J. Physiol. (1982), **328**, *pp.* 295–316
With 9 text-figures
Printed in Great Britain

PASSIVE RUBIDIUM FLUXES MEDIATED BY Na–K-ATPase RECONSTITUTED INTO PHOSPHOLIPID VESICLES WHEN ATP- AND PHOSPHATE-FREE

BY S. J. D. KARLISH* AND W. D. STEIN†

*From the * Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel, and the † Institute of Life Science, Hebrew University, Jerusalem, Israel*

(*Received 19 August 1981*)

SUMMARY

1. Phospholipid vesicles reconstituted with Na–K-ATPase from pig kidney, show slow passive pump-mediated ^{86}Rb fluxes in the complete absence of ATP and phosphate.

2. The Rb fluxes are inhibited in vesicles prepared from enzyme pre-treated with either ouabain or vanadate ions. Rb fluxes through Na–K pumps oriented inside-out or right-side out by comparison with the normal cellular orientation can be distinguished by effects of vanadate on one or both sides of the vesicle.

3. ^{86}Rb uptake into Rb-loaded vesicles represents a ^{86}Rb–Rb exchange. The maximal rate of exchange through inside-out and right-side out oriented pumps is equal, suggesting a random arrangement of the pumps across the vesicle membrane. This Rb–Rb exchange is half-saturated on inside-out and right-side out pumps at about 0·6 and 0·2 mM-external Rb respectively.

4. ^{86}Rb uptake into Rb-free vesicles represents a net Rb flux. The Rb uptake through inside-out pumps has a maximal rate about equal to the Rb–Rb exchange, half-saturates at an external Rb concentration of roughly 0·5 mM, and shows evidence for co-operativity. Net Rb uptake through right-side out pumps is very slow, and half-saturates at roughly 0·1 mM external Rb.

5. K ions at low concentrations in the exterior medium stimulate ^{86}Rb uptake, but at high concentrations, inhibit. Na ions in the exterior medium always inhibit ^{86}Rb uptake. The result suggests that K ions are transported in co-operative fashion together with Rb ions, while Na ions block the Rb fluxes.

6. The presence of Rb congeners at the vesicle interior raises the ^{86}Rb uptake through inside-out pumps with the decreasing order of effectiveness: Li > Na > Cs > K > Rb. Stimulation by Na ions involves a Rb–Na exchange.

7. Turnover numbers were estimated from parallel measurement of Na/K pump mediated fluxes and amount of covalent phosphoenzyme. In units of moles of ion per mole of phosphoenzyme per second at 20 °C the following values were obtained: ATP-dependent Na–Rb exchange, 43; (ATP + phosphate)-stimulated Rb–Rb exchange, 7. For (ATP + phosphate)-independent fluxes: Rb–Rb exchange 0·25; net Rb uptake 0·15 and Rb–Na exchange 0·65.

8. Mg ions in the exterior medium inhibited both net and exchange Rb fluxes

through inside-out pumps in a manner antagonistic with respect to Rb. Mg and vanadate ions inhibit the Rb fluxes in a synergistic fashion.

9. The results are interpreted in terms of a model in which net and exchange ^{86}Rb fluxes occur via conformational transitions between form E_1 which binds Rb at the cytoplasmic face of the protein, the form $E_2\,(Rb)_{occ}$ containing occluded Rb ions and a form E_2 which binds Rb at the extracellular face of the protein. A kinetic analysis allows us to identify rate-limiting steps of the transport cycle by making use of our transport data in combination with values of rate-constants for conformational transitions observed directly in isolated Na–K-ATPase.

INTRODUCTION

It is now widely accepted that active Na and K transport by the Na–K-ATPase involves protein conformational changes. Two major non-phosphorylated conformational forms of the Na–K-ATPase have been detected by a variety of techniques. A form term E_1 or E_1 Na predominates in (Na and K)-free media or in a Na-rich medium respectively. The form $E_2(K)_{occ}$ containing occluded K ions predominates in a K-rich medium, (Post, Hegevary & Kume, 1972; Jorgensen, 1975; Karlish, Yates & Glynn, 1978; Beauge & Glynn, 1979). From studies of the interconversions of these two forms using a number of fluorescent probes we have suggested the following transitions occur, (Karlish *et al.* 1978; Karlish & Yates, 1978; Karlish, 1980).

Scheme I: $E_1Na \rightleftharpoons Na + E_1 + K \rightleftharpoons E_1K \rightleftharpoons E_2(K)_{occ}$.

E_1 is a form with binding sites for Na or K ions, which as we have shown recently, face the cytoplasm (Karlish & Pick, 1981). Upon binding of K to the form E_1 a spontaneous conformation transition $E_1K \rightarrow E_2(K)_{occ}$ traps the K ions on the protein. The reverse conformational transition $E_2(K)_{occ} \rightarrow E_1$ allows release of K at the cytoplasmic surface. The transition $E_2(K)_{occ} \rightarrow E_1$ is very slow but is greatly stimulated by ATP (Post *et al.* 1972; Karlish *et al.* 1978; Karlish & Yates, 1978; Glynn & Richards, 1980). Inward K movements in normal ATP-dependent Na–K exchange and the well-studied $(ATP+P_i)$-stimulated K–K exchange (Glynn, Lew & Luthi, 1970; Simons, 1974), are thought to involve this conformational transition stimulated by ATP (Karlish *et al.* 1978). The outward movement of K in the $(ATP+P_i)$-stimulated K–K exchange has been thought to involve phosphorylation of $E_2(K)_{occ}$ by inorganic phosphate leading to the appearance of K-binding sites at the extracellular surface (Karlish *et al.* 1978; Glynn, Karlish & Yates, 1979).

It occurred to us that a conformational transition of the occluded K form $E_2(K)_{occ}$ carrying the ion-binding sites towards the extracellular face might be possible in the absence of phosphate. Such a conformational transition in combination with that depicted in scheme I might provide a pathway for transport of K ions in the complete absence of both ATP and phosphate. Since the transition $E_2(K)_{occ} \rightarrow E_1K$ is very slow, any fluxes involving this transition could only be slow.

We have recently developed an experimental system of phospholipid vesicles reconstituted with kidney Na–K-ATPase (Karlish & Pick, 1981). The incorporated ATPase molecules show many of the characteristic features of active Na and K transport observed in intact cells. The vesicles are tight to ions and have been used

to study the sidedness of the effects of Na and K ions on the conformational transition described in scheme I. This experimental system seemed to be suitable for testing whether slow pump-mediated Rb fluxes indeed exist in the absence of ATP and phosphate.

In the reconstituted system it is to be expected that the Na–K pumps are oriented randomly by comparison with the normal orientation in intact cells. We refer to the reconstituted pumps as being right-side out or inside-out, and the side to which ligands bind as being cytoplasmic or extracellular with respect to their original orientation in the cell. Ligands may be present either inside or outside the vesicle; we denote their location by the subscripts 'i' or 'o' respectively.

METHODS

Na–K-ATPase was prepared from pig kidney by the simpler of the two procedures described by Jorgensen (1974*a*). The membranes were suspended in a medium containing Imidazole, 25 mM, pH 7·5; EDTA (Tris), 1 mM and sucrose, 250 mM at a protein concentration of 2–3 mg/ml. and stored at −20 °C. Before use 1 ml. of suspension was dialysed overnight against 1 l. of ice cold Imidazole, 25 mM, pH 7·0; EDTA Tris, 1 mM. Na–K-ATPase activity, assayed as in Jorgensen (1974*b*), was 17–21 units/mg protein.

Reconstitution procedure and transport assays

These were as described in detail by Karlish & Pick (1981) with a number of minor modifications. The reconstitution procedure consisted in outline of (*a*) solubilization of the enzyme with cholate (Tris salt) at a ratio of 6 mg cholate/mg protein, (*b*) mixing at 0 °C with soya bean phospholipid vesicles (40 mg/mg protein) in the medium of choice, and freezing rapidly in liquid N_2, (*c*) thawing at room temperature and (*d*) sonication for 1–2 min in a Branson 12 bath sonicator. Since both phospholipid and enzyme were suspended in a solution containing Imidazole, 25 mM, pH 7·0 and EDTA (Tris-form) 1 mM, all vesicle preparations contained these materials in addition to the salts, inhibitors, etc. added to the reconstitution medium.

Some preparations of soyabean phospholipid were found to contain small amounts of K, bound presumably to the acid phospholipids. The phospholipids were therefore treated in the following way to remove contaminating K or Na and convert the acid phospholipids to the Tris salts. Soyabean phospholipid was suspended at 40 mg/ml. in a solution containing Tris-HCl, 500 mM, pH 7·0; Imidazole, 25 mM, pH 7·0; EDTA (Tris), 1 mM and sonicated to near clarity. The suspension was centrifuged briefly on a column of Sephadex G-50 equilibrated with Imidazole, 25 mM, pH 7·0; EDTA (Tris) 1 mM, as described previously (Karlish & Pick, 1981) and was then dialysed overnight against 1000 volumes of Imidazole, 25 mM, pH 7·0; EDTA (Tris), 1 mM. After these procedures, free K or Na in the phospholipid vesicle suspension was shown by flame-photometry to be less than 50 μM. These suspensions were also found to contain less than 3 μM-contaminant phosphate, using the sensitive assay reported by Murphy & Riley (1962).

