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OCCLUSION OF RUBIDIUM IONS BY THE SODIUM–POTASSIUM PUMP: ITS IMPLICATIONS FOR THE MECHANISM OF POTASSIUM TRANSPORT

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SUMMARY

1. The occlusion of rubidium ions by Na, K-ATPase has been investigated by suspending enzyme prepared from pig kidney outer medulla in media containing low concentrations of ^{86}Rb, forcing the suspensions rapidly through small columns of cation-exchange resin, and measuring the amounts of radioactivity emerging from the columns.

2. When the suspension media contained 2 mM-ATP or ADP, or 15 mM-NaCl, the amounts of radioactivity emerging from the columns were greatly (and similarly) reduced, presumably because both nucleotides and sodium ions stabilized the enzyme in the E_1 form. (See p. 19 for definition of E_1 and E_2). The extra radioactivity carried through the columns when nucleotides and sodium were absent was taken as a measure of the amount of rubidium occluded within the enzyme (in the E_2 form) when it emerged from the resin.

3. By varying the flow rate, and therefore the time spent by the enzyme on the resin, and relating this to the amount of radioactivity emerging from the columns, we have been able to estimate the rate constant for the conformational change ($E_2 \rightarrow E_1$) that allows the occluded rubidium ions to escape. At 20 °C, and in the absence of nucleotides, it is about 0·1 s^{-1}.

4. The rate constant for rubidium release was the same in a sodium-containing as in a potassium-containing medium. The opposite effects of sodium and potassium ions on the poise of the equilibrium between the E_1 and the E_2 forms of the enzyme must, therefore, be due solely to opposite effects of these ions on the rate of conversion of E_1 to E_2.

5. The rate constant for rubidium release was greatly increased by ATP and by ADP. Both nucleotides appeared to act at low-affinity sites and without phosphorylating the enzyme.

6. Orthovanadate, in the presence of magnesium ions, stabilized the enzyme in the occluded-rubidium (E_2Rb) form.

7. Ouabain, in the presence of magnesium ions, prevented the occlusion of rubidium ions.

8. We have measured the amount of rubidium occluded by the enzyme as a function of rubidium concentration, and estimate that at saturating rubidium concentrations about three rubidium ions can be occluded per phosphorylation site (or per ouabain-binding site).

9. We have found that the occluded-rubidium form of the enzyme can also be formed by allowing rubidium ions to catalyse the hydrolysis of phosphoenzyme generated by the addition of ATP to enzyme suspended in a high-sodium medium.

10. The properties of the occluded-rubidium form of the enzyme, and of the two routes that can lead to its formation, suggest that an analagous occluded-potassium form plays a central role in the transport of potassium ions through the sodium–potassium pump. This hypothesis is supported by a detailed consideration of the probable magnitudes of the rate constants of the individual reactions making up the two routes.

INTRODUCTION

It has always seemed likely that, in the course of its normal working cycle, the sodium–potassium pump, or Na, K-ATPase, passes through states in which sodium ions or potassium ions are occluded within the pump molecule so that they are unable to exchange with ions in either the extracellular or intracellular medium. In this paper we describe experiments which show (1) that rubidium ions can be occluded within one of the conformations of the unphosphorylated Na, K-ATPase molecule, (2) that the rubidium ions can enter and leave the occluded state by two different routes and (3) that the properties of these two routes make it probable that an analagous occluded-potassium form of the enzyme plays a crucial role in the normal transport of potassium ions by the pump.

In 1972, Post, Hegyvary & Kume described experiments in which they looked at the rate at which ATP was able to rephosphorylate enzyme that had just been dephosphorylated. The dephosphorylation, i.e. the hydrolysis of phosphoenzyme, could be accelerated by potassium, rubidium or lithium ions, and they found that the rate of *re*phosphorylation depended on which of these ions had been used. Furthermore, this was true even when the experiments were done in such a way that the composition of the medium during rephosphorylation was identical; in other words, the enzyme appeared to remember which congener of potassium had accelerated the hydrolysis. To explain this 'memory', Post *et al.* (1972) suggested that, during hydrolysis, the ions became occluded within the dephosphoenzyme, and were released only later after a slow conformational change. Since they also found that the enzyme became capable of rephosphorylation sooner if ATP was present in high concentration, they suggested that the hypothetical slow conformational change was accelerated by the binding of ATP at low-affinity sites.

This hypothesis received strong though indirect support from experiments with the fluorescent ATP and ADP analogues, formycin triphosphate and formycin diphosphate (Karlish, Yates & Glynn, 1978), and from studies of the intrinsic fluorescence of the enzyme (Karlish & Yates, 1978). These fluorescence studies, which were done on Na, K-ATPase prepared from pig kidney outer medulla, showed that the change in conformation ($E_2 \rightarrow E_1$) when the enzyme was transferred from a predominantly potassium to a predominantly sodium medium was remarkably slow ($k \simeq 0{\cdot}2\ s^{-1}$ at 20 °C) and was accelerated by nucleotides at high concentration. Both the slowness of the change and the acceleration by nucleotides suggested that in potassium media the enzyme existed in a form identical with the hypothetical occluded-potassium form.

Beaugé & Glynn (1979*a*) took advantage of the slowness of the $E_2 \rightarrow E_1$ conformational change, in the absence of nucleotides, to test for occlusion directly. They suspended enzyme in a suitable sodium-free medium containing ^{86}Rb and forced the suspension through a cation-exchange column at a rate that was slow enough for the column to remove nearly all of the free rubidium ions yet was fast enough for the enzyme to emerge from the column in less than 1 s, i.e. within a period much less than the time constant of the conformational change. They found that the enzyme did carry ^{86}Rb through the column and that the amount carried through was greatly reduced by the inclusion of ATP or ADP in the suspension medium at concentrations known to be sufficient to convert the enzyme to the E_1 form. The difference between the amounts of radioactivity carried through the column by the enzyme in the absence and in the presence of nucleotides represented, they thought, ^{86}Rb occluded within the E_2 form of the enzyme.

In this paper, we report experiments in which, using a modification of the procedure of Beaugé & Glynn (1979*a*), we have measured the rate of the conformational change ($E_2 \rightarrow E_1$) that precedes rubidium release, and have examined the effects of sodium ions and of nucleotides on that rate. We have also investigated the effects of vanadate and of ouabain on the occlusion of rubidium by the unphosphorylated enzyme, and have attempted to determine the stoichiometry of rubidium occlusion. Finally, we have been able to show that the form of the enzyme that contains occluded rubidium can be produced not only by adding enzyme to a sodium-free medium containing rubidium, but also by allowing rubidium ions to catalyse the hydrolysis of phosphoenzyme generated by the addition of ATP to enzyme suspended in a high-sodium magnesium-containing medium. Since the enzyme is known to treat potassium ions much as it treats rubidium ions, it seems likely that the corresponding two routes to an analogous occluded-potassium form of the dephosphoenzyme would, together, provide a sequence of reactions with many of the properties required to account for the fluxes of potassium through the pump. We discuss the probable magnitudes of the rate constants of the individual reactions making up the two routes and show that they are compatible with this hypothesis.

Preliminary reports of many of the experiments to be described here have already been published (Glynn & Richards, 1980, 1981, 1982).

Definitions

In discussing the experiments reported in this paper, we frequently refer to the E_1 and E_2 forms of the unphosphorylated Na, K-ATPase. By the E_1 form, we mean that form of the dephosphoenzyme that (i) is the stable form of the enzyme in sodium media, (ii) has a high affinity for ATP, ADP and their fluorescent formycin analogues (Hegyvary & Post, 1971; Nørby & Jensen, 1971; Karlish *et al.* 1978), (iii) is attacked in a biphasic fashion by trypsin (Jørgensen, 1975), (iv) can be phosphorylated by ATP but not by orthophosphate (see Post, Toda & Rogers, 1975) and (v) has a low intrinsic fluorescence (Karlish & Yates, 1978). By the E_2 form we mean that form of the dephosphoenzyme that (i) is the stable form of the enzyme in potassium media, (ii) has a lower affinity for ATP, ADP and their fluorescent formycin analogues, (iii) is attacked in a monophasic fashion by trypsin, (iv) can be phosphorylated by orthophosphate but not by ATP and (v) has a higher intrinsic fluorescence.

METHODS

Preparation of enzyme

Na, K-ATPase was prepared from the dark red outer medulla of pig kidney by Jørgensen's (1974) method, in which microsomes treated with Na dodecyl sulphate in the presence of ATP are fractionated on discontinuous sucrose gradients in an angle-head centrifuge. The particles were washed twice in a solution containing 25 mM-histidine (pH 7·5 at 20 °C) and 1 mM-EDTA (Tris salt), to remove traces of ATP and of Na^+, and were then stored in this solution at 0 °C. The specific activity was generally between 12 and 18 μmol (mg protein)$^{-1}$ min^{-1}.

Detection of occluded rubidium by rapid ion exchange

The technique used was based on that described by Beaugé & Glynn (1979*a*), but we found that by substituting the sulphonic resin Dowex 50W (× 8 cross-linked, 100–200 mesh) for the carboxylic resin Biorad Biorex 70 we were able to obtain satisfactory results with five to ten times less enzyme. Each column was prepared by putting the resin in the barrel of a 1 ml disposable tuberculin syringe (cross-sectional area 0·17 cm^2) whose tip was loosely plugged with glass fibre and fitted with a nylon tap. The resin was generally in the Na or K form and suspended in 100 mM-HEPES (Na or K salt, pH 7·4); the final bed volume was 0·5 ml, and the total capacity was 0·85 mequiv. After the resin had packed down, the tap was opened until the surface of the HEPES solution reached the top of the resin. The upper part of the syringe was then filled with water, and acid-washed sand was allowed to settle through the water to form a layer 1 mm thick on top of the resin. This was to prevent later contamination of the enzyme with ions from the resin. The water above the sand was removed by aspiration, and the upper part of the syringe was washed twice with water. Enzyme (30–60 μg protein) suspended in 0·5 ml of a solution containing 100 mM-Tris (pH 7·4), 0·5 mM-EDTA (Tris salt) and 100 μM-[^{86}Rb]RbCl was run slowly into the syringe without disturbing the sand layer, and the syringe was mounted vertically on the moveable platform of a Palmer stand whose coarse screw (pitch 5 mm) could be turned at predetermined rates by a Siemens variable-speed stepping motor (1 AD 5000 OB) acting through a friction clutch and gearbox. The piston was inserted into the barrel so that about 0·25 ml of air was trapped above the enzyme suspension; a small hole drilled through the wall of the barrel, at a point corresponding to 1·3 ml on the (extrapolated) scale, allowed this to be done without any significant rise in pressure. The tap was opened and the motor switched on. Suitable stops had been arranged so that, as the syringe was raised by the motor, the piston was pushed in, the movement beginning only after the motor had reached its set speed, and terminating abruptly just before the piston reached the surface of the resin. Unless otherwise stated, the flow rate was 0·22 ml/s, but adjustment of the stepping motor and gearbox allowed a wide choice of flow rates.

