. of effluent was not measured.

if the ghosts in the different media contained different amounts of radioactivity at the end of the incubation period. Checks showed that in fact total counts did not differ significantly.

(2) The four experiments in Table 1 gave no information about the specific activity of the nucleotides, only about the total radioactivity in each fraction. In the presence of external potassium, extra splitting of ATP through activation of the transport ATPase could have led to a lower concentration of ATP and therefore to fewer ATP counts even if the rate of formation had been the same. With the short period of incubation and the very low internal sodium concentration, particularly in the fourth experiment, the effect is likely to have been small and, in any event, it could not explain the effect of ouabain. Nevertheless, it seemed worth excluding the possibility by doing the experiment on a scale large enough to avoid the need for carriers, and estimating specific activities by a direct comparison of counts and 259 mμ absorption. The results of such an experiment are summarized in Table 2. Figure 3 shows the radioactivity and 259 mμ absorption in each fraction of eluate from the ghosts in the potassium medium. The results show that phosphate incorporation really is greater in a sodium medium.

In this experiment oligomycin was used instead of a cardiac glycoside. At a concentration of 10 μg/ml. (about 60 μg/mg protein) it partly inhibited the extra P_i incorporation associated with external sodium, and this result is in line with the partial inhibition of ATPase activity and of sodium movements caused by similar concentrations of oligomycin (Järnefelt, 1962; Glynn, 1963; Jöbsis & Vreman, 1963; Van Groningen & Slater, 1963; Whittam, Wheeler & Blake, 1964).

(3) Although there is no reason to believe that ouabain inhibits oxidative phosphorylation by triose phosphate dehydrogenase, it seemed worth checking that the ouabain effect observed in the experiments of Table 1 really was an effect on the 'extra' incorporation and not on the 'basal' incorporation. A further experiment was therefore carried out in which the effects of ouabain were tested in both a sodium and a potassium medium. The results are summarized in Table 3. In the sodium medium incorporation was, as usual, reduced in the presence of ouabain. In the potassium medium ouabain appeared to cause a small and probably not significant increase in incorporation, and the amount of radioactivity was exactly the same as in the sodium medium with ouabain. These results make it clear that the ouabain effect observed in the sodium medium was on the 'extra' incorporation.

(4) Another alternative explanation that requires discussion is the following: if the resealed ghosts were much more leaky to potassium than to sodium, they would tend to shrink in a sodium medium though not in a potassium medium; the resulting concentration of metabolites within the ghosts could conceivably lead to faster incorporation of P_i by the glycolytic system. The hypothesis fails to explain the effect of ouabain on the

extra incorporation in the sodium medium. It also seems unlikely because in one experiment, in which the loss of potassium was measured, only about 5% of the estimated potassium in the ghosts escaped during the 15 min incubation period, and much of this loss may well have been caused by lysis. Even if the ghosts were absolutely impermeable to sodium the degree of shrinking brought about by the loss of potassium must have been very small.

TABLE 3. An experiment to test whether the inhibitory effect of ouabain is restricted to the extra incorporation of P_i that occurs in the Na medium

Incubation medium	Fraction of total counts incorporated into 'energy-rich' phosphate in 15 min (%)
'Na medium'	0·357
'Na medium' + ouabain	0·306
'K medium'	0·291
'K medium' + ouabain	0·306

The ghosts were sealed in a medium containing (mM): ATP 1; P_i 5; Mg 7; Na 2; K 145; Tris 5; Cl 150; iodoacetate 2. The incubation media were similar to those used in most of the experiments of Table 1. Before the final incubation the ghosts were in contact with the incubation media for 15 min at 0° C.

