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FACTORS AFFECTING THE RELATIVE MAGNITUDES OF THE SODIUM:POTASSIUM AND SODIUM:SODIUM EXCHANGES CATALYSED BY THE SODIUM PUMP

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SUMMARY

1. The effects of external potassium on sodium:potassium exchange and sodium:sodium exchange in human red cells have been estimated from measurements of ouabain-sensitive potassium influx and ouabain-sensitive sodium influx in media containing different concentrations of potassium.

2. As the external potassium concentration is increased from zero to 5 mM, sodium:sodium exchange—as judged by ouabain-sensitive sodium influx—is progressively suppressed, and sodium:potassium exchange—as judged by ouabain-sensitive potassium influx—is progressively increased. Both exchanges are half-maximal between 1 and 2 mM-K, and at 5 mM-K sodium: sodium exchange becomes very small as sodium: potassium exchange approaches a maximum.

3. Experiments have been carried out, mainly on resealed ghosts, to determine what factors affect the magnitude of the sodium:sodium exchange in potassium-free solutions.

4. Sodium:sodium exchange does not occur in the absence of adenosine triphosphate (ATP).

5. Ghosts containing high concentrations of sodium, no potassium and high concentrations of ATP show no ouabain-sensitive loss of sodium into potassium-free solutions. The ability to carry out sodium:sodium exchange can be restored by replacing most of the internal sodium with potassium or by preparing the cells so that they contain much more orthophosphate (P_i) than ATP.

6. Ghosts containing sodium in low concentration, potassium in high concentration and with a low [ATP]/([ADP].[P_i]) ratio show a greater ouabain-sensitive loss of sodium into potassium-free media than into

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media containing potassium; i.e. external potassium *reduces* ouabain-sensitive sodium efflux.

7. The effect of P_i is not the result of competitive inhibition of the transport ATPase since P_i at the concentrations used does not inhibit ATPase activity in fragmented ghosts.

INTRODUCTION

Experiments reported in the first paper of this series (Garrahan & Glynn, 1967*a*) show that the ouabain-sensitive transport system in red cells can catalyse a one-for-one exchange of sodium ions across the cell membrane. This paper reports experiments designed to define the conditions under which this exchange of sodium occurs, and to find what factors determine whether the transport system exchanges sodium for potassium or sodium for sodium.

The effects of changes in external sodium and potassium concentrations, internal sodium concentration and the concentrations of ATP and P_i have been studied. The effects of external potassium were studied in intact cells. The effects of the other factors were studied mainly in resealed ghosts, though some confirmatory experiments were done on intact cells.

Preliminary accounts of some of the experiments reported here have already been published (Garrahan & Glynn, 1965, 1966).

METHODS

Experiments on intact cells

Fluxes were measured as described in the previous two papers (Garrahan & Glynn, 1967*a*, *b*).

The preparation of resealed ghosts

The methods described here are based on those of Hoffman, Tosteson & Whittam (1960) and of Hoffman (1962).

Spontaneously resealed ghosts (*Hoffman's 'Group I ghosts'*). Red cells from freshly drawn blood were washed 4–5 times with 3–4 volumes of a solution containing (mM): K 105; Mg 30; tris (pH 7·7 at 25° C) 10; Cl 165. The washing was carried out at room temperature using an M.S.E. 'Junior' centrifuge and spinning for 3 min at 1500 *g* after each wash. The cells were packed for 7 min at 1500 *g* and squirted into 60–100 volumes of stirred lysing solution at room temperature. The composition of the lysing solution differed in the different experiments but certain rules were always observed. The total concentration of all solutes was always less than 100 ideal m-osmoles/l. and was usually about 80 ideal m-osmoles/l. Cysteine (1 mM) was always present. The Mg concentration was 0·5–1·0 mM higher than the sum of the concentrations of ATP, ADP and P_i, except in the experiments where the phosphate concentration was very high. In those experiments the Mg concentration was 1 mM greater than the concentration of ATP (ADP was absent). ^{24}Na was added to the lysing solutions *before* the cells were squirted in.

After the addition of the cells the suspension was allowed to stand at room temperature

for 20 min. The ghosts were spun down at 5° C in an M.S.E. 'High Speed 17' centrifuge for 15 min at 17,500 *g* and were washed 4 times with about 15 volumes of an ice cold hypotonic wash solution to get rid of ^{24}Na trapped in unsealed ghosts. After each wash the ghosts were centrifuged for 3 min at 20,000 *g*. The composition of the hypotonic wash solution differed in the different experiments, but it always had an osmolarity 15–25 m-osmoles/l. greater than the osmolarity of the lysing solution. Further details are given in the legends to the figures. After four hypotonic washes the ghosts were washed twice with an ice cold isotonic wash solution containing (mM): Na 140–159; Mg 2–3; Tris (pH 8·3 at 5° C, 7·4 at 37° C) 5–10; Cl 148–164. When ghosts were prepared containing P_i, the isotonic wash solution contained P_i at a similar concentration, and the concentration of Tris was reduced. After the isotonic washes the ghosts were suspended in more of the isotonic wash solution ready for use.

Isotonically resealed ghosts (*Hoffman's 'Group II ghosts'*). Cells from freshly drawn blood were washed as described for the preparation of spontaneously resealed ghosts, and were lysed in the same way except that ^{24}Na was added *after* the cells had been squirted into the lysing solution. (Labelling of any ghosts that sealed spontaneously was therefore avoided.) The haemolysate was stirred, and sufficient 3 M salt solution added to it to restore the tonicity to 315 ideal m-osmolar. The salt solution consisted of a mixture of 3 M-NaCl and 3 M-KCl in proportions chosen to give the desired ratio of Na to K.

The ghost suspension was centrifuged at 5° C for 15 min at 17,500 *g* and most of the supernatant was discarded. The ghosts were resuspended in the remaining supernatant—about 30 ml.—and incubated at 37° C with gentle shaking for 30–40 min. At the end of this period the ghosts were spun down at 5° C for 5 min at 20,000 *g* and washed 5 times with about 15 volumes of an isotonic wash solution similar to that used in the preparation of spontaneously resealed ghosts. In the experiments in which the effects of external sodium concentration were to be studied, the isotonic wash solution was made with choline chloride instead of sodium chloride. After they had been washed, the ghosts were suspended in more of the same wash solution ready for use.

The measurement of sodium efflux from resealed ghosts

Resealed ghosts containing ^{24}Na were suspended in suitable media to give haematocrits of 1–3 %, and 2 or 3 ml. portions of the suspensions were put in stoppered glass tubes in an ice-bath. When required, KCl was added in 0·1 ml. to give a final concentration of 10 mM; the K-free tubes received a similar addition of NaCl. Ouabain was added to the tubes requiring it by including it in the KCl or NaCl solutions added. The final concentration of ouabain was 5×10^{-5} g/ml.

The experiment was started by transferring the tubes to a water-bath at 37° C. At 15 min intervals tubes were removed and replaced in the ice-bath. The chilled contents were transferred to small polythene centrifuge tubes and spun at 5° C for 3 min at 20,000 *g*. Radioactivity was measured in the supernatants and also in the original ghost suspensions. The results were plotted as in the efflux experiments on intact cells described in the first of these papers (Garrahan & Glynn, 1967*a*). With ghosts the initial loss of ^{24}Na was greater than with cells, but the logarithmic plots gave good straight lines showing that efflux followed first order kinetics satisfactorily during the experiments. A similar initial loss from resealed ghosts has been described by Hoffman (1962); its cause is not known.

Incubation media. The incubation media were based on a solution containing (mM): Na 150–164; Mg 2–3; Tris (pH 7·4 at 37° C) 3–10; Cl 157–169. Potassium ions, where required, replaced an equivalent quantity of sodium ions. When experiments were carried out on ghosts containing much P_i the incubation media were prepared with a similar concentration of P_i and the concentrations of Tris and chloride were reduced to maintain isotonicity. Low sodium media were prepared by mixing the basic incubation medium described above with suitable quantities of a similar medium in which choline replaced sodium.

Experiments on fragmented ghosts

The preparation of fragmented ghosts. Cells from freshly drawn heparinized blood were washed 4 times with a wash solution similar to that used in the preliminary stages of the preparation of spontaneously resealed ghosts. Twenty millilitres of cells were lysed at room temperature in 200 ml. of a solution containing (mM): Tris ethylenediaminetetraacetate (Tris EDTA) (pH 7·4 at 25 °C) 1; cysteine 0·1; and the ghosts were spun down and washed 4 times in more of the lysing solution. The volume of solution used for each wash was about 6 times the volume of the ghosts, and centrifuging was for 3 min at 20,000 *g*. After the last wash the packed ghosts were quickly frozen with dry ice and acetone and allowed to thaw at room temperature. The ghost fragments were suspended in 200 ml. of a solution containing (mM): Na 110: K 30; Mg 0·1; Tris (pH 7·4 at 37° C) 20; Cl 154; EDTA 0·1.