Preliminary experiments showed that the elution profile of the Na, K-ATPase used in our experiments was similar to the elution profile of bovine serum albumin, and experiments with larger columns showed that the effective void volume of the resin for albumin was 38% of the total bed volume. With a flow rate of 0·22 ml/s and a bed volume of 0·5 ml, the time spent by the enzyme in passing through the resin was therefore approximately 0·9 s. When enzyme, suspended in a medium containing sufficient sodium or ADP to prevent occlusion of rubidium, was passed through the resin at this flow rate, the resin removed about 99·9% of the rubidium in the suspension. The residual 0·1%, however, represented an amount of rubidium that was too large to be ignored, and in experiments to estimate occluded rubidium we therefore always ran control columns with enzyme suspension containing either 2 mM-ADP or 15 mM-NaCl. Although, in each experiment, the amounts of protein emerging from the different columns were approximately the same, we measured the protein content of the effluents (using Peterson's (1977) modification of the Lowry method) and corrected the ^{86}Rb contents for the slight differences in protein content.

Use of Sephadex as a rapid-mixing device

In several of the experiments reported in this paper, it was necessary to add nucleotide to the enzyme suspension just before the enzyme entered the resin, so that the total period between the enzyme's exposure to nucleotide and its emergence from the resin was small compared with the time constant for the $E_2 \rightarrow E_1$ conformational change (5–10 s). This was achieved by placing, between the resin and the sand layer, about 0·2 ml of Sephadex G25 that had been equilibrated with a solution similar to that used for suspending the enzyme but containing in addition the

nucleotide that was to be added. The flow rates were far too great for the nucleotide to come into equilibrium across the surface of the Sephadex beads, but the experiment summarized in Fig. 1 shows that the concentration of nucleotide in the effluent did not vary too widely with time, and was roughly proportional to the initial concentration in the Sephadex. Calculation of the areas under the curves shows that the amounts of nucleotide eluted could be largely accounted for by the

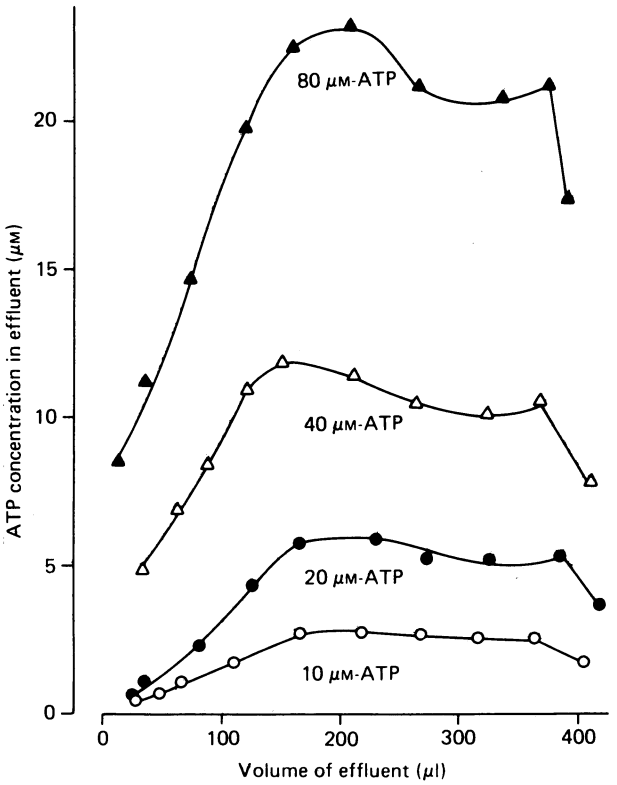

Fig. 1. An experiment to test the use of Sephadex as a rapid-mixing device. Columns were prepared in the usual way but with 0·2 ml portions of G.25 (medium) Sephadex between the resin and the sand layers. The Sephadex had been loaded with solutions containing 100 mM-Tris/Tris Cl (pH 7·4), 0·5 mM-EDTA (Tris salt) and 10, 20, 40 or 80 μM-[γ^{32}P]ATP. Portions (0·3 ml) of a solution similar to those used for loading the Sephadex but lacking ATP were forced through the columns at a rate of 0·22 ml/s. Each effluent was collected in a long piece of narrow-bore plastic tubing so that the successive portions of effluent could be transferred into counting vials, weighed and assayed for radioactivity. (The total volume of effluent was greater than 0·3 ml because of liquid displaced by air at the end of the run.)

nucleotide initially present between the beads, rather little being added by diffusion from the solution within the beads. The Sephadex was, therefore, behaving far from ideally, and it follows that, in experiments using this technique, we cannot assume that the concentration of nucleotide in the effluent indicates the concentration that was experienced by the enzyme during the whole of its passage through the resin; nor can we assume that, in a single run, all of the enzyme would have had a similar exposure to nucleotide. On the other hand, if we compare different runs, we can assume that corresponding portions of enzyme at corresponding stages of their journey would have experienced nucleotide concentrations in the same ratio as that of the initial concentrations

in the Sephadex. For most of the enzyme during most of its journey, however, the actual concentration of nucleotide experienced would have been very much less than the initial concentration in the Sephadex.

Estimation of the number of ouabain-binding sites

(1) *In the presence of Mg^{2+} and orthophosphate.* Enzyme (usually 56 μg) was incubated for 1 h at 37 °C in 200 μl solution containing 3 mM-$MgCl_2$, 3 mM-Tris orthophosphate, 40 mM-Tris/Tris Cl (pH 7·8 at 20 °C) and different concentrations of [^{3}H]ouabain. Ouabain binding was measured by the method of Albers & Krishnan (1979), using a Beckman 'Airfuge' high-speed centrifuge. The maximum binding capacity was estimated from Scatchard plots of the data.

(2) *In the presence of Na^+, Mg^{2+} and ATP.* The procedure was similar to that just described except that the incubation medium contained 120 mM-NaCl, 3·2 mM-$MgCl_2$, 3 mM-ATP (disodium salt), 40 mM-Tris/Tris Cl (pH 7·8 at 20 °C), 0·2 mM-EGTA, 0·2 mM-EDTA, and different concentrations of [^{3}H]ouabain.

Estimation of the number of phosphorylation sites

(*a*) *Phosphorylation by [γ^{32}P]ATP.* The procedure was that described by Beaugé & Glynn (1979*b*) except that the wash solution contained 5% (w/v) trichloracetic acid, 50 mM-phosphoric acid, and 80 mM-$Na_4P_2O_7$. The Whatman glass-fibre filters were placed in plastic counting vials and their ^{32}P content estimated by measurement of the Cerenkov radiation.

(*b*) *Phosphorylation by [^{32}P]orthophosphate.* Enzyme (usually 56 μg) was incubated for 1 h at 20 °C in 120 μl solution containing 1·7 mM-$MgCl_2$, 0·5 mM-ouabain, 0·2 mM-EGTA, 0·2 mM-EDTA, and 40 mM-Tris/Tris Cl (pH 7·0 at 20 °C). The suspension was chilled to 0 °C and [^{32}P]orthophosphate (Tris salt) was added, in a volume of 100 μl, to give a final phosphate concentration of 0·1 μM. After a further hour, the reaction was stopped with 23 volumes of an ice-cold solution containing 1·5 mM-$HClO_4$, 1 M-H_3PO_4 and 20% (w/v) polyphosphoric acid. Blank tubes were prepared by adding the quench solution to the enzyme suspension before the addition of [^{32}P]orthophosphate. After the addition of the quench solution, the enzyme suspension was allowed to stand for 10 min at 0 °C and was then filtered by suction through Whatman GF/C glass-fibre filters. The filters were washed ten times with an ice-cold wash solution containing 5% (w/v) trichloracetic acid, 1 M-H_3PO_4 and 20% (w/v) polyphosphoric acid. They were then placed in plastic counting vials and their ^{32}P content determined by measurement of the Cerenkov radiation.

Sources of materials

NaCl and KCl were the 'spectroscopically pure' salts supplied by Johnson Matthey, London. $MgCl_2$ (analytical reagent grade) was from Fisons, Loughborough. RbCl was from Hopkin & Williams, Chadwell Heath, Essex. ATP, ADP and AMP-PCP were from Boehringer, Mannheim. Na_3VO_4 was from B.D.H., Poole. Histidine, EDTA (free acid), EGTA (free acid), HEPES, Tris, ouabain, polyphosphoric acid, $Na_4P_2O_7$ and bovine serum albumin (fatty acid free) were from Sigma, London. [^{86}Rb]RbCl, [^{3}H]ouabain, [^{32}P]orthophosphate and [γ^{32}P]ATP were from Amersham International.

RESULTS

Prevention of rubidium occlusion by sodium ions

If Beaugé & Glynn (1979*a*) were correct in their assumption that the extra ^{86}Rb carried through their ion-exchange columns when nucleotides were absent represented ^{86}Rb occluded within the E_2 form of the enzyme, it should be possible to produce a similar reduction in the amount of ^{86}Rb emerging from the columns by adding sufficient sodium ions to the enzyme suspension to hold the enzyme in the E_1 form even in the presence of a low concentration of rubidium ions. Fig. 2 summarizes the results of an experiment to test this point. Enzyme was suspended in buffered solutions containing 100 μM-[^{86}Rb]RbCl and different concentrations of NaCl or 2 mM-ADP. As expected, the presence of sodium reduced the amount of ^{86}Rb carried

through the columns by the enzyme, and the maximum reduction was similar to that produced by 2 mM-ADP.