The rate of the reversed reaction

In the second experiment of Table 1, 0·159% of the total counts were incorporated into energy-rich phosphate through the reversed action of the pump in 15 min. If the P_i concentration in the ghosts was 5 mM, this is equivalent to an incorporation of 31·8 μmoles of P_i into ATP per litre of sealed ghosts per hour. A slight correction must be made to this figure. If the ghosts sealed to P_i at a volume greater than the volume at which they sealed to potassium, the concentration of P_i after sealing would have been higher than 5 mM. And if some ghosts sealed to P_i but remained leaky to potassium, they would not have incorporated P_i into ATP through the pump mechanism and the incorporation in the fully sealed ghosts would therefore have been higher than the calculated figure. There is no way of estimating each of these factors separately but their combined effect can be allowed for in the following way.

It is invariably found that resealed ghosts contain a little less sodium or potassium relative to their content of nucleotide than would be expected from the composition of the haemolysing medium; on the other hand the ratio P_i/nucleotide in the ghosts is the same as in the haemolysing medium. It is not clear whether the discrepancy comes about because some of the ghosts that seal to the nucleotides fail to seal to the smaller cations, or because the volume at which the ghosts seal to the cations is smaller

than the volume at which they seal to the nucleotides, but the magnitude of the discrepancy gives an estimate of the sum of the two effects. In the cells used in the experiment of Table 4, which were from the same batch as those used in the second experiment of Table 1, the ratio of potassium to nucleotide after resealing and washing was 0·76 times the ratio of potassium to nucleotide in the haemolysing medium. The figure of 31·8 μmoles P_i incorporated per litre of sealed ghosts per hour has therefore been divided by 0·76 to give a figure of 41·8 μmoles/l. sealed ghosts/hr.

Another small correction worth considering is that made necessary by the loss of P_i from the ghosts during the final incubation. The loss was measured in one experiment and accounted for 15 % of the intracellular P_i, but as the loss of potassium in the same experiment was only 5 % it seems likely that most of the loss of P_i was by lysis of ghosts that were rather leaky and had already lost their potassium during the preliminary washing. If ghosts lysed during the incubation they would contribute neither radioactive ATP nor total counts and so would not affect the results.

TABLE 4. An experiment to measure the *forward* rate of the pump when ghosts prepared like those in the second experiment of Table 1 were incubated in a medium containing 3 mM-K

	Rate constant for Na efflux (hr^{-1})	Na efflux (m-mole/l sealed ghosts/hr)
Control tubes	0·215	1·03
Ouabain tubes	0·158	0·758
Ouabain-sensitive efflux	0·057	0·274

Ghosts prepared from the same batch of cells as those used in the second experiment of Table 1 were sealed in a medium containing (mM): ATP 1; ADP 2; P_i 5; Mg 9; Na (labelled with ^{24}Na) 4·8; K 134; Tris 9·4; Cl 143; iodoacetate 2. The resealed ghosts were incubated for 30 min at 37° C in the presence and absence of ouabain in a medium containing (mM): K 3; Na 134; Mg 9; Tris (pH 7·4 at 37° C) 7·8; Cl 158; iodoacetate 2. The ouabain concentration was 5×10^{-5} g/ml.

Comparison of reversed and forward rates

It is interesting to compare the rate of the reversed reaction estimated in the previous section with the rate of the forward reaction in similar ghosts immersed in a medium like the sodium medium but with 3 mM-KCl replacing an equivalent amount of NaCl. To measure the forward rate, cells from the same batch as those used in the second experiment of Table 1 were prepared as in that experiment but with ^{24}Na inside, and the loss of ^{24}Na during a 30 min incubation was measured. The results are shown in Table 4. Ouabain-sensitive ATPase activity may be estimated from the ouabain sensitive efflux by dividing by 3 to give a figure of 91 μmoles/l. sealed ghosts/hr. This figure will be an upper limit as with only 3 mM-K outside some of the sodium efflux probably represented

sodium:sodium exchange (see Fig. 3 of Garrahan & Glynn, 1967*c*). The reversed rate in the potassium-free medium was therefore at least $(41{\cdot}8/91{\cdot}0) \times 100\,\% = 46\,\%$ of the forward rate in the medium with 3 mM-K.