Measurement of the ATPase activity of fragmented ghosts in the presence and absence of P_i. Two 90 ml. portions of ghost suspension were prepared. To one was added 10 ml. of 50 mM sodium phosphate buffer (pH 7·4) to give a final phosphate concentration of 5 mM. To the other was added 10 ml. of 90 mM-NaCl to give the same final concentration of Na. Three millilitre portions of these suspensions were put into stoppered glass tubes in an ice-bath. Small volumes of stock [γ-^{32}P]ATP solution (see below) were added to give final ATP concentrations of 0·5–2·5 mM, and the sodium concentration was brought up to the same level in all tubes by the addition of suitable amounts of NaCl. The stock [γ-^{32}P]ATP solution contained 33·5 mM [γ-^{32}P]ATP disodium salt, buffered with Tris base to pH 7·4 (25° C), and 33·5 mM-$MgCl_2$. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). Ouabain was added, when required, in 0·1 ml. of distilled water to give a final concentration of 5×10^{-5} g/ml., and the same amount of distilled water was added to the ouabain-free tubes.

The tubes were transferred to a water-bath at 37° C for 1 hr and then returned to the ice-bath. Ice-cold trichloracetic acid (55 g/100 ml.) was added to give a final concentration of 5 g/100 ml., and the precipitated material was removed by centrifuging. P_i in the supernatants was converted to phosphomolybdate and extracted into isobutanol as in the phosphate estmation method of Weil-Malherbe & Green (1951). The radioactivity contained in the isobutanol was estimated by liquid scintillation counting using Bray's (1960) solution.

Sources of materials

ATP was obtained from Sigma, London, Ltd. as the crystalline disodium salt (Sigma grade) and, where necessary, was converted to the dipotassium salt as follows. About 6 g of Amberlite resin IR-120(H) (British Drug Houses Ltd.) was prepared as a column of 12 mm diameter and converted to the potassium form. Up to 4 m-moles of ATP disodium salt were dissolved in 10 ml. of water and allowed to run through the column at 0·2 ml./min. The ATP solution was followed through with an equal volume of water and the mixed effluents were adjusted to pH 7·4 with Tris base.

ADP was obtained as the sodium salt from Sigma, London, Ltd. or from the Boehringer Corporation, London. When necessary it was converted to the potassium salt with Amberlite IR-120 resin as described above for ATP.

^{32}P-labelled P_i was obtained from the Radiochemical Centre, Amersham, as a sterile solution of very high specific activity in dilute HCl, pH 2–3 (reference PBS. 1).

Cysteine was obtained from Sigma, London, Ltd. or from the Nutritional Biochemical Corporation, Cleveland, Ohio.

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

RESULTS

The effects of external potassium

The ouabain-sensitive exchange of sodium ions described by Garrahan & Glynn (1967*a*) was observed in red cells incubated in potassium-free solutions. Since (i) little or no ouabain-sensitive sodium influx is detected in red cells incubated in the presence of 10 mM-K (Glynn, 1956) and (ii)

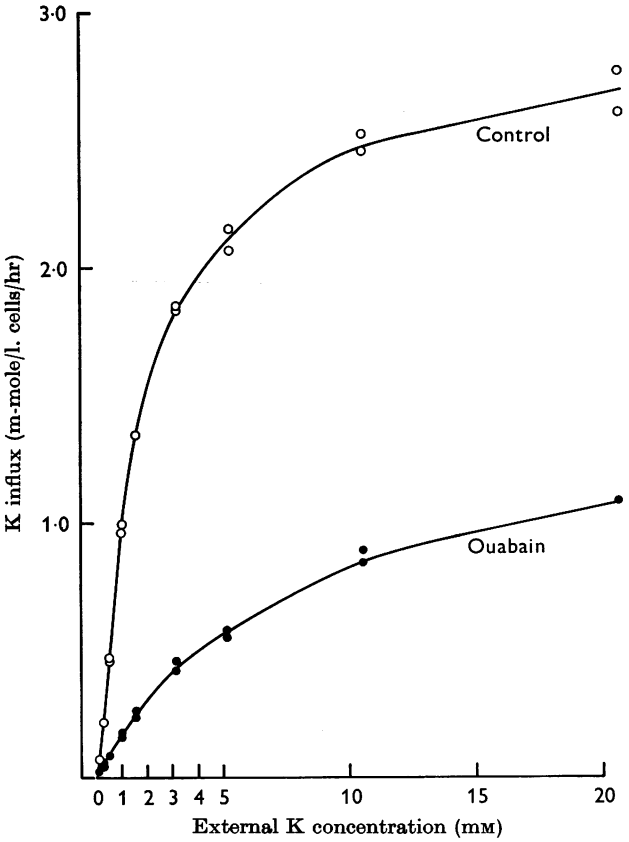

Fig. 1. The K flux into fresh red cells in the presence and absence of ouabain as a function of the external K concentration. The cells were incubated for 1 hr at 37° C. The incubation media were based on a medium containing (mM): Na 129; choline 20; Mg 1; Ca 2·2; Cl 151; phosphate (pH 7·4) 2·5; glucose 11. K was substituted for choline to get media with different K concentrations. ○, control tubes; ● tubes containing ouabain (5×10^{-5} g/ml.).

sodium efflux into solutions containing 10 mM-K is not reduced by the removal of external sodium (Garrahan & Glynn, 1967*a*), it is clear that potassium at this concentration must abolish the sodium : sodium exchange.

If sodium: sodium exchange in potassium-free media involves the mechanism responsible for sodium: potassium exchange under physiological conditions, and if potassium ions affect both processes by acting at the same sites, then we might expect potassium at any level to be equally effective in blocking the sodium: sodium exchange and in activating the sodium: potassium exchange. More precisely, we might expect the concentration of potassium at which sodium: sodium exchange is 50% inhibited to be just the concentration required for half-maximal activation

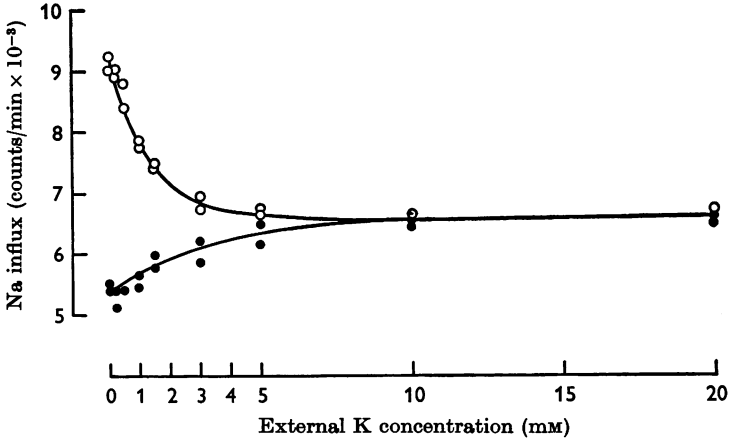

Fig. 2. The Na flux into fresh red cells in the presence and in the absence of ouabain as a function of external K concentration. The cells were incubated for 30 min at 37° C in media similar to those used in the experiment of Fig. 1. To calculate the influxes it was necessary to apply a small correction to the figures for ^{24}Na uptake, to allow for efflux of some of the ^{24}Na that had entered. The correction was made assuming that the rate constant for Na efflux was 0·1 hr^{-1} in the presence of ouabain and 0·3 hr^{-1} in the absence of ouabain and the presence of K. ○, control tubes; ●, tubes containing ouabain (5×10^{-5} g/ml.).

of the sodium: potassium exchange. Similarly, the minimum potassium concentration at which the sodium: sodium exchange is fully inhibited should be just the concentration required for full activation of the sodium: potassium exchange. The relation between the absolute values of sodium: sodium exchange and sodium: potassium exchange at any given external potassium concentration will be complicated, but if ouabain-sensitive sodium influx (used as a measure of sodium: sodium exchange) is expressed as a fraction of its magnitude in potassium-free solution, and ouabain-sensitive potassium influx (used as a measure of sodium: potassium exchange) is expressed as a fraction of its magnitude at saturating levels of potassium, we should expect the curve of *sodium* influx plotted against

$[K]_o$ to be the mirror image of the curve of *potassium* influx plotted against $[K]_o$.

The results of experiments to test these predictions are shown in Figs. 1, 2 and 3. Figure 1 shows potassium influx into fresh red cells in the presence and absence of ouabain as a function of external potassium concentration. The difference between the two curves gives the ouabain-sensitive potassium influx. Figure 2 shows sodium influx into fresh red cells in the presence and absence of ouabain as a function of external *potassium* concentration. The difference between the two curves gives the ouabain-sensitive sodium influx. The measurements of sodium and

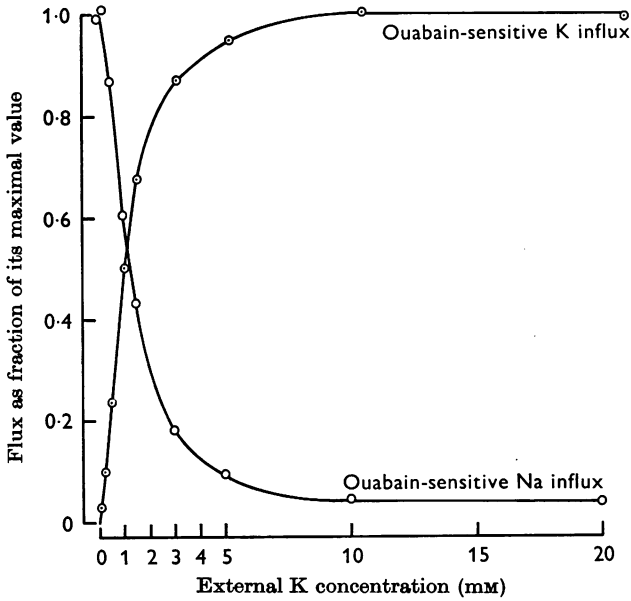

Fig. 3. The effects of external K on Na:Na exchange—judged by the ouabain-sensitive influx of Na—and on Na:K exchange—judged by the ouabain-sensitive influx of K. Each of the ouabain-sensitive influxes has been expressed as a fraction of its maximal value. This figure is based on data from the experiments of Figs. 1 and 2.

potassium fluxes were made on different batches of red cells, and the conditions of incubation were not identical, but the treatment of the cells and the compositions of the incubation media were similar enough to make comparison of the results reasonable. In Fig. 3 the ouabain-sensitive influxes of sodium and of potassium each expressed as a fraction of its maximal value are plotted against external potassium concentration. Except that there is a trace of ouabain-sensitive sodium influx at high potassium concentrations, the predictions are fulfilled remarkably well.