After reconstitution from the phospholipid and Na–K-ATPase, the vesicles were always centrifuged at 0 °C on short columns of Sephadex G-50-40 equilibrated with Tris-HCl 150 mM, pH 7·0 (cf. Karlish & Pick, 1981). In experiments involving the use of vesicles loaded with RbCl, the suspensions were usually centrifuged twice on such columns. Thus, unless stated otherwise, the exterior medium of all vesicle suspensions consisted, initially, only of Tris-HCl 150 mM, pH 7·0.

For transport assays, the vesicle suspensions were warmed to room temperature (20–24 °C), and ^{86}Rb or ^{22}Na uptake was measured at room temperature in duplicate, in the conditions described in the figure legends. Dowex 50 columns and elution with 1·5 ml. cold sucrose solutions were used to separate vesicles from the medium, as described by Karlish & Pick (1981). In all cases, the total ^{86}Rb or ^{22}Na radioactivity in the reaction mixtures was measured. The absolute rates of Rb or Na fluxes were usually calculated as n-mole taken up per 1 or 2 min per 10 μl. of original vesicles. Results given are means of duplicate measurements which generally agreed to within 5–7 %.

Source of Materials

^{86}Rb was obtained from New England, Nuclear, Boston, MA, U.S.A. The stock isotope solution (5 mc) was diluted with Tris-HCl 150 mM, pH 7·5 so as to give a solution containing 2 mM-RbCl. ^{22}Na was obtained from the Radiochemical Centre, Amersham, Bucks. ^{32}P was obtained from the Israel Nuclear Centre, Beersheba, Israel. [^{32}P]ATP was prepared by the method of Avron (1961). ATP was purchased from Boehringer. Dowex-50 X 8, 50–100 mesh, and ammonium ortho-vanadate were obtained from Merck. Ouabain, soyabean phospholipid, valinomycin and Sephadex G-50-40

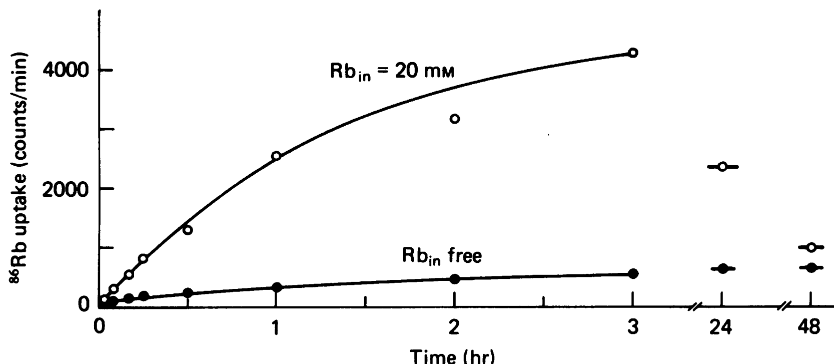


Fig. 1. Equilibration or counter-transport of ^{86}Rb into Rb-free or Rb-loaded vesicles. Two sets of reconstituted vesicles were prepared, containing either Tris-HCl, 150 mM; $MgCl_2$, 3 mM or Tris-HCl, 130 mM; $MgCl_2$, 3 mM and RbCl, 20 mM. 200 μl. of each vesicle suspension was mixed with 400 μl. of Tris-HCl, 150 mM containing RbCl (+^{86}Rb), 150 μM (final concentration of 100 μM). At the times indicated 50 μl. samples were removed to Dowex columns for analysis of the vesicle radioactivity content.

were purchased from Sigma. ATP (Na salt), ammonium ortho-vanadate and the cholate solution used in the reconstitution (see Karlish & Pick, 1981) were converted to the Tris salts by passage through small columns of the Dowex-50 (Tris-form). Choline chloride was recrystallized from hot ethanol. All salts and other materials were of analytical grade.

RESULTS

Rb–Rb exchange

Fig. 1 shows the time-course of passive equilibration of Rb (100 μM) from the external medium into vesicles either without internal Rb or with internal Rb at 20 mM. In the absence of internal Rb (lower curve) uptake of ^{86}Rb was relatively slow and could be resolved into two phases as reported previously (Karlish & Pick, 1981). The presence of internal Rb (upper curve) however, greatly increased the extent of isotope uptake. At its maximum the isotope was concentrated in these vesicles by some 10-fold, compared to the equilibrium value in vesicles lacking Rb. After reaching its transient maximal value the ^{86}Rb began to leave the Rb-loaded vesicles and by 48 hr almost reached the equilibrium value. In a separate experiment (Table 1, exp. 29780) valinomycin (3 μM) was added to vesicles after they had already concentrated ^{86}Rb. After elution on the Dowex columns these vesicles contained only 10% of the original radioactivity, showing that accumulated ^{86}Rb was not bound to the vesicles but must have been transported into them. Behaviour of the sort apparent in Fig. 1 is typical counter-transport and suggested that we are dealing with

a carrier-mediated process. In order to test whether this ^{86}Rb transport is mediated by the Na–K pump we have looked at the effect of ouabain. Fig. 2 shows how the rate of the flux depends on the external Rb concentration, in control vesicles (upper curve) and in vesicles made with enzyme pre-treated with ouabain (lower curve). It is clear from the figure that there is a ouabain-sensitive saturable component of the

TABLE 1. Transport or binding of ^{86}Rb to vesicles

Experiment	Condition	^{86}Rb uptake (counts/min)
29780	Uptake of ^{86}Rb into Rb-loaded vesicles at peak of accumulation	63750
	Ditto – after subsequent addition of valinomycin, 3 μM	6670
19280	Net uptake of ^{86}Rb (0·2 mM) in 8 min	772
	Ditto – after subsequent addition of valinomycin	105
	Net uptake of ^{86}Rb (0·2 mM) in 8 min + vanadate$_0$	341
	Ditto + subsequent addition of valinomycin and FCCP, 5 μM	57
8381	Net uptake of ^{86}Rb (0·2 mM) in 2 min	1316
	Ditto + ATP in eluting solution	1509
	Net uptake of ^{86}Rb, (0·2 mM) + vanadate$_0$	666
	Ditto + ATP in eluting solution	628

Expt. 29780 was similar to that in Fig. 1. After 3 hr of incubation, valinomycin was added and after 5 min the vesicles were removed to Dowex columns. Expts. 19280 and 8381 were similar to experiment 4D. After 8 min of incubation, valinomycin + FCCP was added to the vesicle suspension in exp. 19280, and after 3 min more the vesicles were applied to the Dowex columns.

Rb flux (inset). This is superimposed on a ouabain-resistant linear component which is probably attributable to a passive leak. Essentially, only this latter component is seen in the ouabain-treated vesicles. The maximal rate of the ouabain-sensitive flux in Fig. 2 (inset) is about 0·05 n-mole/min . 10 μl. vesicles, amounting to some 1 % of the maximal rate of ATP-dependent Na–K exchange in such vesicles (Karlish & Pick, 1981). The concentration of Rb$_0$ for half-maximal stimulation is about 0·5 mM.

In experiments such as those of Figs. 1 and 2, the Rb flux could be mediated by Na–K pumps oriented either right-side out or inside-out compared with the normal cellular orientation. Inhibition by vanadate which is known to occur at the cytoplasmic face of the Na–K pump (Cantly, Resh & Guidotti, 1978) is a useful tool for discriminating between fluxes through right-side out and inside-out oriented pumps. Fig. 3*A* shows how the rate of ^{86}Rb uptake depends on the external Rb concentration in control vesicles in the absence of vanadate (upper curve) or in the presence of vanadate added to the external medium (middle curve) or into vesicles made with enzyme pre-treated with vanadate and suspended in an external medium containing also vanadate (lower curve). Fig. 3*B* is derived from Fig. 3*A*, and shows how the separate components through inside-out or right-side out pumps depend on the external Rb concentration. The 'inside-out component' is obtained by subtracting the flux in the presence of vanadate, only outside, from the control flux. The 'right-side out component' is obtained by subtracting from the latter flux, the flux with vanadate on both sides of the vesicle. Since the maximal rate through the pumps in either orientation is essentially the same, it is apparent that as many pumps are

oriented inside-out as right-side out, as might have been expected. The absolute value of the Rb uptake inhibitable by vanadate on both sides of the membrane, 0·084 n-mole/2 min . 10 μl. is similar to the value of the ouabain sensitive flux of Fig. 2. A striking feature of the ^{86}Rb fluxes are their high apparent affinities for Rb. The concentration of Rb_0 for half-maximal saturation of the flux through inside-out