It is also interesting to compare the rate of the reversed reaction observed in these experiments with the rate of the forward reaction in cells liberally supplied with ATP and doing no osmotic or electrical work. Dunham & Glynn (1961) found that the rate of ouabain-sensitive hydrolysis by fragmented ghosts under more or less optimal conditions was about 1 m-mole/l. original cells/hr, equivalent to about 3 m-mole/l. ghosts/hr since ghosts generally occupy about one-third of the original volume of the cells. The reversed rate of 41·8 μmoles/l. sealed ghosts/hr is therefore about 1·4 % of the maximum forward rate in fragmented cells doing no work.

The relation between driving force and rate

As shown in the Appendix, ΔG for the forward reaction under conditions like those existing in the experiments of Table 1 is in the region of + 6000 cal. Estimates of ΔG are not very reliable, because the conditions in the ghosts and the relative numbers of sodium and potassium ions transported are not accurately known, but it seems likely that the driving force in the reversed direction in these experiments was rather greater than the force driving the system forwards in fresh cells under physiological conditions. The fact that the reversed rates in the experiments of Table 1 were much less than the forward rate under physiological conditions presents no thermodynamic difficulty, because the free energy change associated with a reaction gives no information about absolute reaction rates—it only defines the ratio between forward and backward rates. An interesting test of the validity of the whole concept of a transport ATPase is to measure both forward and backward rates under conditions chosen so that ΔG is approximately zero, when the two rates should be equal. The experiment with 3 mM-K shown in Table 4 was part of an attempt to carry out this test, but unfortunately in the part of the experiment to measure the reversed rate, the solution for chromatography was lost and an estimate was got only by the less reliable extraction method. For what it is worth, the estimated incorporation was 19 μmoles/l. sealed ghosts/hr. This has to be compared with the figure of 91 μmoles/l. sealed ghosts/hr, which was the upper limit calculated for the forward reaction. As the former figure is unreliable and the latter an upper limit, too much importance should not be attached to the ratio, but at its face value it is equivalent to a ΔG of − 960 cal compared with the theoretical + 424 cal. In view of the uncertainties the agreement is satisfactory.

DISCUSSION

The first question that needs discussion is: does the extra incorporation of P_i observed in the experiments represent a net synthesis of ATP? If the ATP concentration in the ghosts at the end of the 15 min incubation is something like 0·67 mM, a net synthesis of 42 μmoles/l. sealed ghosts/hr would represent an increase of only 1·56 % per hour and would be more than offset by the ouabain-resistant hydrolysis of ATP. We saw on p. 248, however, that the forward rate of the ouabain-sensitive ATPase when similar ghosts were incubated in a medium containing 3 mM-K was not more than 91 μmoles/l. sealed ghosts/hr. The potassium concentrations in the nominally potassium-free media in the experiments of Table 1 were almost certainly less than 0·1 mM, and judging from the curve in Fig. 1 of Garrahan & Glynn (1967*c*) this concentration of potassium would give a ouabain-sensitive potassium influx equal to, at most, 3·2 % of the potassium influx with 3 mM-K outside. It follows that the rate of ouabain-sensitive ATP breakdown in the potassium-free solutions in the experiments of Table 1 is unlikely to have been more than $(91 \times 3{\cdot}2)/100 = 2{\cdot}9$ μmoles/l. sealed ghosts/hr, and may have been much less than this. It is possible that the potassium influx curve for cells with low concentrations of ATP and high concentrations of ADP and P_i differs from the curve in Fig. 1 of Garrahan & Glynn (1967*c*) in being shifted slightly to the right, but the effect cannot be large since in experiments on stored cells potassium influx was half maximal at about 2 mM-K (Glynn, 1956). It is therefore safe to conclude that in the experiments of Table 1 the rates of the reverse reaction in the potassium-free media were considerably more than the rates of the forward reaction; in other words, running the pump backwards led to a net synthesis of ATP.