The rate of release of occluded rubidium

If the release of rubidium ions from the occluded-rubidium form of the enzyme is preceded by the conversion of that form to the E_1 form, the rate of release cannot be faster than the rate of the conformational change and is likely to be equal to it.

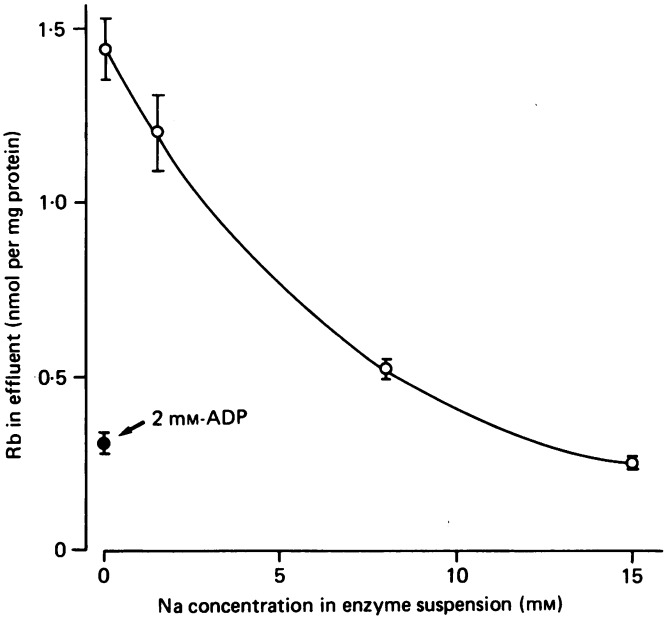

Fig. 2. An experiment showing that sodium ions prevent the occlusion of rubidium ions. Na, K-ATPase (specific activity 16 μmol mg^{-1} min^{-1}) was suspended at a concentration of 120 μg/ml in solutions containing 100 mM-Tris/Tris Cl (pH 7·4), 100 μM-[^{86}Rb]RbCl, 1 mM-$MgCl_2$, 1 mM-histidine and the concentrations of NaCl shown in the Figure. A similar suspension was prepared containing 2 mM-ADP but no sodium. Portions (0·5 ml) of the various enzyme suspensions were forced through columns of sulphonic resin in the K form at a flow rate of 0·22 ml/s as described in the Methods, and the effluents were assayed for radioactivity and for total protein. The temperature was 24 °C. Each point in the Figure is the mean of four ± S.E. of the mean. ($MgCl_2$ was included in the suspending media in this experiment because another part of the experiment (not shown) involved the use of ouabain.)

We have therefore done a number of experiments in which the rate of release of occluded rubidium has been measured by forcing enzyme, suspended in suitable ^{86}Rb-containing media, down ion-exchange resin columns at different flow rates and comparing the amounts of radioactivity emerging from the columns. For each flow rate, control runs in which ADP was present at a concentration sufficient to hold the enzyme in the E_1 form were used to determine the amounts of unoccluded rubidium carried through the columns.

The results of two similar experiments are shown in Fig. 3. The loss of ^{86}Rb followed

an exponential course, with a rate constant of 0·09 s^{-1} in one experiment and of 0·12 s^{-1} in the other. In both of these experiments, the resin had been equilibrated with Tris buffer, or a Tris/Mg/EDTA solution, of the same pH (7·4) and the same composition (except that it lacked rubidium) as that used for suspending the enzyme. In a third experiment, in which the resin was loaded with potassium instead of Tris, the rate constant for ^{86}Rb release was 0·2 s^{-1} (Temp. = 19 °C; see Fig. 1 of Glynn &

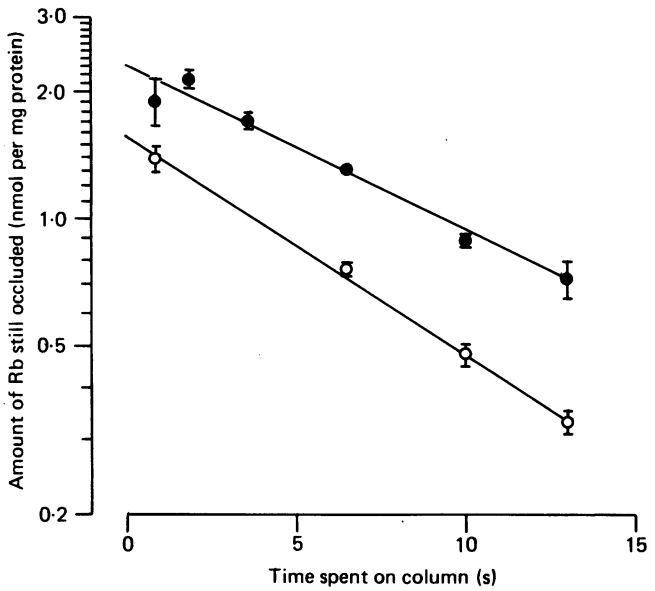

Fig. 3. Two experiments to measure the rate of release of rubidium from the occluded state. Each graph shows the amount of occluded rubidium carried through the resin column by the enzyme as a function of the time spent by the enzyme in passing down the column. Na, K-ATPase (specific activity 17 μmol mg^{-1} min^{-1}) was suspended at a concentration of 100 μg/ml in a solution containing 100 mM-Tris/Tris Cl (pH 7·4), 100 μM-[^{86}Rb]RbCl, 0·5 mM-EDTA (Tris salt), 0 (●) or 2 mM (○)-$MgCl_2$, 1 mM-histidine and either 0 or 2 mM-ADP (Tris salt). Portions (0·5 ml) of the enzyme suspensions were passed at different speeds through columns of sulphonic resin which had been equilibrated with 100 mM-Tris/Tris Cl (pH 7·4) (●) or with 100 mM-Tris/Tris Cl (pH 7·4), 2 mM-$MgCl_2$, 0·5 mM-EDTA, (○). The effluents were analysed for radioactivity and total protein. The temperature was 20 °C (filled circles) or 21 °C (○). Each point represents the difference (±S.E.) between the mean of three determinations with no ADP in the suspending medium and three determinations with 2 mM-ADP in the suspending medium. The slopes of the regression lines, which give the rate constants for the release of occluded rubidium in the two experiments, are 0·09 s^{-1} (●) and 0·12 s^{-1} (○).

Richards, 1982). The slightly faster release of rubidium in that experiment probably resulted from a change in pH (see Skou & Esmann, 1980), since exchange of potassium ions for cationic Tris would have led to a small increase in pH (about 0·3 of a pH unit) during the passage of the enzyme down the resin.

Magnesium chloride was included in the enzyme suspension in one of the experiments in Fig. 3, because another part of the experiment (not shown) involved the use of vanadate. We do not know whether the presence of magnesium ions contributed to the small difference between the rate

constants in the two experiments, but it is clear that magnesium ions cannot have a large effect. This is what would be predicted from the lack of any effect of magnesium on the rate of the conformational change from E_2 to E_1 measured by observations of formycin diphosphate fluorescence (Karlish *et al.* 1978).

Lack of effect of sodium ions on the rate of release of occluded rubidium. Sodium ions in moderate or high concentration hold the enzyme in the E_1 form even in the presence of low concentrations of potassium or rubidium ions. This effect of sodium could be

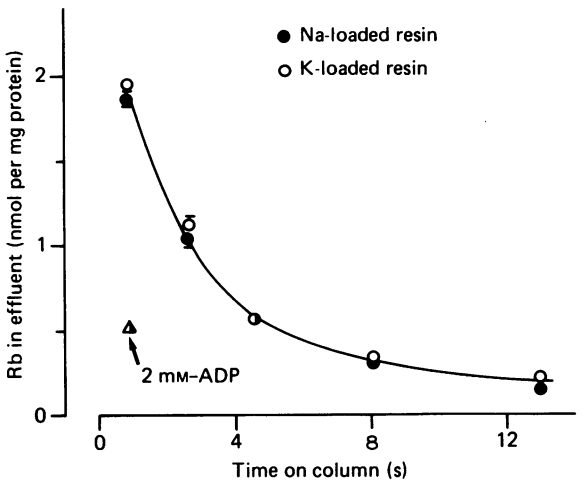

Fig. 4. A comparison of the release of occluded rubidium from the enzyme in sodium-containing and potassium-containing media. Na, K-ATPase (specific activity 12 μmol mg^{-1} min^{-1}) was suspended at a concentration of 80 μg/ml in a solution containing 100 mM-Tris/Tris Cl (pH 7·4), 100 μM-[^{86}Rb]RbCl, 0·5 mM-EDTA (Tris salt), 1 mM-histidine, and 0 or 2 mM-ADP (Tris salt). Portions (0·5 ml) of enzyme suspension were passed at different speeds through columns of sulphonic resin in either the Na form (filled symbols) or the K form (open symbols) and analysed for radioactivity and for total protein. The temperature was 20 °C. (Because the amount of rubidium carried through the columns in the presence of 2 mM-ADP was determined only for a single flow rate, it was not possible to estimate the occluded rubidium emerging from the columns at each flow rate by subtracting the 'ADP-insensitive' rubidium; there was therefore no point in plotting the amounts of rubidium in the effluents on a logarithmic scale.) Preliminary experiments with similar Na-loaded columns that had been washed with water showed that passage of 0·5 ml of a solution similar to the enzyme suspension medium released sufficient sodium to make the concentration in the effluent about 45 mM, irrespective of flow rate. ●, ○, no ADP in the enzyme suspension; ▲, △, 2 mM-ADP in the enzyme suspension. Each point in the Figure is the mean of four ± S.E. of the mean.

explained by supposing *either* that sodium ions act by displacing potassium or rubidium ions from sites on E_1, *or* that they accelerate the conversion of E_2 to E_1. The second explanation is excluded by the experiment illustrated by Fig. 4. Enzyme in a 100 mM-Tris medium containing 100 μM-[^{86}Rb]RbCl was passed at different flow rates down columns of sulphonic resin in either the sodium or the potassium form. Preliminary experiments showed that, during the passage of the enzyme suspension down the sodium-loaded resin, the Tris would displace sufficient sodium from the resin to make the sodium concentration in the effluent fluid about 45 mM, irrespective of

flow rate. During most of its passage down the sodium-loaded column, the enzyme must therefore have been exposed to sodium at concentrations sufficient, given enough time, to displace the equilibrium between E_1 and E_2 forms well over to E_1. In spite of this, the rate of release of ^{86}Rb was not significantly different from the rate of release observed when the enzyme was passed down a potassium-loaded column. Sodium ions do not, therefore, accelerate the change in conformation from E_2 to E_1.