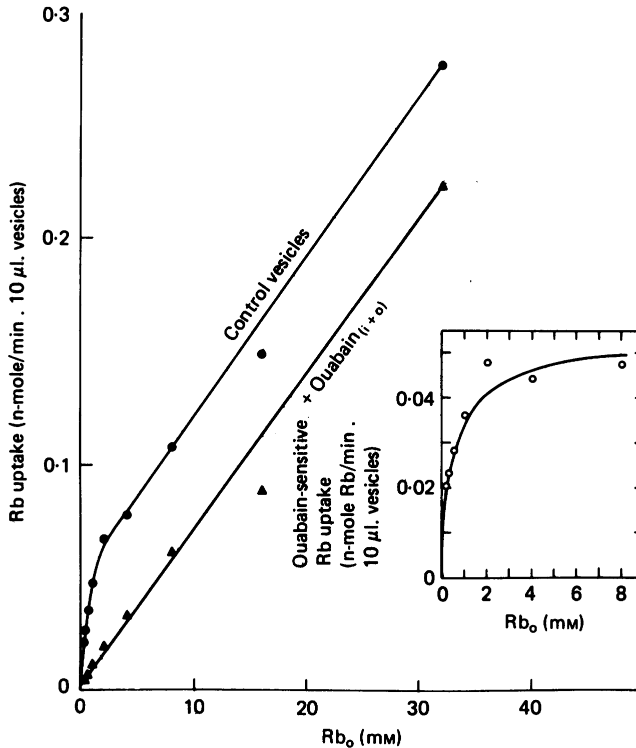


Fig. 2. Ouabain-sensitive ^{86}Rb uptake into Rb-loaded vesicles at different Rb_0 concentrations. Two sets of reconstituted vesicles were prepared, both containing RbCl, 100 mM and $MgCl_2$, 2·5 mM; but one set was prepared from Na–K-ATPase pre-incubated for 1 hr at 20 °C with ouabain, 2 mM; $MgCl_2$, 2 mM and phosphate (Tris), 1 mM. The ^{86}Rb uptake assay was initiated by adding 40 μl. of the vesicles suspension to 40 μl. of a solution containing RbCl + Tris-HCl (total concentration 128 mM) mixed in proportions to produce the desired final concentration of RbCl, a fixed amount of ^{86}Rb, and $MgCl_2$, 2 mM (final concentration of 1 mM). For assay of the vesicles prepared with enzyme pre-incubated with ouabain, the reaction mixture also contained ouabain, 1 mM. After 4 min, the suspensions were removed to the Dowex columns.

pumps is about 0·6 mM. This represents interaction of Rb at the cytoplasmic face of the pump. The concentration of Rb_0 for half-maximum saturation of the flux through right-side out pumps is about 0·2 mM. This represents interaction of Rb_0 at the extracellular face of the pump.

Notice that a high concentration of vanadate has been used in this and subsequent experiment. With 1–10 mM Rb_0 and Mg_0 ions the $K_{0.5}$ for $vanadate_0$ was about 0·5–1 μM. But for experiments involving the use of Rb_0 as low as 50 μM, much higher concentrations of vanadate were necessary to obtain maximal inhibition. Experiments showed that in the presence of 100 μM-Rb and 100 μM-Mg,

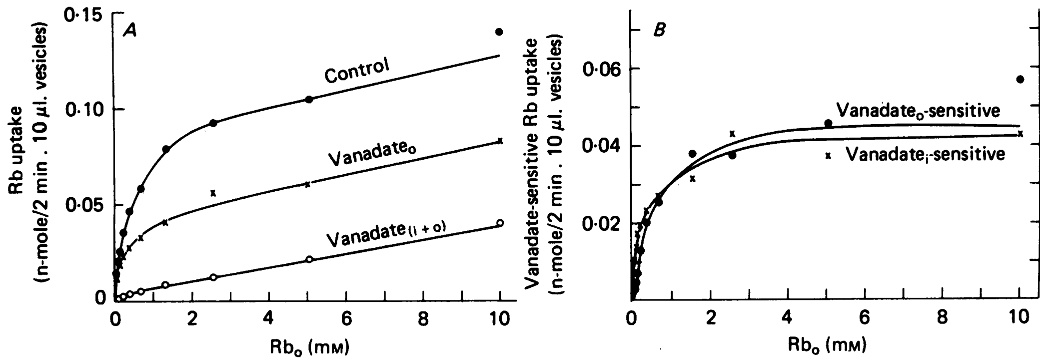


Fig. 3. Vanadate$_0$-sensitive ^{86}Rb–Rb exchange through inside-out and right-side out pumps: dependence on Rb_0 concentration. Two sets of reconstituted vesicles were prepared, one containing RbCl, 150 mM; the other containing RbCl, 150 mM; vanadate (Tris), 50 μM and $MgCl_2$, 1·5 mM. After centrifugation twice on columns of Sephadex G-50 the first set was divided and $MgCl_2$, 200 μM + vanadate (Tris) 600 μM was added to one half set (i.e. the + vanadate$_0$ sample) and also to the set of vesicles containing vanadate$_i$ (i.e. the + vanadate$_{i+o}$ sample). The assay was initiated by adding 40 μl. of vesicles (control or + vanadate$_0$ or + vanadate$_{i+o}$), to 40 μl. of the appropriate RbCl + Tris-HCl mixture (total concentration 150 mM) containing also a fixed amount of ^{86}Rb. After 2 min incubation the suspension was applied to the Dowex columns for analysis.

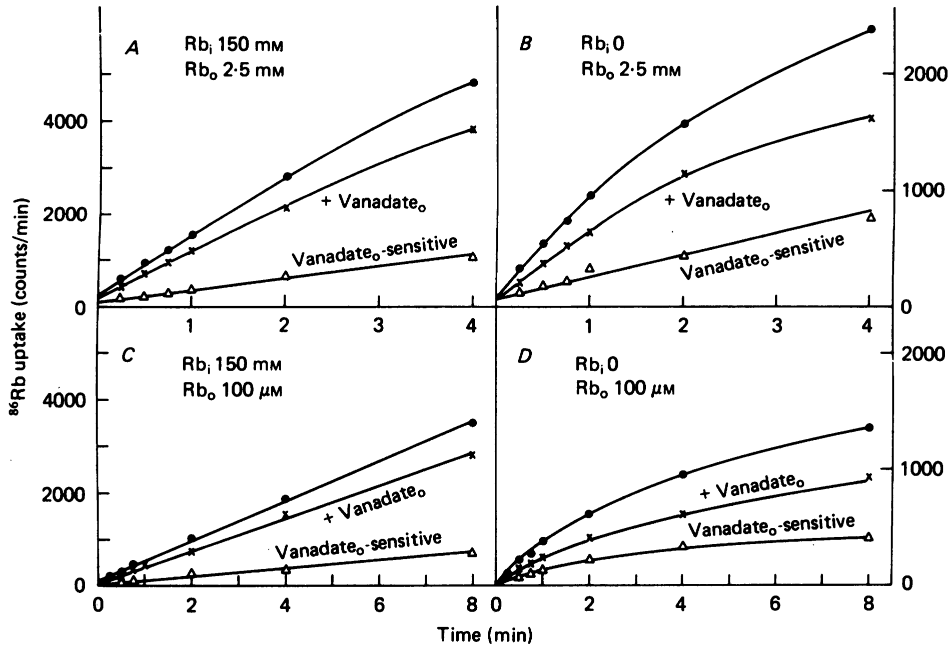


Fig. 4. Time course of vanadate$_0$-sensitive ^{86}Rb uptake into Rb-free and Rb-loaded vesicles. Two sets of reconstituted vesicles were prepared, containing either Tris-HCl, 150 mM or RbCl, 150 mM. Each set was divided, and $MgCl_2$, 10 mM + vanadate (Tris), 1 mM were added to one half of each set. The assay was initiated by mixing 200 μl. of vesicles with 200 μl. of a solution of Tris-HCl, 150 mM containing RbCl (+ ^{86}Rb), 5 mM or 200 μM, respectively. Final concentrations were: Rb_0, 2·5 mM or 100 μM, respectively, and when present, $MgCl_2$, 5 mM + vanadate$_0$, 500 μM. At the times indicated single samples of 50 μl. were removed to Dowex columns for analysis.

100–200 μM vanadate gave maximal inhibition of Rb uptake. High concentrations of vanadate were therefore used throughout. (cf. Cantley, Cantley & Josephson (1978) for a description of the synergistic effects of Rb, Mg and vanadate, and also Figs. 8 and 9).