The effects of substances inside the cell

To study the effects of substances inside the cell it is necessary to be able to control the composition of the cell interior. This can be done only to a very limited extent with intact cells, so most of the experiments have been carried out on resealed ghosts.

Two types of resealed ghosts have been used: (i) *spontaneously resealed ghosts*, i.e. those sealing spontaneously in the hypotonic lysing solution; (ii) *isotonically resealed ghosts*, i.e. those in which sealing is induced by adding sufficient salt to restore isotonicity and incubating for 30 min at 37° C (see Hoffman *et al.* 1960; Hoffman, 1962). The spontaneously resealed ghosts have the advantage that, because no incubation is necessary, the relative concentrations of different metabolites in the ghosts at the beginning of the experiment may be known fairly accurately. They have two disadvantages. They do not seal satisfactorily to potassium ions, and the total amount of sodium in the ghosts is rather small. The yield of spontaneously resealed ghosts is always smaller than the yield of isotonically resealed ghosts—1–10% compared with about 40%—and is less predictable.

The effects of internal sodium concentraton

Work on frog muscle and sodium-depleted squid nerve (Keynes & Swan, 1959; Keynes, 1965; Frumento & Mullins, 1964) has demonstrated that a fraction of the sodium efflux in these tissues can depend on the presence of sodium in the external solution and that the size of this fraction may be greatly reduced by procedures that raise the internal sodium concentration. The sodium-dependent sodium efflux in frog muscle seems to be insensitive to ouabain (Horowicz, 1965), and in neither tissue is it clear that this flux represents an exchange of sodium ions across the membrane. Nevertheless, it seemed worth investigating the effect of internal sodium concentration on the sodium-dependent sodium efflux in red cells.

Figure 4 shows the efflux of ^{24}Na from spontaneously resealed ghosts containing sodium as the only univalent cation into: (i) a high sodium medium containing 10 mM-K, (ii) a potassium-free high-sodium medium, and (iii) a potassium-free high-sodium medium containing ouabain. In the lysing solution, sodium ions accounted for 29 ideal m-osmoles of a total 82 ideal m-osmoles/l., so that the concentration inside the sealed ghosts under isotonic conditions must have been something like $(315 \times 29)/82 = 111$ mM. The curves in Fig. 4 show that there was no detectable ouabain-sensitive sodium efflux into a potassium-free medium. A similar result has been obtained in four other similar experiments.

Figure 5*a–e* shows the efflux of ^{24}Na from batches of isotonically resealed ghosts containing different concentrations of sodium ranging from 2·8 to 144 mM. Isotonicity inside the ghosts was maintained with potassium. It is clear that, compared with the efflux in the presence of external potassium, the ouabain-sensitive efflux in potassium-free solutions was very low in the high-sodium ghosts and increased as the ghost sodium was decreased.

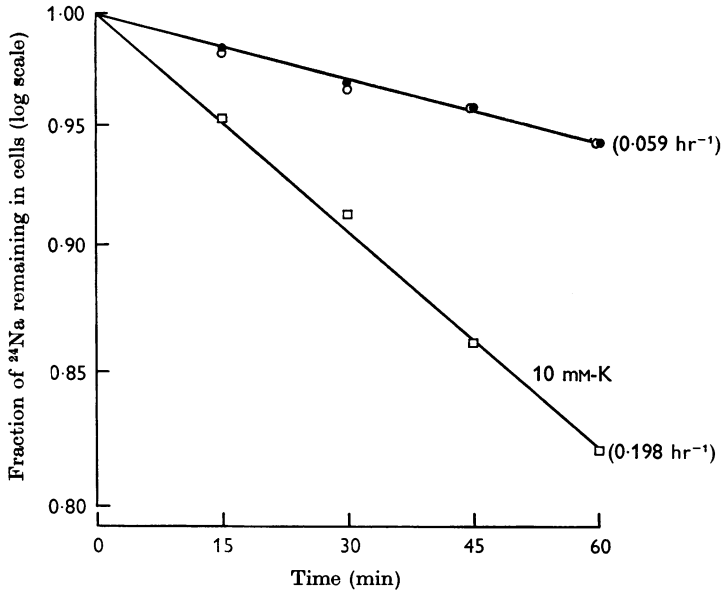

Fig. 4. The efflux of Na from spontaneously sealed ghosts rich in ATP and containing Na as the only univalent cation. This experiment and those of Figs. 11 and 12 were done on cells from the same batch and at the same time. The lysing solution contained (mM): ATP 4·9; Mg 5·9; Na 29; Tris 10; Cl 30·5; cysteine 1; pH 7·4; and had an osmolarity of 82 ideal m-osmole/l, so that after isotonicity has been restored the concentration of Na inside the sealed ghosts must have been about 111 mM, and the concentration of ATP at least 18·8 mM. The hypotonic wash solution contained (mM): Na 29; Mg 10; Tris 10; Cl 56. The K-free incubation medium contained (mM): Na 150; Mg 2; Tris (pH 7·4 at 37° C) 5; Cl 15. 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. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

The absolute magnitude of the ouabain-sensitive sodium efflux into potassium-free solutions is difficult to determine accurately when the internal sodium concentration is high. In the experiment of Fig. 4, on spontaneously resealed ghosts, it appears to have been zero, but an immeasurably small rate constant might be equivalent to a significant flux when multiplied by a high internal sodium concentration. In the experiment on isotonically resealed ghosts there appears to have been a very

small ouabain-sensitive efflux of sodium into the potassium-free solution from the ghosts containing 134 mM-Na (Fig. 5*b*). These ghosts, however, also contained 11 mM-K, and leakage of potassium into the external medium would have allowed some sodium : potassium exchange to take place. The potassium concentration at the end of the experiment was 72 μM and, on the assumption that the potassium influx curve in Fig. 3 applies also to high sodium cells, this would account for about two thirds of the observed sodium efflux into the potassium-free medium. External potassium would of course also have tended to reduce the sodium : sodium exchange so partly offsetting the effect just discussed.

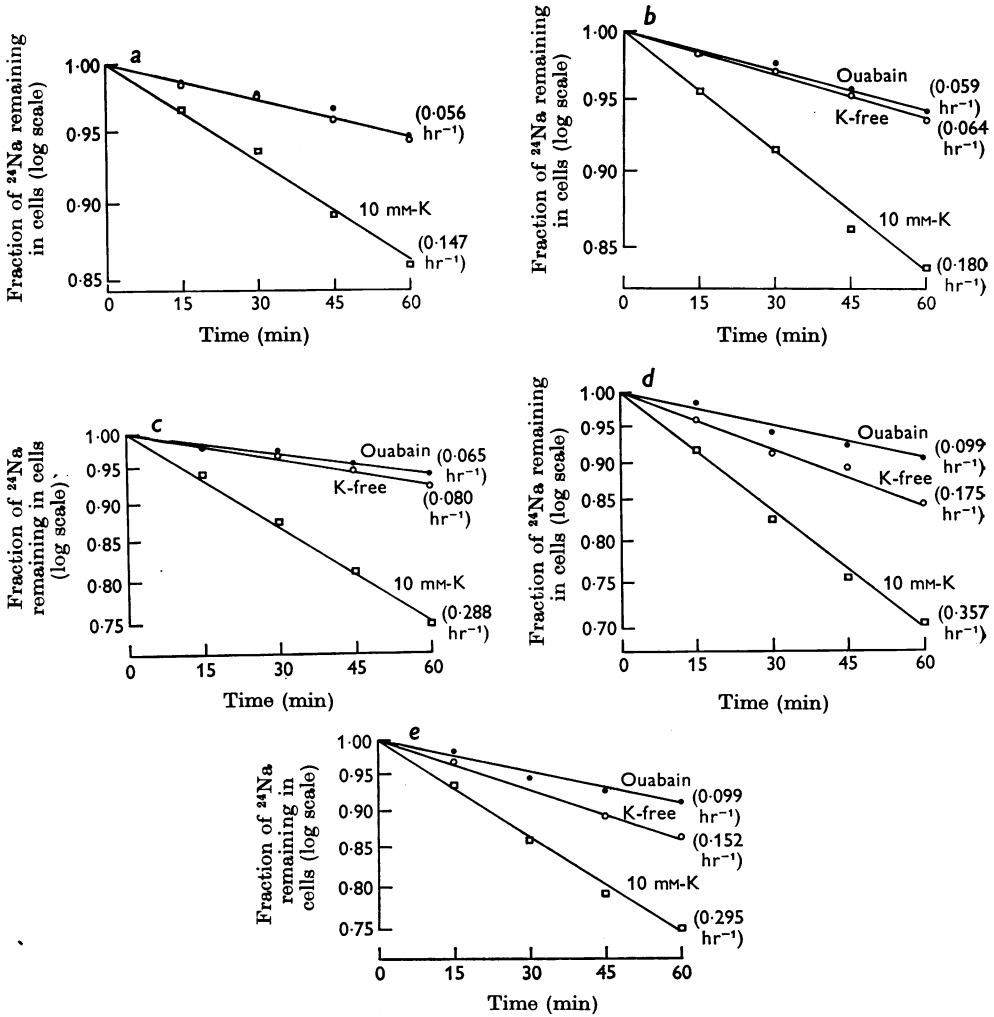

Fig. 5. For legend see opposite page.