The effects of ATP and ADP on rubidium release

If rubidium release is preceded by the conversion of the E_2 form of the enzyme to the E_1 form, it should be accelerated by the presence of nucleotides at concentrations at which they have been shown to accelerate this conformational change (Post *et al.* 1972; Karlish *et al.* 1978; Karlish & Yates, 1978). Furthermore, because the acceleration of the conformational change by nucleotides does not involve phosphorylation, the effect of nucleotides on the rate of rubidium release should occur in the absence of magnesium ions. The difficulty in testing these predictions is that the presence of nucleotides at adequate concentrations will displace the equilibrium between the E_1 and E_2 forms towards E_1 (see Beaugé & Glynn, 1980); it is therefore not sufficient to demonstrate that the inclusion of nucleotides in the enzyme suspension leads to a reduction in the amount of ^{86}Rb that is carried through the columns, since such a reduction could merely reflect the lower concentration of E_2Rb in the enzyme suspension initially. To prove an effect on the rate of conversion of E_2Rb to $E_1 + Rb^+$, it is necessary to add the nucleotide to the enzyme suspension only just before it encounters the resin, so that the enzyme is exposed to nucleotide for a period that is short compared with the time constant for the conformational change. To achieve this brief exposure, we used the procedure described in the Methods, in which the enzyme suspension is passed through a layer of G 25 Sephadex loaded with the nucleotide and placed immediately above the resin. As explained in the Methods, with this procedure, the nucleotide does not have time to equilibrate across the surface of the Sephadex, so that the concentration of nucleotide to which the enzyme is exposed will vary with the flow rate. For this reason, the flow rate, and therefore the duration of exposure of the enzyme to the resin, was kept constant, and the nucleotide concentration in the Sephadex was varied. The rate of rubidium release determined the amount of occluded rubidium remaining after about 0·4 s on the Sephadex and 0·9 s on the resin. This amount was calculated, for each nucleotide concentration, by measuring the amounts of ^{86}Rb emerging from the columns, and subtracting the amount emerging when 2 mM-nucleotide was included in the original enzyme suspension.

The results of two experiments, one with ADP and one with ATP are summarized in Figs. 5 and 6. In both experiments, the amounts of occluded ^{86}Rb have been plotted logarithmically against the nucleotide concentration in the Sephadex. If the loss of ^{86}Rb is exponential with time and the rate constant is proportional to the nucleotide concentration (which is what we should expect if the nucleotide acts at a site with a very low affinity) the resulting graph should be a straight line. With ADP, this is just what was observed. With ATP, the graph was slightly curved; nevertheless, it is clear that ATP was acting with a fairly low affinity.

Before the above interpretation of Figs. 5 and 6 can be accepted, an alternative explanation of the results must be excluded. Because E_1 has a high affinity for nucleotides and a low affinity for potassium ions, at low potassium concentrations the equilibrium between E_1 and E_2 is readily displaced by quite low concentrations of nucleotides (Beaugé & Glynn, 1980). With 1 mM-potassium, for example, Beaugé & Glynn (1980) found that about 15 μM-ATP, or about 25 μM-ADP, was

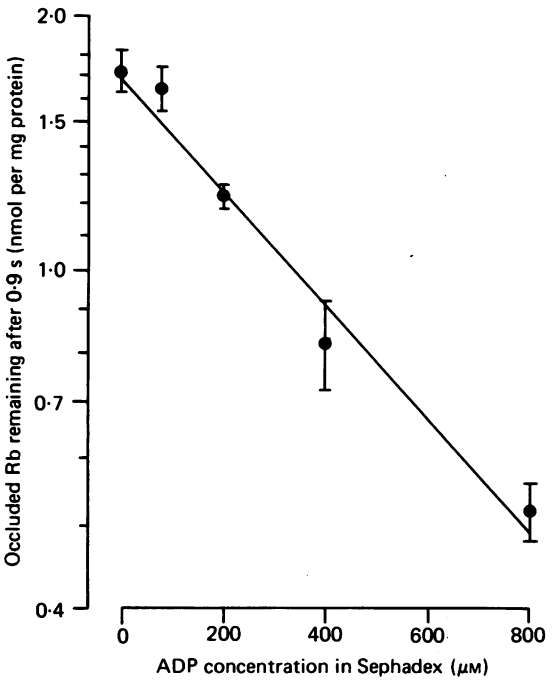

Fig. 5. An experiment showing that ADP accelerates the release of occluded rubidium. Na, K-ATPase (specific activity 18 μmol mg^{-1} min^{-1}) was suspended at a concentration of 90 μg/ml in a solution containing 100 mM-Tris/Tris Cl (pH 7·4), 200 μM-[^{86}Rb]RbCl, 1 mM-histidine and 0·5 mM-EDTA (Tris salt). Portions of the enzyme suspension, 0·3 ml in volume, were forced at a flow rate of 0·22 ml/s down Sephadex/sulphonic-resin (K form) columns as described in the Methods. (At this flow rate, the enzyme spent about 0·4 s in the Sephadex and 0·9 s in the resin.) The Sephadex had been equilibrated with solutions similar to that used for suspending the enzyme but containing in addition ADP (Tris salt) at different concentrations. The effluents were analysed for radioactivity and total protein. For each ADP concentration, the amount of rubidium in the effluent was corrected by subtracting the amount of rubidium in the effluent in a similar experiment in which 2 mM-ADP had been included in the solution used to suspend the enzyme. The corrected amounts have been plotted as a function of the ADP concentration in the Sephadex. Each point in the Figure is the mean of four ± S.E. of the mean. Note that, for reasons explained on p. 20, the concentrations of ADP experienced by the enzyme in passing down the columns would have been much less than the initial concentrations in the Sephadex.

sufficient to convert half of the enzyme to the E_1 form, as judged by its intrinsic fluorescence. If rubidium ions behave like potassium ions, then with only 100 μM-rubidium we should expect even greater sensitivity to nucleotide. Since ADP at these low concentrations appears to have only a very small effect on the *rate* of release of occluded rubidium, we have been able to investigate the effect of ADP on the equilibrium by measuring the amount of occluded ^{86}Rb as a function of the concentration of ADP in the enzyme suspension. It turned out that about 1·4 μM-ADP was sufficient

to reduce the amount of occluded rubidium to one half. This striking sensitivity made it necessary to consider whether the effects observed in the experiments of Figs. 5 and 6 could have been the result of a decrease in the equilibrium concentration of E_2Rb caused by contamination of the enzyme suspension with small quantities of nucleotide from the Sephadex. Although we had attempted to prevent such contamination by washing the syringe above the sand layer before introducing the enzyme suspension, it was necessary to check that our precautions were effective. We did this in two ways. In one experiment, in which the Sephadex had been loaded with 800 μM-[γ^{32}P]ATP, the enzyme suspension was carefully removed from the syringe and examined for radioactivity. The concentration of ATP detected was only $0{\cdot}17 \pm 0{\cdot}07$ μM. In another experiment, enzyme suspension which had been loaded onto a column containing 800 μM-ADP in the Sephadex layer was carefully withdrawn and loaded onto a similar column with no nucleotide in the Sephadex layer. When this enzyme suspension was forced through the second column in the usual way, the amount of ^{86}Rb

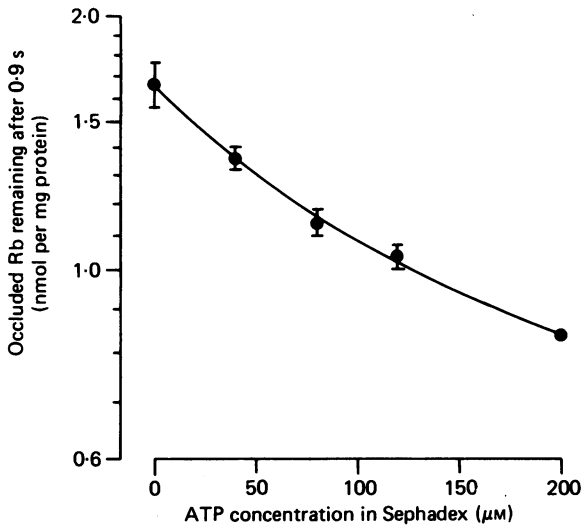

Fig. 6. An experiment showing that ATP accelerates the release of occluded rubidium. The procedure was similar to that described in the legend to Fig. 5, but ATP was used instead of ADP, the range of concentrations investigated was lower, and the enzyme preparation was a different one (specific activity 15 μmol mg^{-1} min^{-1}).

which emerged from the resin was not significantly less than the amount which emerged in a control experiment with fresh enzyme suspension. The effects of ATP and ADP in Figs. 5 and 6 cannot therefore be explained by a reduction in the amount of occluded rubidium in the enzyme suspension initially.

