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OCCLUSION OF COBALT IONS WITHIN THE PHOSPHORYLATED FORMS OF THE Na^+–K^+ PUMP ISOLATED FROM DOG KIDNEY

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SUMMARY

1. Co^{2+} ions can replace Mg^{2+} ions as co-factors for the Na^+–K^+ pump purified from dog kidney outer medulla. The evidence comes from (*a*) measurement of ouabain-sensitive Na^+,K^+-ATPase activity, (*b*) measurement of ATP-dependent ^{22}Na uptake catalysed by the Na^+–K^+ pump reconstituted into phospholipid vesicles, (*c*) measurements of phosphorylation of the Na^+–K^+ pump either in the presence of ATP and sodium ions or in the presence of inorganic phosphate, and (*d*) measurement of occlusion of rubidium ions through the route involving phosphorylation and dephosphorylation.

2. Purified Na^+,K^+-ATPase incubated in the presence of ATP, Na^+ ions and $[^{60}Co]CoCl_2$, can carry occluded Co^{2+} ions through a cation-exchange resin. The enzyme fails to occlude the divalent cation (i) if ADP replaces ATP, (ii) if the enzyme is heat-inactivated, (iii) if the enzyme is inactivated by treatment with fluorescein isothiocyanate, (iv) if K^+ replaces Na^+ in the incubation medium, (v) if Na^+ ions are omitted, and (vi) if Mg^{2+} ions are added in a sufficient concentration.

3. The amount of occluded Co^{2+} ions is unaffected by pre-treatment of the Na^+,K^+-ATPase with oligomycin, which stabilizes the phosphoenzyme in the E_1P form.

4. The addition of K^+ ions to Na^+,K^+-ATPase that has been phosphorylated in the presence of ATP, Na^+ ions and $[^{60}Co]CoCl_2$ releases the occluded Co^{2+} ions from the enzyme. Under those conditions, K^+ ions accelerate the hydrolysis of the phosphoenzyme, and become occluded in the resulting dephosphoenzyme.

5. The stoichiometry of Co^{2+} ion occlusion is about one occluded Co^{2+} ion per phosphorylation site.

6. These results support the hypothesis that, in the normal working of the Na^+–K^+ pump, Mg^{2+} ions are trapped in the phosphorylated forms of the enzyme, and are released by a K^+-dependent dephosphorylation reaction.

INTRODUCTION

It is generally accepted that, in its normal sequence, the Na^+,K^+-ATPase goes through a sequence of phosphorylation and dephosphorylation reactions (for references see Glynn, 1985). Figure 1 shows a model of the functioning of the

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Na^+,K^+-ATPase (Karlish, Yates & Glynn, 1978; Glynn, 1985) to which have been added proposals about the role of Mg^{2+} ions (see Fukushima & Post, 1978; Cantley, 1981, and Results and Discussion). In the presence of Mg^{2+} ions and intracellular Na^+ ions, the enzyme is phosphorylated by ATP to give E_1P, the first form of the phosphoenzyme; the phosphorylation reaction is reversible in the presence of ADP, so E_1P is referred to as an ADP-sensitive phosphoenzyme. This reaction is followed by a conformational change in which E_2P, a second form of the phosphoenzyme, is produced; this phosphoenzyme cannot react with ADP, but K^+ ions accelerate its hydrolysis, so E_2P is a K^+-sensitive phosphoenzyme. The resulting dephosphoenzyme, $E_2(K)$, containing occluded K^+ ions, releases K^+ to the interior after a

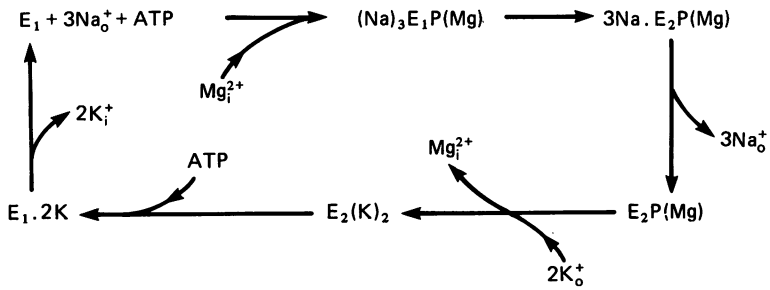

Fig. 1. The scheme is based on a model for Na^+,K^+-ATPase put forward by Karlish, Yates & Glynn (1978), with additional proposals about the role of Mg^{2+} ions (Post *et al.* 1975; Fukushima & Post, 1978; Cantley, 1981, and this paper). For clarity, some intermediate steps in the cycle have not been shown. For further details see Glynn (1985), and Results and Discussion.

second conformational change, which is accelerated by ATP acting at a low-affinity site (for references see Glynn, 1985). Free Mg^{2+} ions are required for the phosphorylation reaction, but apparently not for dephosphorylation of the enzyme, either in the presence or absence of K^+ ions; consequently, Post, Toda & Rogers (1975) suggested that the divalent cations required for phosphorylation normally remain tightly bound to the phosphoenzyme. Fukushima & Post (1978), arguing from phosphorylation experiments in which Ca^{2+} ions replaced Mg^{2+} ions, reached the same conclusion, and suggested, furthermore, that the tightly bound divalent cation was required for the normal reactivity of the phosphoenzyme. In addition, Smith, Zinn & Cantley (1980), on the basis of their experiments on Mn^{2+} binding to vanadate-inhibited Na^+,K^+-ATPase, suggested that a single divalent cation site is involved in vanadate (and probably phosphate) binding.

The experiments described in this paper were done to test directly the hypothesis that the phosphorylated forms of the Na^+,K^+-ATPase contain a tightly bound divalent cation. The method employed was a modification of the rapid ion-exchange technique (Beauge & Glynn, 1979; Glynn & Richards, 1982; Glynn, Hara & Richards, 1984) used previously to detect forms of the Na^+,K^+-ATPase occluding monovalent ions. In this technique the enzyme is mixed with the radioactive ion under appropriate conditions, and then the suspension is passed rapidly through a cation-exchange resin; the radioactive ion, occluded in the enzyme molecule, is detected in the effluent from the columns.

Long-life radioactive isotopes of Mg^{2+} are not available. There were some reports in the literature which suggested that Co^{2+} ions could replace Mg^{2+} ions in the partial reactions catalysed by the Na^+–K^+ pump (Rendi & Uhr, 1964; Fukushima & Post, 1978; Gache, Rossi, Leone & Lazdunski, 1979; Perez, Miara & Dahms, 1979; Fukushima & Nakao, 1980). It is essential for the purpose of the present investigation that the divalent cation used to replace Mg^{2+} ions is capable of supporting all the enzymic activities catalysed by the Na^+,K^+-ATPase which require Mg^{2+} ions, since otherwise it would not be justifiable to use these experiments as a guide to the behaviour of Mg^{2+} ions in the normal working of the Na^+–K^+ pump.

The first part of this paper discusses experiments, using a purified Na^+,K^+-ATPase preparation from dog kidney outer medulla, which confirm and extend some of the earlier reports, showing that Co^{2+} ions can indeed replace Mg^{2+} ions in the normal working of the Na^+–K^+ pump. The second part describes experiments that provide direct evidence for the existence of Co^{2+} ions trapped or occluded in the phosphorylated, but not in the dephosphorylated, forms of the Na^+,K^+-ATPase; these experiments, therefore, support the hypothesis of Fukushima & Post (1978) that Mg^{2+} ions are cyclically bound and released during the normal working of the Na^+–K^+ pump.