The effects of ATP, ADP and P_i

In experiments on cyanide-poisoned squid axons by Caldwell, Hodgkin, Keynes & Shaw (1960*a*) it was found that the efflux of sodium could be restored by injecting either ATP or arginine phosphate. When arginine phosphate was injected, the restored efflux was like the normal efflux in being very sensitive to the level of external potassium. When ATP was injected the restored efflux was unaffected by the removal of external potassium. It was suggested that the difference between the effects of arginine phosphate and of ATP came about because arginine phosphate removed ADP in forming ATP, so that the ATP/ADP ratio was higher after the arginine phosphate injections; and this interpretation was supported by the effect of injected ADP (but cf. Caldwell, Hodgkin, Keynes & Shaw, 1964). The sodium efflux from ATP-injected fibres was sensitive to ouabain, and though there was no proof that the efflux represented a sodium:sodium exchange there was a little evidence in this direction. The resemblance between this behaviour of squid axons and our findings in red cells suggested that it would be worth looking at the effects of ATP, ADP and P_i in resealed red cell ghosts.

The effect of a low ATP/ADP ratio on sodium efflux from high-sodium spontaneously resealed ghosts. Since spontaneously resealed ghosts rich in sodium and containing ATP in high concentration showed no ouabain-

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Legend for Fig. 5

Fig. 5. The efflux of Na from isotonically resealed ghosts rich in ATP and containing different concentration of Na. The incubation media were the same as those used for the experiment of Fig. 4. In (*a*) the ghosts contained 144 mM-Na and about 1·5 mM-K, having been sealed in a solution containing (mM): ATP 4·6; Mg 5·6; Na 144; K approx. 1·5; Tris 9·3; Cl 146; cysteine 0·9; pH 7·4. In (*b*) the ghosts contained 134 mM-Na and 11 mM-K but were otherwise similar. At the end of the incubation the K-free medium contained 72 μM-K, and this K could have accounted for perhaps two thirds of the difference between the 'K-free' curve and the 'ouabain' curve. In (*c*) the ghosts contained 61 mM-Na and 85 mM-K. At the end of the incubation the K-free medium contained 108 μM-K and this K could have accounted for rather more than half of the difference between the 'K-free' and 'ouabain' curves. In (*d*) the ghosts contained 6·6 mM-Na and 137 mM-K. At the end of the incubation the K-free medium contained 152 μM-K, but this K could have accounted for less than one quarter of the difference between the 'K-free' and 'ouabain' curves. In (*e*) the ghosts contained 2·8 mM-Na and 139 mM-K. At the end of the incubation the K-free medium contained 148 μM-K, but this K could have accounted for only about one quarter of the difference between the 'K-free 'and 'ouabain' curves. □, 10 mM-K medium; ○, K-free medium; ● K-free medium with ouabain. The figures in brackets are rate constants for efflux. (Note the change in scale of the ordinate.)

sensitive loss of sodium into a potassium-free medium (Fig. 4), we first tried the effect of ADP in ghosts of this kind. Figure 6 shows the results of an experiment in which the ATP and ADP concentrations were initially equal. There is a little scatter in the results but no significant ouabain-sensitive loss of sodium into the potassium-free medium. Figure 7 summarizes the results of another experiment, on cells from a different donor, in which ATP, ADP and P_i were incorporated into the ghosts in equal concentrations. The ouabain-resistant loss of sodium was somewhat greater—a feature often noticed in ghosts containing relatively high

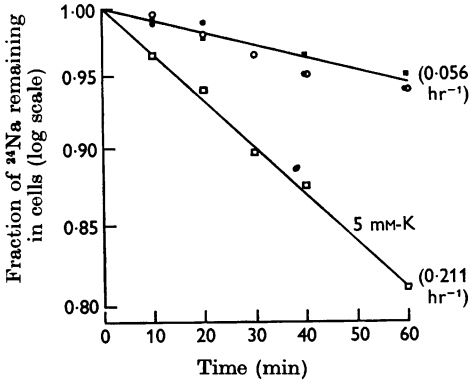

Fig. 6

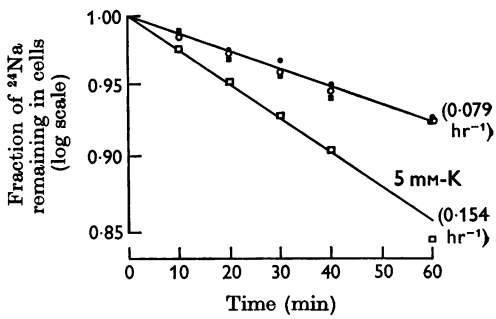

Fig. 7

Fig. 6. The efflux of Na from spontaneously resealed ghosts rich in Na and containing ATP and ADP in initially equal concentrations. The lysing solution contained (mM): ATP 4·9; ADP 4·9; Mg 10·3; Na 21·3; Tris 22; Cl 24·3; cysteine 1; pH 7·4; and had an osmolarity of 85 ideal m-osmoles/l., so that after isotonicity had been restored the concentration of Na inside the sealed ghosts must have been about 79 mM, and the concentrations of ATP and ADP at least 18·2 mM. The hypotonic wash solution contained (mM): Mg 26; Tris 12; Cl 62. The K-free incubation medium contained (mM): Na 155; Mg 3; Tris (pH 7·4 at 37° C) 10; Cl 169. In the 5 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was $5{\cdot}5 \times 10^{-5}$ g/ml. □ 5 mM-K medium; ○, K-free medium; ■, 5 mM-K medium with ouabain; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

Fig. 7. The efflux of Na from spontaneously sealed ghosts rich in Na and containing ATP, ADP and P_i in initially equal concentrations. The lysing solution contained (mM): ATP 3·9; ADP 3·9; P_i 3·9; Mg 12·3; Na 30; Tris 17; Cl 28; cysteine 1; pH 7·4; and had an osmolarity of 85 ideal m-osmoles/l. so that after isotonicity had been restored the concentration of Na inside the sealed ghosts must have been about 111 mM and the concentrations of ATP, ADP and P_i at least 14·5 mM. The hypotonic wash solution contained (mM): Na 9; Mg 30; P_i 5; Cl 60. The K-free incubation medium contained (mM): Na 164; Mg 2; Cl 158; P_i (pH 7·4) 5. In the 5 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. □, 5 mM-K medium; ○, K-free medium; ■, 5 mM-K medium with ouabain; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

concentrations of non-penetrating anions—but again there was no significant ouabain-sensitive loss into the potassium-free medium. The ouabain-sensitive loss of sodium into the 5 mM-K medium was rather low, suggesting perhaps that the pump was partly inhibited.

The effects of ATP, ADP and P_i on sodium efflux from low-sodium isotonically resealed ghosts. Having failed to produce a ouabain-sensitive sodium efflux into potassium-free media in experiments on spontaneously resealed ghosts rich in sodium, we turned to isotonically resealed ghosts

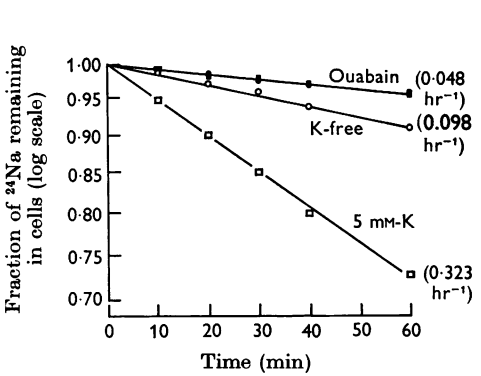

Fig. 8

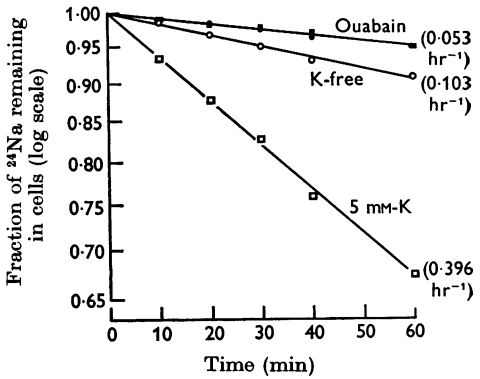

Fig. 9

Fig. 8. The efflux of Na from low-Na, isotonically resealed ghosts with a rather low ATP:ADP ratio and containing some P_i; This experiment and that of Fig. 9 were done on cells from the same batch and at the same time. The solution in which the ghosts were sealed contained (mM): ATP 3·8; ADP 3·8; P_i 0·94; Mg 9·4; Na 6·6; K 130; Tris 17; Cl 136; cysteine 1; pH 7·4. The K-free incubation medium contained (mM): Na 152; Mg 2; Tris (pH 7·4 at 37° C) 9; Cl 159; P_i (pH 7·4) 1. In the 5 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the incubation the K-free medium contained 115 μM-K, but this K could have accounted for only about one fifth of the difference between the 'K-free' curve and the 'ouabain' curve. □, 5 mM-K medium; ○, K-free medium; ■, 5 mM-K medium with ouabain; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