Zero-trans net Rb flux

The ^{86}Rb flux into Rb-loaded vesicles represents a ^{86}Rb–Rb exchange. It was of interest to test whether the ^{86}Rb uptake in zero-trans conditions, i.e. into Rb-free vesicles was, in part, also mediated by the Na–K pump. Fig. 4*B* shows the first 4 min

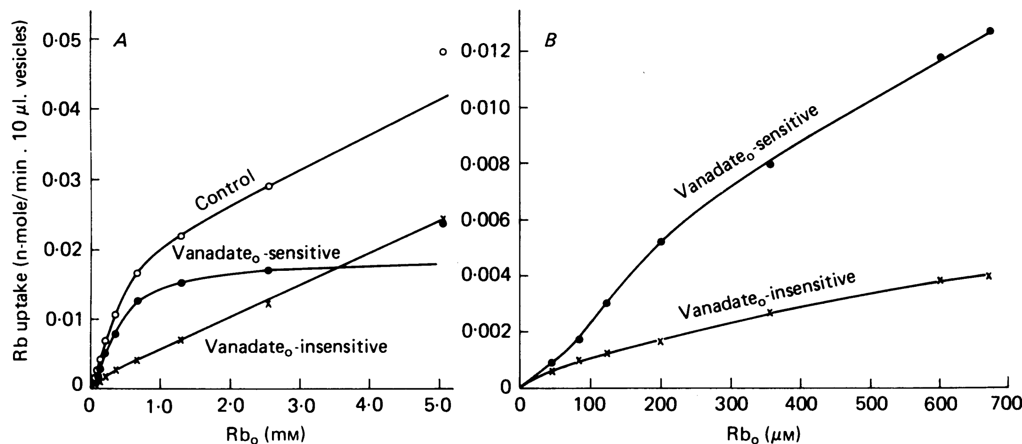


Fig. 5. Vanadate$_0$-sensitive net Rb flux into Rb-free vesicles: dependence on Rb$_0$. Reconstituted vesicles were prepared containing Tris-HCl, 150 mM, pH 7·0. After centrifugation on Sephadex G-50 the suspension was divided and $MgCl_2$, 2 mM + vanadate (Tris), 1 mM was added to one half set. The assay was performed as in Fig. 4, except that the flux time was reduced to 1 min in order to ensure that ^{86}Rb uptake was linear with time (cf. Fig. 4).

of a time-course for ^{86}Rb uptake, from a medium containing 2·5 mM-RbCl, into Rb-free vesicles, in the presence and absence of vanadate. The vanadate$_0$-sensitive component in Fig. 4*B* clearly represents a pump-mediated net flux from the cytoplasmic surface (of rate 0·015 n-mole Rb/min . 10 μl. vesicles). The parallel experiment for vesicles initially loaded with 150 mM-RbCl is shown in Fig. 4*A*. The rate of vanadate$_0$-sensitive Rb–Rb exchange was 0·019 n-mole Rb/min. 10 μl . vesicles. In this experiment the rate of exchange was a little higher than the rate of net Rb flux through the Na–K pump, but one cannot exclude the possibility that the difference results merely from variability in the parallel vesicle preparations. At all events the rates are not very different. Fig. 4*C* and *D* presents data from a similar experiment in which a much lower Rb$_0$ concentration was used, 100 μM. Essentially similar phenomena are observed as in Fig. 4*A* and *B*, but the rate of the vanadate$_0$-sensitive net Rb flux was maintained linear for no longer than one minute.

Fig. 5*A* shows how Rb uptake (measured at 1 min) into vesicles lacking Rb$_i$ depends on the external Rb concentration in media without or with vanadate. The vanadate$_0$-sensitive zero-trans net Rb flux saturates at the millimolar concentration range and shows a sigmoid dependence on Rb$_0$ concentration (see Fig. 5*B*, upper

curve). The maximal flux in this experiment was about 0·02 n-mole/min . 10 μl. vesicles, and the concentration of Rb_0 for a half-maximal stimulation was about 0·5 mM. This vanadate$_0$-sensitive component must represent the net Rb flux from the cytoplasmic face of inside-out oriented pumps. The vanadate$_0$-insensitive flux consisted of a small saturable component (seen more clearly in Fig. 5*B*, lower curve) superimposed on a linear flux, which is probably a simple leak. The size of this saturable component was estimated by subtracting from the total vanadate$_0$-insensitive flux a linear component having a slope equal to that of the points at the five highest Rb_0 concentrations. The maximal rate of the saturable flux, so calculated, was about 0·0013 n-mole Rb/min . 10 μl. vesicles, and the concentration of Rb_0 for half-maximal saturation was roughly 100 μM. This component may represent zero-trans net Rb flux from the extracellular surface of the right-side out oriented pumps. Further evidence for the identity of this flux is presented in Fig. 6*A* and *B*. In the further analysis of the net Rb fluxes given below it is worth bearing in mind that one is dealing with three components, the vanadate$_0$-sensitive flux, the vanadate$_0$-insensitive saturable component and a simple leak (which too is vanadate$_0$-insensitive). The maximal velocity of the vanadate$_0$-sensitive flux is roughly 15-fold that of the vanadate$_0$-insensitive saturable component. Since the apparent affinity of Rb_0 for the latter is some 5-fold that of the former flux the relative contribution of the vanadate$_0$-insensitive component becomes appreciable at low Rb_0 concentrations.

Effects of monovalent cations

Monovalent cations should compete with Rb for the binding sites on the pump. In view of the co-operative activation of ^{86}Rb uptake by the Rb ions, seen clearly in Fig. 5*B*, cations which are transported could, at low concentrations, stimulate Rb uptake, but at high concentrations should inhibit the flux. Cations which are not transported should only inhibit.

Fig. 6*A* (upper curve) shows the effect of increasing concentrations of external K ions on the net Rb uptake (Rb_0, 50 μM). The vanadate$_0$-sensitive Rb uptake is indeed stimulated by low concentrations of K_0 and is inhibited as K_0 is raised above about 1 mM. The vanadate$_0$-insensitive ^{86}Rb flux is inhibited by K_0 ions, indicating that a substantial fraction of this flux is carrier-mediated at this low concentration of Rb ion and confirming the evidence of Fig. 5. It is apparent from Fig. 6*A* that at 50 μM-Rb_0 the K_0-inhibitable part of the vanadate$_0$-insensitive net Rb flux amounts to about 70 % of the vanadate$_0$-sensitive net Rb flux. The fact that only inhibition of the vanadate$_0$-insensitive flux is seen does not necessarily exclude co-operativity between Rb_0 and K_0 at these extracellular sites, for it might only mean that the Rb_0 concentration used was too high.

As seen in Fig. 6*B* externally added Na ions only inhibit net ^{86}Rb uptake both at 50 and 500 μM-total Rb_0 concentrations. The data for 50 μM-Rb_0 show two ranges over which the Na_0 ions inhibit. That component inhibited at low concentrations ($K_i \sim 100$ μM) amounts to about 40 % of the total net Rb uptake. High concentrations of Na_0 ions ($K_i \sim 8$ mM) reduce the net Rb flux to well below the value in the presence of externally added vanadate. Hence both vanadate$_0$-sensitive and insensitive pump mediated net Rb fluxes are inhibited by Na_0 ions. But from this curve alone one cannot determine whether it is the vanadate$_0$-sensitive or insensitive component

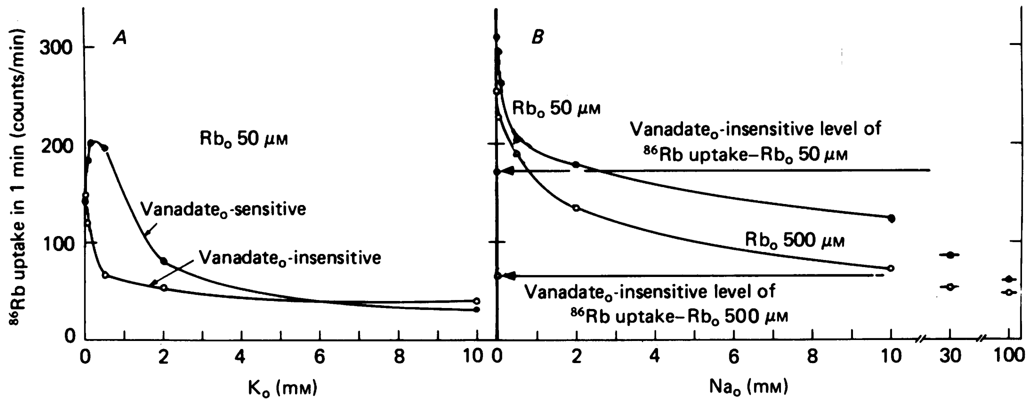


Fig. 6. Effects of K_0 and Na_0 on vanadate$_0$-sensitive and insensitive net ^{86}Rb uptake. Reconstituted vesicles were prepared as in Fig. 5. The reaction mixture contained RbCl ($+^{86}Rb$) sufficient to give a final concentration of 50 or 500 μM as indicated, and mixtures of KCl+Tris-HCl or NaCl+Tris-HCl, (total concentrations 200 mM) producing the desired final concentrations of KCl and NaCl. The flux time was 1 min as in Fig. 5.

TABLE 2. Effects of different internal cations on vanadate$_0$-sensitive and insensitive ^{86}Rb uptake

	^{86}Rb uptake per minute (counts/min)	
Internal cation	Vanadate$_0$-sensitive	Vanadate$_0$-insensitive
Tris	255	312
Choline	317	380
Lysine	297	208
Rb	429	638
K	798	692
Cs	867	1352
Na	1406	430
Li	1611	600

Eight sets of reconstituted vesicles were prepared, containing 150 mM-Tris-HCl, pH 7·0, choline-Cl, lysine-HCl, pH 7·0, RbCl, KCl, CsCl, NaCl or LiCl, respectively. The vanadate$_0$-sensitive and insensitive fluxes were measured as in Fig. 4, over 1 min, at a final external Rb_0 ($+^{86}Rb$) concentration of 2 mM. Results are the mean of duplicate samples which agreed within 5%.

which is inhibited at low concentrations. This question can be resolved, however, by considering the curve for 500 μM-Rb_0. Here the vanadate$_0$-sensitive component constitutes the overwhelming contributor to the net Rb flux. The concentration of Na_0 for half inhibition of the net flux at 500 μM-Rb_0 is about 1 mM. Thus, that component inhibited by Na_0 with a K_i of about 8 mM in the presence of 50 μM-Rb_0 cannot be the vanadate$_0$-sensitive component. We conclude that it is the cytoplasmic sites which bind Na with a high affinity, while the extracellular sites bind Na with a far lower affinity.