A preliminary report of some of these results has already been published (Richards, 1987).

METHODS

Preparation of the enzyme

Dog kidneys were obtained, frozen, from Pel-Freeze, WI, U.S.A., and stored at −70 °C for up to 9 months. The Na^+,K^+-ATPase was prepared from the dark outer medulla by Jorgensen's (1974) simpler method, in which microsomes treated with sodium dodecyl sulphate (SDS) in the presence of ATP are fractionated in discontinuous sucrose gradients. The kidneys were sliced frozen, and the pieces of outer medulla were stored for up to 48 h at −70 °C in a solution containing 250 mM-sucrose, 1 mM-EDTA and 30 mM-histidine. The pieces of outer medulla were then treated for 15 s at maximum speed in a Politron homogenizer. This procedure had no effect on the specific activity of the enzyme, but made the subsequent homogenization in a glass homogenizer with a tight-fitting Teflon pestle much easier (M. Steinberg, personal communication). The Na^+,K^+-ATPase particles were washed twice in a solution containing 25 mM-imidazol (pH 7·5 at 20 °C) and 1 mM-EDTA (Tris salt) (wash solution) to remove traces of ATP and of sodium, and were then stored at concentrations of 1·5–4 mg protein/ml at −70 °C in a solution containing 10% (w/v) sucrose, 25 mM-imidazol (pH 7·5 at 20 °C) and 1 mM-EDTA (Tris salt). Before use, the enzyme suspension was washed once in about 40 volumes of wash solution lacking EDTA, and resuspended in a small volume of the same solution. The specific activities of the enzyme preparations were assayed at 37 °C as described by Glynn *et al.* (1984), and varied between 9·8 and 27 μmol mg protein^{-1} min^{-1}

Measurement of occluded Co^{2+} ions

Normally the whole procedure was carried out at 2–4 °C. Na^+,K^+-ATPase (50–100 μg) was suspended in a medium containing 100 mM-Tris chloride (pH 7·0 at 20 °C), and 30 mM-NaCl. The suspension was mixed with ATP (Tris salt) to a final concentration of 10 μM, and 5 s later [^{60}Co]$CoCl_2$ was added to a final concentration of 10 μM. Fifty seconds later the suspension was forced through a column of cation-exchange resin Dowex 50W (×8, 200–400 mesh) in the Tris form, at a speed such that the enzyme spent about 0·9 s in contact with the column (for details of the apparatus see Glynn & Richards, 1982). The effluents of the columns were collected and analysed for radioactivity (by scintillation counting) and for protein. The columns were prepared in 1 ml plastic disposable syringes and the syringes were immersed up to the 1·0 ml mark in circulating ice-cold water in a purpose-built bath. Perforations had been drilled in the bottom of

the bath, and the tips of the syringes fitted tightly into these perforations, preventing any leakage of water from the bath. The effluents of the columns were collected from beneath the bath.

For each condition experiments were performed in quadruplicate; the results are averages ± S.E.M.

In experiment 1130 of Table 5, performed at 20 °C, the enzyme suspended in a medium containing Tris chloride and Na^+ at the above concentrations was passed rapidly through a small layer of Sephadex G-25 before it entered the resin. The Sephadex layer, which had been previously equilibrated with [^{60}Co]$CoCl_2$ (40 μM), ATP (Tris salt, 40 μM) and Tris chloride and Na^+ at the same concentrations as above, acted as a 'rapid mixing device' (Glynn & Richards, 1982). This procedure prevented long contact between low concentrations of ATP and high concentrations of enzyme, which may have caused a substantial hydrolysis of ATP.

In both experiments of Table 6 the sodium concentration was 200 mM to allow oligomycin to act (see Glynn *et al.* 1984). The Dowex resin in these experiments was replaced by a Chelex-70 resin (200–400 mesh), which allowed the experiments to be performed at the higher sodium concentration. The enzyme was pre-incubated for 30 min at 0 °C in a medium containing NaCl (200 mM) Tris choloride (30 mM) pH 7·4 at 0 °C, with or without oligomycin (20 μg/ml). The resin columns were pre-washed with an ice-cold solution containing NaCl (200 mM), Tris chloride (30 mM), pH 7·4 at 0 °C, with or without oligomycin (20 μg/ml).

In the experiment of Table 7, the enzyme was suspended in a medium containing 30 mM-NaCl and 100 mM-Tris chloride, pH 7·4 at 20 °C; it was then passed quickly through a small Sephadex layer (Glynn & Richards, 1982), equilibrated with the same medium containing also [^{60}Co]$CoCl_2$ (40 μM) and either 40 μM-Tris-ATP or 40 μM-Tris-ADP, and the suspension was immediately mixed in a mixing chamber (Glynn, Howland & Richards, 1985) with an equal volume of a solution containing Tris (100 mM), pH 7·4 at 20 °C, and *either* 30 mM-NaCl *or* 30 mM-KCl. After about 0·5 s, the effluent of the mixing chamber was allowed to pass through a column of cation-exchange resin, in the Tris form, at a speed such that the enzyme spent about 0·9 s in contact with the resin. The whole procedure was carried out at room temperature. The effluents of the resin columns were analysed for radioactivity and protein content.

In the results presented in Table 8, occluded Co^{2+} was measured by the usual procedure (see above) and the amount of Co^{2+} found in the effluents in the presence of ADP was subtracted from the amount found in the presence of ATP.

Phosphorylation of the enzyme from ATP

All procedures were done at 2–4 °C. Na^+,K^+-ATPase (50 μg) was incubated in a medium containing Tris chloride (100 mM) and NaCl. [γ-^{32}P]ATP (Tris salt) was added to a final concentration of 10 μM, and 5 s later either $MgCl_2$ or $CoCl_2$ or water was added. Ten or fifty seconds later 10 volumes of quench solution containing 1·4 mM-perchloric acid, 2 mM-sodium phosphate and 20 mM-ATP (sodium salt) were added. The suspension was left for 20 min in ice, and was then filtered through glass-fibre filters. The filters were washed 5 times – 10 volumes each time – with wash solution containing 5% trichloroacetic acid (TCA), 47 mM-phosphoric acid and 82·7 mM-sodium pyrophosphate. The washed filters were placed in plastic counting vials, and ^{32}P was counted by Cerenkov radiation. The level of phosphorylation in acid-denatured enzyme was taken as a blank. The level of phosphorylation measured in the absence of added divalent cations was 5–10% of the maximum level of phosphorylation and was subtracted from all values shown. The level of phosphoenzyme in experiment 939 of Table 1 is about 20–30% lower than expected from its ATPase activity (9·8 μmol mg protein^{-1} min^{-1}). It is not clear what is the reason for this.

To characterize phosphoenzyme as E_1P or E_2P the procedure of Kuriki & Racker (1976) was used. In short, after 10 s of phosphorylation at 0 °C, we added either CDTA and ADP to a final concentration of 10 mM for each, or CDTA and K^+ ions to a final concentration of 10 mM for each. The reaction was stopped with acid and the denatured protein treated as above.

In the experiments of Table 7 the phosphorylation procedure was exactly as described for the Co^{2+} ion occlusion protocol, except that (i) [^{60}Co]$CoCl_2$ was replaced by $CoCl_2$, (ii) ATP was replaced by [^{32}P]ATP, (iii) the effluents were received in 10 volumes of ice-cold quench solution and the suspension filtered, washed and counted as above, and (iv) the blanks contained 0·4 mM-Tris-CDTA (pH 7·0 at *RT*), and no added divalent cations. As there was a slight delay between the moment when the effluent fluid left the resin bed and the moment when it was mixed with the cold quench

solution (see 'Measurement of occluded Co^{2+} ions', above), it is possible that a small proportion of the phosphoenzyme would have suffered hydrolysis before the enzyme was denatured by the quench solution.