Fig. 9. The efflux of Na from low-Na isotonically resealed ghosts with a high ATP:ADP ratio (cf. Fig. 8). The solution in which the ghosts were sealed contained (mM): ATP 4·7; creatine phosphate 1·9; Mg 7·5; Na 6·6; K 131; Tris 12; Cl 142; cysteine 1; and 40 mg/l. of a creatine phosphokinase preparation containing 15–50 units/mg. (One unit converts 1 μmole of substrate per minute at 30° C). The pH was 7·4. The K-free incubation medium contained (mM): Na 152; Mg 2; Tris (pH 7·4 at 37° C) 10; Cl 164. In the 5 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the incubation the K-free medium contained 130 μM-K, but this K could have accounted for only about one quarter of the difference between the 'K-free' curve and the 'ouabain' curve. □, 5 mM-K medium; ○, K-free medium; ■, 5 mM-K medium with ouabain; ●, K-free medium with ouabain. The figures in brackets are rate constants for efflux.

containing only 6·6 mM-Na. Figures 8 and 9 show the loss of sodium from two lots of ghosts of this kind both prepared from the same batch of cells. One lot contained initially 3·8 mM ATP, 3·8 mM ADP, and 0·94 mM P_i; the other lot contained 4·7 mM ATP, 1·9 mM creatine phosphate and a suitable quantity of creatine phosphokinase, the idea being to keep the ratio [ATP]/([ADP].[P_i]) at a high level by reconverting any ADP formed back into ATP. Comparison of the figures shows that the ouabain-sensitive efflux of sodium into the 5 mM-K medium, but not into the zero-K medium, was greater in the ghosts with creatine phosphate. The difference is in the direction that would be expected from the squid experiments but is small.

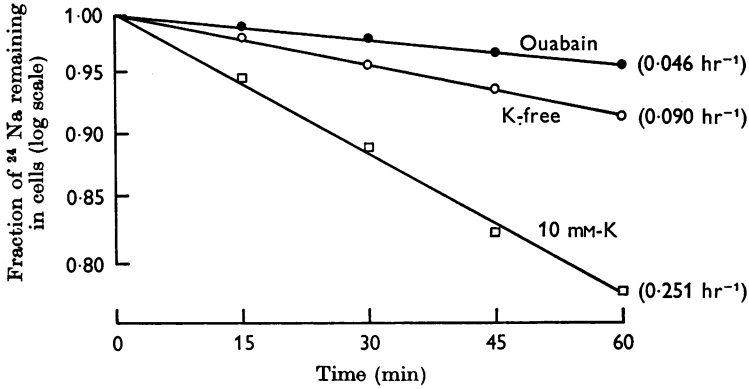

Fig. 10. An indirect test for adenylate kinase activity. The efflux of Na from low-Na isotonically resealed ghosts prepared by lysing cells in a solution containing ADP as the only nucleotide. The solution in which the ghosts were sealed contained (mM): ADP 3·7; Mg 4·6; Na 6·5; K 133; Tris 8; Cl 147; cysteine 1; pH 7·4. The K-free incubation medium contained Na 150; Mg 2; Tris (pH 7·4 at 37° C) 3; Cl 157. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the incubation the K-free medium contained 60 μM-K, but this K could have accounted for less than one tenth of the difference between the 'K-free' curve and the 'ouabain' curve. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are rate constants for efflux.

The next logical step would have been to try the effects of a still lower [ATP]/[ADP] ratio, but although there is no difficulty in preparing ghosts in which this ratio is low initially, it will not remain low if the ghosts contain appreciable amounts of adenylate kinase. As an indirect test of adenylate kinase activity, we prepared ghosts containing ADP as the only nucleotide and measured the efflux of sodium into 10 mM-K and potassium-free media, and also into the potassium-free medium in the presence of ouabain. The results are shown in Fig. 10. It is clear that ADP alone can support active sodium:potassium exchange quite well, presum-

ably by being converted to ATP through adenylate kinase activity (see Sen & Post, 1964). Instead of trying the effects of still higher ADP levels we therefore tried the effect of increasing the concentration of P_i.

The effects of high internal P_i concentrations. Three lots of spontaneously resealed ghosts were prepared from the same batch of intact cells. For the first lot the lysing solution contained 4·9 mM ATP; for the second 1·5 mM ATP; and for the third 1·5 mM ATP and 7·5 mM P_i. The total concentration of solutes in each lysing solution was about 82 ideal m-osmoles/l., so that the concentration of ATP and P_i in the resealed ghosts under isotonic conditions must have been (315/82) times the concentrations in the lysing solutions. The concentration of sodium in each lysing solution was 29 mM,

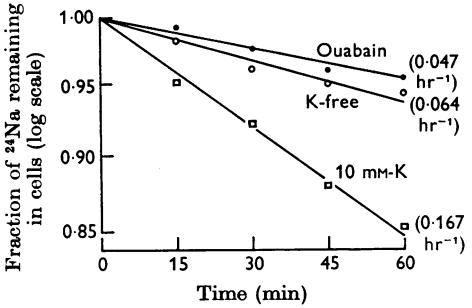

Fig. 11

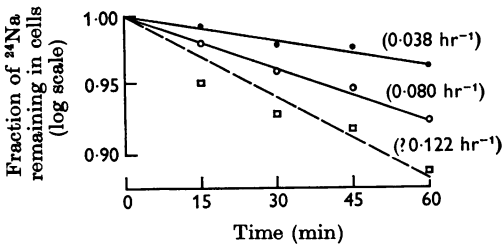

Fig. 12

Fig. 11. The efflux of Na from high-Na spontaneously sealed ghosts containing a relatively small amount of ATP (cf. Figs. 4 and 12). The lysing solution contained (mM): ATP 1·5; Mg 2·5; Na 29; Tris 11; Cl 38; pH 7·4, and had an osmolarity of 82 ideal m-osmoles/l., so that after isotonicity has been restored the concentration of Na inside the sealed ghosts must have been about 111 mM, and the initial concentration of ATP at least 5·7 mM. Although the initial concentration of ATP was therefore quite high the amount of ATP in each highly shrunken ghost was small, and a relatively large fraction of this ATP must have been hydrolysed by the ouabain-resistant ATPase during the course of the incubation. The hypotonic wash solution contained (mM): Na 29; Mg 10; Tris 10; Cl 56. The incubation media were the same as those used in the experiment of Fig. 4. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

Fig. 12. The efflux of Na from high-Na spontaneously resealed ghosts containing much more P_i than ATP. The lysing solution contained (mM): ATP 1·5; P_i 7·5; Mg 2·5; Na 29; Tris 13; Cl 25·5; cysteine 1; pH 7·4, and had an osmolarity of 80 ideal m-osmoles/l., so that after isotonicity had been restored the concentration of Na inside the sealed ghosts must have been about 111 mM, the concentration of ATP at least 5·9 mM and the concentration of P_i at least 29·5 mM. The K-free incubation medium contained (mM): Na 150; Mg 2; P_i (pH 7·4) 7·5; Cl 131. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was $5·5 \times 10^{-5}$ g/ml. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

so the final concentration must have been about 111 mM; sodium was the sole univalent cation in the ghosts.

The efflux of ^{24}Na from the three lots of ghosts into a 10 mM-K medium, a potassium-free medium, and a potassium-free medium containing ouabain are shown in Figs. 4, 11 and 12. From the three Figures it is obvious that only the ghosts with low ATP and high P_i concentrations showed a large ouabain-sensitive efflux of sodium into the potassium-free medium. The high-ATP ghosts showed none at all; the low ATP, initially P_i-free ghosts appeared to show a small flux but it is of doubtful significance. In the presence of potassium the sodium efflux was highest in the ghosts with a high ATP concentration and lowest in the ghosts with low ATP and high P_i concentrations. It is not clear whether the lowest curve in Fig. 12 shows more than the normal scatter, or whether the efflux really had decreased after the first 15 min.

It is interesting to consider whether P_i and ADP both act by lowering the ratio [ATP]/([ADP].[P_i]), perhaps in this way lowering the amount of free energy available from the hydrolysis of each molecule of ATP, or whether they have independent actions. Theoretically, the question can be answered by seeing whether the behaviour of the ghosts is independent of the concentration of ADP and of P_i provided their product is constant. In fact, because ADP is being formed by hydrolysis of ATP and is being removed by adenylate kinase activity, the concentrations of the different phosphate compounds is difficult to calculate except at the very beginning of the experiment. It is possible, however, to make some sort of estimate of the amount of hydrolysis and hence to calculate the concentrations half-way through an experiment, assuming either that there is no adenylate kinase activity or, alternatively, that there is so much activity that ([ATP].[AMP])/[ADP]^2 is held at 0·44 (Eggleston & Hems, 1952). For what they are worth, such calculations have been carried out for the ghosts in potassium-free media in the experiments shown in Figs. 7 and 12. In ghosts immersed in potassium-free media, ADP will be formed at an appreciable rate only by the (Na+K)-independent ATPase (Dunham & Glynn, 1961; Garrahan & Glynn, 1967*d*), which has a maximal velocity of about 1 m-mole/l. cells/hr. After 30 min the amounts of ADP and P_i formed are unlikely to be more than 0·5 m-mole/l. cells. If adenylate kinase activity is absent this figure gives an [ATP]/([ADP].[P_i]) ratio of 0·25 mM^{-1} for the experiment of Fig. 12 and 0·176 mM^{-1} for the experiment of Fig. 7. If adenylate kinase activity is very great the ratio is 0·34 mM^{-1} for both experiments. From these figures it seems unlikely that the [ATP]/([ADP].[P_i]) ratio can have been lower in the experiment of Fig. 12 than in the experiment of Fig. 7, yet it is Fig. 12 that shows the ouabain-sensitive flux into a potassium-free medium. The tentative conclusion is that ADP and P_i do not act simply by their effects on the [ATP]/([ADP].[P_i]) ratio.