Table 2 presents data showing the trans-effects of various cations on ^{86}Rb uptake. (Rb_0 2 mM). Consider first the vanadate$_0$-sensitive flux. Although differences of, say, 20% in rates can be the result of variability between parallel vesicle preparations,

there are nevertheless very striking differences in the effects of various cations. It seems that $Tris_i$, $choline_i$ and $lysine_i$ form a class of cations which do not interact significantly with the pump at the extracellular surface. Rb_i stimulates slightly (see also Fig. 4*A* and B), K_i and Cs_i stimulate more, while Na_i and Li_i stimulate markedly (about 5-fold). These stimulations must result from interactions at the extracellular

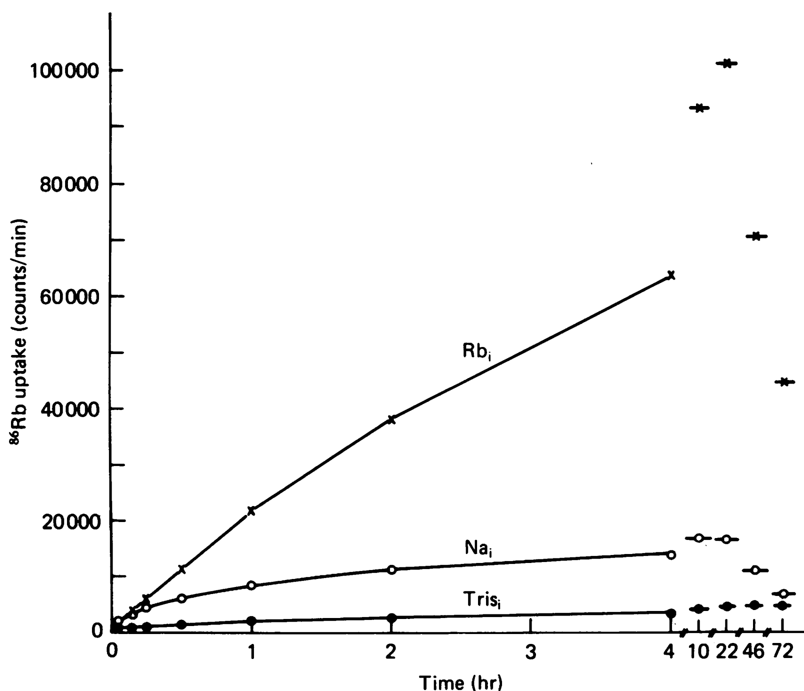


Fig. 7. Equilibration of ^{86}Rb into Rb-free and counter-transport of ^{86}Rb into Rb-and Na-loaded vesicles. Three sets of reconstituted vesicles were prepared, containing 150 mM-Tris-HCl, RbCl or NaCl respectively. The vesicles were centrifuged twice on Sephadex G-50 columns. 300 μl. of each vesicle suspension was mixed with 750 μl. of a solution containing Tris-HCl 150 mM, pH 7·0 and RbCl (+ ^{86}Rb) at 1·4 mM (final concentration of 1 mM). At the indicated times single 80 μl. samples were removed to Dowex columns for analysis.

surface of the pump. Consider now the $vanadate_0$-insensitive flux. Here the effects of cations within the vesicles result from interactions at the cytoplasmic surface of the pump. Again $Tris_i$ and $choline_i$ do not seem to interact significantly. $Lysine_i$ may inhibit. Rb_i, K_i and Li_i definitely stimulate Rb uptake. Cs_i gives a striking stimulation. Interestingly, Na_i produces little or no stimulation. One might ask if the observed trans-effects of the Rb congeners reflect transport, that is exchange of the congener for ^{86}Rb, or whether stimulation of ^{86}Rb by the congeners is allosteric and does not involve congener–^{86}Rb exchange. In order to answer this question we have tested whether internal Na, like Rb (see Fig. 1), can sustain counter-transport of ^{86}Rb. Fig. 7 shows the time-course of ^{86}Rb uptake at 1 mM-Rb into vesicles containing 150 mM-Tris-HCl, RbCl or NaCl respectively. ^{86}Rb uptake into the RbCl and NaCl-containing vesicles does show the counter-transport effect, for the maximal level reached is substantially higher than the uptake of ^{86}Rb into Tris-containing vesicles

at equilibrium. The fact that the maximal accumulation of ^{86}Rb into the Na-loaded vesicles is less than into Rb-loaded vesicles can be attributed to two factors. Firstly, in Rb-loaded vesicles counter-transport is being sustained by pumps oriented both inside-out and right-side out, whereas in Na-loaded vesicles essentially only inside-out oriented pumps should be sustaining a ^{86}Rb flux (see Table 2). In addition, ^{86}Rb

TABLE 3. Parallel measurements of phosphoenzyme and ^{86}Rb and ^{22}Na fluxes

	Tris-containing vesicles	Na-containing vesicles	Rb-containing vesicles
p-mole of phosphoenzyme/10 μl. vesicles	1·45	1·45	0·026
	(n-mole/min . 10 μl. vesicles)		
ATP-dependent Na uptake	3·49	—	3·32
(ATP + P_i)-stimulated ^{86}Rb–Rb exchange	—	—	0·49
Vanadate$_0$-sensitive ^{86}Rb-uptake	0·010	0·044	0·017

Covalent incorporation of ^{32}P from [γ-^{32}P]-ATP was measured at 0 °C, as follows: vesicles (containing Tris-HCl, 150 mM, NaCl, 150 mM, or RbCl, 150 mM) were reconstituted from phospholipid and Na–K-ATPase. For these experiments cholate solubilized Na–K-ATPase was separated from undissolved membranes by centrifugation in a Beckman Airfuge, 5 min at 30 Lb/sq. in. 200 μl. of reconstituted vesicles were rapidly mixed at 0 °C with 50 μl. of a reaction solution producing finally, a suspension with Tris-HCl, 150 mM, pH 7·0; NaCl, 10 mM; $MgCl_2$, 3 mM and ATP (containing [γ-^{32}P]-ATP], 10 μM. After 30 sec of incubation, 5 ml. of an ice-cold stopping solution containing perchloric acid 5 % w/v; ATP, 5 mM and phosphate, 5 mM, was rapidly added. The tubes were placed on ice for about 30 min to allow complete denaturation of the protein. The cloudy suspension was then filtered on Whatman GF/C paper filters and these were washed with 30 ml. of trichloracetic acid 5 % w/v. Blank samples to account for radioactivity adhering non-specifically to denatured protein, or the filters, were obtained by adding 200 μl. of vesicles to 5 ml. of the stopping solution prior to the 50 μl. of reaction solution. The washed filters were placed in counting vials, 7·5 ml. of a toluene-Triton scintillation solution was added, and the radioactivity counted. Total radioactivity was also measured in a GF/C filter to which 50 μl. of the reaction solution was added. The maximum incorporation of ^{32}P, i.e. in vesicles containing Tris-HCl or NaCl, was about 10-fold the blank value. Control experiments showed that 15 or 30 sec incubations gave the same level of incorporation. Phospholipid vesicles, not containing Na–K-ATPase, showed no incorporation of ^{32}P.

ATP-dependent Na uptake into Tris containing vesicles was estimated in a medium containing Tris-HCl, 100 mM, pH 7·0; NaCl (+^{22}Na), 30 mM; KCl, 10 mM and $MgCl_2$, 3 mM, with or without ATP, 3 mM, using ionophores valinomycin + FCCP, 5 μM, to make the vesicles permeable to K ions (see Karlish & Pick, 1981). ATP-dependent ^{22}Na uptake into Rb-loaded vesicles was measured in a medium containing only Tris-HCl, 100 mM, pH 7·0; NaCl (+^{22}Na), 30 mM; $MgCl_2$, 3 mM, with or without ATP (Tris), 3 mM. (ATP + P_i) dependent ^{86}Rb–Rb exchange was measured in the Rb-loaded vesicles suspended in a medium containing RbCl, 25 mM; $MgCl_2$, 6 mM, phosphate (Tris) (pH 7·0) 20 mM, with or without ATP (Tris), 5 mM, (see also Karlish *et al.* 1982).

Vanadate$_0$-sensitive ^{86}Rb uptake into Tris-, Na- or Rb-loaded vesicles was assayed as in Table 2 and Fig. 4, with a final RbCl concentration in the medium of 2 mM.

efflux from the Na-loaded vesicles should be less inhibited than the Rb-loaded vesicles.

