J. Physiol. (1967), **192**, *pp.* 217–235
With 2 text-figures
Printed in Great Britain

THE STOICHEIOMETRY OF THE SODIUM PUMP

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(*Received* 3 *April* 1967)

SUMMARY

1. When resealed ghosts containing adenosine triphosphate (ATP), magnesium and sodium were incubated in a medium containing potassium, ATP was hydrolysed vigorously by a ouabain-sensitive mechanism. If the ghosts contained potassium instead of or in addition to sodium, and the external solution contained sodium but no potassium, there was little ouabain-sensitive hydrolysis of ATP. As it is known that the ouabain-sensitive ATPase in fragmented ghosts requires both sodium and potassium ions, these results show that the ATPase is activated by potassium externally and by sodium internally, and suggest that the ions activating the ATPase are the ions that are transported.

2. Resealed ghosts containing ATP, magnesium and sodium were incubated in sodium-free media containing potassium, with and without ouabain, and the rate of loss of sodium and rate of hydrolysis of ATP were measured. The hydrolysis of 1 molecule of ATP by the ouabain-sensitive mechanism was accompanied by the ouabain-sensitive loss of about 3 sodium ions.

3. ^{24}Na and ^{42}K were used to measure sodium efflux and potassium influx in identical batches of fresh red cells under the same conditions and at the same time. Each flux was measured in the presence and absence of ouabain. The ratio (ouabain-sensitive sodium efflux)/(ouabain-sensitive potassium influx) was significantly greater than 1 ($1{\cdot}20 \pm 0{\cdot}01$ and $1{\cdot}35 \pm 0{\cdot}01$ in two experiments). If a small fraction of the potassium influx represented a ouabain-sensitive potassium : potassium exchange, the ratio of the numbers of ions moved in the sodium : potassium exchange catalysed by the pump must have been even further from unity.

4. Resealed ghosts containing [γ-^{32}P]ATP, magnesium, ^{24}Na and orthophosphate were incubated in balanced salt solutions with and without potassium and with and without ouabain. A comparison of sodium efflux, estimated from ^{24}Na loss, with ATP hydrolysis, estimated from the for-

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mation of [^{32}P]orthophosphate, showed that the sodium:sodium exchange in a potassium-free medium was accompanied by little or no ouabain-sensitive hydrolysis of ATP.

5. Experiments on intact red cells loaded with ^{24}Na showed that both sodium:sodium exchange in a potassium-free medium, and sodium:potassium exchange in a medium containing potassium, were partially inhibited by oligomycin (1–10 μg/ml.). Inhibition of the sodium:potassium exchange was not affected by raising the external potassium concentration.

INTRODUCTION

This paper reports experiments of several different kinds, mostly dealing with the stoicheiometry of the cation transport system in the red cell membrane. The first two experiments were done about 6 years ago, the remainder more recently. Preliminary accounts of all the experiments have been published (Glynn, 1962; Garrahan & Glynn, 1965, 1966).

The first experiment was not concerned with stoicheiometry but was to determine at which surface of the membrane sodium ions and potassium ions activated the ouabain-sensitive ATPase. The results showed that sodium was required at the inner surface and potassium at the outer surface and these findings, since confirmed by Whittam (1962) and by Laris & Letchworth (1962), make it almost certain that the ions activating the ATPase are the ions that are pumped (see also Baker, 1965).

The second experiment was designed to find how much ATP was used when cells pumped sodium into a solution free of sodium but containing potassium. Very nearly three sodium ions appeared to leave resealed ghosts by a ouabain-sensitive pathway for each molecule of ATP hydrolysed by the ouabain-sensitive ATPase.

The third and fourth experiments determined the ratio between active potassium influx and active sodium efflux when red cells were incubated under fairly physiological conditions. The ratio was significantly greater than 1.

The next two experiments were designed to compare the ratio between sodium efflux and associated hydrolysis of ATP during sodium:sodium exchange with the same ratio during sodium:potassium exchange. It turned out that little or no hydrolysis of ATP was associated with sodium:sodium exchange. This lack of hydrolysis of ATP made it interesting to test the sensitivity of sodium:sodium exchange to oligomycin. Like the sodium:potassium exchange it was partially inhibited.

METHODS

Procedure to test whether the ouabain-sensitive ATPase was activated by internal sodium ions and external potassium ions

The preparation of resealed ghosts. Red cells from 40 ml. of fresh heparinized blood were washed 4 times with a solution containing (mM): K 105; Mg 30; Tris (pH 7·3 at 25° C) 10; Cl 172; and once with a solution containing (mM): K 150; Tris (pH 7·3 at 25° C) 10; Cl 157. The washed cells were packed at 1500 *g* for 10 min at room temperature and were separated into two portions each of which was squirted into 82 volumes of a lysing solution containing 5 mM ATP disodium or dipotassium salt, 5·5 mM-$MgCl_2$ and 1 mM cysteine, adjusted to pH 7 (20° C) with Tris base. The haemolysate (202 ml.) containing potassium ATP was restored to isotonicity by the addition of 47·6 ml. of 786 mM Tris chloride buffer (pH 7·3 at 20° C) giving final concentrations of (mM): Tris 147; K 9 (including 1 from the K originally in the cells); Mg 4·4; ATP 4; cysteine 0·8; Cl 134. The haemolysate containing sodium ATP was divided into two portions. The first, 200 ml. in volume, was brought back to isotonicity by the addition of 47·6 ml. of a solution containing (mM): Na 91; Tris (pH 7·3 at 20° C) 795; Cl 801, to give final concentrations of (mM): Tris 130; Na 25; K 1 (from the K originally in the cells); Mg 4·4; ATP 4; cysteine 0·8; Cl 135. The second portion, 309 ml. in volume, was brought back to isotonicity by the addition of 73·5 ml. of a solution containing (mM): Na 89; K 42; Tris (pH 7·3 at 20° C) 64; Cl 188, to give final concentrations of (mM): Tris 122; Na 25; K 9 (including 1 from the K originally in the cells); Mg 4·4; ATP 4; cysteine 0·8; Cl 137.