For each condition the experiments were performed in quadruplicate; the results are averages ± S.E.M.

Phosphorylation of the enzyme from inorganic phosphate (P_i)

Experiment 14*N of Table* 2. The procedure was carried out at 0–2 °C. In a total volume of 30 μl, 50 μg of Na^+,K^+-ATPase were resuspended in a medium containing Tris chloride (30 mM), pH 7·4 at the temperature of the experiment, $CoCl_2$ or $MgCl_2$ (2 mM), or Tris-CDTA (0·4 mM), and with or without 10^{-3} M-ouabain. After 10 min incubation [^{32}P]P_i (Tris salt) to a final concentration of 0·4 mM was added, and the suspension incubated for different times. At the end of the incubation 2 ml of solution A, containing 10% polyphosphoric acid, 5% TCA, 47 mM-phosphoric acid and 82·7 mM-sodium pyrophosphate, was added. After 20 min in ice the suspension was filtered as described above and washed 5 times with 5 ml each time of solution A. The washed filters were counted as described above.

Experiment 20*N of Table* 2. Twenty micrograms of Na^+,K^+-ATPase were suspended in 0·5 ml of a solution containing 100 mM-Tris chloride (pH 7·4 at 20 °C) and 0·5 mM-RbCl, and were passed quickly through a small layer of cation-exchange resin in the Tris form (which reduced the free rubidium concentration to less than 0·5 μM), before being mixed in a rapid mixing device (Glynn *et al.* 1985) with an equal volume of a solution containing Tris phosphate (2 mM), Tris chloride (100 mM), pH 7·4 at 20 °C, and either $MgCl_2$ (2 mM), $CoCl_2$ (2 mM) or Tris-CDTA (0·8 mM). This procedure was carried out at 20 °C. After about 0·5 s, the effluent from the mixing chamber was mixed with 5 volumes of ice-cold solution A. After 20 min in ice, the denatured enzyme was washed 5 times by centrifugation (2750 *g*, for 15 min) with solution A. The final pellet was dissolved in 0·5 ml of 4% SDS, incubated for 30 min at 37 °C, and 200 μl aliquots were used for measuring ^{32}P radioactivity (by scintillation counting) and protein content.

In all experiments each determination was carried out in quadruplicate; the values shown have been corrected for the values obtained in the presence of CDTA and are expressed as averages ± S.E.M.

Measurement of Na^+,K^+-ATPase activity

The Na^+,K^+-ATPase activity was assayed at 20 °C in a medium containing 25 mM-imidazol (pH 7·5 at 20 °C), 120 mM-NaCl, 20 mM-KCl and various concentrations of [^{32}P]ATP and divalent cation. The reaction was stopped with TCA (final concentration 5%). The amount of [^{32}P]P_i produced was determined according to Brown (1982). No ouabain-sensitive ATPase activity was detected in the absence of added divalent cations. Each experimental point was performed in quadruplicate, and the points are represented as average ± S.E.M.

^{22}Na uptake catalysed by Na^+,K^+-ATPase incorporated into phospholipid vesicles

The Na^+,K^+-ATPase was incorporated into phospholipid vesicles by the procedure of freeze–thaw sonication of Karlish & Pick (1981). The crude soybean lecithin was resuspended in 500 mM-Tris chloride (pH 7·0 at 20 °C), and after sonication it was dialysed overnight against 1000 volumes of 25 mM-imidazol (pH 7·0 at 20 °C) and 1 mM-EDTA (Tris salt).

For the uptake experiments, the vesicles contained 200 mM-KCl, and the extravesicular medium contained 190 mM-Tris (pH 7·0 at 20 °C), 10 mM-[^{22}Na]NaCl, Tris-ATP or Tris-ADP (3 mM), and either $MgCl_2$ or $CoCl_2$ (3 mM). The ATP-dependent uptake was obtained by subtracting the uptake in the presence of ADP from that in the presence of ATP. In the absence of added divalent cations there was no detectable ATP-dependent uptake. When vanadate was present, it was added to the extravesicular medium.

Each experimental point was performed in triplicate, and the results are expressed as average ± S.E.M.

Protein determination

Protein was determined by a modification (Peterson, 1977) of the method of Lowry, using bovine serum albumin as a standard.

Sources of materials

Soybean lecithin, sodium pyrophosphate, polyphosphoric acid, Tris (Trizma grade), imidazol (grade III), Sephadex G-25, Sephadex G-50-40, fluorescein isothiocyanate, CDTA (trans-1,2-cyclohexilenedinitrilo-tetraacetic acid) (free acid), EDTA (ethylenediamine-tetraacetic acid) (free acid), histidine, molybdic acid (ammonium salt), ouabain, and bovine serum albumin (fatty acid free) were from Sigma, London. NaCl, KCl, $CoCl_2$ and RbCl were the 'Specpure salts' from Johnson & Matthey, Royston, Hertfordshire. $MgCl_2$, amidol (laboratory reagent grade), sucrose (AR grade) and TCA (trichloroacetic acid, AR grade) were from Fisons, Loughborough. [^{22}Na]NaCl, [γ^{32}P]ATP, [^{32}P]P_i, [^{86}Rb]RbCl and [^{60}Co]$CoCl_2$ were from Amersham International. Crystalline disodium ATP and ADP were from Boehringer Mannheim; they were freed from sodium by passage through a Dowex 50W cation-exchange resin, and the solutions were neutralized with Tris base. Phosphoric acid ('Aristar'), perchloric acid ('Aristar'), sodium dodecyl sulphate ('specially purified for biochemical work'), and sodium vanadate were from BDH, Poole. Dowex 50 (×8, 200–400 mesh) and 'Chelex' cation-exchange resins were of analytical reagent grade from Biorad, Watford, Hertfordshire. Glass-fibre filters, GF/C, were obtained from Whatman. All other reagents were of 'Analytical Reagent' grade, if available.

RESULTS

The Na^+,K^+-ATPase can be phosphorylated in the presence of Na^+, ATP and Mg^{2+} (for references, see Glynn, 1985). Table 1 shows that Co^{2+} ions can replace Mg^{2+} ions for phosphorylation in canine kidney ATPase, as is also the case with other kidney preparations of Na^+,K^+-ATPase (Fukushima & Post, 1978; Fukushima & Nakao, 1980). Reducing the concentration of Na^+ from 100 to 30 mM has very little effect on the steady-state level of phosphorylation. Table 1 also shows (experiment 939) that Co^{2+} ions are effective at quite low concentrations, 5 μM giving the same level of phosphorylation as 1 mM, in the presence of 10 μM-ATP.

In Fig. 2 the sensitivity to ADP and K^+ ions of phosphoenzyme formed in the presence of either Co^{2+} ions or Mg^{2+} ions has been compared. It is clear that the addition of K^+ ions causes a rapid dephosphorylation of the enzyme, while the addition of ADP has a much smaller effect on the level of phosphoenzyme, and this is seen with both Mg^{2+} and Co^{2+} ions. The results indicate that the phosphoenzyme formed in the presence of Co^{2+} ions has similar properties to the one formed in the presence of Mg^{2+} ions, and furthermore that it is mostly in the K^+-sensitive E_2P form.