The experiments described so far show that ouabain-sensitive efflux of sodium into a potassium-free medium occurs in ghosts with a low internal sodium concentration and a relatively high concentration of ATP, and also in ghosts with a high internal sodium concentration provided the ration of ATP to P_i is low. What happens in ghosts with a low internal sodium concentration and a low ratio of ATP to P_i? Figures 13, 14 and 15 show the efflux of ^{24}Na from three lots of isotonically resealed ghosts

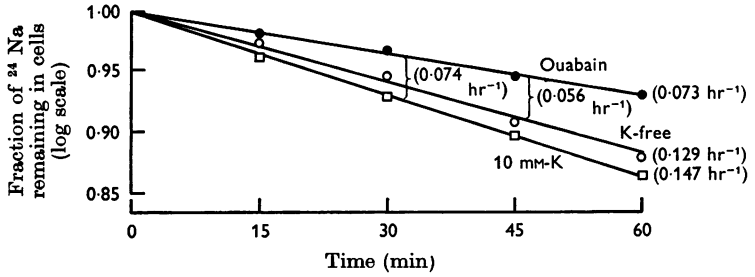

Fig. 13. The efflux of Na from low-Na, isotonically resealed ghosts containing ATP at a rather low concentration together with some ADP and P_i. This experiment and those of Figs. 14 and 15 were done on cells from the same batch and at the same time. The solution in which the ghosts were sealed contained (mM): ATP 0·92; Mg 5·5; Na 6·4; K 142; Tris 6; Cl 161; cysteine 1; pH 7·4, but some of the ATP trapped in the ghosts would have been hydrolysed during the pre-incubation. The K-free incubation medium contained (mM): Na 150; Mg 2; Tris (pH 7·4 at 37° C) 3; Cl 157. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the incubation the K-free medium contained 122 μM-K, but this K could have accounted for less than one twentieth of the difference between the 'K-free' curve and the 'ouabain' curve. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

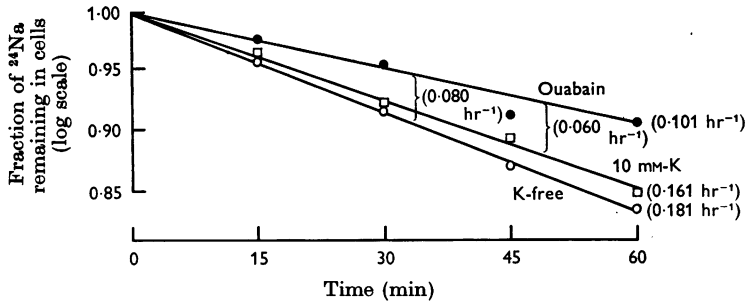

Fig. 14. The efflux of Na from low-Na isotonically resealed ghosts containing much more P_i than ATP (cf. Figs. 13 and 15). The solution in which the ghosts were sealed contained (mM): ATP 0·92; P_i 4·6; Mg 6·4; Na 6·4; K 140; Tris 2; Cl 149; cysteine 1; pH 7·4, but some of the ATP trapped in the ghosts would have been hydrolysed during the preincubation. The K-free medium contained (mM): Na 150; Mg 2; P_i (pH 7·4) 5; Cl 145. In the 10 mM-K medium, K replaced an equivalent quantity of Na. The ouabain concentration was 5×10^{-5} g/ml. At the end of the incubation the K-free medium contained 139 μM-K but this K cannot have accounted for a significant fraction of the difference between the 'K-free' curve and the 'ouabain' curve. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figures in brackets are the rate constants for efflux.

prepared from the same batch of intact cells. Figure 13 refers to ghosts containing a low concentration of ATP, Figure 14 to ghosts containing the same low concentration of ATP and at least 5 times this concentration of P_i, and Fig. 15 to ghosts containing a similar concentration of P_i but no ATP. Some hydrolysis of ATP will have taken place during the pre-incubation necessary for sealing, so both lots of ghosts containing ATP will also contain appreciable amounts of ADP, and the ghosts initially free of P_i will contain a little P_i. The following conclusions may be drawn: (i) The ghosts containing P_i and no ATP show no ouabain-sensitive sodium efflux under any conditions; (ii) the ghosts with low ATP and high P_i concentrations show a *greater* efflux of sodium into the potassium-free

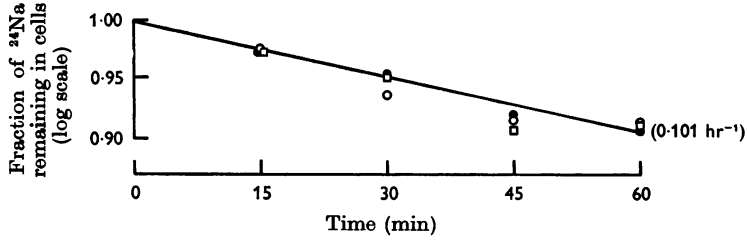

Fig. 15. The efflux of Na from low-Na, isotonically resealed ghosts containing P_i but no ATP or ADP (cf. Figs. 13 and 14). The solution in which the ghosts were sealed contained (mM): P_i (pH 7·4) 4·6; Mg 5·5; Na 6·4; K 142; Cl 151; cysteine 1. The incubation media were the same as those used in the experiment of Fig. 14. At the end of the incubation the K-free medium contained 102 μM-K. □, 10 mM-K medium; ○, K-free medium; ●, K-free medium with ouabain. The figure in brackets is the rate constant for efflux.

medium than into the medium containing 10 mM-K; (iii) comparison of Figs. 13 and 14 shows that the ouabain-sensitive loss into the potassium-free medium is greater when the ghosts are rich in P_i, whereas the loss into the 10 mM-K medium is greater when they are not.

It is useful to compare also Fig. 13 with Figs. 5*d* and 9 from earlier experiments on ghosts similarly poor in sodium but rich in ATP. As these experiments were done on different batches of cells small differences are not significant, but it is clear that the ouabain-sensitive sodium efflux into potassium-free media is little affected by raising the ATP concentration, whereas the ouabain-sensitive efflux into the 5 or 10 mM-K media is very greatly increased.

In most experiments the total magnesium concentration in the ghosts was 1 mM greater than the sum of the concentrations of P_i and nucleotides. In the experiment of Fig. 12 there was a considerable excess of free magnesium, but behaviour rather similar to that shown in the figure has been observed in another experiment in which a large excess of magnesium was not present—see the first experiment of Table 1.

The effects of external sodium concentration

A detailed study of the effects of external sodium concentration on the efflux of sodium from intact cells immersed in potassium-free media has been reported in the first and second papers of this series (Garrahan & Glynn, 1967*a*, *b*). The experiments to be reported here serve merely to confirm that the ouabain-sensitive sodium efflux observed when suitably prepared ghosts are immersed in potassium-free media is similarly affected by the external sodium concentration.

TABLE 1. The effects of external Na concentration on the efflux of Na from isotonically resealed, low-Na ghosts

Na concn. in medium (mM)	Rate constants for sodium efflux: Efflux into a 10 mM-K medium (hr^{-1})	Efflux into a K-free medium (hr^{-1})	Efflux into a K-free medium containing ouabain (hr^{-1})	Ouabain-sensitive efflux into a K-free medium (hr^{-1})
		*Experiment 1		
140	0·186	0·186	0·086	0·100
25	0·196	0·094	0·058	0·036
		†Experiment 2		
140	0·175	0·198	0·133	0·065
25	0·170	0·143	0·109	0·034
5	0·179	0·115	0·115	0·000

* The solution in which the ghosts were sealed contained (mM): ATP 0·94; Mg 1·9; Na 6·6; K 141; Tris 7; Cl 150; cysteine 1; pH 7·4; but some of the ATP trapped in the ghosts would have been hydrolysed during the preincubation. The 25 mM-Na, K-free incubation medium contained (mM): Na 25; choline 125; Mg 2; Tris 5; Cl 161; pH 7·4. In the other incubation media K and Na replaced equivalent quantities of choline. The ouabain concentration was 5×10^{-5} g/ml.

† The solution in which the ghosts were sealed contained (mM): ATP 0·94; P_i 4·7; Mg 6·6; K 146; Cl 153; cysteine 1; pH 7·4; but some of the ATP trapped in the ghosts would have been hydrolysed during the preincubation. The 5 mM-Na, K-free incubation medium contained (mM): Na 5; choline 145; Mg 2; Tris 11; P_i 5; Cl 154; pH 7·4. In the other incubation media K and Na replaced equivalent quantities of choline. The ouabain concentration was 5×10^{-5} g/ml.