The three suspensions were centrifuged at 16,000 *g* for 15 min at 5° C and most of the supernatant was discarded from each. The ghosts were resuspended in the remaining supernatants (about 30 ml.) and were incubated at 37° C for 30 min with gentle shaking. After this incubation the ghosts were centrifuged at 20,000 *g* for 5 min at 5° C and were washed 5 times with about 15 volumes of an ice-cold wash solution containing (mM): Tris (pH 7·9 at 5° C) 155; Mg 5; cysteine 1; Cl 148. All the ghosts of each type were then suspended in more of the cold wash solution ready for use.

The measurement of ATPase activity. Ghost suspensions were prepared, as described below, in a series of stoppered glass tubes immersed in an ice-bath. The ghosts containing 9 mM-K with virtually no Na were suspended in a solution containing (mM): Na 25; Tris (pH 7·9 at 5° C, 7·1 at 37° C) 125; Mg 4; cysteine 0·8; Cl 145. The ghosts containing 25 mM-Na and only 1 mM-K were suspended in a solution containing (mM): K 8; Tris (pH 7·9 at 5° C, 7·1 at 37° C) 142; Mg 4; cysteine 0·8; Cl 143. The ghosts containing 25 mM-Na and 9 mM-K were suspended in a solution containing (mM): Na 25; Tris (pH 7·9 at 5° C, 7·1 at 37° C) 125; Mg 4; cysteine 0·8; Cl 145.

These suspensions were prepared by adding suitable quantities of ice-cold isotonic solutions of NaCl, KCl, or Tris buffered to pH 7·3 (at 20° C) with HCl, to suspensions of the ghosts in the wash solutions. The tubes were divided into two groups. Ouabain dissolved in 3 % ethanol was added to one group to give a final concentration of $5{\cdot}6 \times 10^{-5}$ g/ml.; an equal amount of 3 % ethanol was added to the tubes of the other group. The final ethanol concentration was 0·3 % and the final volume of solution in each tube was 8 ml. The haematocrit was approximately 3 % based on the original volume of the cells.

Tubes of each kind were incubated at 37° C for 1 hr and were returned to the ice-bath. Trichloracetic acid (55 g/100 ml.) was added to give a final concentration of 5 g/100 ml., and phosphate was estimated in the supernatants by the method of Weil-Malherbe & Green (1951). The experiment was carried out in triplicate and phosphate was also measured in control tubes that were not incubated.

Procedure to measure the ratio between ouabain-sensitive sodium efflux and ouabain-sensitive hydrolysis of ATP in resealed ghosts exchanging sodium for potassium

The preparation of resealed ghosts. Cells from 40 ml. of fresh heparinized blood were washed as described on p. 219, packed at 1500 *g* for 10 min at room temperature and squirted into 69 volumes of a solution containing 5·5 mM ATP disodium salt, 6 mM-$MgCl_2$ and 1 mM cysteine, adjusted to pH 7·0 (at 20° C) with Tris base. Haemolysate (731 ml.) was restored to isotonicity by the addition of 71 ml. of a solution containing (mM): Na 453; K 900; Tris (pH 7·3 at 20° C) 248; Cl 1574, to give final concentrations of (mM): Na 50; K 80; Tris (pH 7·3 at 20° C) 30; Mg 5·4; ATP 4·9; cysteine 0·9; Cl 148. The suspension was centrifuged at 16,000 *g* for 15 min at 5° C and most of the supernatant was discarded. The ghosts were resuspended in the remaining supernatant (about 30 ml.) and were incubated at 37° C for 36 min with gentle shaking. After this incubation the ghosts were centrifuged at 20,000 *g* for 5 min at 5° C and were washed 4 times with about 15 volumes of an ice-cold wash solution containing (mM): Tris (pH 7·9 at 5° C) 155; Mg 5; cysteine 0·5; Cl 148. The washed ghosts were resuspended in more of the same wash solution to give a haematocrit of about 3 % (based on the original volume of the cells) and 160 mM-KCl was added to give a final concentration of 5 mM.

The measurement of ATPase activity and of sodium loss. Portions of the ghost suspension were added to a series of stoppered glass tubes immersed in an ice-bath. Ouabain in 3 % ethanol was added to some to give a final concentration of 5×10^{-5} g/ml., and an equivalent amount of ethanol was added to the others. The final ethanol concentration was about 0·1 % and the volume of the solutions 8·0 ml. Ouabain and control tubes were incubated at 37° C for 75 min and returned to the ice-bath. Half were deproteinized with trichloracetic acid (final concentration 5 g/100 ml.) and phosphate was estimated in the supernatants. The contents of the remainder were transferred to small polythene tubes and centrifuged at 20,000 *g* for 5 min at 5° C. The supernatants were collected and the sodium contents were estimated by flame photometry using an 'Eel' flame photometer (Evans Electroselenium Ltd.). Because the ratio of sodium to potassium was low, care was taken to use sodium standards containing suitable concentrations of potassium. The experiment was carried out in quintuplicate and further pairs of ouabain and ouabain-free tubes were treated similarly but without incubation to determine the initial ATP hydrolysis and initial sodium loss.

Corrections for changes in volume and in internal sodium concentration. It is not permissible to equate active sodium loss with the difference between the sodium losses with and without ouabain. The ouabain-treated ghosts will have contained more sodium at any moment than the control ghosts so that passive efflux will have been greater. A further complication is caused by progressive swelling of the ghosts brought about by the entry of Tris during the incubation. To correct for the change in passive sodium efflux it is necessary to know the relative change in internal concentration and the relative change in volume that occurred during the incubation. The average initial sodium concentration for the whole mass of ghosts was estimated from direct measurements of sodium concentration and haematocrit in portions of ghost suspension put aside for this purpose. If it is assumed that the ghosts that resealed contained sodium at a concentration equal to that in the lysing solution, the fraction of ghosts that resealed may also be calculated. Relative changes in volume during the incubation were found by packing, separately, (i) ghosts from the unincubated tubes, (ii) ghosts from the tubes incubated with ouabain, and (iii) ghosts from the tubes incubated without ouabain. Each group of ghosts was centrifuged under standard conditions and the heights of the columns of packed ghosts were compared. Since it was presumably only the sealed ghosts that swelled, the relative changes in volume were divided by the fraction of ghosts that had sealed to give the relative changes in volume of the sealed ghosts.