The next question needing discussion is: why was the incorporation of P_i significantly less in the high-potassium media than in the potassium-free media containing ouabain? A difference in the right direction would be caused by loss of ATP through hydrolysis by the transport ATPase when potassium was present in the outside medium, but the magnitude of the difference that could arise in this way is certainly too small to explain the results in the second, third and fourth experiments of Table 1. A more likely explanation is that ouabain failed to inhibit completely during the initial part of the incubation. The design of most of the experiments did not allow any pre-treatment with ouabain and the total incubation time was only 15 min, so that failure to achieve complete inhibition at once would cause an appreciable effect. In the experiment of Table 3 the cells were exposed to ouabain in the cold for 15 min before the incubation and the discrepancy is almost absent.

The nature of the transport ATPase system

It is convenient to divide all hypothetical mechanisms for the sodium pump into two formal kinds, in some way analogous to the two alternative mechanisms for oxidative phosphorylation put forward by Slater (see Lehninger, 1964, ch. 6). The two kinds of mechanism, reduced to their simplest forms, are:

$$\text{I.}\quad \mathrm{ATP}+\mathrm{X}+\mathrm{Y} \leftrightharpoons \mathrm{ADP}+\mathrm{P}_i+\mathrm{X}\sim\mathrm{Y},$$
$$\mathrm{X}\sim\mathrm{Y}+m\mathrm{Na}_\mathrm{I}+n\mathrm{K}_\mathrm{O} \leftrightharpoons \mathrm{X}+\mathrm{Y}+m\mathrm{Na}_\mathrm{O}+n\mathrm{K}_\mathrm{I}.$$

$$\text{II.}\quad \mathrm{ATP}+\mathrm{X} \leftrightharpoons \mathrm{ADP}+\mathrm{X}\sim\mathrm{P},$$
$$\mathrm{X}\sim\mathrm{P}+m\mathrm{Na}_\mathrm{I}+n\mathrm{K}_\mathrm{O} \leftrightharpoons \mathrm{X}+\mathrm{P}_i+m\mathrm{Na}_\mathrm{O}+n\mathrm{K}_\mathrm{I}.$$

In mechanisms of the first kind, energy from the hydrolysis of ATP is used to form some non-phosphorylated compound, or is stored in some other way, and this energy is then used to drive the reactions immediately associated with the movement of ions. The crucial point is that the release of P_i occurs *before* the consumption of energy by the ion movements. The alternative scheme assumes that the phosphorylated intermediates are themselves involved in the transport of ions, and that it is not until Na^+ and K^+ are moved that phosphate is released. It is, of course, possible to design a mixed scheme in which phosphate energy is used directly for moving, say, Na^+ but energy in some other form is used for moving K^+.

An attraction of the type I scheme is that in mitochondria there is evidence that cation accumulation can be driven *either* by ATP, through an oligomycin-sensitive step, *or* by oxidative processes without the involvement of phosphate (Brierley, Murer, Bachmann & Green, 1963; Chappell, Greville & Bicknell, 1962; Chappell, Cohn & Greville, 1963; Lehninger, 1964). But though on classical oxidative phosphorylation theory, this suggests that ion transport is driven by a non-phosphate energy-rich intermediate, if oxidation and phosphorylation are coupled only by the generation of an electrochemical potential gradient for hydrogen ions (Mitchell, 1966) the lack of involvement of phosphate in the oxidation-driven cation movements is irrelevant. In any case, the existence of type I mechanisms in cell membranes is difficult to reconcile with:

(1) recent work on ^{32}P incorporation into cell membrane preparations exposed to [^{32}P]ATP (Post, Sen & Rosenthal, 1965; Albers, Fahn & Koval, 1963; Whittam, Wheeler & Blake, 1964; Nagano, Kanazawa, Mizuno, Tashima, Nakao & Nakao, 1965; Ahmed & Judah, 1965; Rodnight, Hems & Lavin, 1966; but cf. Skou, 1965);

(2) the Na-dependent ATP–ADP exchange reaction demonstrated by Fahn, Koval & Albers (1966) and by Fahn, Hurley, Koval & Albers (1966);

(3) the absence of ATP–P_i exchange in resealed high-Na ghosts catalysing sodium:sodium exchange—ghosts which were prevented from catalysing sodium:potassium exchange only by the absence of external potassium (Garrahan & Glynn, 1967*d*).