Table 1 summarizes the results of two experiments on isotonically resealed ghosts containing about 7 mM-Na. In one experiment the ghosts contained a low concentration of ATP; in the other a low concentration of ATP and a high concentration or P_i. In these experiments the ghosts were washed with sodium-free buffered choline chloride solutions and such ghosts tend to give slightly erratic results. The time curves for loss of ^{24}Na show rather more than the usual amount of scatter, and small differences between rate constants are not significant. Nevertheless, it is clear in both experiments that lowering the external sodium concentration to 25 mM reduced the ouabain-sensitive efflux of sodium into a potassium-free medium. In the second experiment, this flux appears to

have been abolished by further lowering the external sodium concentration to 5 mM.

Because lowering the external sodium concentration reduces sodium efflux into a potassium-free medium but has little effect on the efflux into a 10 mM-K medium, the effect of adding potassium to the medium may be reversed as the external sodium concentration is lowered. This effect is seen in the second experiment in Table 1: with 140 mM-Na, efflux was greater in the absence of potassium; with 25 or 5 mM-Na, efflux was greater when potassium was present.

The results of the first experiment in Table 1, showing no effect of external potassium on sodium efflux into a medium containing 140 mM-Na, represents behaviour intermediate between that shown in Fig. 13 for low-ATP ghosts and that shown in Fig. 14 for low-ATP high-P_i ghosts. The interpretation is not that potassium has no effect on the transport system, but merely that conditions happen to be such that the rate of sodium loss by sodium:sodium exchange in a potassium-free medium just equals the rate of sodium loss by sodium:potassium exchange in a 10 mM-K medium.

The apparent decrease in ouabain-resistant sodium efflux when the external sodium was low, seen in both experiments, is puzzling, since no such effect has been observed in intact cells or in high-sodium spontaneously resealed ghosts. If the effect is real, the explanation may be that some ghosts are moderately leaky to sodium but tight to choline. These ghosts would survive the choline wash intact but might suffer osmotic lysis in a solution containing 140 mM-Na.

Confirmatory experiments on intact cells

Although the extent to which the concentrations of ions and metabolites can be manipulated in intact cells is rather limited, it seemed worth while to try to confirm some of the findings of the ghost experiments.

Table 2 contrasts the behaviour of fresh and cold-stored red cells. Ouabain-sensitive sodium efflux into a potassium-free medium is expressed both as m-mole/l. cells/hr and as a fraction of the ouabain-sensitive sodium efflux from the same cells in the presence of 5 or 10 mM-K. In the experiments on fresh cells the flux varied between 0·55 and 1·04 m-mole/l. cells/hr and was never less than 39% of the flux into the potassium-containing medium. The first experiment on stored cells was done on cells that had been kept for 6 days at 5° C in 154 mM-NaCl, and had then been incubated for 6 hr in a potassium-free balanced glucose salt solution containing ^{24}Na (Garrahan & Glynn, 1967*a*). The cells contained 33 mM-Na and showed a ouabain-sensitive sodium efflux of 0·62 m-mole/l. cells/hr into a potassium-free medium, equivalent to 17% of the ouabain-sensitive efflux into a 5 mM-K medium. Although the absolute value of the efflux

was not less than in fresh cells, the value relative to the efflux into a 5 mM-K medium was greatly reduced. The second stored cell experiment was done on 2-week old blood bank cells loaded with ^{24}Na by preincubation for 5 hr in a balanced glucose salt solution containing 30 mM-P_i. The cells at the end of the final incubation contained 33 mM-Na and 15 mM-P_i, and they showed a ouabain-sensitive sodium efflux of 1·94 m-mole/l. cells/hr

TABLE 2. The relative magnitudes of Na:Na exchange and Na:K exchange in different preparations of intact cells

Type of cell	Ouabain-sensitive Na efflux into a high-Na, K-free medium (m-mole/l. cells/hr)[1]	Flux in previous column as a fraction of ouabain-sensitive Na efflux into a 10 mM-K medium (%)
Fresh	0·61	43
Fresh	0·76	45
Fresh	0·65	39[2]
Fresh	0·73	44
Fresh	1·04	—[3]
Fresh	0·64	53
Fresh	0·99	—[3]
Fresh	0·55	40[2]
Fresh	0·68	52
Cold-stored[4] $[Na]_i$ was 33 mM	0·62	17
Cold-stored[5] $[Na]_i$ was 33 mM, $[P_i]_i$ was 15 mM	1·95[6]	40

Notes

[1] In most of the experiments on fresh cells the sodium content of the cells was not measured, and the effluxes are calculated on the assumption that the internal sodium concentration was 6 m-mole/l. cells. The figures in the last column are calculated directly from rate constants and do not depend on this assumption.

[2] The K-medium contained 5 mM-K instead of 10 mM-K in these experiments.

[3] The flux into a medium containing K was not measured.

[4] These cells were stored for 6 days in 154 mM-NaCl and were preincubated for 6 hr in a K-free glucose Ringer solution so that the internal P_i concentration was presumably low during the final incubation.

[5] These cells were from 2-week-old blood and were pre-incubated at 37° C for 5 hr in a solution containing (mM): Na 144; choline 21; Mg 1; P_i 30; Cl 113; glucose 11; pH 7·4. The K-free medium for the final incubation contained (mM): Na 127; choline 40; Tris 7·7; Mg 1; P_i 30; Cl 113; glucose 11; pH 7·4. In the 10 mM-K medium, K replaced an equivalent quantity of choline.

[6] The whole of this efflux disappeared when the external Na concentration was reduced to 5 mM.

into a potassium-free medium, equivalent to 40 % of the ouabain-sensitive efflux into a 10 mM-K medium. This very large efflux was abolished by replacing all but 5 mM of the external sodium with choline. It is clear that in this experiment the high concentration of P_i more than offset the effect of the high concentration of sodium inside the cells, confirming that P_i, as in the ghost experiments, facilitated sodium:sodium exchange.

In the above discussion it has been assumed that the ouabain-sensitive

efflux of sodium from sodium-rich cells immersed in potassium-free media, like the corresponding efflux from normal cells, represents a sodium: sodium exchange. The effect of replacing most of the external sodium in the second stored cell experiment is fairly good evidence for this, but confirmation is provided by a further experiment in which ouabain-sensitive influx and efflux were measured simultaneously in cells containing 55 mM-Na and probably about 20 mM-P_i. The efflux was 0·926 ± 0·039 m-mole/l. cells/hr and the influx was 1·080 ± 0·093 m-mole/l. cells/hr.

TABLE 3. An experiment to see whether fresh cells starved for 7 hr behaved like low-Na, high-P_i ghosts

	Rate constants for sodium efflux					Ratio of ouabain-sensitive effluxes into K-free and 10 mM-K media (%)
Substrate in incubation medium	Efflux into a 10 mM-K medium (hr^{-1})	Efflux into a K-free medium (hr^{-1})	Efflux into a K-free medium containing ouabain (hr^{-1})	Ouabain-sensitive efflux into a 10 mM-K medium (hr^{-1})	Ouabain-sensitive efflux into a K-free medium (hr^{-1})	
10 mM inosine	0·190	0·137	0·082	0·108	0·055	51
None	0·132	0·139	0·092	0·040	0·047	117

Cells were washed free of glucose and incubated for 7 hr at 37° C in a 6·7 mM-K Ringer solution (Garrahan & Glynn, 1967*a*) containing ^{24}Na. The loaded, starved cells were washed 5 times in a choline Ringer solution (Garrahan & Glynn, 1967*a*) and were then incubated for 30 min at 37° C with and without inosine, in (*a*) a 10 mM-K Ringer solution, (*b*) a K-free Ringer solution, or (*c*) a K-free Ringer solution containing ouabain (5×10^{-5} g/ml.). Rate constants were calculated from the ^{24}Na losses in 30 min, after small corrections for haemolysis had been made.

The results of another confirmatory experiment are shown in Table 3. Fresh cells were washed free of glucose and incubated for 7 hr in a glucose-free balanced salt solution containing ^{24}Na (Garrahan & Glynn, 1967*a*). Sodium efflux was measured in the usual media with and without the addition of inosine (10 mM). Although P_i levels were not measured, it can be assumed that the cells incubated for 7 hr without substrate must have had a low level of ATP and a high level of P_i (Whittam, 1958); in the presence of inosine the levels would have returned rapidly towards normal (Glynn & Lüthi, 1967; Whittam & Wiley, 1967). The results show that in the presence of inosine the ratio

$$\frac{\text{ouabain-sensitive Na efflux into a K-free medium}}{\text{ouabain-sensitive Na efflux into a 10 mM-K medium}}$$

was about normal at 0·51, though the absolute values of the fluxes were a little low. In the absence of inosine, the ratio was a little greater than 1. This is exactly the behaviour found in low-Na, low-ATP, high-P_i ghosts—see Fig. 14 and the second experiment in Table 1.