Procedure to measure the ratio between ouabain-sensitive sodium efflux and ouabain-sensitive potassium influx in intact cells

The measurement of sodium efflux. Fresh cells were loaded with ^{24}Na as described by Garrahan & Glynn (1967*a*) and were washed 5 times with a sodium-free salt solution containing (mM): choline 150; Mg 1; Ca 1; orthophosphoric acid adjusted to pH 7·4 with Tris base 2·5; Cl 154; glucose 11. The washed cells were resuspended in about 10 volumes of the same solution and a small portion of the suspension was put aside for estimations of radioactivity, total sodium and haemoglobin. One millilitre portions of suspension were added to 9 ml. portions of an ice-cold solution containing (mM): Na 145; K 10; Mg 1; Ca 1; phosphate (pH 7·4) 2·5; Cl 154; glucose 11. Ouabain was added, where required, in 0·3 ml. of the sodium-free wash solution, and a similar amount of this solution was added to the control tubes. The final ouabain concentration was $5{\cdot}5 \times 10^{-5}$ g/ml. From this point the procedure was as described by Garrahan & Glynn (1967*a*). The incubation lasted 30 min. In the second experiment the procedure was similar except that the sodium-free wash solution contained (mM): Tris (pH 8·3 at 5° C, 7·4 at 37° C) 172; Mg 1; Ca 1; phosphate (pH 7·4) 2·5; Cl 117.

The measurement of potassium influx. Cells from the same batch of blood were treated identically except that ^{24}Na was not present during the pre-incubation and ^{42}K was present during the final incubation. Again the final incubation was for 30 min. For further details see Garrahan & Glynn (1967*a*).

The measurement of potassium efflux. In the second experiment potassium efflux was measured from cells loaded with ^{42}K during the pre-incubation. Treatment of the cells was identical with the treatment of the ^{24}Na-loaded cells except that the final incubation was for 1 hr.

Procedure for the comparison of the rates of hydrolysis of ATP associated with sodium:potassium and with sodium:sodium exchange

The preparation of resealed ghosts. Cells from 40 ml. of fresh heparinized blood were washed 4 times with a solution containing (mM): K 105; Mg 30; Tris (pH 7·3 at 20° C) 10; Cl 172. They were then packed at 1500 *g* for 10 min at room temperature and squirted into 125 volumes of a lysing solution containing (mM): Na, labelled with ^{24}Na, 44; Tris 7; Mg 4; [γ-^{32}P]ATP 3; orthophosphate 15; cysteine 1. The pH of the lysing solution was 7·4 at room temperature. The haemolysate was allowed to stand at room temperature for 20 min and was then centrifuged at 16,000 *g* for 15 min at 5° C. The supernatant was discarded and the ghosts were washed 4 times with a hypotonic wash solution containing (mM): Na 44; Mg 13; phosphate (pH 7·4) 15; Cl 43; then twice with an isotonic wash solution containing (mM): Na 140; Mg 3; Tris (pH 7·4 at 20° C) 14; phosphate (pH 7·4) 15; Cl 132. The washed ghosts were suspended in more of the isotonic wash solution to give a haematocrit of about 1% (based on original cell volume).

The measurement of sodium efflux and of ATP hydrolysis. Two millilitre portions of ghost suspension were put in thirty-six stoppered glass tubes immersed in an ice-bath. One-third of the tubes received 0·1 ml. of 213 mM-KCl, giving a final K concentration of 10·1 mM. Another third received a similar amount of NaCl. The remainder received a similar amount of NaCl together with ouabain to give a final concentration of $5{\cdot}3 \times 10^{-5}$ g/ml. The tubes were transferred to a water-bath at 37° C. Half were returned to the ice-bath after 16 min, and the remainder after 66 min. The measurements of sodium efflux and of ATP hydrolysis were based on the differences between these 'initial' and 'final' samples.

The 16 min and 66 min tubes were treated similarly. Half of each group were deproteinized with trichloracetic acid (final concentration 5 g/100 ml.), and the phosphate in the supernatants was converted to phosphomolybdate and extracted into isobutanol by the method of Weil-Malherbe & Green (1951). The radioactivity of each isobutanol solution was

measured with a liquid scintillation counter, using Bray's (1960) solution, and gave an estimate of ATP hydrolysis. The contents of the remaining tubes in each group were transferred to small polythene tubes and centrifuged at 20,000 *g* for 3 min at 5° C. The radioactivity in each supernatant was measured with a well-type sodium iodide crystal scintillation counter and gave an estimate of sodium loss.

In any double labelling experiment it is necessary to be sure that the measurement of each isotope is not affected by contamination with the other. Preliminary checks showed that no appreciable amount of ^{24}Na was extracted into the isobutanol with the phosphomolybdate. The risk that radioactivity from traces of ^{32}P might lead to false estimates of ^{24}Na was reduced by counting the ^{24}Na in a solid scintillation counter, which is relatively insensitive to the softer radiation from ^{32}P. Recounting of the supernatants after 48 hr confirmed that no appreciable amounts of ^{32}P were present.

Modified procedure in the second experiment. Although the first experiment gave reasonably satisfactory results, the efflux of sodium by the ouabain-*insensitive* pathway was relatively high because the initial internal sodium concentration was high. In the second experiment the cells were therefore lysed in a solution made up with Tris phosphate instead of sodium phosphate so that the sodium concentration was only 12 mM. Sodium was also replaced by Tris in the hypotonic wash solution. Determination of sodium efflux and of ATP hydrolysis were carried out in quadruplicate instead of triplicate, and the timing was different. Loss of ^{24}Na was measured after 6, 36 and 66 min, and the formation of [^{32}P]orthophosphate after 6 and 66 min.

Testing the effect of oligomycin on sodium efflux from intact cells

Oligomycin was obtained from Sigma, London, Ltd. and consisted of approximately 15% oligomycin A and 85% oligomycin B. It was added to the incubation media as a concentrated ethanolic solution (2 mg/ml.) prepared on the day of use.

The procedure for measuring sodium efflux was slightly different from that described by Garrahan & Glynn (1967*a*), and was designed to avoid having to add very small quantities of inhibitor to a large number of different tubes. ^{24}Na-loaded cells were prepared in the usual way, but the experiment was carried out in 50 ml. flasks, and samples were withdrawn from these flasks at convenient intervals of time.