The work on ^{32}P incorporation, on the ATP–ADP exchange reaction and on the properties of the sodium:sodium exchange all tends to suggest that sodium ions are associated with a phosphorylation step and potassium ions with dephosphorylation.

In designing hypothetical models, four features of the sodium:sodium exchange deserve particular attention. These are:

(i) the increase in the rate of exchange with increased external sodium concentration over a surprisingly wide range;

(ii) the need for ATP, despite the absence of appreciable associated hydrolysis;

(iii) the absence of sodium:sodium exchange in high-Na resealed cells unless they contain much more P_i than ATP;

(iv) the sensitivity to oligomycin.

If the low apparent affinity of the sodium:sodium exchange mechanism for external sodium is really the result of an obligatory modification of the carrier molecule during its outward passage—see discussion by Garrahan & Glynn (1967*a*)—it is economical to suppose that this same modification replaces a selective affinity for sodium ions by a selective affinity for potassium ions. There is no evidence to decide whether such a modification is likely to be the result of phosphorylation of the carrier by ATP, as suggested by Baker & Connelly (1966), or of a reaction subsequent to phosphorylation. Unless there are confusing species differences, a reaction subsequent to phosphorylation does seem to be involved in sodium:sodium exchange, since oligomycin inhibits this exchange in red cells but has no effect on the Na-dependent formation of a phosphorylated intermediate by a kidney membrane ATPase preparation (Whittam *et al.* 1964), or on the Na-dependent ATP–ADP exchange reaction catalysed by electric organ preparations (Fahn, Koval & Albers, 1966; Fahn, Hurley, Koval & Albers, 1966). If an enzyme-catalysed step is obligatory in the outward movement of sodium, the reversal of this step necessary for sodium entry—and therefore for sodium:sodium exchange—will not occur unless there is a reasonable chance that the carrier on its way back finds the enzyme free. It may be that with high internal levels of both sodium and ATP, the enzyme is kept saturated from the inside and sodium:sodium exchange is blocked. Reducing the level of either sodium or ATP would release the block by decreasing the rate of Na-dependent phosphorylation.

This effect might be achieved in another way if a very high level of P_i could reverse the final step in the over-all ATPase reaction—the release of inorganic phosphate to the cell interior—and so reduce the amount of carrier available for phosphorylation by ATP. The reversal of the entire ATPase system, demonstrated in this paper, makes such an explanation of the effect of internal P_i plausible, and further support for the idea comes from recent work on the relation between internal P_i level and ouabain-sensitive potassium efflux (Glynn & Lüthi, 1967). A rather similar explanation of the blocking of Na:Na exchange by high levels of ATP or of internal sodium has been suggested by Baker & Connelly (1966).

It would be interesting to know more about the relation between sodium:sodium exchange and the Na-dependent ATP–ADP exchange reaction, in particular to know whether the shuttling of sodium ions necessarily involves the exchange of phosphate between ATP and the enzyme system.

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APPENDIX

Calculation of ΔG under physiological conditions assuming that the hydrolysis of each molecule of ATP is associated with the extrusion of three Na^+ ions and the uptake of two K^+ ions.

Assume:

$$\begin{aligned}
[Na]_i &= 10 \text{ m-equiv/l. cell water},\\
[Na]_o &= 145 \text{ m-equiv/l.},\\
[K]_i &= 150 \text{ m-equiv/l. cell water},\\
[K]_o &= 5 \text{ m-equivl/l.},\\
[ATP] &= 1{\cdot}5 \times 10^{-3} \text{ M},\\
[ADP] &= 0{\cdot}32 \times 10^{-3} \text{ M},\\
[P_i] &= 0{\cdot}36 \times 10^{-3} \text{ M}.
\end{aligned}$$

ΔG^0 for the reaction: $ATP + H_2O \rightarrow ADP + P_i$ at pH 7·4 and in the presence of excess Mg^{2+} ions is 7200 cal (see Benzinger, Kitzinger, Hems & Burton, 1959).