The Na^+,K^+-ATPase can also be phosphorylated by inorganic phosphate, in the presence of Mg^{2+} (for references, see Glynn, 1985). Table 2 shows results of experiments where the enzyme was phosphorylated in the presence of (^{32}P) P_i, and either Mg^{2+} ions or Co^{2+} ions. In experiment 14N, in the presence of ouabain, the amount of phosphoenzyme in the presence of Co^{2+} ions, after 1 h incubation at 0 °C, is about 17 % of the level obtained in the presence of Mg^{2+}. At room temperature, on the other hand, the amount in the presence of Co^{2+} ions is about 85 % of the amount obtained in the presence of Mg^{2+} ions, and therefore Co^{2+} ions may replace Mg^{2+} ions under these conditions.

The long time required to obtain full phosphorylation, even in the presence of Mg^{2+}, is supposed to represent the slow conversion of the Na^+,K^+-ATPase from a form E_1, with low affinity for P_i, to a form E_2 that reacts readily with P_i. The failure of Co^{2+} ions in supporting phosphorylation in the absence of ouabain, compared to a partial effect of Mg^{2+} ions (which is almost independent of the length of incubation,

TABLE 1. Effects of Mg^{2+} or Co^{2+} ions on the phosphorylation of Na^+,K^+-ATPase with ATP

Concentration (mM)	Phosphorylation time (s)	Phosphoenzyme (nmol/mg)
Expt 869		
Na^+ 100, Mg^{2+} 1	10	2·14±0·02
Na^+ 30, Mg^{2+} 1	10	1·92±0·10
Na^+ 30, Co^{2+} 1	10	1·87±0·13
Na^+ 30, Co^{2+} 1	50	1·82±0·02
Na^+ 30, Co^{2+} 0·16	10	2·01±0·09
Na^+ 30, Co^{2+} 0·16	50	1·88±0·11
Expt 939		
Na^+ 30, Co^{2+} 0·005	50	0·75±0·02
Na^+ 30, Co^{2+} 0·010	50	0·79±0·05
Na^+ 30, Co^{2+} 0·100	50	0·80±0·02
Na^+ 30, Co^{2+} 0·160	50	0·76±0·03
Na^+ 30, Co^{2+} 1	50	0·76±0·02
Expt 7N		
Na^+ 30, Mg^{2+} 3	20	1·58±0·05
Na^+ 30, Co^{2+} 0·01	20	1·57±0·03

The enzyme is phosphorylated in the presence of Na^+, [γ^{32}P]ATP and either Mg^{2+} or Co^{2+} ions. For further details see Methods.

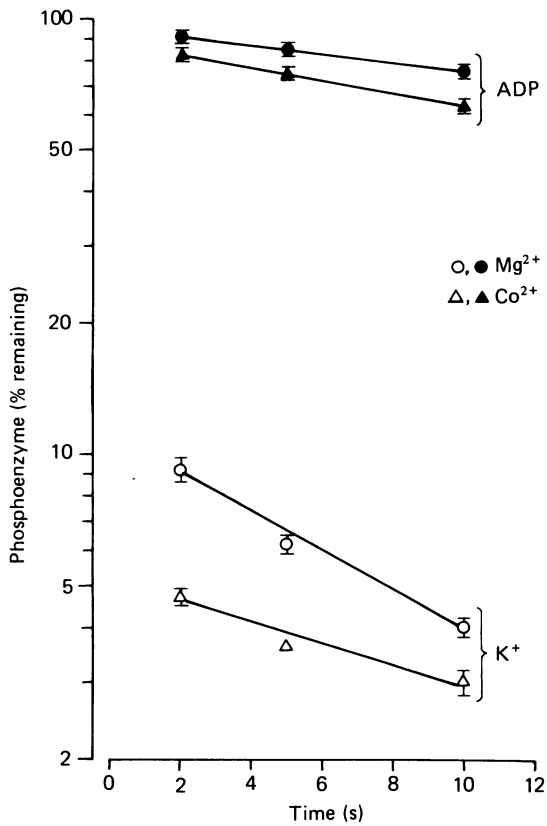

Fig. 2. Measurement of content of E_1 P and E_2 P in Na^+,K^+-ATPase phosphorylated in the presence of Na^+, ATP and either Mg^{2+} or Co^{2+}. For further details see Methods.

see experiment 14N of Table 2), could be taken to mean that Co^{2+} ions are markedly different from Mg^{2+} ions in their properties, and will not support phosphorylation in the presence of inorganic phosphate unless ouabain is present; another possibility is that the conformational change $E_1 \rightarrow E_2$ is slower in the presence of Co^{2+} ions. To decide between these possibilities, we decided to put the enzyme in its E_2 form before P_i and the divalent cation had time to act. This was accomplished, in experiment

TABLE 2. Effects of Mg^{2+} or Co^{2+} ions on phosphorylation of Na^+,K^+-ATPase with inorganic phosphate

Expt 14N

Incubation time	Temperature of incubation (°C)	Ouabain (M)	Phosphoenzyme (nmol/mg) Mg^{2+}	Phosphoenzyme (nmol/mg) Co^{2+}
1 h	0–2	10^{-3}	1·90 ± 0·01	0·32 ± 0·02
30 s	20	10^{-3}	1·49 ± 0·08	1·34 ± 0·07
1 min	20	10^{-3}	1·83 ± 0·03	1·40 ± 0·03
10 min	20	10^{-3}	1·82 ± 0·15	1·56 ± 0·03
30 s	20	—	0·50 ± 0·01	0·05 ± 0·03
1 min	20	—	0·56 ± 0·02	0·01 ± 0·01
10 min	20	—	0·56 ± 0·01	0·02 ± 0·01
1 h	20	—	0·58 ± 0·0	0·02 ± 0·01

Expt 20N

Conditions	Phosphoenzyme (nmol/mg)
1 mM-Mg^{2+}	0·88 ± 0·21
1 mM-Co^{2+}	0·98 ± 0·20
100 μM-Co^{2+}	0·58 ± 0·06

In experiment 14N, the enzyme should be (mostly) in the E_1 conformation before being mixed with radioactive inorganic phosphate. In experiment 20N, the enzyme should be (mostly) in the E_2(Rb) form, containing occluded Rb^+, before being mixed with radioactive inorganic phosphate. For further details see Methods.

20N, by pre-incubating the enzyme with Rb^+ ions, at 20 °C, to form E_2(Rb) (Beauge & Glynn, 1979; Glynn & Richards, 1982) and passing the suspension rapidly through a short column of cation-exchange resin (which reduces the concentration of free Rb^+ ions to nanomolar levels; Glynn *et al.* 1985) before being mixed with radioactive P_i and the divalent cation. After about a second the reaction was stopped with acid. It can be seen that under these conditions Co^{2+} ions are as effective as Mg^{2+} ions as co-factors for phosphorylation of the Na^+,K^+-ATPase from inorganic phosphate. The failure of Co^{2+} ions to support phosphorylation in the conditions of experiment 14N may not then be attributed to a fundamental difference between Co^{2+} ions and Mg^{2+} ions as co-factors of the Na^+,K^+-ATPase.

If the Na,K-ATPase is phosphorylated in the presence of ATP, Mg^{2+} ions and Na^+, and then Rb^+ ions are added, the Rb^+ ions catalyse the hydrolysis of the phosphoenzyme, and themselves become trapped, or occluded, in the resulting dephosphoenzyme (Post, Hegyvary & Kume, 1972; Glynn & Richards 1982). Table 3 shows results of an experiment where it was tested whether Co^{2+} ions could replace Mg^{2+} ions in this reaction. As can be seen, the amount of occluded rubidium obtained in the presence of Co^{2+} ions is about 90% of the amount obtained in the presence of magnesium.