RESULTS

Activation of the transport ATPase by internal sodium and external potassium

At the time this experiment was carried out it was known that activation of the ouabain-sensitive ATPase required the simultaneous presence of sodium and potassium ions (Post, Merritt, Kinsolving & Albright, 1960; Dunham & Glynn, 1961), but there was no evidence about the site of action of sodium and only indirect evidence about the site of action of potassium (Dunham & Glynn, 1961). Ideally, it would have been desirable to compare ouabain-sensitive ATP hydrolysis in ghosts containing sodium but no potassium incubated in a medium containing potassium but no sodium, with the hydrolysis in ghosts containing potassium but no sodium incubated in a medium containing sodium but no potassium. Unfortunately, it is difficult to prepare resealed ghosts free of potassium because of the

TABLE 1. Activation of the ouabain-sensitive ATPase of resealed ghosts by internal Na and external K

Composition of the solution in which the ghosts were sealed (mM)	ATP hydrolysed in 25 mM-Na medium (m-mole/l. orig. cells/hr)	ATP hydrolysed in 25 mM-Na medium containing ouabain (m-mole/l. orig. cells/hr)	Ouabain-sensitive hydrolysis of ATP in 25 mM-Na medium (m-mole/l. orig. cells/hr)	ATP hydrolysed in 8 mM-K medium (m-mole/l. orig. cells/hr)	ATP hydrolysed in 8 mM-K medium containing ouabain (m-mole/l. orig. cells/hr)	Ouabain-sensitive hydrolysis of ATP in 8 mM-K medium (m-mole/l. orig. cells/hr)
'K' ghosts K 9; Mg 4·4; Tris 147; ATP 4; Cl 134; cysteine 0·8; pH 7·1 at 37° C	0·401 ± 0·015	0·353 ± 0·013	0·048 ± 0·020	—	—	—
'Na' ghosts Na 25; K approx. 1; Mg 4·4; Tris 130; ATP 4; Cl 135; cysteine 0·8; pH 7·1 at 37° C	—	—	—	0·851 ± 0·010	0·404 ± 0·007	0·447 ± 0·012
'Na+K' ghosts Na 25; K 9; Mg 4·4; Tris 122; ATP 4; Cl 137; cysteine 0·8; pH 7·1 at 37° C	0·338 ± 0·007	0·301 ± 0·009	0·037 ± 0·012	—	—	—

The 25 mM-Na medium contained (mM): Na 25; Mg 4; Tris (pH 7·1 at 37° C) 125; Cl 145; cysteine 0·8.

The 8 mM-K medium contained (mM): K 8; Mg 4; Tris (pH 7·1 at 37° C) 142; Cl 143; cysteine 0·8.

The ouabain concentration was $5{\cdot}6 \times 10^{-5}$ g/ml. The ghosts were incubated at 37° C for 1 hr. Only one lot of ghosts of each kind was prepared, but the incubations were done in triplicate and the results in the Table are the means ± S.E.M.

high concentration of potassium originally present in the cells. Three types of ghost were therefore prepared with the compositions shown in the first column of Table 1. The 'Na' ghosts were incubated in a medium containing 8 mM-K, the 'K' ghosts and the 'Na+K' ghosts in a medium containing 25 mM-Na. The results show that appreciable ouabain-sensitive hydrolysis occurred only when sodium was inside and potassium outside.

The ratio between the number of sodium ions expelled and the number of ATP molecules hydrolysed by the transport ATPase during sodium: potassium exchange in a sodium-free medium

To determine this ratio it is necessary to compare the rate of loss of sodium by the ouabain-sensitive pathway with the rate of hydrolysis of ATP by the ouabain-sensitive ATPase, both measurements being made under identical conditions at the same time and on cells from the same batch. The methods used are described in detail in the Methods section and the results are given in Table 2.

TABLE 2. The ratio between the number of Na ions expelled and the number of molecules of ATP hydrolysed by the transport ATPase during Na:K exchange in a Na-free medium

	Release of orthophosphate in 75 min per litre of sealed ghosts (m-mole)	Loss of Na in 75 min per litre of sealed ghosts (m-mole)
Control tubes	5·63 ± 0·05	13·48 ± 0·23
Ouabain tubes	2·79 ± 0·06	5·71 ± 0·32
Ouabain-sensitive hydrolysis of ATP	2·84 ± 0·07	—

Resealed ghosts containing 50 mM-Na were incubated for 75 min at 37° C in Na-free media with and without ouabain. Details are given in the Methods section. 41·8% of the ghosts had sealed to Na. The figures for orthophosphate release and for Na loss are each the mean of 5 estimates ± S.E.M.

To calculate the ouabain-sensitive sodium efflux from the observed losses of sodium in the presence and absence of ouabain, it is necessary to allow for the differences in internal sodium concentration that must have developed during the incubation. An accurate method for doing this is given in the Appendix, but a simpler method—and one that more easily gives an estimate of the probable error—is the following:

The initial sodium concentration in the sealed ghosts was 50 mM.

The sodium concentration in the control ghosts at the end of the incubation was 36·5 mM.

The sodium concentration in the ghosts in the ouabain medium at the end of the incubation was 44·3 mM.

Therefore during the incubation the mean sodium concentration in the control ghosts was 43·3 mM, and the mean sodium concentration in the ghosts in the ouabain medium was 47·2 mM.

If ouabain-*insensitive* sodium efflux is proportional to the internal sodium concentration, the ouabain-*insensitive* loss of sodium from the control ghosts during the 75 min incubation period must have been

$$(5{\cdot}71 \pm 0{\cdot}32) \times 43{\cdot}3/47{\cdot}2 = 5{\cdot}23 \pm 0{\cdot}30 \text{ m-mole/l. sealed ghosts.}$$

The ouabain-*sensitive* loss must therefore have been

$$(13{\cdot}48 \pm 0{\cdot}23) - (5{\cdot}23 \pm 0{\cdot}30) = 8{\cdot}25 \pm 0{\cdot}37 \text{ m-mole/l. sealed ghosts.}$$

This is equivalent to $6{\cdot}60 \pm 0{\cdot}30$ m-mole/l. sealed ghosts/hr, a figure not significantly different from that obtained by the method given in the Appendix.

The ouabain-sensitive hydrolysis of ATP in 75 min shown in Table 2 is equivalent to a rate of $2{\cdot}27 \pm 0{\cdot}06$ m-mole/l. sealed ghosts/hr. The Na:ATP ratio is therefore

$$\frac{6{\cdot}61 \pm 0{\cdot}03}{2{\cdot}27 \pm 0{\cdot}06} = 2{\cdot}91 \pm 0{\cdot}15,$$

which is not significantly different from 3.