The effect of orthovanadate on rubidium release

Experiments with fluorescein-labelled Na, K-ATPase by Karlish, Beaugé & Glynn (1979) showed that vanadate, in the presence of magnesium ions, stabilizes the E_2 form of the unphosphorylated enzyme. We therefore expected that vanadate would allow the enzyme to occlude potassium or rubidium ions in conditions in which occlusion would otherwise not occur. To test this prediction, we compared the ability of normal and of vanadate-treated enzyme to carry ^{86}Rb through columns of cation-exchange resin under various conditions. The results of three experiments are summarized in Table 1. The first of these experiments showed that exposure of the enzyme to 10 μM-vanadate for about 1 min allowed occlusion of rubidium ions to

occur even in the presence of ADP or sodium at concentrations that prevented occlusion of rubidium by the untreated enzyme. The second experiment confirmed that vanadate permits occlusion of rubidium to occur in the presence of a high concentration of ADP, and showed that vanadate is effective only in the presence of magnesium ions. In both of these experiments, vanadate also increased the amount of rubidium occluded by the enzyme in the absence of ADP and of sodium, but the augmented amount was still much less than could be achieved by increasing the rubidium concentration (see below). The third experiment in Table 1 shows that increasing the

TABLE 1. The effects of vanadate on rubidium occlusion

Experiment	Suspension medium	Rb in effluent from columns (nmol/mg protein)
1	Control	1·5 ± 0·07
	+ vanadate* (10 μM)	2·1 ± 0·12
	+ ADP (2 mM)	0·4 ± 0·01
	+ ADP (2 mM) + vanadate* (10 μM)	1·6 ± 0·04
	+ Na (10 mM)	0·3 ± 0·01
	+ Na (10 mM) + vanadate* (10 μM)	1·7 ± 0·08
2	Control	2·21 ± 0·06
	+ vanadate* (10 μM)	2·52 ± 0·17
	+ ADP (2 mM)	0·48 ± 0·02
	+ ADP (2 mM) + vanadate* (10 μM)	2·24 ± 0·09
	Mg-free, + ADP (2 mM) + vanadate* (10 μM)	0·57 ± 0·04
3	Control	1·79 ± 0·07
	+ ADP (2 mM)	0·25 ± 0·05
	+ vanadate (100 μM) for 4 min	4·90 ± 0·16
	+ vanadate (100 μM) for 35 min	5·07 ± 0·13

* Unless otherwise stated, the enzyme was exposed to the vanadate for 2–4 min at room temperature before being forced down the column.

Na, K-ATPase (specific activity 14–18 μmol mg^{-1} min^{-1}) was suspended at a concentration of 60–120 μg/ml in a solution containing 100 mM-Tris/Tris Cl (pH 7·4), 100 μM-[^{86}Rb]RbCl, 1·5 mM-$MgCl_2$, 0·5 mM-EDTA (Tris salt), ± ADP and Na orthovanadate as shown. Portions (0·5 ml) of the enzyme suspensions were forced down columns of sulphonic resin in the K form at a flow rate such that the enzyme spent about 0·9 s in contact with the resin. Each figure in the Table is the mean of four ± S.E. of the mean.

vanadate concentration and prolonging the period of exposure increased the amount of rubidium occluded from a solution containing 100 μM-RbCl to about 4·9 nmol/mg. This is roughly the amount we should expect to be occluded at saturating rubidium concentrations (see below) though a precise figure for the stoichiometry cannot be given because we did not estimate the number of phosphorylation sites or of ouabain-binding sites in the preparation used in this experiment. We suspect that the relative ineffectiveness of low concentrations of vanadate and of short periods of exposure is the result of the low concentration of rubidium ions in these experiments (cf. Beaugé, Cavieres, Glynn & Grantham, 1980).

In the three experiments of Table 1 the duration of exposure of the enzyme to the resin was fixed at about 0·9 s. We also did one experiment in which enzyme that had been suspended for 30 min in a medium containing 100 mM-Tris/Tris Cl (pH 7·4),

2 mM-$MgCl_2$, 0·5 mM-EDTA (Tris salt), 100 μM-[^{86}Rb]RbCl, with or without 10^{-4} M-Na_3VO_4, was forced at different speeds through columns of resin that had been equilibrated with a similar medium lacking both RbCl and vanadate. From the amounts of ^{86}Rb in the effluents, corrected for non-occluded rubidium by subtraction of the amount of ^{86}Rb that emerged when 2 mM-ADP was present (without vanadate) in the enzyme suspension, we calculated the rate constant for rubidium release to be 0·12 s^{-1} when vanadate was absent, and 0·06 s^{-1} when vanadate was present (Temp. = 20–21 °C).

TABLE 2. The effect of ouabain on rubidium occlusion

Experiment	Suspension medium	Rb in effluent from columns (nmol/mg protein)
1	Control	1·44 ± 0·09
	+ ADP (2 mM)	0·31 ± 0·03
	+ ouabain (10^{-4} M)	0·33 ± 0·02
2	Control	0·95 ± 0·04
	+ NaCl (10 mM)	0·23 ± 0·01
	+ ouabain (10^{-4} M)	0·29 ± 0·04

The procedure was similar to that used for the experiments in Table 1, except that the enzyme was exposed to ouabain for 10–15 min at room temperature before being forced down the resin columns. Each figure in the Table is the mean of four ± S.E. of the mean.

Vanadate had a greater effect in reducing the acceleration of rubidium release by ADP. When ADP (final concentration 1·5 mM) was added to enzyme that had been exposed to 100 μM-vanadate at 14 °C for 30 min in a solution containing 100 μM-RbCl, 2 mM-$MgCl_2$ and 100 mM-Tris/Tris Cl (pH 7·4), it increased the rate of rubidium release by a factor of only 2·5 (not shown). The vanadate-treated enzyme therefore seemed to be very much less sensitive to ADP than the enzyme in the experiment of Fig. 5, though the difference in temperature between the experiments and the uncertainty about the effective ADP concentration in the experiment of Fig. 5 make a strict comparison impossible.

The effect of ouabain on rubidium occlusion

Because ouabain is thought to stabilize the unphosphorylated enzyme in the E_2 conformation (see Karlish *et al.* 1978; Karlish, 1980), we expected that ouabain would resemble vanadate in its effects on rubidium occlusion. In the event, the effect turned out to be quite different, as shown in Table 2. Addition of ouabain to the enzyme suspension was as effective as 2 mM-ADP or 10 mM-NaCl in reducing the amounts of ^{86}Rb emerging from the columns; in other words, no occluded rubidium was detectable in the presence of ouabain. The effect of ouabain was half-maximal at about 2×10^{-5} M, and ouabain was ineffective in the absence of magnesium ions (not shown).

Stoichiometry

In order to discover how many rubidium ions are occluded per molecule of enzyme, it is necessary to estimate the number of phosphorylation sites or of ouabain-binding sites per mg enzyme protein, and also to correct the measurements of occluded

TABLE 3. The stoichiometry of rubidium occlusion

Experiment	Ouabain-binding sites: In presence of P_i (nmol/mg)	Ouabain-binding sites: In presence of ATP + Na (nmol/mg)	Phosphorylation sites: Phosphorylation by ATP + Na (nmol/mg)	Phosphorylation sites: Phosphorylation by P_i + ouabain (nmol/mg)	Rb occluded (estimated maximum) (nmol/mg)	Mol Rb occluded per mol ouabain-binding sites or per mol phosphorylation sites
1	—	—	1·55	—	4·45	2·87
2	—	1·65	1·10	—	4·01	2·43; 3·65
3	—	1·05	—	—	3·93	3·74
4	0·93	—	—	—	2·02	2·17
5	1·09	1·21	1·15	1·30	3·54	3·25; 2·93; 3·08; 2·72
6	1·47	1·44	1·36	1·62	4·88	3·32; 3·39; 3·59; 3·01

$$\text{Mean } (\pm \text{S.E. of the mean}) \frac{\text{mol Rb occluded}}{\text{mol sites}} = 2{\cdot}92 \pm 0{\cdot}23.$$

The maximum amounts of rubidium occluded were estimated from graphs such as that of Fig. 7, as described in the text. The numbers of ouabain-binding sites and of phosphorylation sites were estimated as described in the Methods.

rubidium for the losses that occur during the passage of the enzyme down the ion-exchange column. To discover the maximum number of rubidium ions that can be occluded, it is also necessary to measure the amounts of rubidium occluded at different rubidium concentrations, so as to be able to extrapolate to saturating concentrations.

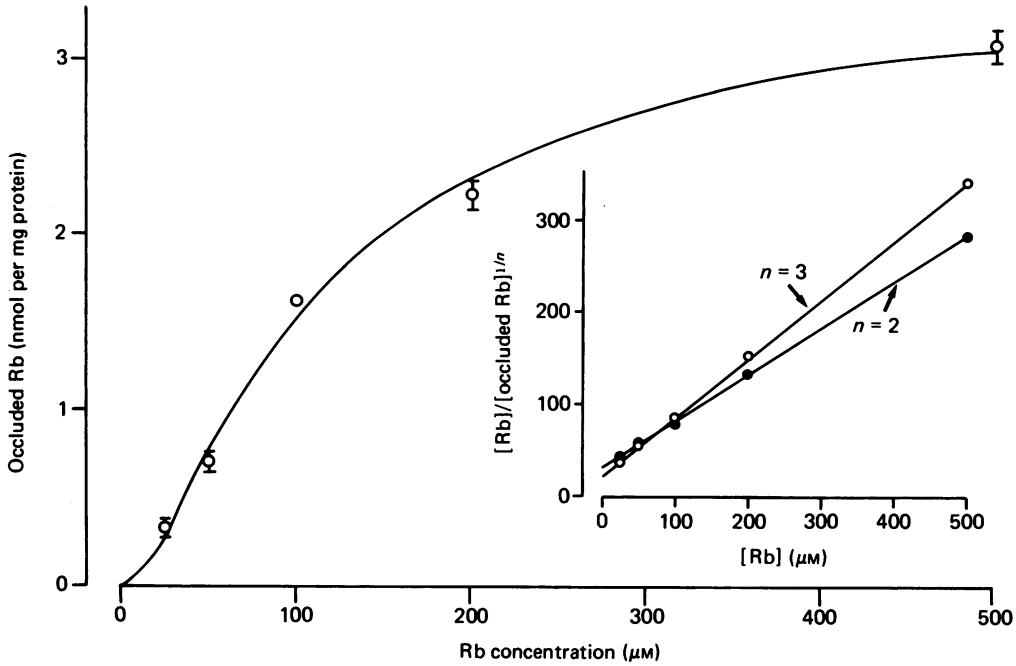

Fig. 7. The relation between the amount of rubidium occluded (uncorrected for loss during passage through the resin) and the concentration of rubidium in the suspending medium. Na, K-ATPase (specific activity 18 μmol mg^{-1} min^{-1}) was suspended at a concentration of 54 μg/ml in a solution containing 100 mM-Tris/Tris Cl (pH 7·4), 0·5 mM-EDTA (Tris salt), 0·5 mM-histidine, the concentrations of [^{86}Rb]RbCl shown in the Figure, and 0 or 2 mM-ADP (Tris salt). Portions (0·5 ml) of the enzyme suspensions were forced down columns of sulphonic resin in the K form, as described in the Methods, at a flow rate such that the enzyme spent about 0·9 s in contact with the resin. The effluents were analysed for radioactivity and for total protein. For each rubidium concentration, the amount of 'ADP-sensitive' rubidium carried through the columns per mg protein has been calculated and is plotted in the Figure as a function of the rubidium concentration in the medium. Each point in the Figure represents the difference (±S.E.) between the mean of three determinations with no ADP in the suspending medium and three determinations with 2 mM-ADP in the suspending medium. The continuous line represents the equation $y = \alpha(1+\beta/x)^{-3}$, where x is the concentration of rubidium (μM), y is the amount of rubidium occluded (nmol/mg), $\alpha = 3{\cdot}76$ nmol/mg, and $\beta = 34{\cdot}8$ μM. *Inset.* [Rb]/[occluded Rb]$^{1/n}$ is plotted as a function of [Rb] for $n = 2$ and $n = 3$.