The effect of P_i on the ATPase activity of fragmented ghosts

Since (i) low concentrations of ATP and high concentrations of P_i both appear to facilitate sodium:sodium exchange in potassium-free media, and (ii) there is a little evidence that a high concentration of P_i inhibits sodium:potassium exchange in potassium-containing media (cf. Figs. 13 and 14, and Figs. 11 and 12), it is interesting to ask: does P_i act by competing with ATP and so lowering its effective concentration? To test this, the ouabain-sensitive ATPase activity of a preparation of fragmented ghosts was measured at different levels of ATP in the presence and

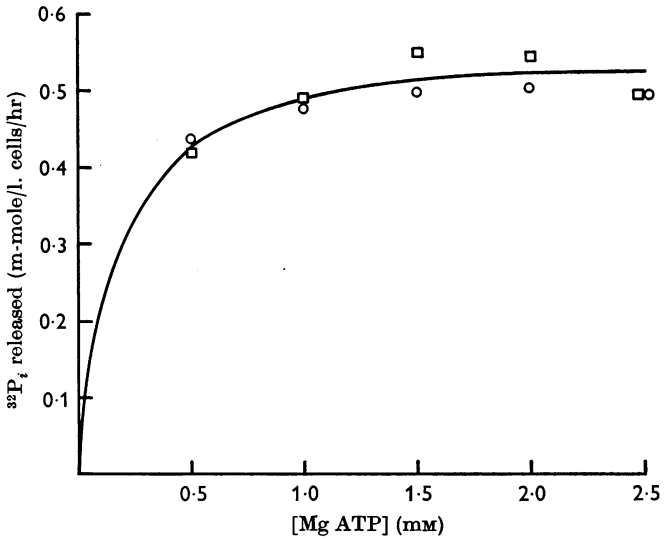

Fig. 16. The ouabain-sensitive hydrolysis of [γ^{32}-P]-ATP by fragmented ghosts in the presence and absence of P_i. ○, P_i initially absent from incubation medium; □, 4·5 mM P_i in incubation medium. All tubes contained equimolar quantities of Mg and ATP, and optimal concentrations of Na and K.

absence of P_i. [γ-^{32}P]ATP was used and hydrolysis was estimated from the radioactivity of the P_i at the end of the experiment. The results are shown in Fig. 16. P_i seemed to have no inhibitory effect on the ouabain-sensitive ATPase even at a concentration 9 times that of the ATP.

DISCUSSION

The first conclusion to be drawn from the experiments reported in this paper is that the same system is responsible for the exchange of sodium for potassium under physiological conditions and for the abnormal sodium: sodium exchange that occurs in low-potassium media. Not only do both exchanges require the presence of ATP, and are both inhibited by ouabain, but

both show precisely the same sensitivity to potassium ions in the outside solution. The two exchanges should therefore be regarded as alternative modes of behaviour of the cation transport system. Under physiological conditions the system will exchange sodium for potassium almost exclusively; in potassium-free media only sodium:sodium exchange will occur; at potassium concentrations less than physiological both exchanges will go on at the same time.

Under one set of conditions—high internal sodium and ATP concentrations and low internal P_i concentration—the transport system is in such a state that it will catalyse ouabain-sensitive sodium:potassium exchange if potassium is present, but it will not catalyse ouabain-sensitive sodium:sodium exchange in potassium-free media. Under these conditions sodium efflux by the transport system appears to be tightly coupled to potassium influx. 'Loosening' of the coupling and facilitation of sodium:sodium exchange can be achieved by one of the following procedures:

(i) reducing the internal sodium concentration to a fairly low level,

(ii) greatly reducing the internal concentration of ATP,

(iii) raising the internal concentration of P_i.

If the cells contain a low concentration of sodium and much more P_i than ATP, sodium efflux by exchange for sodium in high-sodium, potassium-free media is faster than sodium efflux in exchange for potassium in potassium-containing media, and with such cells external potassium *reduces* sodium efflux.

A feature common to all the procedures that permit sodium:sodium exchange is that by increasing the amount of osmotic work involved in sodium efflux, or by decreasing the amount of free energy theoretically available from the hydrolysis of each molecule of ATP, they tend to decrease the margin between the energy available and the energy required for sodium:potassium exchange. In squid nerves, Caldwell & Schirmer (1965), developing the ideas of Caldwell, Hodgkin, Keynes & Shaw (1960*a*, *b*; 1964), suggest that the coupling of sodium efflux to potassium influx is progressively reduced as the margin of free energy is diminished. The behaviour in red cells can obviously be described in this way, but it is not clear how helpful such a thermodynamic approach is and it may be misleading. If what matters is the free energy available, we should get equivalent effects whether this free energy is reduced by increasing the level of P_i or by increasing the level of ADP, whereas we have seen that there is a little evidence—certainly not conclusive—that P_i and ADP are not equivalent. If what matters is not the energy available but the concentration of some intermediate in the system, then, provided that ADP and P_i are released at different steps, there is no reason why their effects should be equivalent.

Whether P_i acts by altering the amount of energy available, or in some other way, there is good evidence that it does not act by competition with ATP. At moderately high concentrations (i) it does not inhibit ATP hydrolysis by a preparation of fragmented ghosts (this paper), (ii) it does not inhibit the formation of intermediates in the transport system from [γ^{32}-P] ATP (R. L. Post, personal communication), and (iii) it does not inhibit the ATP:ADP exchange probably associated with the transport system (Skou, 1960).

The problem of the facilitation of sodium:sodium exchange by low sodium, low ATP and high P_i levels will be considered further in the last paper in this series (Garrahan & Glynn, 1967*e*).

The relation between the behaviour seen in red cells and the behaviour in other tissues

As many of the experiments in this paper were prompted by the earlier observations of other workers on nerve and muscle, it is interesting to see to what extent the relatively simple picture that has emerged from the red cell experiments can clarify the observations on the other tissues.

The position is slightly clearer in nerve. Fresh squid nerves, and nerves poisoned with cyanide and injected with arginine phosphate, seem to be analogous to resealed ghosts with moderately high internal sodium and ATP concentrations in that the efflux of sodium is fairly tightly coupled to potassium influx (Caldwell *et al.* 1960*a*). In axons partially poisoned with dinitrophenol (0·2 mM at pH 8) (Caldwell *et al.* 1960*b*), or in cyanide poisoned axons injected with ATP, the sodium efflux is less affected, or unaffected, by the removal of external potassium, suggesting perhaps that under these conditions the pump can switch over to sodium:sodium exchange. Unfortunately sodium influx into partially poisoned axons has not been studied,* and sodium influx into cyanide-poisoned axons injected with ATP has been measured only in the presence of external potassium. Caldwell *et al.* (1960*b*) have pointed out that the transient increase in sodium efflux observed when squid nerve fibres in potassium free solutions are poisoned with cyanide, does not occur in sodium-free media and may therefore represent a transient stimulation of sodium:sodium exchange.

Another set of observations on squid nerve that are more readily understandable in the light of the red cell experiments are the observations of Frumento & Mullins (1964) on the behaviour of squid fibres that had been depleted of sodium by being stimulated in a lithium medium. Lowering the internal sodium decreased the sensitivity of sodium efflux to external potassium, and changed the effect of the removal of external sodium from

* See note added in proof.

a stimulation of sodium efflux to an inhibition. Furthermore, the onset of the lack of sensitivity to external potassium coincided with the reversal of the effect of removing external sodium. Analogy with red cells would suggest that as the internal sodium was lowered the capacity of the pump to exchange sodium for potassium progressively decreased and the capacity for sodium:sodium exchange progressively increased.

In frog muscle, where the dependence of sodium efflux on external sodium has been much better established than in nerve, the position has recently become very much more complicated following the demonstration by Horowicz (1965), confirmed by Keynes (1966), that the effects of a cardiac glycoside and of sodium-free solutions in reducing the efflux of labelled sodium are independent and additive. Whether sodium:sodium exchange in frog muscle is for some reason not sensitive to cardiac glycosides, or whether the sodium-dependent sodium efflux represents quite a different process, is not clear, but the fact that it is affected by *internal* sodium concentration in a way similar to the sodium:sodium exchange in red cells makes it seem less likely that the flux involves some quite different mechanism. It would be interesting to know whether any ouabain-sensitive efflux or influx is detectable in the absence of external potassium.

The ouabain-resistant sodium efflux in red cells

We have tended to assume that the sodium efflux from red cells in the presence of ouabain is irrelevant to the pump mechanism, but this assumption needs some qualification. In red cells the ouabain-resistant efflux is almost insensitive to the levels of sodium or potassium present externally and seems to be unaffected by the absence of ATP internally (cf. Figs. 14 and 15). Comparison of rate constants from different experiments on resealed ghosts shows a slight tendency for the rate constants to be higher at lower internal sodium concentrations, as though the efflux mechanism saturates, but this tendency may come about simply because cells rich in potassium lyse more readily.

Nevertheless, even if the efflux is linear with internal sodium concentration, the sodium fluxes across the membrane in the presence of ouabain cannot be explained by simple leaks. For one thing, the ratio of efflux to influx is much too high; furthermore, the influx is not linear with external sodium concentrations (Glynn, 1957), has a high temperature coefficient (Glynn, 1957) and is somewhat increased by external potassium (Fig. 2). This curious effect of potassium may be related to the small but non-linear potassium influx that is seen even in the presence of high concentrations of a cardiac glycoside (Glynn, 1957). The mechanisms responsible for the ouabain-resistant sodium fluxes, and their relation to the mechanism responsible for the ouabain-sensitive fluxes, remain obscure though some

interesting results have recently been reported by Hoffman & Kregenow (1966).

Note added in proof. Recent and unpublished work on squid axons by P. F. Baker, M. P. Blaustein, R. D. Keynes, Jacqueline Manil, T. I. Shaw & R. A. Steinhardt has shown that the sodium efflux can be divided into ouabain-sensitive and ouabain-insensitive components. In normal axons the ouabain-sensitive sodium efflux is completely dependent on external potassium; in axons partially poisoned with alkaline DNP there is a ouabain-sensitive sodium efflux in the absence of external potassium, and this efflux is accompanied by an equal ouabain-sensitive sodium influx.

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