It is perhaps worth pointing out that the observed values of ouabain-sensitive sodium efflux and ouabain-sensitive hydrolysis of ATP are not unreasonably high, because the volume of the ghosts was rather less than one half of the volume of the cells from which they had been formed.

The ratio between the number of sodium ions expelled and the number of potassium ions taken up during sodium:potassium exchange

This ratio has been the subject of some controversy. Using cold-stored red cells Post & Jolly (1957) measured net movements of sodium and potassium in the presence and absence of strophanthin-*k*, at the same time minimizing passive movements by keeping the concentrations of sodium and potassium outside the cells similar to the concentrations inside. They calculated that 1·5 sodium ions were pumped out of the cell for each potassium ion pumped in, but their argument has been criticized by McConaghey & Maizels (1962) on a number of grounds. In lactose-treated cells McConaghey & Maizels found the ratio to be nearer 1:1. Since it now appears that (i) tracer movements of sodium efflux are not complicated by a large amount of 'slowly exchanging sodium' and (ii) no sodium:sodium exchange occurs in a 10 mM-K medium (Garrahan & Glynn, 1967*a*), the sodium:potassium ratio can be estimated from simultaneous measurements of ^{24}Na loss and ^{42}K uptake. Two experiments of this kind have been carried out.

^{24}Na and ^{42}K were used to measure sodium efflux and potassium influx, with and without ouabain, in separate lots of cells from the same batch.

Both sets of measurements were made under identical conditions and at the same time. The methods are described in the Methods section. Each flux measurement was carried out in quadruplicate or quintuplicate and systematic errors were reduced to a minimum by the use of automatic syringes and the measurement of small volumes of fluid by weight. In the second experiment, potassium efflux was also measured; as this measurement is liable to serious error from haemolysis, it was made in septuplicate. The results of the two experiments are summarized in Table 3.

TABLE 3. The ratio between the number of Na ions expelled and the number of K ions taken up during Na:K exchange

Experiment 1

	Na efflux (m-mole/l. cells/hr)	K influx (m-mole/l. cells/hr)
Control	$2{\cdot}557 \pm 0{\cdot}012$	$2{\cdot}058 \pm 0{\cdot}011$
Ouabain	$0{\cdot}823 \pm 0{\cdot}004$	$0{\cdot}611 \pm 0{\cdot}006$
Ouabain-sensitive flux	$1{\cdot}734 \pm 0{\cdot}013$	$1{\cdot}447 \pm 0{\cdot}013$

$$\frac{\text{ouabain-sensitive Na efflux}}{\text{ouabain-sensitive K influx}} = 1{\cdot}20 \pm 0{\cdot}01.$$

Experiment 2

	Na efflux (m-mole/l. cells/hr)	K influx (m-mole/l. cells/hr)	K efflux (m-mole/l. cells/hr)
Control	$3{\cdot}434 \pm 0{\cdot}003$	$1{\cdot}636 \pm 0{\cdot}007$	$2{\cdot}205 \pm 0{\cdot}019$
Ouabain	$2{\cdot}084 \pm 0{\cdot}004$	$0{\cdot}634 \pm 0{\cdot}003$	$1{\cdot}938 \pm 0{\cdot}015$
Ouabain-sensitive flux	$1{\cdot}350 \pm 0{\cdot}005$	$1{\cdot}002 \pm 0{\cdot}008$	$0{\cdot}267 \pm 0{\cdot}024$

$$\frac{\text{ouabain-sensitive Na efflux}}{\text{ouabain-sensitive K influx}} = 1{\cdot}35 \pm 0{\cdot}01,$$

$$\frac{\text{ouabain-sensitive Na efflux}}{\text{ouabain-sensitive K influx} - \text{ouabain-sensitive K efflux}} = 1{\cdot}84 \pm 0{\cdot}06.$$

Cells were prepared as described in the Methods section and were incubated at 37° C. For the measurement of Na efflux and K influx the cells were incubated for 30 min; K efflux was measured during an incubation of 1 hr. In the first experiment the fluxes given in the Table are each the mean of 4 estimates ± S.E.M. In the second experiment the values of Na efflux and K influx are each the mean of 5 estimates, and the values for K efflux are each the mean of 7 estimates. In the first experiment the incubation medium contained (mM): Na 126; K 8·7; choline 17; Ca 1; Mg 1; Tris 0·6; phosphate 2·5; Cl 154; glucose 11; pH 7·4. The incubation medium in the second experiment was similar but contained 20 mM Tris (pH 7·4 at 37° C) and no choline. The ouabain concentration was $5{\cdot}5 \times 10^{-5}$ g/ml.

To estimate the ouabain-sensitive sodium efflux, the losses of ^{24}Na in the presence and in the absence of ouabain were converted to rate constants (see Garrahan & Glynn, 1967*a*) and each rate constant was multiplied by the sodium content of the cells at the beginning of the final incubation. The figures obtained in this way are shown in the table, and the differences between the figures with and without ouabain give the ouabain-sensitive fluxes. The advantage of this procedure is that no error is introduced by the small increase in sodium content that must have

occurred in the ouabain-treated cells. The procedure assumes that the sodium content in the cells without ouabain did not change, and that the ouabain-resistant efflux varied linearly with internal sodium concentration over the range through which this concentration changed in the cells with ouabain.

The results show that in both experiments ouabain-sensitive sodium efflux was significantly greater than ouabain-sensitive potassium influx. A small part of the ouabain-sensitive potassium influx in red cells may represent a potassium:potassium exchange since (i) potassium efflux is a little reduced in potassium-free media (Glynn, 1956, Table 8), and (ii) potassium efflux into a medium containing potassium is somewhat inhibited by ouabain (Glynn, 1957, Table 1). If a part of the potassium influx representing potassium:potassium exchange must be deducted from the total ouabain-sensitive potassium influx, the discrepancy between potassium influx and sodium efflux will be even greater. Table 3 shows the ratio between the fluxes in the second experiment calculated on the assumption that the ouabain-sensitive potassium efflux represents a one-for-one exchange of potassium ions.