Turnover numbers

Turnover numbers have been estimated by measuring in parallel, the level of phosphoenzyme and the rates of various cation fluxes, assuming only one ion per phosphorylation site (Tables 3 and 4). The phosphoenzyme level was measured at

0 °C in a medium containing Na ions, Mg ions and 10 μM-ATP ([^{32}P]ATP). The level of phosphoenzyme found in the Tris- or Na-loaded vesicles was the same, but as expected for pumps oriented inside out, the Rb-loaded vesicles showed essentially no phosphoenzyme formation. Other experiments with Na-loaded vesicles showed that addition of 1 mM-K to the phosphorylating medium did not reduce the EP level,

TABLE 4. Derived turnover numbers

ATP-dependent ^{22}Na–Rb exchange	(ATP + P_i)-stimulated ^{86}Rb–Rb exchange	Vanadate$_o$-sensitive ^{86}Rb–Rb exchange	Vanadate$_o$-sensitive net ^{86}Rb uptake	Vanadate$_o$-sensitive ^{86}Rb–Na exchange
(mole of Rb or Na per second per mole of phosphoenzyme)				
43	7	0·25	0·15	0·63

The values have been calculated from the experimental data in Table 3, and corrected slightly as follows to take into account lack of full saturation or inhibition by ions: ATP dependent Na uptake is activated only 91 % by 30 mM-NaCl (see Karlish & Pick, 1981). (ATP + phosphate)-stimulated Rb–Rb exchange is activated 90 % at 25 mM-RbCl and is inhibited by about 15 % at 1 mM-free Mg_o ions (see Karlish *et al.* 1982). The slow vanadate$_o$-sensitive Rb fluxes are activated only by about 80 % at 2 mM-external RbCl (see Figs. 3 and 5).

unless valinomycin was also added, when a large reduction occurred (data not shown). Also, pre-incubation of Na-loaded vesicles with ouabain 1 mM and $MgCl_2$ did not reduce EP level upon subsequent addition of the phosphorylating reaction mixture (data not shown). The latter two experiments exclude the possibility that we are measuring phosphoenzyme formation in un-reconstituted ATPase molecules.

Parallel measurements in these Tris-, Na- or Rb-loaded vesicles of ^{86}Rb or ^{22}Na uptake enabled us to calculate the turnover numbers recorded in Table 4.

Compared to the maximal rate of ATP dependent Na–Rb exchange, the rate of (ATP + P_i)-stimulated Rb–Rb exchange is about 17 % (this flux is discussed in detail in the third paper of this series). The newly described fluxes have rates which are the following percentage of the ATP-dependent Na–Rb exchange: ^{86}Rb–Rb exchange, 0·64 %; net Rb uptake, 0·38 % and ^{86}Rb–Na exchange, 1·6 %.

Mg effects

Previously we have reported effects of Mg ions on the rates and apparent K affinities for inducing E_1 to $E_2(K)_{occ}$ conformational transitions (Karlish *et al.* 1978; Karlish, 1980). It was therefore of some interest to look for Mg effects on the Rb fluxes. It can be seen in Fig. 8 that Mg_o ions inhibit net Rb fluxes into Rb-free vesicles. At 200 μM-Rb_o, Mg_o ions at high enough concentrations reduce the flux to nearly the same level as did vanadate$_o$ (in the presence of much lower concentrations of Mg_o, 50 μM). The $K_{0·5}$ for Mg_o inhibition was about 1·3 mM. At 1 mM-Rb_o, Mg_o ions reduced the flux but not as much as vanadate$_o$. The $K_{0·5}$ for Mg_o inhibition of 3·2 mM was significantly if moderately raised. Fig. 9*A* and *B* shows that with 100 μM-Rb_o both the net Rb flux and ^{86}Rb–Rb exchange are inhibited by Mg_o ions. In the presence of vanadate$_o$ ions, a low Mg_o concentration was strictly necessary for inhibition. The extent of inhibition is the same as that reached in the absence of vanadate$_o$, at high

enough Mg_0. In the exchange situation (Fig. 9*A*) the extent of inhibition is proportionately smaller, consistent with the greater contribution here of flux carried by vanadate$_0$-insensitive pumps. Since the vanadate$_0$-insensitive flux is not inhibited by Mg_0 ions, inhibition by Mg is clearly restricted to an action on the cytoplasmic face of the pump.

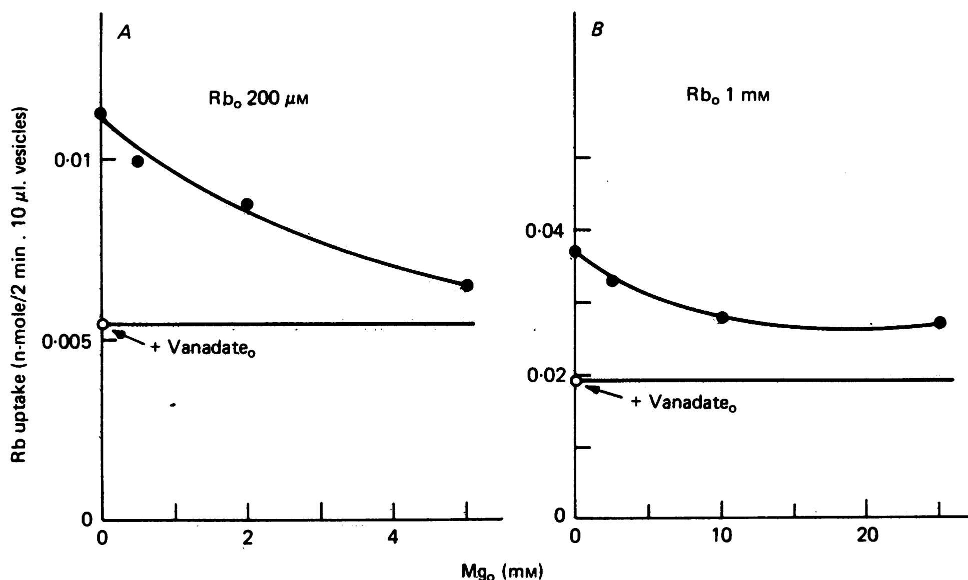


Fig. 8. Inhibition by Mg_0 of net Rb uptake at two different Rb_0 concentrations. Reconstituted vesicles were prepared and assayed as in Fig. 4. The 40 μl. reaction medium consisted of the appropriate Tris-HCl + $MgCl_2$ mixture, and RbCl (+ ^{86}Rb) at twice the final concentrations of 0·2 or 1 mM respectively.

DISCUSSION

Na–K pump associated cation transport phenomena

It is now well established that Rb can be bound and/or occluded to the isolated Na–K-ATPase (Matsui, Hayashi, Homareda & Kimimura, 1977; Cantley *et al.* 1978; Beauge & Glynn, 1979; Glynn & Richards, 1980). To what extent, if any, can the phenomena described in this paper, particularly those observed at very low Rb concentrations, be attributed to binding rather than transport? The following observations suggest that we are measuring exclusively transport.

(1) Ionophores such as valinomycin release the accumulated ^{86}Rb on to the Dowex columns (Table 1). (2) Eluting the vesicles off the Dowex columns with sucrose solutions containing ATP did not reduce the radioactivity associated with the vesicles (Table 1, exp. 8381). Had an appreciable fraction of vesicle associated ^{86}Rb been occluded, it should have been rapidly released by the ATP, trapped on the Dowex, and seen as a reduced ^{86}Rb uptake. This was not the case. (3) The relatively slow time-course of ^{86}Rb uptake (Figs. 1 and 4), the counter transport phenomenon (Figs. 1 and 7) and the trans-effects of congeners (Table 2) are phenomena to be expected of transport and not of binding.

The following properties suggest very strongly that the fluxes we have been measuring are associated with the pump: (*a*) inhibition by ouabain (Fig. 2) and vanadate (Figs. 3, 4 and 5); (*b*) high apparent affinities and co-operative effects of Rb (Figs. 3, 5 and 6); (*c*) congener effects, (Table 2) interpretable in terms of properties of the Na–K pump (see below).

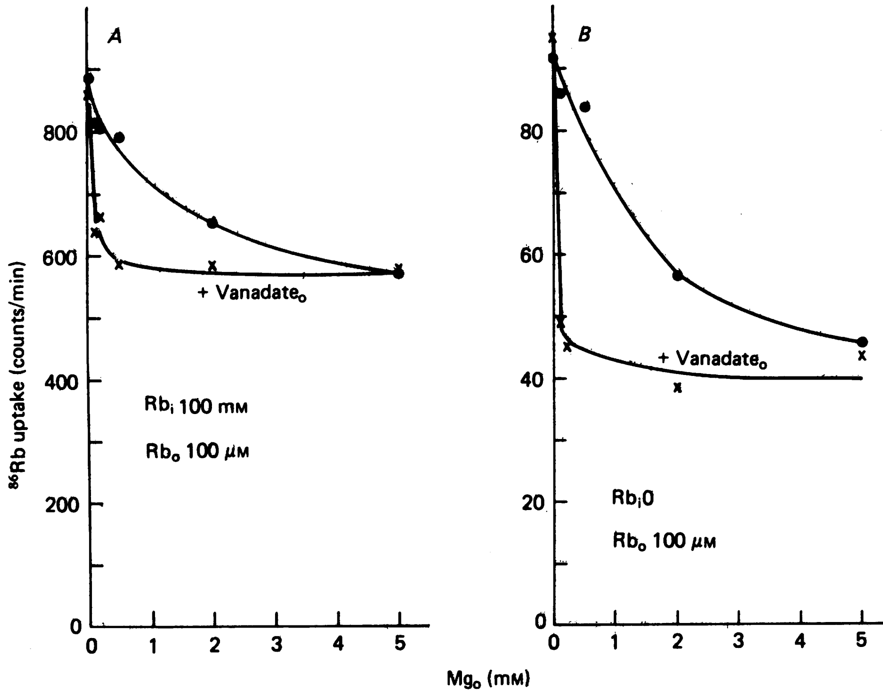
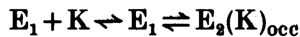


Fig. 9. Synergistic inhibition of net Rb uptake and Rb–Rb exchange by Mg_0 and $vanadate_0$. Two sets of reconstituted vesicles were prepared containing either Tris-HCl, 150 mM or RbCl, 150 mM. ^{86}Rb uptake was measured as in Fig. 4 with the following final ligand concentrations: RbCl ($+^{86}Rb$), 0·2 mM; vanadate (Tris), 0·1 mM and either CDTA, 2 mM ($Mg_0 = 0$), or $MgCl_2$ at the indicated final concentration. The flux time in this experiment was 4 min.