TABLE 3. Effects of Mg^{2+} or Co^{2+} ions on the occlusion of Rb^+ ions through the route involving phosphorylation and dephosphorylation

Conditions	Rb^+ in effluent (nmol/mg)	Occluded Rb^+ (nmol/mg)
Mg^{2+}, ATP	$3{\cdot}98 \pm 0{\cdot}15$	$3{\cdot}82 \pm 0{\cdot}15$
Mg^{2+}, ADP	$0{\cdot}16 \pm 0{\cdot}01$	
Co^{2+}, ATP	$3{\cdot}68 \pm 0{\cdot}07$	$3{\cdot}52 \pm 0{\cdot}07$
Co^{2+}, ADP	$0{\cdot}16 \pm 0{\cdot}01$	

The enzyme was mixed with Na^+, ATP (or ADP) and either Mg^{2+} or Co^{2+} ions; [^{86}Rb]RbCl was then added, and the level of occlusion of Rb^+ by the enzyme was measured. For further details see Glynn & Richards (1982).

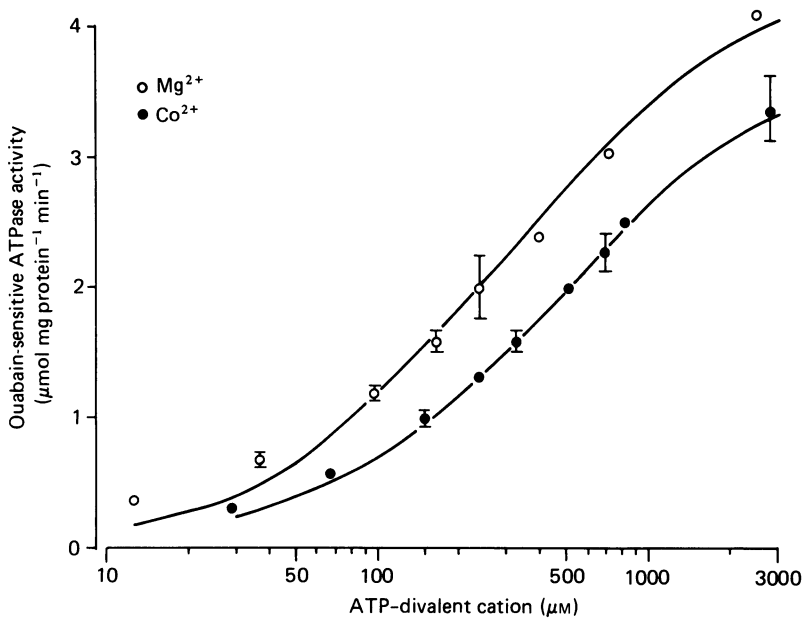

Fig. 3. Ouabain-sensitive (Na^+,K^+-ATPase activity in the presence of either Mg^{2+} or Co^{2+} ions. The abscissa shows the calculated concentrations of the complex of ATP with the divalent cation (Sillen & Martell, 1971). The drawn lines have been obtained by fitting the experimental points to a curve of the form $A = B/(1+C/D)$, where A is the ouabain-sensitive ATPase activity, D is the concentration of the complex of ATP with the divalent cation, and B and C are constants (Wilkinson, 1961). For Mg^{2+}, $B = 4{\cdot}4 \pm 0{\cdot}2$ μmol min^{-1} mg^{-1} and $C = 298 \pm 28$ μM. For Co^{2+} ions, $B = 3{\cdot}8 \pm 0{\cdot}2$ μmol min^{-1} mg^{-1} and $C = 453 \pm 50$ μM. For further details see Methods.

Figure 3 shows the result of an experiment where the ouabain-sensitive Na^+,K^+-ATPase activity of the enzyme purified from dog kidney outer medulla has been measured in the presence of either Co^{2+} ions or Mg^{2+} ions. The activity measured in the presence of Co^{2+} ions, at all concentrations tested (from 50 μM to 3 mM, added in equimolar concentrations with ATP), is 50–80% of the same enzymic activity measured in the presence of Mg^{2+} ions.

I have also tested whether Co^{2+} ions can replace Mg^{2+} ions as co-factors for the

transport of Na^+ and K^+ ions catalysed by the Na^+–K^+ pump. Na^+,K^+-ATPase purified from dog kidney outer medulla was incorporated into phospholipid vesicles, by the method of freeze–thaw sonication (Karlish & Pick, 1981), and the ATP-dependent uptake of radioactive Na^+ into K^+-filled vesicles was measured. (In this case, only the pump molecules which have their substrate site facing the outside of the vesicles are effective in catalysing ATP-dependent transport.) The results of

TABLE 4. Effects of Mg^{2+} or Co^{2+} ions on ATP-dependent ^{22}Na uptake catalysed by the Na^+–K^+ pump incorporated into phospholipid vesicles

	Conditions (mM)	^{22}Na uptake (nmol min^{-1} 10 μl $vesicles^{-1}$)	ATP-dependent ^{22}Na uptake (nmol min^{-1} 10 μl $vesicles^{-1}$)
Expt 1154	Mg^{2+} 3, ATP 3	1·00±0·05	0·93±0·05
	Mg^{2+} 3, ADP 3	0·07±0·01	
	Co^{2+} 3, ATP 3	0·81±0·03	0·74±0·03
	Co^{2+} 3, ADP 3	0·07±0·01	
Expt 208N	Mg^{2+} 3, ATP 3	1·80±0·08	1·62±0·08
	Mg^{2+} 3, ADP 3	0·18±0·01	
	Co^{2+} 3, ATP 3	1·68±0·03	1·52±0·03
	Co^{2+} 3, ADP 3	0·16±0·01	
	Mg^{2+} 3, ATP 3, vanadate 0·1	0·14±0·01	0·03±0·01
	Mg^{2+} 3, ADP 3, vanadate 0·1	0·11±0·01	
	Co^{2+} 3, ATP 3, vanadate 0·1	0·12±0·02	0·02±0·02
	Co^{2+} 3, ADP 3, vanadate 0·1	0·10±0·01	

The method used was essentially as described by Karlish & Pick (1981). For further details see Methods.

experiments of this kind are shown in Table 4, where it can be seen that Co^{2+} ions can replace Mg^{2+} ions for active transport of Na^+ into K^+-containing vesicles, and that the transport is inhibited by vanadate at a concentration of 10^{-4} M. The value of the ATP-dependent uptake of ^{22}Na in the presence of Co^{2+} ions is between 10 and 20% lower than the corresponding value in the presence of Mg^{2+} ions.