The relation between sodium movements and the associated hydrolysis of ATP during sodium:sodium exchange

The technique used to determine the ratio between the number of sodium ions expelled and the number of molecules of ATP hydrolysed during sodium:potassium exchange cannot be used to determine this ratio during sodium:sodium exchange. If ghosts with a low internal sodium concentration are used, the absolute magnitude of the sodium:sodium exchange is low, so that any associated hydrolysis of ATP will represent only a small increase over the hydrolysis brought about by the ouabain-resistant ATPase. If ghosts rich in sodium are used, sodium:sodium exchange is absent or very small unless the concentration of orthophosphate is very high (Garrahan & Glynn, 1967*c*). A high concentration of orthophosphate makes it impossible to estimate ATP hydrolysis by measuring the orthophosphate formed.

These difficulties were overcome by using spontaneously resealed high-sodium ghosts containing [γ-^{32}P]ATP, ^{24}Na and a high concentration of orthophosphate. ATP hydrolysis was measured by the appearance of radioactivity in the orthophosphate fraction, and sodium efflux by the appearance of radioactivity in the suspending media. A full description of the methods used and the particular precautions necessary to avoid errors from the two sources of radioactivity is given in the Methods section.

The results of two experiments are summarized in Table 4. In both

experiments a large ouabain-sensitive efflux of sodium into the potassium-free medium was accompanied by little or no ouabain-sensitive hydrolysis of ATP. Parallel measurements in the presence of potassium showed a good rate of ouabain-sensitive hydrolysis associated with the sodium: potassium exchange.

TABLE 4. A comparison of the rates of hydrolysis of ATP associated with Na:K and with Na:Na exchange

	External K concentration (mM)	Ouabain-sensitive loss of ^{24}Na (counts/min/hr)	Ouabain-sensitive orthophosphate from [γ-^{32}P]ATP (m-mole/l. sealed ghosts/hr)
Expt. 1	10	474 ± 42	0·328 ± 0·004
	0	224 ± 32	0·015 ± 0·020
Expt. 2	10	296 ± 20	0·266 ± 0·008
	0	135 ± 11	0·022 ± 0·013

Spontaneously resealed ghosts containing [γ-^{32}P]ATP and ^{24}Na were prepared as described in the Methods section, and were incubated at 37° C for up to 66 min in suitable media with and without K and with and without ouabain. ATP hydrolysis was estimated from the appearance of radioactivity in the inorganic phosphate fraction. For Expt. 1, the figures in the Table are each the mean of 3 estimates ± S.E.M.; for Expt. 2 they are the mean of 4 estimates. The specific activity of the intracellular sodium was not the same in the two experiments.

This method is satisfactory for comparing the stoicheiometries during sodium:sodium exchange and sodium:potassium exchange but cannot be used to give reliable figures for sodium efflux or for ATP hydrolysis. Sodium efflux cannot be estimated accurately because the concentration of sodium inside the spontaneously sealed ghosts cannot be measured. ATP hydrolysis cannot be estimated accurately because adenylate kinase activity may lead to an exchange of the β and γ phosphorus atoms in the ATP. A further difficulty is that the fraction of ghosts that have sealed is estimated on the assumption that ghosts seal to ATP and to sodium at the same volume. This may not be true; it is possible that sealing to ATP occurs at the haemolytic volume and sealing to sodium only after the ghosts have shrunk.

A possible fallacy in the conclusion drawn from the experiments of Table 4 needs to be discussed. If there is a large fraction of ghosts that have sealed to ATP but not to sodium, these ghosts will contribute to the ATP hydrolysis but not to the ^{24}Na efflux. As we are not interested in the absolute rates of hydrolysis, this will not matter provided that the relative rates of hydrolysis by the leaky ghosts with and without ouabain and with and without potassium are similar to the relative rates of hydrolysis by the ghosts containing ^{24}Na. In the presence of potassium, both leaky and unleaky ghosts should show full activation of the (Na + K)-activated

ATPase. The crucial question is whether in the absence of potassium the leaky ghosts would be expected to carry out sodium:sodium exchange, so that any extra hydrolysis associated with this exchange could be detected. To answer this question a subsidiary experiment was carried out. Isotonically resealed ghosts were prepared with the composition that

TABLE 5. Na efflux from isotonically resealed ghosts very rich in Na and containing much more orthophosphate than ATP

Incubation medium	Total Na efflux (m-mole/l. sealed ghosts/hr)	Ouabain-sensitive Na efflux (m-mole/l. sealed ghosts/hr)
K-free	38·52 ± 0·42	4·33 ± 0·42
Ouabain	34·19 ± 0·55	—
10 mM-K	39·58 ± 0·53	5·39 ± 0·53

The ghosts were sealed in a medium containing (mM): ATP 3; orthophosphate 13; Mg 3·9; Na 153; K approx. 1; Tris 6·6; Cl 132; cysteine 0·8; pH 7·4. Incubation was at 37° C for 47 min. The K-free incubation medium contained (mM): Na 150; Mg 3; Tris 14; orthophosphate 15; Cl 129; pH 7·4. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was $5{\cdot}5 \times 10^{-5}$ g/ml. The values in the column headed 'total Na efflux' are each the mean of 4 estimates ± S.E.M. In calculating standard errors of the ouabain-sensitive fluxes, the possible error in the estimate of the ouabain-resistant efflux has been ignored because it would affect the calculated values of both oubain-sensitive fluxes equally.

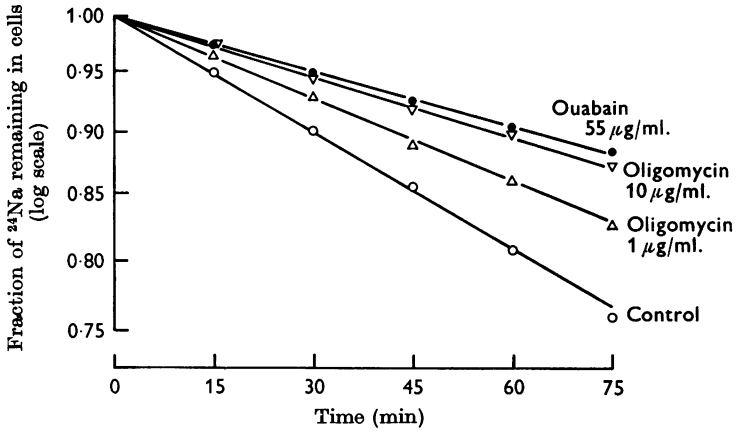

Fig. 1. The effect of oligomycin on Na efflux into a K-free medium. Cells loaded with ^{24}Na were incubated at 37° C in a medium containing (mM): Na 149; Mg 1; Ca 2·2; orthophosphate (pH 7·4) 2·5; glucose 11; ethanol 109. The haematocrit was 2·3 %.