Table 3 summarizes the results of six separate experiments. The number of phosphorylation sites and the number of ouabain-binding sites were each determined in two ways, as described in the Methods, though all of the procedures were not used in all of the experiments. The correction for loss of rubidium during passage of the enzyme down the resin columns was made assuming a rate constant for rubidium

release of $0{\cdot}2\ s^{-1}$; this figure was chosen because sodium- or potassium-loaded columns, rather than Tris-loaded columns, were used in these experiments (see p. 24, above). The magnitude of the correction was, anyway, only about 16 %, so that a large error in the rate constant would have caused only a small error in the corrected figure. Extrapolation from the amounts of rubidium occluded at concentrations that permit easy measurement of occlusion to the amount occluded at saturating concentrations presents a greater difficulty, since the form of the relation between the amount occluded and the concentration is not precisely known. Fig. 7 shows the results of one of a number of very similar experiments in which rubidium concentrations up to 500 μM were used. The results could be fitted satisfactorily by assuming *either* that, for occlusion to occur, rubidium ions had to bind to two identical sites, *or* that binding to three identical sites was necessary (see inset to Fig. 7). The figures for rubidium occlusion in Table 3 are calculated on the assumption that three identical sites had to be filled, but the two-site assumption gives figures that do not differ systematically from those in the Table, and that lead to a mean figue for the stoichiometry that is not significantly different ($2{\cdot}99 \pm 0{\cdot}11$ compared with $2{\cdot}92 \pm 0{\cdot}23$).

At very low rubidium concentrations, the relation between rubidium concentration and the amount of rubidium occluded is very sensitive to the presence of magnesium ions. The experiment of Fig. 7 was done with a nominally magnesium-free medium containing 0·5 mM-EDTA, and the curve in the Figure shows a slight but significant degree of inflexion. This inflexion was more marked in the presence of 2 mM-magnesium, and was not detectable in an experiment in which the medium contained 2 mM-(*trans*-1,2-cyclohexylenedinitrilo) tetraacetic acid (CDTA) and no magnesium (not shown). However, although the presence of magnesium ions reduced the amount of rubidium occluded from a 12 μM-Rb^+ medium to less than one half of the amount occluded from a similar medium containing 2 mM-CDTA and no magnesium, magnesium ions had little effect on the amount of rubidium occluded at rubidium concentrations approaching saturation (not shown).

A second route to the occluded-rubidium form of Na, K-ATPase

In the experiments described so far, the occluded-rubidium form of the enzyme was generated by the addition of rubidium to the unphosphorylated enzyme in appropriate conditions (i.e. the absence of sodium ions and of nucleotides in high concentration, or the presence of vanadate). If the occluded rubidium form generated in this way is (substituting potassium for rubidium) identical with the hypothetical occluded-potassium form of Post *et al.* (1972), it should be possible to use the rapid ion-exchange technique to demonstrate occlusion of rubidium in dephosphoenzyme formed by allowing $^{86}Rb^+$ ions to catalyse the hydrolysis of phosphoenzyme. There is, however, a technical difficulty. To phosphorylate the enzyme, some ATP must be present; but if the concentration is too high the conformational change will be accelerated sufficiently to make the detection of any occluded rubidium impossible. If, to avoid this acceleration, one attempts to use very low concentrations of ATP in the enzyme suspension, all of the ATP will be hydrolysed before the experiment starts, because the enzyme concentration is necessarily high and sodium, magnesium and rubidium ions are all present. To limit the rapid hydrolysis of the ATP, we have therefore used the Sephadex method to add ATP to the enzyme suspension just before it enters the resin. Enzyme suspended in a solution containing 15 mM-NaCl, 100 mM-Tris/Tris Cl buffer (pH 7·4), 1 mM-$MgCl_2$ and 100 μM-[^{86}Rb]RbCl, was passed first through Sephadex and then immediately through the resin. The Sephadex was loaded with

TABLE 4. The generation of the occluded-rubidium form of the enzyme by hydrolysis of phosphoenzyme

Experiment	Nucleotide in Sephadex	Rb in effluent from columns (nmol/mg protein)
1	None	0·30±0·05
	ATP	1·15±0·01
	ADP	0·31±0·01
2	ATP	1·15±0·04*
	ADP	0·30±0·05*
3	ATP	0·92±0·02*
	ADP	0·13±0·01*
4	None	0·31±0·05
	ATP	1·10±0·03
	AMP-PCP	0·20±0·01
	ATP but Mg-free	0·22±0·03

* There is some uncertainty about the absolute amounts of Rb shown for expts. 2 and 3, but the relative amounts within each experiment are reliable.

Na, K-ATPase (specific activity 9–17 μmol mg^{-1} min^{-1}) was suspended at a concentration of 60–100 μg/ml in a medium containing 15 mM-NaCl, 85 mM-Tris/Tris Cl (pH 7·4), 100 μM-[^{86}Rb]RbCl, 1·5 mM-$MgCl_2$ and 0·5 mM-EDTA. Portions of the enzyme suspension (0·3 ml) were forced through Sephadex/sulphonic-resin (Na form) columns as described in the Methods section. The Sephadex layer had been equilibrated with a solution similar to that used for suspending the enzyme but containing also, where appropriate, 40 μM-nucleotide as shown in the Table. For the Mg-free part of expt. 4, $MgCl_2$ was omitted from the solutions used for suspending the enzyme and for loading the Sephadex. AMP-PCP was used as the tetralithium salt, but in a separate experiment (not shown) 160 μM-lithium in the Sephadex did not reduce the amount of rubidium occluded in the presence of ATP. The flow rate through the columns was 0·22 ml/s, giving an estimated contact time with the Sephadex of about 0·4 s and with the resin of about 0·9 s. Each figure in the Table is the mean of four±S.E. of the mean.

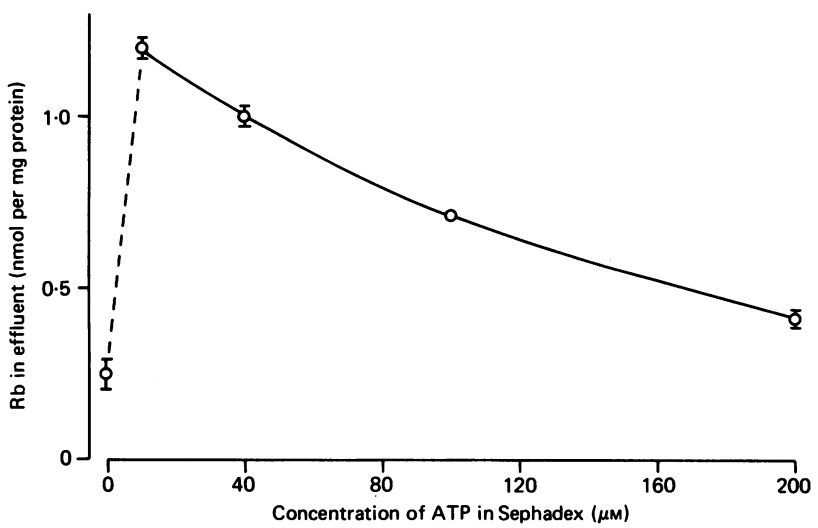

Fig. 8. The effect of ATP concentration on the amount of rubidium occluded through the route that involves phosphorylation and dephosphorylation. The procedure was similar to that described in Table 4 except that the concentration of ATP in the Sephadex was varied. Each point in the Figure is the mean of four±S.E. of the mean. Note that, for reasons explained on p. 20, the concentrations of ATP experienced by the enzyme would have been much less than the initial concentrations in the Sephadex.

a solution similar in composition to that used for suspending the enzyme but containing in addition 40 μM-ATP (Tris salt) or, in the control columns, 40 μM-ADP (Tris salt).