The orientation of pumps catalysing particular Rb fluxes can be determined by looking at inhibition by vanadate on either one or both sides of the vesicles. The observation that the maximal rate of Rb–Rb exchange sensitive to the external vanadate is one half of the exchange sensitive to vanadate on both sides (Fig. 3), suggests that the pumps are randomly oriented across the vesicle membrane.

A model for Rb fluxes

Consider again scheme I

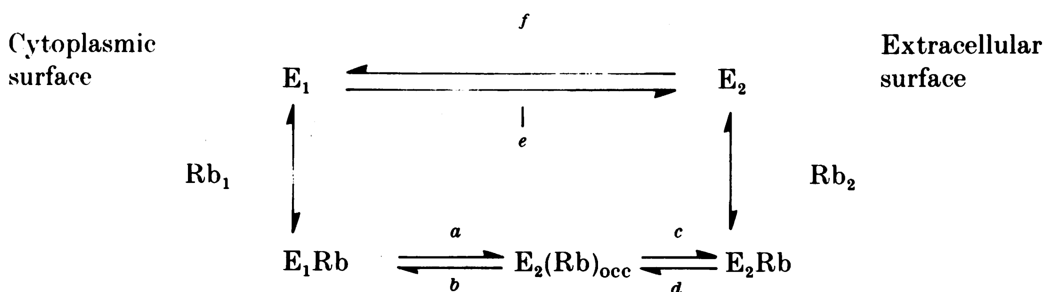


$$E_1 + K \rightleftharpoons E_1 \rightleftharpoons E_2(K)_{occ}$$

E_1 is the form which binds K ions at the cytoplasmic face. The intrinsic affinity of E_1 for K is low, ~ 75 mM (Karlish *et al.* 1978; Karlish, 1980; Beauge & Glynn, 1980). Titration of the form E_1 to the form $E_2(K)_{occ}$ by K or Rb ions gives a high apparent

affinity, 0·1–0·2 mM or 0·1 mM respectively (Karlish *et al.* 1978; Beauge & Glynn, 1980; Jorgensen & Karlish, 1980; Skou & Esman, 1980). The high apparent affinity is accounted for by coupling between the weak intrinsic binding and the conformational equilibrium poised about 1000-fold in the direction of $E_2(K)_{occ}$. Direct measurement of the rates of the conformational transitions show that the rate of $E_1K \rightarrow E_2(K)_{occ}$ is about 300/sec and that of $E_2(K)_{occ} \rightarrow E_1K$ is slow, some 0·3/sec at 20 °C (Karlish, 1980). $E_2(K)_{occ}$ is the form containing occluded K (or Rb ions) (Beauge & Glynn, 1979). Direct measurements show that occluded Rb is lost from Na–K-ATPase at a rate of about 0·2/sec at 20 °C (Glynn & Richards, 1980). About 3 moles of Rb ions are occluded per mole of phosphorylated enzyme (Glynn & Richards, 1982).

An obvious minimal extension of the above scheme, which accounts for trans-membrane transport of Rb, both zero-trans net and exchange fluxes, is the following: Scheme II:



We propose that the occluded form $E_2(Rb)_{occ}$ can undergo a transition to the form E_2Rb which is in equilibrium with the form E_2 and Rb ions at the extracellular face of the pump. The unloaded forms E_1 and E_2 are interconvertible. Zero-trans, net Rb flux from the cytoplasmic to extracellular face involves the sequence: $E_1 \rightarrow E_1Rb \rightarrow E_2(Rb)_{occ} \rightarrow E_2Rb \rightarrow E_2 \rightarrow E_1$ while zero-trans net Rb flux in the opposite direction involves the sequence $E_2 \rightarrow E_2Rb \rightarrow E_2(Rb)_{occ} \rightarrow E_1Rb \rightarrow E_1 \rightarrow E_2$. ^{86}Rb–Rb exchange, on the other hand involves the sequence: $E_1 \rightleftharpoons E_1Rb \rightleftharpoons E_2(Rb)_{occ} \rightleftharpoons E_2Rb \rightleftharpoons E_2$. E_2Rb and E_2 are designated '2' forms of the enzyme by direct analogy with the conventional phosphoenzyme E_2-P in which the cation binding sites also face the extracellular surface (see also the models in Karlish & Stein, 1982; Karlish, Lieb & Stein, 1982). E_2-P and the occluded Rb form $E_2(Rb)_{occ}$ are both '2' forms as judged by tryptic digestion (Jorgensen, 1975) and fluorescence evidence (Jorgensen & Karlish, 1980). Previously the occluded Rb form has been referred to only as E_2(Rb). We now add the subscript 'occ' to re-emphasize the ion occlusion, and distinguish $E_2(Rb)_{occ}$ from the other E_2 forms in which the ion binding sites face the extracellular surface.

Kinetic analysis

Our experiments show that the rates of ^{86}Rb–Rb exchange and net Rb flux from the cytoplasmic to extracellular surface (direction $1 \rightarrow 2$) are similar, with turnover numbers 0·25 and 0·15/sec respectively (Table 4). The maximal rate of net Rb flux from extracellular to cytoplasmic surface (direction $2 \rightarrow 1$) is at most one fifteenth of the net flux in the opposite direction, and so must have a turnover number of some

0·01/sec. In the following kinetic analysis our aim is to identify the rate-limiting steps operative in the various transport modes and attempt to set bounds to the rate constants of scheme II. We have, however, some prior information allowing us to set limits to some rate-constants independently of transport measurements.

(1) For K the rate-constant $a \simeq 300$/sec and $b \simeq 0{\cdot}3$/sec (Karlish *et al.* 1978; Karlish & Yates, 1978; Karlish, 1980). The rates of the transition with Rb are unlikely to be very different than with K, and at all events a is much greater than b.

(2) The measured rate-constant of loss of occluded ^{86}Rb from Na–K-ATPase at 20 °C is about 0·2/sec (Glynn & Richards, 1980). This rate must reflect $b+c$, and thus a is clearly much greater than both b and c.

(3) The measured stoichiometry of Rb occlusion is three Rb ions per phosphorylated enzyme (Glynn & Richards, 1982). Were rate-constants c and d to be of comparable magnitude, the equilibrium between $E_2(Rb)_{occ}$ and E_2Rb would be about unity, and the real stoichometry of Rb occlusion would be six ions occluded per pump. This seems unlikely. Therefore it is likely that $d > c$. We shall show independently in Karlish *et al.* (1982) that this assumption is very likely correct.

From eqn. (A 1) of the Appendix the maximum velocity of zero-trans net Rb flux from the cytoplasmic to extracellular face (direction $1 \rightarrow 2$) is given by:

$$\frac{V_{max}\ 1 \rightarrow 2}{E_{Total}} = \frac{acf}{(b+a)f+c(a+f)}.$$

Since $a \gg b+c$, the rate will be determined by $cf/c+f$. The rate-limiting step of this net transport could be c or f or both steps. The corresponding expression for net Rb flux in direction $2 \rightarrow 1$ is obtained by interchanging in eqn. (A 1) the symbols a for d, b for c and e for f.

$$\frac{V_{max}\ 2 \rightarrow 1}{E_{Total}} = \frac{dbe}{(c+d)\ e+b(d+e)}.$$

We have argued that d is greater than c. Therefore in this case

$$\frac{V_{max}\ 2 \rightarrow 1}{E_{Total}} = \frac{dbe}{e(d+b)+db}.$$

In principle the rate-limiting step could be either b, d or e. It is unlikely to be b which has a rate 0·2–0·3/sec (Karlish *et al.* 1978). If d were rate-limiting, d would be about 0·01/sec. We have argued that d is greater than c. But as shown just above, c itself cannot be less than $V_{1\rightarrow 2}$ or about 0·15/sec. Therefore it is very unlikely that d is rate limiting. Thus it is probably e which is rate-limiting. Since also $V_{1\rightarrow 2}$ is limited by the smaller of the two rate-constants c or f, f itself must be greater than or equal to $V_{1\rightarrow 2}$ i.e. 0·15/sec. Since e must be of the order of $V_{2\rightarrow 1}$ i.e. 0·01/sec, the equilibrium between E_1 and E_2 must be far in the direction of E_1. This is of course consistent with studies on the conformational changes of the isolated Na–K-ATPase, showing that in the absence of Na or K (and the presence of Tris ions) the protein is essentially all in the form E_1 (Jorgensen, 1975; Karlish & Yates, 1978; Skou & Esman, 1980). The expression for the maximal rate or ^{86}Rb–Rb exchange given in eqn. (A 3) of the Appendix:

$$\frac{V_{max,\ exch.}}{E_{Total}} = \frac{abcd}{(b+c)\ (bd+ca+ad)}.$$

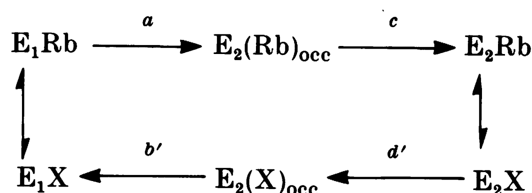
Since $a \gg b$, and as we have argued $d > c$, essentially

$$\frac{V_{\text{max, exch.}}}{e_{\text{Total}}} = \frac{bc}{b+c}.$$

Here the rate-limiting step could be b or c or both.