The results shown in Figs 2 and 3, and Tables 1–4, taken together show that Co^{2+} ions can replace Mg^{2+} ions in the normal working of the Na^+–K^+ pump, and that we may use Co^{2+} ions to obtain information about the role of Mg^{2+} ions in this enzyme. According to the hypothesis put forward by Post *et al.* (1975) and Fukushima & Post (1978), Mg^{2+} ions, which are required for phosphorylation of the Na^+,K^+-ATPase, bind tightly to the resulting phosphoenzyme (see Introduction). If this is so, then if the enzyme is phosphorylated in the presence of radioactive Co^{2+} ions, under the appropriate conditions it should be possible to detect these trapped, or occluded, ions; for example, as an extra amount of radioactivity carried through a cation-exchange resin, as compared with the amount carried under conditions that do not allow phosphorylation. This was tested by mixing enzyme with ATP, Na^+ ions and [^{60}Co]$CoCl_2$ for 50 s, and passing the suspension through a cation-exchange resin; the enzyme spent about 0·9 s in contact with the resin, and the whole procedure was

carried out at 0–4 °C. The effluents were collected and analysed for radioactivity and protein content. As a control we replaced ATP by ADP, which does not phosphorylate the enzyme. Table 5, experiment 873, shows that in the presence of

TABLE 5. Occlusion of Co^{2+} ions by the Na^+,K^+-ATPase

	Conditions	Co^{2+} ions in effluent (nmol/mg)	Occluded Co^{2+} (nmol/mg)
Expt 873	ATP, Na^+	$7{\cdot}08 \pm 0{\cdot}13$	$2{\cdot}52 \pm 0{\cdot}26$
	ADP, Na^+	$4{\cdot}57 \pm 0{\cdot}23$	
Expt 1130 (done at *RT*)	ATP, Na^+	$2{\cdot}70 \pm 0{\cdot}06$	$1{\cdot}19 \pm 0{\cdot}06$
	ADP, Na^+	$1{\cdot}51 \pm 0{\cdot}02$	
Expt 163N	ATP, Na^+	$3{\cdot}63 \pm 0{\cdot}05$	$2{\cdot}46 \pm 0{\cdot}06$
	ADP, Na^+	$0{\cdot}17 \pm 0{\cdot}04$	
	No added nucleotide, Na^+	$1{\cdot}18 \pm 0{\cdot}02$	$0{\cdot}01 \pm 0{\cdot}05$
	ATP, no added Na^+	$1{\cdot}27 \pm 0{\cdot}03$	$0{\cdot}10 \pm 0{\cdot}05$
Expt 932	ATP, Na^+, native enzyme	$3{\cdot}35 \pm 0{\cdot}02$	$1{\cdot}15 \pm 0{\cdot}20$
	ADP, Na^+, native enzyme	$2{\cdot}19 \pm 0{\cdot}03$	
	ATP, Na^+, heat-inactivated enzyme	$2{\cdot}87 \pm 0{\cdot}13$	$-0{\cdot}27 \pm 0{\cdot}13$
	ADP, Na^+, heat-inactivated enzyme	$3{\cdot}14 \pm 0{\cdot}03$	
Expt 161N	ATP, Na^+, native enzyme	$3{\cdot}67 \pm 0{\cdot}04$	$2{\cdot}42 \pm 0{\cdot}06$
	ADP, Na^+, native enzyme	$1{\cdot}25 \pm 0{\cdot}04$	
	ATP, Na^+, FITC-treated enzyme	$1{\cdot}27 \pm 0{\cdot}02$	$0{\cdot}06 \pm 0{\cdot}05$
	ADP, Na^+, FITC-treated enzyme	$1{\cdot}21 \pm 0{\cdot}04$	
Expt 937	ATP, Na^+	$3{\cdot}69 \pm 0{\cdot}15$	$1{\cdot}28 \pm 0{\cdot}17$
	ADP, Na^+	$2{\cdot}41 \pm 0{\cdot}08$	
	ATP, K^+	$2{\cdot}30 \pm 90{\cdot}14$	$0{\cdot}11 \pm 0{\cdot}20$
	ADP, K^+	$2{\cdot}19 \pm 0{\cdot}14$	
Expt 231N	ATP, Na^+, Co^{2+} 10 μM	$3{\cdot}85 \pm 0{\cdot}07$	$2{\cdot}62 \pm 0{\cdot}08$
	ADP, Na^+, Co^{2+} 10 μM	$1{\cdot}23 \pm 0{\cdot}04$	
	ATP, Na^+, Co^{2+} 1 μM	$1{\cdot}70 \pm 0{\cdot}10$	$1{\cdot}47 \pm 0{\cdot}10$
	ADP, Na^+, Co^{2+} 1 μM	$0{\cdot}23 \pm 0{\cdot}01$	
	ATP, Na^+, Co^{2+} 0·1 μM	$0{\cdot}30 \pm 0{\cdot}03$	$0{\cdot}27 \pm 0{\cdot}03$
	ADP, Na^+, Co^{2+} 0·1 μM	$0{\cdot}03 \pm 0{\cdot}01$	
	ATP,Na^+,Co^{2+} 10 μM,Mg^{2+} 1 mM	$1{\cdot}08 \pm 0{\cdot}01$	$-0{\cdot}04 \pm 0{\cdot}07$
	ADP,Na^+,Co^{2+} 10 μM,Mg^{2+} 1 mM	$1{\cdot}12 \pm 0{\cdot}07$	
	ATP,Na^+,Co^{2+} 10 μM,Mg^{2+} 0·1 mM	$2{\cdot}03 \pm 0{\cdot}09$	$0{\cdot}90 \pm 0{\cdot}09$
	ADP,Na^+,Co^{2+} 10 μM,Mg^{2+} 0·1 mM	$1{\cdot}13 \pm 0{\cdot}01$	
	ATP,Na^{2+},Co^{2+} 10 μM,Mg^{2+} 10 μM	$3{\cdot}25 \pm 0{\cdot}06$	$2{\cdot}18 \pm 0{\cdot}07$
	ADP,Na^+,Co^{2+} 10 μM,Mg^{2+} 10 μM	$1{\cdot}07 \pm 0{\cdot}04$	

For details of each experiment, see text and Methods.

ADP there is a small amount of radioactivity coming through the resin, but that this amount is increased to about double when the enzyme is phosphorylated. This extra amount carried through the resin in the presence of ATP is taken to be occluded Co^{2+} ions. Experiment 1130 shows that the extra amount is also present when the experiment is done at 20 °C. In experiment 163N, lines 2 and 3, it can be seen that

the same basal level of radioactivity in the effluents is obtained in the presence of ADP as in the absence of nucleotides, indicating that ADP can be used as an adequate control.

Before one can be sure that this extra amount of Co^{2+} ions in the effluents is carried by the phosphorylated form(s) of the enzyme, we have to discard a trivial explanation for these results, namely that the extra amount of Co^{2+} ions appearing in the effluents of the columns in the presence of ATP is the result of the binding of Co^{2+} ions to ATP, and not, as proposed, to the phosphorylated enzyme (ATP can bind Co^{2+} ions, log $K = 4{\cdot}8$ at 0·4 °C; Sillen & Martell, 1971). This possibility seems unlikely because of the results of several experiments, also shown in Table 5. (i) Inactivation of the enzyme, by heating for 30 min at 90 °C in a water bath, totally abolishes the Na^+,K^+-ATPase activity (not shown), and also the Co^{2+} ion occlusion (experiment 932). (ii) Fluorescein isothyocyanate (FITC) is a reagent which binds covalently to the Na^+,K^+-ATPase and inhibits the enzyme by blocking phosphorylation from ATP (Karlish, 1980). Treatment of purified dog kidney Na^+,K^+-ATPase with FITC so that only 5% of the original Na^+,K^+-ATPase activity remained (not shown) prevents the enzyme from occluding Co^{2+} ions (experiment 161N). (iii) If the enzyme is incubated in the presence of $[^{60}Co]CoCl_2$ and ATP but in the absence of Na^+ ions (which are an essential requirement for phosphorylation of the enzyme), we can detect no occluded Co^{2+} ions (line 4 of experiment 163N).