would be expected in the hypothetical leaky ghosts but containing ^{24}Na, and these ghosts were incubated in the same potassium-free incubation medium with and without ouabain. The sodium effluxes are shown in Table 5. The ouabain-resistant fluxes were very large, partly because of

the high internal sodium concentration, but the important point is that the ouabain-sensitive sodium loss in the potassium-free medium was nearly as great as in the 10 mM-K medium. If the hypothetical leaky ghosts behaved in this way, any hydrolysis of ATP associated with the ouabain-sensitive loss of sodium into the potassium-free medium should have been detected. The conclusion from the main experiments is therefore justified.

The effects of oligomycin on sodium:potassium exchange and sodium:sodium exchange

The effects of oligomycin at 1 and 10 μg/ml. were tried on the sodium efflux from ^{24}Na-loaded cells incubated in potassium-free, 10 and 100 mM-K

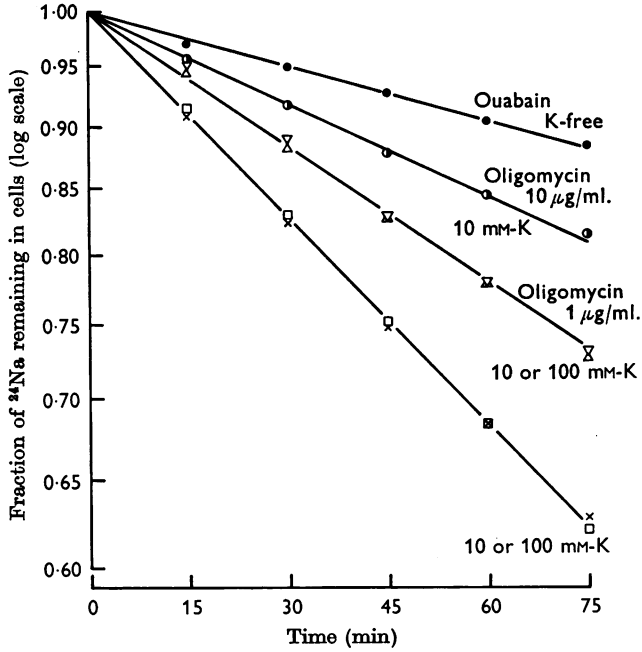

Fig. 2. The effects of oligomycin on Na efflux into 10 mM-K and 100 mM-K media. ^{24}Na-loaded cells from the same batch were used in this experiment and in the experiment of Fig. 1, and the ouabain curve is taken from that figure. The conditions of incubation were the same as in the experiment of Fig. 1, and the incubation media were similar but with suitable fractions of Na replaced by K. ◑, oligomycin 10 μg/ml., K 10 mM; △, oligomycin 1 μg/ml., K 10 mM; ▽, oligomycin 1 μg/ml., K 100 mM; □, K 10 mM; ×, K 100 mM.

media. The procedure is described in the Methods section and the results are shown in Figs. 1 and 2. As a preliminary experiment had shown that inhibition by oligomycin at low concentrations had a slow onset, the cells were allowed to remain in contact with the inhibitor for 1 hr at 0° C

before the incubation was started. In the presence of 10 mM-K, ouabain-sensitive sodium efflux was inhibited 47% by 1 μg oligomycin/ml. and 74% by 10 μg oligomycin/ml. Increasing the potassium concentration to 100 mM did not affect the degree of inhibition. In the absence of potassium, ouabain-sensitive sodium efflux was inhibited 48% by 1 μg oligomycin/ml. and 91% by 10 μg oligomycin/ml.

DISCUSSION

The experiment in which three sodium ions were expelled for each molecule of ATP hydrolysed by the ouabain-sensitive ATPase was, of course, carried out under conditions in which pumping was 'downhill', since the drop in free energy associated with the outward movement of sodium into the sodium-free solution would have more than out-weighed the increase in free energy associated with the linked uptake of potassium. It would be interesting to repeat the experiment using ^{24}Na-loaded ghosts immersed in a high sodium medium, to get a direct estimate of the stoicheiometry of the pump during the 'uphill' movement of sodium. Unfortunately, there are technical difficulties. The ratio of ATP to sodium in isotonically resealed ghosts is usually about 1·5 times the ratio of ATP to sodium in the lysing solution. It is possible that this difference comes about because the cells seal to ATP at the haemolytic volume and seal to sodium only after they have shrunk, but it is also possible that some ghosts seal to ATP but remain leaky to sodium. Such ghosts would show no ouabain-sensitive hydrolysis of ATP in a sodium-free medium, but they would show ouabain-sensitive hydrolysis if both sodium and potassium were present in the medium. This difficulty might be overcome by incorporating some non-penetrating molecule in the ghosts before resealing, so that ghosts that remained permeable to sodium would undergo osmotic haemolysis. It might be thought that ATP itself would make ghosts that were leaky to sodium osmotically unstable, but probably the concentration of Mg-ATP complex inside is largely offset by the free magnesium ions outside. A further difficulty in using ^{24}Na-loaded ghosts is that the entry of unlabelled sodium during the incubation would cause changes in specific activity and total sodium concentration. In experiments on intact cells such changes are relatively small and can be allowed for, but in resealed ghosts they would be relatively large and difficult to allow for because the volume of the ghosts is small and not accurately known.

Using rather different methods, however, Sen & Post (1964) were able to measure the number of sodium ions expelled per molecule of ATP hydrolysed, when the concentrations of sodium and of potassium outside the cells were the same as inside—in other words, when pumping was 'on

the level'—and Whittam & Ager (1965) were able to get estimates of the ratio in intact cells when the pump was pumping 'uphill' to a greater or lesser extent. 'Downhill', 'on the level', 'slightly uphill' and 'steeply uphill' pumping all seem to give a stoicheiometric ratio of about 3:1, suggesting that the pump uses the same amount of ATP however little or however much work it has to do in expelling each sodium ion. Although the various estimates are not significantly different from 3·0 it is probably not justified to assume that the ratio is necessarily integral.