The results of three experiments of this kind are summarized in Table 4. They show that in the presence of ATP, extra radioactivity was carried through the columns. ADP was ineffective. A further experiment summarized in Table 4 shows that the effect of ATP was not seen in the absence of magnesium ions and that ATP could

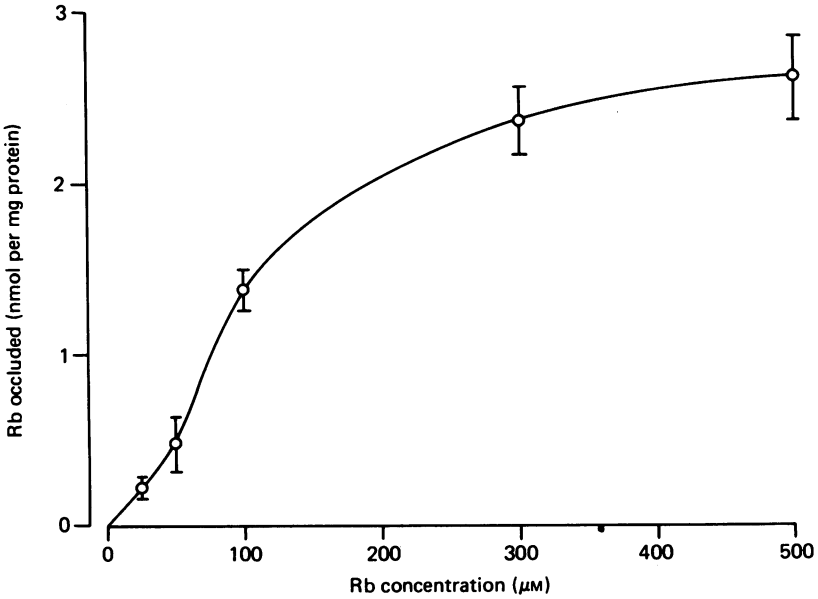

Fig. 9. An experiment showing how the amount of rubidium occluded through the route that involves phosphorylation and dephosphorylation varies with the rubidium concentration. The procedure was similar to that described in Table 4 except that: (i) the rubidium concentration was varied, (ii) the sodium concentration was 30 mM and the Tris concentration only 70 mM, and (iii) the concentration of ATP in the Sephadex was only 10 μM. (The increase in the concentration of sodium compared with the earlier experiments was necessary because, with only 15 mM-sodium, some rubidium would have been occluded from a 500 μM-rubidium medium even in the absence of ATP. The concentration of ATP was made 10 μM because that concentration gave the greatest occlusion of rubidium in the experiment of Fig. 8.) Each point in the Figure represents the difference ($\pm$S.E.) between the mean of three determinations with 10 μM-ATP in the Sephadex and three determinations with no ATP in the Sephadex.

not be replaced by the non-phosphorylating analogue adenylyl (β, γ-methylene)-diphosphonate (AMP-PCP).

Fig. 8 shows the results of an experiment in which the concentration of ATP in the Sephadex was varied. As expected, the amount of occluded rubidium was maximal with a low concentration of ATP, presumably because higher concentrations accelerated the conformational change to E_1 that allows the rubidium to escape.

Fig. 9 shows the results of an experiment in which the concentration of rubidium in the enzyme suspension (and in the Sephadex) was varied between 25 and 500 μM. The increase in ATP-dependent rubidium-occlusion as the rubidium concentration

was increased shows that the rubidium-catalysed hydrolysis of the phosphoenzyme must have been rate-limiting. With 500 μM-Rb, the amount of occluded rubidium that emerged from the resin was 2·62 nmol per mg enzyme protein, which for that particular preparation is equivalent to 1·79 ions per phosphorylation or ouabain-binding site. Correcting for the loss of rubidium during the passage down the resin gives a figure of 2·14 ions per site. Since (1) there is likely to have been some loss of occluded rubidium while the enzyme was still in the Sephadex layer, (2) the graph of Fig. 9 suggests that 500 μM-Rb was not quite enough to give maximal occlusion, and (3) we cannot be sure that all of the enzyme was phosphorylated, there seems to be no reason to suppose that the maximal amount of rubidium that can be occluded through the route that involves phosphorylation and dephosphorylation is less than the maximal amount than can be occluded by the direct route. At the same time, the uncertainties are such that we cannot be sure that the number of ions occluded through the route involving phosphorylation and dephosphorylation exceeds two.

DISCUSSION

The experiments just described show that the form (E_2Rb) in which the unphosphorylated Na, K-ATPase mainly exists in sodium-free, rubidium-containing media contains occluded rubidium ions, and that this form of the enzyme can be generated either by adding rubidium ions to the unphosphorylated enzyme in appropriate conditions or by allowing rubidium ions to catalyse the hydrolysis of the potassium-sensitive form of the phosphoenzyme. If we ignore multiple ion binding, and assume that both routes are reversible, the two routes coupled back-to-back may be written:

$$Rb^+ + E_1 \rightleftharpoons E_1Rb \rightleftharpoons E_2Rb \underset{P_i}{\rightleftharpoons} E_2P \,.\, Rb \rightleftharpoons E_2P + Rb^+. \qquad (1)$$

The justification for assuming that it is the E_1 rather than the E_2 form of the dephosphoenzyme that binds rubidium ions, and that the equilibrium between E_1Rb and E_2Rb is poised far to the right, is that potassium ions appear to act with a low affinity when one examines their effect on the rate of the conversion of E_1 to E_2 but with a high affinity when one examines their effect on the equilibrium between E_1 and E_2 (see Karlish *et al.* (1978) p. 259). The justification for assuming that phosphorylation of E_2Rb by inorganic phosphate allows the occluded rubidium ions to escape is that the experiments of Table 4 and Fig. 8 show that the rubidium-catalysed hydrolysis of E_2P leads to the trapping of the rubidium ions, and we know that the hydrolysis is reversible (Garrahan & Glynn, 1967; Glynn & Lew, 1970; Taniguchi & Post, 1975). (We have attempted to demonstrate, directly, that the addition of inorganic phosphate promotes rubidium release, but there are technical difficulties and we have succeeded in seeing only a partial release.)

In discussing the various experiments concerned with the rate of rubidium release from the occluded-rubidium form of the dephosphoenzyme, we have assumed that, in the absence of phosphate, the rate of release of rubidium is determined by the rate of the conformational change from E_2Rb to E_1Rb. Very recently, Karlish & Stein (1982) demonstrated a very small flux of rubidium ions through unphosphorylated Na, K-ATPase incorporated into artificial lipid vesicles, and concluded that there must be a slow loss of occluded rubidium directly from E_2Rb, i.e. without the preliminary formation of E_1Rb. They point out that, if there is such a direct loss, the rate constant for rubidium release measured in experiments like that of Fig. 3 would represent the sum of the rate constant for the conformational change and the

rate constant for the direct loss. From their flux data, they argue that the rate constant for the direct loss is likely to be several times greater than the rate constant for the conformational change (in the absence of nucleotides) and they conclude that the good agreement between the rate constant for rubidium loss reported by Glynn & Richards (1980) and the rate constant for the conformational change from E_2K to E_1K deduced from the fluorescence data (Karlish *et al.* 1978; Karlish & Yates, 1978) is fortuitous. Whatever may be true of Na, K-ATPase incorporated into artifical lipid vesicles, however, we doubt whether a direct loss of rubidium from E_2Rb several times faster than the conformational change can be taking place in our experiments. In the first place, our best estimate for the rate constant for rubidium release (about 0·1 s^{-1}) is lower, not higher, than the rate constant for the conformational change from E_2K to E_1K deduced from the earlier fluorescence experiments. Secondly, the figure of 0·1 s^{-1} agrees well with a recent estimate of the rate constant for the conformational change from E_2Rb to E_1Rb made using the eosin technique of Skou & Esmann (1981) and employing the same enzyme preparation as that used for the experiment of Fig. 3 (unpublished experiments of A. Fersht, I. M. Glynn, J. L. Howland & D. E. Richards).

The possible role of an occluded-potassium form of dephosphoenzyme in potassium transport

Karlish *et al.* (1978) and Beaugé & Glynn (1979*a*) pointed out that, if the ion binding sites on E_1 have a low affinity for potassium ions and are accessible from the intracellular surface, and the ion binding sites on E_2P have a high affinity for potassium ions and are accessible from the extracellular surface, the sequence of reactions summarized by eqn. (1), but with potassium replacing rubidium, could account, qualitatively at least, for many of the features of potassium movements through the Na, K-ATPase. There is good evidence (see especially Blostein & Chu, 1977) that the ion-binding sites on E_2P have a high affinity for potassium ions and are accessible from the extracellular surface. The fluorescence studies of Karlish *et al.* (1978), of Beaugé & Glynn (1980) and of Jørgensen & Karlish (1980) provide indirect evidence that E_1 binds potassium ions at low-affinity sites. The experiments of Karlish & Pick (1981) provide indirect evidence that these sites are accessible from the cell interior. Qualitatively then, a sequence of reactions like that shown in eqn. (1) could account for the movements of potassium ions in each direction across the cell membrane, and for many of the features characteristic of those movements.

Glynn & Karlish (1982) considered in some detail whether the rate constants of the individual reactions making up the sequence were likely to be big enough to account for the observed fluxes, and concluded that they were. It is, however, not enough to show that the rate constants are likely to be big enough: they must also not be too big. Table 5 lists the various estimates made by Glynn & Karlish (1982). For potassium influx (associated with sodium–potassium exchange), the estimated maximal turnover rate at room temperature is about 30 s^{-1}. This fits with the relevant rate constants if, in the steady state, about half of the enzyme is in the E_2K form, which is not unlikely (see Karlish & Yates, 1978). For potassium efflux, however, there is a difficulty. The estimated maximal turnover rate for rubidium–rubidium exchange at room temperature is only about 7 s^{-1} (Karlish & Stein, 1982), and measurements

of potassium–potassium exchange in red cells at 37 °C (Glynn, Lew & Lüthi, 1970) suggest that, at room temperature, potassium–potassium exchange would occur at a similar rate. Furthermore, if we assume that there is only one route by which potassium ions can move inwards through the Na, K-ATPase, the rate of exchange

TABLE 5. Estimates of turnover rates and of rate constants relevant to potassium transport. (Based on Glynn & Karlish, 1982.) Note that all estimates are for room temperature. No figures are available for the rates of binding of potassium ions to E_1 or to E_2P, or for the rates of dissociation of potassium ions from E_1K or from E_2PK, but all four processes are likely to be extremely fast since, by hypothesis, they involve neither major conformational nor chemical changes

Reaction	Estimated rate constant (or maximal turnover)	Basis of estimate*
K^+ influx	30 s^{-1}	Na,K-ATPase activity (Karlish & Yates, 1978)†
$K^+ + E_2P \rightarrow E_2K + P_i$	$\lesssim$ 230 s^{-1} (pseudo-first-order rate constant when $[K^+] = 10$ mM)	Quench-flow experiments on brain Na, K-ATPase (Mårdh, 1975)
$E_2K \xrightarrow[ATP]{\text{saturating}} E_1K$	60 s^{-1}	Stopped-flow measurements of intrinsic fluorescence at ATP concentrations up to 100 μM (Karlish & Yates, 1978)
K^+ efflux	7 s^{-1}	Rb^+–Rb^+ exchange‡ by Na, K-ATPase incorporated into artificial lipid vesicles (Karlish & Stein, 1982)
$E_1K \rightarrow E_2K$	290 s^{-1}§	Stopped-flow measurements using fluorescein-labelled Na, K-ATPase at K^+ concentrations up to 80 mM (Karlish, 1980)
$E_2K + P_i \rightarrow E_2PK$	$\lesssim$ 77 s^{-1} (pseudo-first-order rate constant when $[P_i] = 1$ mM)	Steady-state phosphorylation of kidney cortex enzyme at 0° C (Post *et al.* 1975), and the figure of $\lesssim$ 230 s^{-1} for the hydrolysis of E_2P in brain enzyme (Mårdh, 1975)

* Unless otherwise stated, the Na, K-ATPase used in the experiments referred to was from pig kidney outer medulla.