In the presence of ADP and Na^+ the enzyme is in the E_1 conformation, so these results suggest that in the E_1 form the enzyme does not occlude divalent cations. Experiment 937 of Table 5 suggests that neither does the E_2 form of the enzyme occlude divalent cations: if the enzyme is mixed with ATP, $[^{60}Co]CoCl_2$ and K^+ ions, there is no extra amount of radioactivity appearing in the effluent of the columns. Taking into consideration the concentrations of ATP and K^+ ions in this experiment one can calculate that more than 95% of the enzyme should have been in the E_2 unphosphorylated form (Beauge & Glynn, 1980).

Also in Table 5 it is shown (experiment 231N) that half the maximum level of occluded Co^{2+} ions is obtained at a concentration of Co^{2+} ions of about 1 μM, in the presence of 10 μM-ATP.

If Co^{2+} ions are indeed bound to (and subsequently occluded in) a site that is physiologically occupied by Mg^{2+} ions, it is to be expected that the amount of occluded Co^{2+} ions should be reduced if Mg^{2+} ions are included in the incubation medium. As can be seen also in experiment 231N of Table 5, in the presence of 1 mM-Mg^{2+} ions (with 10 μM of both $[^{60}Co]CoCl_2$ and ATP) there are no detectable occluded Co^{2+} ions, and this effect is half-saturated at between 10 and 100 μM of added magnesium.

The results of Table 5 therefore suggest that the phosphorylated forms of the Na^+K^+-ATPase can carry occluded divalent cations, and that the dephosphorylated forms of the enzyme do not. As has already been shown (Fig. 2), under the conditions of the experiments of Table 5 most of the phosphoenzyme is in the K^+-sensitive form, E_2P. It is of interest to know if the ADP-sensitive phosphoenzyme, E_1P, does also occlude divalent ions. For this purpose, purified Na^+,K^+-ATPase was inhibited with oligomycin, a treatment that blocks the conversion of E_1P to E_2P (for references see Glynn, 1985). The results of two experiments (Table 6) show that the

phosphorylated intermediate formed in the presence of oligomycin also carries an excess of radioactivity through the resin columns, and that the amount of occluded Co^{2+} ions is very similar in the presence and in the absence of oligomycin. A control to experiment 1N performed identically, but with [^{32}P]ATP and $CoCl_2$ ions replacing ATP and [^{60}Co]$CoCl_2$, showed that in the oligomycin-treated enzyme, more than 95% of the phosphoenzyme was sensitive to ADP, but insensitive to K^+ ions, i.e. was

TABLE 6. Effect of oligomycin on Co^{2+} occlusion by the Na^+,K^+-ATPase

	Conditions	Co^{2+} in effluent (nmol/mg)	Occluded Co^{2+} (nmol/mg)
Expt 985	ATP	4·85±0·13	2·12±0·22
	ADP	2·73±0·18	
	ATP, oligomycin	4·76±0·09	2·18±0·12
	ADP, oligomycin	2·58±0·07	
Expt 1N	ATP	6·22±0·12	2·94±0·20
	ADP	3·28±0·16	
	ATP, oligomycin	6·59±0·28	3·49±0·29
	ADP, oligomycin	3·10±0·09	

For details, see Methods.

TABLE 7. Effect of adding K^+ to Na^+,K^+-ATPase containing occluded Co^{2+} (experiment 1132)

Conditions	Mixed rapidly with	Co^{2+} in effluent (nmol/mg)	Occluded Co^{2+} (nmol/mg)
Na^+, ATP	Na^+	3·57±0·25	1·81±0·32
Na^+, ADP	Na^+	1·76±0·20	
Na^+, ATP	K^+	1·84±0·08	0·01±0·15
Na^+, ADP	K^+	1·83±0·13	

The enzyme was mixed with Na^+, ATP (or ADP) and [^{60}Co]$CoCl_2$, and then Na^+ or K^+ ions were added for about 0·5 s; the suspension was then passed through columns of cation-exchange resin, and the level of occluded Co^{2+} was determined. For further details see Methods.

in the E_1P form (not shown). Therefore, these results, together with those of Table 4, show that both forms of the phosphoenzyme, E_1P and E_2P, contain occluded divalent cations, but that the dephosphorylated forms of the enzyme, E_1 and E_2, do not.

In Table 4 it was shown that if the enzyme is in the $E_2(K)$ form it does not occlude divalent ions. One may expect, therefore, that in the normal working of the pump, when extracellular K^+ ions catalyse the dephosphorylation of E_2P to produce $E_2(K)$, the occluded divalent cation should be released. This point has been tested by allowing the enzyme to be phosphorylated in the presence of ATP, Na^+ and [^{60}Co]$CoCl_2$, for 50 s at 0 °C, adding *either* 30 mM-K^+ ions *or* 30 mM-Na^+ ions to the resulting phosphoenzyme, and about 1 s later passing the suspension through a column of cation-exchange resin (see Methods). The effluents were analysed for radioactivity and protein content. The results are shown in Table 7, where we can see that if K^+ ions are added after phosphorylation, the enzyme does not carry occluded Co^{2+} ions, which agrees with the prediction made above.

In order to measure the stoichiometry of Co^{2+} occlusion, that is the number of occluded Co^{2+} ions per pump molecule, I compared the amount of phosphoenzyme formed in the presence of Na^+, ATP and Co^{2+} ions (a measure of the total amount of enzyme present in the preparation used) with the amount of occluded Co^{2+} obtained, both measured under the same conditions (see Methods). Table 8 shows the results

TABLE 8. Stoichiometry of Co^{2+} occlusion by the Na^+,K^+–ATPase

Expt	Co^{2+} occlusion (a)	Phosphoenzyme (b)	Stoichiometry (a/b)
1139	$2{\cdot}18 \pm 0{\cdot}20$	$1{\cdot}73 \pm 0{\cdot}05$	$1{\cdot}26 \pm 0{\cdot}12$
11N	$1{\cdot}70 \pm 0{\cdot}13$	$1{\cdot}22 \pm 0{\cdot}04$	$1{\cdot}39 \pm 0{\cdot}14$
171N	$3{\cdot}67 \pm 0{\cdot}07$	$2{\cdot}99 \pm 0{\cdot}07$	$1{\cdot}23 \pm 0{\cdot}04$
181N	$3{\cdot}09 \pm 0{\cdot}07$	$2{\cdot}54 \pm 0{\cdot}03$	$1{\cdot}22 \pm 0{\cdot}03$

For details, see Methods.

of four experiments, in each of which the occlusion of Co^{2+} ions and the phosphoenzyme level in the presence of Co^{2+} ions have been measured, and the ratio between these two values, i.e. the stoichiometry of Co^{2+} ion occlusion, has been calculated. The results, taken at face value, show that slightly more than one Co^{2+} ion is occluded per phosphorylation site (see Discussion).

DISCUSSION

Co^{2+} ions as replacement for Mg^{2+} ions in the Na^+–K^+ pump

The objective of this investigation was to obtain information about the role that Mg^{2+} ions play in the normal working of the Na^+–K^+ pump. As the experimental approach relied on replacing Mg^{2+} ions with another divalent cation, it was essential that the replacing divalent cation could substitute for Mg^{2+} ions in supporting the enzymic activities catalysed by the Na^+–K^+ pump, because only then would the results obtained have physiological significance.