Also assume that chloride ions are passively distributed so that the membrane potential is given by

$$E = \frac{RT}{F} \ln \frac{[Cl]_o}{[Cl]_i} = \frac{RT}{F} \ln 1{\cdot}4.$$

The osmotic work done in expelling three equivalents of sodium ions is

$$3RT \ln \tfrac{145}{10} = 4927 \text{ cal.}$$

The osmotic work done in taking up two equivalents of potassium ions is

$$2RT \ln \tfrac{150}{5} = 4177 \text{ cal.}$$

The electrical work done in moving one Faraday of positive charge outwards is

$$EF = RT \ln 1{\cdot}4 = 207 \text{ cal.}$$

Therefore the total work done is 9311 cal.

For the hydrolysis of ATP under the conditions existing in the cell

$$\Delta G = \Delta G^0 - RT \ln \frac{[\text{ATP}]}{[\text{ADP}].[\text{P}_i]} \text{ cal.}$$

$$= -13{,}017 \text{ cal.}$$

For the over-all reaction the free energy change is therefore $-13{,}017 + 9311 = -3706$ cal.

Calculation of ΔG for the ghosts in the third experiment of Table 1

Assume:

$$[\text{Na}]_i = 2 \text{ m-equiv/l. cell water,}$$
$$[\text{Na}]_o = 140 \text{ m-equiv/l.}$$
$$[\text{K}]_i = 147 \text{ m-equiv/l. cell water,}$$
$$[\text{K}]_o = 0{\cdot}1 \text{ m-equiv/l.,}$$
$$[\text{Cl}]_o/[\text{Cl}]_i \simeq 1,$$
$$[\text{ATP}] = 0{\cdot}67 \times 10^{-3} \text{ M,}$$
$$[\text{ADP}] = 0{\cdot}33 \times 10^{-3} \text{ M,}$$
$$[\text{P}_i] = 5{\cdot}33 \times 10^{-3} \text{ M.}$$

Then, ΔG for the outward movement of three equivalents of sodium ions and the inward movement of two equivalents of potassium ions is $+16{,}784$ cal.

ΔG for the hydrolysis of ATP is $-10{,}853$ cal, and ΔG for the over-all reaction is $-10{,}853 + 16{,}784 = +5931$ cal.

Calculation of ΔG for the ghosts in the experiment of Table 4

Assume:

$$[\text{Na}]_i = 4{\cdot}8 \text{ m-equiv/l. cell water,}$$
$$[\text{Na}]_o = 137 \text{ m-equiv/l.}$$
$$[\text{K}]_i = 134 \text{ m-equiv/l. cell water,}$$
$$[\text{K}]_o = 3 \text{ m-equiv/l.,}$$
$$[\text{Cl}]_o/[\text{Cl}]_i \simeq 1.$$

Also assume that one third of the ATP is hydrolysed during the pre-incubation and that there is sufficient adenylate kinase activity to maintain $[ATP].[AMP]/[ATP]^2 = 0{\cdot}44$ (Eggleston & Hems, 1952).

$$\text{Then, } [ATP] = 1{\cdot}22 \times 10^{-3} \text{ M},$$
$$[ADP] = 1{\cdot}22 \times 10^{-3} \text{ M},$$
$$[AMP] = 0{\cdot}53 \times 10^{-3} \text{ M},$$
$$[P_i] = 5{\cdot}33 \times 10^{-3} \text{ M}.$$

ΔG for the outward movement of three equivalents of sodium ions and the inward movement of two equivalents of potassium ions is +10,838 cal.

ΔG for the hydrolysis of ATP is −10,414 cal, and ΔG for the over-all reaction is $-10{,}414 + 10{,}838 = +424$ cal.

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