† A figure of 43 s^{-1} has recently been reported by Karlish & Stein (1982) for Na–Rb exchange by Na, K-ATPase incorporated into artificial lipid vesicles.

‡ Exchange rather than pump reversal was chosen as the basis for the calculation because in red cells (the only cells in which both have been measured) K^+–K^+ exchange under optimal conditions is faster than pump reversal under optimal conditions (Glynn *et al.* 1970).

§ This figure is based on experiments done in the absence of magnesium and nucleotides. For reasons given in the text, we suspect that at physiological magnesium and ATP concentrations the rate constant may be very much lower.

of potassium must be limited by a step or steps associated with the outward movement, since the inward movement can proceed at a much faster rate when potassium is being exchanged for sodium. Yet the two reactions that might be thought likely to limit the rate of the outward movement are shown in Table 5 as

having estimated rate constants of 290 s^{-1} and $\lesssim 77$ s^{-1}. If eqn. (1) (with K^+ substituted for Rb^+) represents the reactions responsible for potassium–potassium exchange, why is that exchange so slow?

We suspect that the explanation is that the rate constant for the conformational change from E_1K to E_2K in the conditions in which potassium–potassium exchange was measured is very much lower than the estimated figure of 290 s^{-1}. In the first place, the experiments with fluorescein-labelled enzyme that led to the figure of 290 s^{-1} were done in the absence of magnesium ions, and there is evidence from experiments with formycin diphosphate that, at least at low potassium concentrations, the presence of magnesium ions slows the conformational change from E_1K to E_2K several-fold (Karlish *et al.* 1978). Secondly, we suspect that the binding of nucleotide to the enzyme, which we know greatly accelerates the conversion of E_2K to E_1K, may also greatly slow the conversion of E_1K to E_2K. At first sight, this hypothetical slowing might appear unlikely because, in the restricted range of potassium concentrations in which measurements were possible, the experiments with formycin diphosphate gave estimated rate constants that were not very different from those obtained at similar potassium concentrations using the fluorescein-labelled enzyme in the absence of nucleotide (cf. Karlish *et al.* 1978 and Karlish, 1980). However, the concentration of formycin diphosphate that was used (4 μM) was so low that at any time about half of the enzyme would have been free of nucleotide. Furthermore, although, largely for technical reasons, the effect of nucleotides on the rate of conversion of E_1K to E_2K has never been measured, the following argument points to the existence of a large effect. When Na, K-ATPase is suspended in a sodium-free 100 mM-potassium medium containing 5 mM-ATP, and exposed to trypsin, the pattern of tryptic digestion is biphasic and resembles that observed in the absence of ATP in a sodium rather than a potassium medium (see Fig. 7 of Jørgensen, 1975). This implies that at saturating levels of ATP the equilibrium between E_1K and E_2K is poised well over to E_1K (see also Beaugé & Glynn, 1980; Jørgensen & Karlish, 1980). It follows that, if the rate constant for the conversion of E_2K to E_1K at saturating levels of ATP and at room temperature is about 50–60 s^{-1}, the rate constant for the conversion in the reverse direction must be much less than 50 s^{-1}, and could well be slow enough to limit the over-all reaction rate to 7 s^{-1}.

The effects of vanadate and of ouabain

The experiments with vanadate suggest that the inhibitory effects of vanadate on sodium–potassium exchange, potassium–potassium exchange, and Na, K-ATPase activity are mainly due to a great reduction in the accelerating effect of ATP on the conformational change $E_2K \rightarrow E_1K$. This effect cannot, however, explain the observed stabilization of E_2Rb in high-sodium media containing no nucleotides. That stabilization cannot be explained, either, by the small reduction in the rate constant for the conversion of E_2Rb to E_1Rb that was seen in the absence of nucleotides. It could reflect a change in the relative affinities of the E_1 form of the enzyme for sodium and rubidium ions, *or* an increase in the rate constant for the conversion of E_1Rb to E_2Rb, *or* a combination of both effects.

The lack of rubidium occlusion in the presence of ouabain is interesting, since the effects of ouabain on the fluorescence of fluorescein-labelled enzyme (Karlish, 1980) and on the way in which the enzyme is attacked by trypsin (Castro & Farley, 1979)

both suggest that ouabain stabilizes the dephosphoenzyme in the E_2 form. Castro & Farley (1979) showed, however, that the conformation of the α chain, at least, must be different in the ouabain-bound dephosphoenzyme, because chymotrypsin was able to attack a peptide bond that was otherwise not vulnerable. It is possible that both ouabain and phosphorylation produce a similar change in conformation, which allows occluded rubidium or potassium ions to escape, and the facts that ouabain promotes phosphorylation by orthophosphate and that phosphorylation promotes ouabain binding are in line with this hypothesis. Unfortunately, Castro & Farley do not describe the effect of chymotrypsin on phosphoenzyme in the absence of ouabain, so we do not know whether phosphorylation exposes the same peptide bond on the α chain. It is also worth pointing out that we cannot be certain that ouabain prevents rubidium occlusion by allowing free access to the sites at which the occluded ions are normally bound. If ouabain blocked access to those sites, and were able to act only when the sites were empty, it would in effect lock the stable door when the horses had bolted. That too would lead to an empty stable.

Stoichiometry

Our finding that the unphosphorylated Na, K-ATPase appears to be capable of occluding three rubidium ions per phosphorylation site or ouabain binding site is surprising, and not only because two potassium ions are believed to be transported each cycle. There have been several studies of the equilibrium binding of potassium or rubidium ions, at low concentrations, and though not all bound ions need be occluded there cannot be more occluded than are bound. Yet, though Hastings & Skou (1980) working with Na, K-ATPase from the salt gland of the spiny dogfish, found that about five potassium ions could be bound per phosphorylation site, and Jensen & Ottolenghi (1982) working with Na, K-ATPase from pig kidney outer medulla, found a figure of three rubidium ions per ouabain-binding site, most workers have found figures near two. Thus in Na, K-ATPase from dog kidney outer medulla, Matsui, Hayashi, Homareda & Kimimura (1977) found that 1·7 potassium ions (displaceable by ouabain) were bound per ouabain-binding site; Matsui, Hayashi, Homareda & Taguchi (1982) found that 1·9 potassium ions (displaceable by ouabain) were bound per ATP-binding site; and Cantley, Cantley & Josephson (1978) found that about two rubidium ions were bound per vanadate-binding site. In Na, K-ATPase from pig kidney outer medulla, Yamaguchi & Tonomura (1979, 1980) and Jørgensen (1982) found that close to two rubidium ions were bound per phosphorylation site.

Because our estimates of ouabain-binding sites and of phosphorylation sites were each made in two ways, and all four estimates agreed reasonably well, a systematic error in the estimated number of sites seems unlikely. The estimates of the maximum amount of rubidium that can be occluded depended on both correction and extrapolation, as explained in the Results; but though both processes were subject to error, the likely errors seem too small to have changed the apparent stoichiometry from two to three. An error in the assumed specific activity of the [^{86}Rb]RbCl could, of course, have given rise to consistently wrong answers, but, in an experiment to check this point, dilution of the [^{86}Rb]RbCl 10-fold with cold RbCl did not lead to a different estimate for the amount of rubidium occluded.

One possibility that we cannot exclude is that our enzyme preparation contained

damaged enzyme which was capable of occluding rubidium ions but whose phosphorylation and ouabain-binding sites were not counted. The surprising resistance of the rubidium-occluding capability of the enzyme to radiation (Richards, Ellory & Glynn, 1981) makes this suggestion plausible, but the fact that ouabain, albeit under conditions different from those used in estimating ouabain-binding sites, totally prevented rubidium occlusion makes it awkward to suppose that the damaged enzyme had lost its ouabain-binding sites.

Whether the true stoichiometry is two or three, it is certainly greater than one: and that is true both when magnesium ions are present (and the curve showing the relation between rubidium occlusion and rubidium concentration is inflected) and when magnesium ions are rigorously excluded (and the saturation curve approximates to a rectangular hyperbola). The effect of magnesium ions on the saturation curve (see p. 33) parallels an effect recently described by Hegyvary & Jørgensen (1981), who found that the degree of quenching of the fluorescence of fluorescein-labelled enzyme varied with the potassium concentration in a way which could be described by a rectangular hyperbola when magnesium was absent but by an inflected curve when magnesium was present. There would seem to be two possible explanations. (1) In the absence of magnesium the binding of a single potassium or rubidium ion is sufficient to convert the E_1 form of the dephosphoenzyme to the E_2 form. This is the explanation suggested by Hegyvary & Jørgensen (1981); but since, in our experiments, the amount of rubidium occluded at saturating rubidium concentrations was not much affected by the presence of magnesium ions, one must assume that even if the binding of one rubidium ion is sufficient to promote the conformational change, more than one ion can be bound. (2) For the conformational change to occur two (or three?) rubidium ions must be bound, but in the absence of magnesium all but one of the binding sites have such a high affinity that the saturation curve reflects binding at a single site. This could be the result of a high intrinsic affinity, so that the saturation curve reflected the filling of the last site, or of strong positive co-operativity, so that the saturation curve reflected the filling of the first site.

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