There have been several reports on the effects of replacing Mg^{2+} ions by Co^{2+} ions in some of the partial reactions catalysed by the Na^+–K^+ pump (Rendi & Uhr, 1964; Gache *et al.* 1979; Perez *et al.* 1979; Fukushima & Nakao, 1980). The results presented in the first part of this paper show that Co^{2+} ions can replace Mg^{2+} ions in the normal functioning of the Na^+–K^+ pump purified from dog kidney outer medulla. This statement is based on several lines of evidence. In steady-state experiments in the presence of ATP, both qualitatively and quantitatively the phosphoenzyme formed in the presence of Co^{2+} ions is very similar to the one formed in the presence of Mg^{2+} ions: it is mostly in the E_2P form, as indicated by its low sensitivity to ADP and its high sensitivity to K^+ ions (Fig. 2) and the monovalent cation accelerating the dephosphorylation of the enzyme becomes occluded in the dephosphoenzyme (Table 3). In the presence or absence of ouabain, Co^{2+} ions can replace Mg^{2+} ions as a co-factor in the phosphorylation from P_i (Table 2). In Fig. 3, it is shown that the Na^+,K^+-ATPase activity in the presence of Co^{2+} ions is about 50–80% of the same activity when Mg^{2+} ions are present. And finally, in the presence of Co^{2+} ions, the purified Na^+,K^+-pump, inserted into artificial phospholipid vesicles, can catalyse the

ATP-dependent and vanadate-sensitive uptake of ^{22}Na at a rate that is only about 10–20% less than the rate observed in the presence of Mg^{2+} ions (Table 4).

These results, taken together, indicate that the Na^+,K^+-ATPase purified from dog kidney outer medulla reacts similarly to Co^{2+} ions and Mg^{2+} ions, and that any results obtained with Co^{2+} ions as co-factor of the Na^+–K^+ pump is relevant to the behaviour of the enzyme in the presence of Mg^{2+} ions.

Occlusion of Co^{2+} ions in the Na^+,K^+-ATPase

The experiments of Fukushima & Post (1978), using calcium as a replacement of Mg^{2+} ions for phosphorylation studies (calcium does not support Na^+,K^+-ATPase activity), and the experiments of Smith *et al.* (1980), measuring binding of Mn^{2+} ions to an enzyme preparation inhibited by vanadate, suggested that the divalent cation necessary for phosphorylation of the enzyme (either in the presence of ATP or of P_i) became tightly bound to the phosphoenzyme. We have tested this prediction by allowing the enzyme to be phosphorylated in the presence of radioactive isotopes of Co^{2+} ions, and attempting to measure the trapped ion by the method of rapid ion exchange (Beauge & Glynn, 1979; Glynn & Richards, 1982). The results (Table 5) have shown that in the presence of ATP, but not ADP, the enzyme carries an extra amount of Co^{2+} ions through the ion-exchange resin. This extra amount of radioactivity in the presence of ATP has been taken to be occluded Co^{2+} ions. The concept has its equivalent in the occlusion of monovalent cations by the Na^+–K^+ pump (Post *et al.* 1972; Beauge & Glynn, 1979; Glynn & Richards, 1982; Glynn *et al.* 1984; Forbush, 1985), and implicit in its definition is the idea that the occluded ion is hidden or trapped in a cavity in one of the conformations of the transport protein, and has no access to either the intracellular or the extracellular media until the enzyme adopts a different conformation.

Conformations of the enzyme that occlude divalent cations

In experiments in which there was no phosphorylation (either because the Na^+,K^+-ATPase had been inactivated by heat treatment, or by treatment with FITC, or because Na^+ ions had been omitted from the incubation media), the enzyme failed to occlude Co^{2+} ions. Therefore the occlusion of the divalent cation requires phosphorylation of the enzyme. Because it was shown in Fig. 2 that more than 90% of the phosphoenzyme formed in the presence of ATP is in the K^+-sensitive E_2P form, the experiments of Table 5 suggest that the E_2P form of the enzyme occludes divalent cations. As shown in the same table, neither of the dephosphorylated forms of the enzyme, E_1 or E_2, can occlude Co^{2+} ions.

In the original proposal of Fukushima & Post (1978), both forms of the phosphoenzyme trapped Mg^{2+} ions. In the experiments described in Table 6 it can be seen that if the Na^+,K^+-ATPase is in the E_1P form (following treatment with oligomycin, which blocks the transition from E_1P to E_2P) (see Glynn, 1985, for references), the amount of occluded Co^{2+} ions is not significantly different from the amount obtained when the enzyme is in the E_2P form. This indicates that both phosphorylated forms of the Na^+,K^+-ATPase can occlude divalent cations in the normal reaction sequence. The results do not allow one to say whether there is any difference in affinity for the divalent cation between the two forms of the

phosphoenzyme; such a difference was found by Fukushima & Nakao (1980) in experiments where calcium ions replaced Mg^{2+} ions.

The stoichiometry of divalent cation occlusion

The results shown in Table 7 address the question of how many divalent cations are occluded per molecule of enzyme. The calculated ratio between the phosphoenzyme levels obtained in the presence of ATP (a measure of the number of enzyme molecules present in the enzyme preparation) and the amount of occluded Co^{2+} ions gives a value slightly higher than 1 ($1{\cdot}28 \pm 0{\cdot}8$) for the stoichiometry of Co^{2+} ion occlusion. The results could be explained either by proposing a very unlikely stoichiometry for divalent cation occlusion in the Na^+–K^+ pump, or they could be the result of a systematic error. Since there are reasons to believe (see Methods) that the value of phosphoenzyme level may be underestimated in these experiments, it is likely that the stoichiometry of divalent cation binding is one Co^{2+} ion occluded per phosphorylation site.

The release of the occluded divalent cation

It was shown that if the enzyme is in the E_2 form, it fails to occlude Co^{2+} ions. This suggests that, in the normal reaction when the extracellular K^+ ions catalyse the hydrolysis of E_2P to produce $E_2(K)$, the occluded divalent cation is released. That this is indeed the case is suggested by the results shown in Table 7: if the enzyme is allowed to occlude divalent cations in the E_2P form, and then K^+ ions are added, the resulting dephosphoenzyme does not carry occluded Co^{2+} ions through the ion-exchange resin.

The phosphorylation reaction requires Mg^{2+} ions acting at an intracellular site (see Glynn, 1985, for references). As far as I am aware there has never been any suggestion that the divalent cation that supports phosphorylation of the Na^+,K^+-ATPase is itself transported across the membrane. Nevertheless, the experiments mentioned above on release of the occluded divalent cation by a dephosphorylation reaction catalysed by (presumably) extracellular K^+ ions, prompts the question where is the divalent cation released? In experiments with Na^+,K^+-ATPase incorporated into phospholipid vesicles, similar to the ones described in Table 4 but where [^{22}Na]NaCl was replaced by NaCl and $CoCl_2$ was replaced by [^{60}Co]$CoCl_2$, there was no detectable ATP-dependent [^{60}Co]$CoCl_2$ uptake (not shown). Presumably, then, in the normal working of the Na^+–K^+ pump (see Fig. 1), one intracellular Mg^{2+} ion becomes occluded (this paper) together with three Na^+ ions (Glynn *et al.* 1985) in each phosphorylated pump molecule. After the Na^+ ions are released to the outside, extracellular K^+ ions catalyse the dephosphorylation of E_2P; the occluded Mg^{2+} ions are driven off the enzyme, and are released to the interior of the cell.

The present results represent an initial characterization of the physiological Mg^{2+}-occluded state of the Na^+,K^+-ATPase, using [^{60}Co]Co^{2+} ions as a substitute for Mg^{2+} ions. This should enable future studies on the kinetics of divalent cation occlusion, concentration dependence, the kinetics of divalent cation de-occlusion, and comparative studies with other Mg^{2+}-requiring ATPases.

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