As mentioned earlier, the ratio between the number of sodium ions expelled and the number of potassium ions taken up has been the subject of some controversy. The present results show that under physiological conditions the potassium ions taken up are fewer than the sodium ions expelled, though uncertainties about potassium:potassium exchange make it impossible to give a definite figure for the ratio. The discrepancy between the numbers of sodium and of potassium ions transported is interesting because it stands in marked contrast to the equality of ion movements during sodium:sodium exchange, and because of its relevance to the electrogenic nature of the sodium pump, for which, in frog muscle at any rate, there is now substantial evidence (Kernan, 1962; Hashimoto, 1964; Cross, Keynes & Rybová, 1965; Mullins & Awad, 1965; Frumento, 1965; Adrian & Slayman, 1966).

A number of attempts were made to find out how many potassium ions were taken up for each molecule of ATP hydrolysed, from a direct comparison of ouabain-sensitive potassium accumulation and ouabain-sensitive hydrolysis of ATP by resealed ghosts incubated in media containing potassium. Unfortunately potassium influx measurements in resealed ghosts are not satisfactory. By combining the information on the Na:ATP ratio and on the Na:K ratio, we can derive an indirect estimate of the K:ATP ratio, which will be nearer 2 than 3. This agrees with the estimates of Sen & Post (1964), Gárdos (1964) and Whittam & Ager (1965).

Perhaps the most interesting observation reported in this paper is the observation that sodium:sodium exchange by ghosts containing [γ-^{32}P]-ATP is not accompanied by the appearance of appreciable radioactivity in the inorganic phosphate fraction. This means that sodium:sodium exchange is accompanied by little or no hydrolysis of ATP; nor is there any considerable ATP–orthophosphate exchange. Yet, we have seen that ATP must be present. Recent work on erythrocyte AMP deaminase has shown that ATP alters the affinity of this enzyme for its substrate by a process which does not involve phosphorylation of the enzyme by the ATP (Askari & Franklin, 1965). It is possible that a similar explanation accounts for the need for ATP in sodium:sodium exchange, but a more likely explanation is that ATP is needed to make some phosphorylated inter-

mediate that is required for the sodium:sodium exchange but is not broken down, or at any rate not broken down irreversibly, during the course of that exchange. Slow loss of this intermediate would lead to a requirement for ATP, but the rate of break-down could be very slow compared with the turnover of sodium ions. A scheme of this kind fits nicely with recent experimental work on phosphorylated intermediates (Charnock, Rosenthal & Post, 1963; Post, Sen & Rosenthal, 1965; Albers, Fahn & Koval, 1963; Fahn, Albers & Koval, 1965; Whittam, Wheeler & Blake, 1964; Ahmed & Judah, 1965), since the conditions required for the formation of a phosphorylated intermediate—the presence of magnesium, ATP and sodium—are necessary, though not sufficient conditions, for sodium:sodium exchange. There is, however, an interesting difference between the effects of inhibitors on the sodium:sodium exchange and on the formation of a phosphorylated intermediate. Ouabain inhibits both processes—though rather high concentrations are necessary to prevent the formation of the intermediate, and the sensitivity of the sodium:sodium exchange to low concentrations of ouabain has not been tested. Oligomycin inhibits the sodium:sodium exchange but has no effect on the formation of the intermediate. The implication is, presumably, that sodium:sodium exchange requires not only the formation of the intermediate but also its behaviour in a way that is affected by oligomycin.

We are grateful to Dr P. F. Baker and Professor A. L. Hodgkin for helpful discussion, to the Medical Research Council for a grant for apparatus, and to the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, for a personal grant to one of us (P.J.G.).

APPENDIX

Calculation of active sodium efflux from the losses of ^{24}Na with and without ouabain

It is assumed that:

(i) the active efflux of sodium does not change during the incubation (the fall in internal sodium concentration will have little effect if the pump is near saturation);

(ii) the passive efflux of sodium is proportional to the internal sodium concentration;

(iii) the increase in the total volume of the ghosts, caused by the entry of Tris, occurs at a constant rate;

(iv) it is only the resealed ghosts that increase in volume.

For the sealed ghosts in the presence of ouabain:

$$\frac{dx}{dt} = \frac{-kx}{v_0(1+\alpha t)}, \qquad (1)$$

where x is the internal sodium concentration, k is the rate constant for the passive efflux of sodium, v_0 is the initial volume of the sealed ghosts and α is a constant defining the rate of swelling of the sealed ghosts in the presence of ouabain.

If v_0 is arbitrarily put at unity and $x = x_0$ when $t = 0$, the solution of (1) is

$$k = \frac{\alpha \ln (x/x_0)}{\ln(1/(1+\alpha t))}. \tag{2}$$

In the presence of ouabain the total ghost volume increased by 17·4% in 75 min, so that the sealed ghosts must have swollen by $(17{\cdot}4 \times 100)/41{\cdot}8 = 41{\cdot}6\%$. This gives a value of 0·00553 min^{-1} for α. Substitution of the initial and final sodium concentrations for x_0 and x gives $k = 1{\cdot}91 \times 10^{-3}$ min^{-1}.

For the sealed ghosts in the absence of ouabain

$$\frac{dx}{dt} = -\frac{kx}{1+\beta t} - m, \tag{3}$$

where m is the active sodium efflux and β is a constant defining the rate of swelling of the sealed ghosts in the absence of ouabain. The integrating factor is

$$\exp \int \frac{k}{1+\beta t} dt = (1+\beta t)^{k/\beta}.$$

Hence

$$x(1+\beta t)^{k/\beta} = -m \int (1+\beta t)^{k/\beta} \, d(1+\beta t). \tag{4}$$

Integrating, putting $x = x_0$ when $t = 0$ and solving for m:

$$m = (k+\beta) \frac{x(1+\beta t)^{k/\beta} - x_0}{1-(1+\beta t)^{(k+\beta)/\beta}}. \tag{5}$$

In the absence of ouabain the total ghost volume increased by 16·8% and this gives a value of 0·00535 min^{-1} for β. Substitution of the initial and final sodium concentrations for x_0 and x gives $m = 6{\cdot}61$ m-moles/l. sealed ghosts/hr.

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