A consideration of the trans effect of Rb congeners on Rb uptake allows us to make further likely assignments as to which of the alternative steps are rate-limiting for a particular transport mode. Fig. 7 shows that the trans-effects of congeners (see also Table 2) are transport effects, that is they reflect exchange of Rb with the congeners as shown in scheme III for exchange of Rb with congener X.

Scheme III:



Consider first the vanadate$_0$-sensitive fluxes. The flux into Tris, choline or lysine containing vesicles should, on the basis of scheme II, be limited by rate-constants c and/or f. The flux into vesicles containing high concentrations of Rb should be limited by constants b and/or c of scheme II, and the Rb flux into high trans concentrations of congeners X should be limited by c or by b', or d' of scheme III. Since the rate has increased along the series Rb to Li, step c cannot be rate-limiting for all exchanges. If c was limiting for Rb–Rb exchange it could not also be rate-limiting for the faster Rb–X exchanges. b' or d' would then have to be rate-limiting, and hence slower than c. Thus the rate of Rb–X exchange could not be faster than Rb–Rb exchange. Hence Rb–Rb exchange must be limited primarily by b, and Rb–X exchange by b', d' or c. In the original experiments to demonstrate the occluded Rb form, Post *et al.* (1972) showed that the rate of the transition between a Li bound species $E_2(Li)_{occ}$ to E_1 was much faster than the transition betwen the $E_2(Rb)_{occ}$ and E_1. This is consistent with our finding, and suggests that the reason for the increased influx is due to an increase in the rate of the conformational transition $E_2(X)_{occ} \rightarrow E_1X$ along the series Rb, K, Cs, Na and Li. The highest rates of Rb flux into vesicles containing congeners could be limited by the rate of the transition $E_2(Rb)_{occ} \rightarrow E_2Rb$, giving a lower limit for c.

To what extent are the rate-constants deduced from our transport experiments compatible with rates that can be deduced from results in the literature (given that estimates of all rate-constants cannot be considered accurate to better than 2–3-fold). A relevant experiment is that of Glynn & Richards (1980) showing that occluded Rb is lost from Na–K-ATPase at a rate of about 0·2/sec. On the basis of the model in scheme II this rate should equal $b+c$, divided by the number of Rb ions occluded per phosphorylated enzyme. This equals 1–1·25 divided by 3, or 0·3–0·4/sec. Very good agreement. In principle one should be able to obtain a lower limit for the transition $E_2 \rightarrow E_1$, (f), from measurements of Na-dependent ATPase activity which is thought to involve the following steps. $E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_2 \rightarrow E_1$ (Glynn &

Karlish, 1975). The maximal velocity of this reaction is usually 5–10 % of the maximal rate of Na–K-ATPase (Blostein, 1970; Post *et al.* 1972; Glynn & Karlish, 1976). Given a turnover number of the latter of roughly 30/sec, the turnover number of Na-ATPase should be 1·5–3·0/sec. No step on the pathway can be slower than this rate. By this criterion the rate of the transition $E_2 \rightarrow E_1$, f must be greater than or equal to 1·5–3·0/sec. However, the value of f we obtain from our transport experiments is only 0·15/sec. A possible explanation for this discrepancy is given in the next paper of this series (Karlish & Stein, 1982).

Apparent Rb affinities

The apparent affinities for Rb ions in stimulating both net and exchange fluxes (in the direction $1 \rightarrow 2$) are high, (Figs. 3 and 5). These apparent affinities are related to the intrinsic Rb affinity at the cytoplasmic surface by eqns. (A 2) and (A 4) of the Appendix. Using the relations between the rate constants of scheme II which we have established, we can reduce the expressions in eqns. (A 2) and (A 4) to the following:

$$\text{for net flux}: \underset{1\rightarrow 2}{K_{\mathrm{m}}^{\mathrm{Rb}}} = K_{1}^{\mathrm{Rb}}.\frac{f}{a}$$

$$\text{for exchange}: K_{\mathrm{m}}^{\mathrm{Rb}} = K_{1}^{\mathrm{Rb}}.\frac{b}{a}$$

Since f and b are both very much smaller than a it is clear that the measured apparent affinity of, say, 0·5 mM is far higher than the intrinsic binding affinity of Rb_1 to E_1. Thus, the intrinsic binding affinity of Rb ions to E_1 is low, a conclusion in agreement with that drawn previously from the fluorescence experiments (Karlish *et al.* 1978; Karlish, 1980; Beauge & Glynn, 1980). One might have thought *a priori* that the occluded $E_2(K)_{occ}$ or $E_2(Rb)_{occ}$ form was not on the transport pathway (see for example Repke & Dittrich, 1979). But these considerations of the Rb affinities make the contrary assumption by far the more likely. The good agreement we have found between our deduced rate constants and those determined directly by monitoring conformational transitions lends further support to that view.

Relevance of these net and exchange fluxes to Na–K pump-mediated fluxes in cells

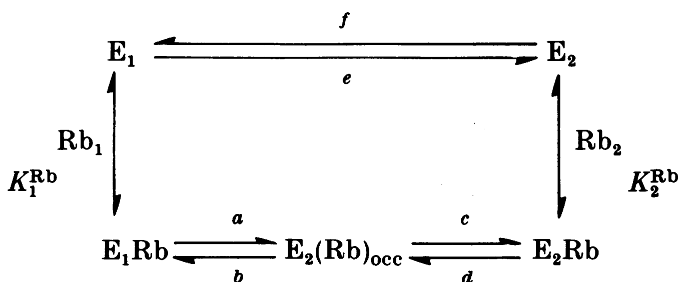
The human red cell is the experimental system which has been used most extensively to study abnormal (Na–K) pump mediated cation fluxes (Glynn & Karlish, 1975). In the red cell the active ATP-dependent Na–K exchange, measured as the ouabain-sensitive efflux, amounts to not more than about 60 % of the total Na efflux. The fluxes we have studied are between 0·5 and 2 % of the maximal ATP-dependent Na–K exchange measured in the same vesicles (Table 4). It seems clear that such small fluxes would not be detected in red cells and certainly not in other cells which are more leaky to Na and K. Appearance of new flux modes in the vesicles might be considered *a priori* to result from the cholate treatment of the enzyme before reconstitution. But the excellent agreement between the deduced transport rate-constant (b) and that measured directly on isolated Na–K-ATPase for the transition $E_2(K)_{occ} \rightarrow E_1$ makes it unlikely that the initial solubilization by cholate affected appreciably the properties of the reconstituted enzyme.

It is of some interest to consider whether the fluxes we have measured could have any physiological significance. Any such role could only involve either net movement of K from the cytoplasm to the exterior or its exchange against movement of Na from the exterior to the cytoplasm. In either case this would lead to dissipation of the gradients built up by the pump. But the very small size of these fluxes presumably excludes a significant role in dissipation of the Na and K gradients, unless one invokes stimulation by a physiological control mechanism. If, with control mechanisms, these dissipations became a significant fraction of the forward pumping rate, gearing of ion movements to ATP hydrolysis could be regulated.

It is more likely, however, that the passive fluxes we have described have no physiological significance and represent inevitable slippage of a molecular machine which has evolved to effect rapid pumping of Na and K. Their special interest would seem to be more in the fact of their very small size and in what they can tell us of the Na–K pump mechanism.

APPENDIX

We calculate the unidirectional flux of Rb from side 1 (cytoplasmic) to side 2 (extracellular) for the following scheme:



We use the method of Cha (1968), assuming Rb binding and dissociation is rapid, and King & Altman (1956). K_1^{Rb} and K_2^{Rb} represent equilibrium constants. For simplicity, the equation is derived for the case where one Rb ion is transported per cycle in each direction.

The unidirectional flux from side 1 to 2

$$V = E_T . \alpha . \frac{\gamma}{\beta+\gamma} . \frac{(\beta+\gamma)\phi+\beta\delta}{\phi(\alpha+\beta+\gamma)+(\delta+\epsilon)\beta+(\alpha+\epsilon).(\gamma+\delta)},$$

where
$$\alpha = \frac{a\,Rb_1}{Rb_1+K_1^{Rb}}, \quad \beta = b, \quad \gamma = c,$$

$$\delta = \frac{d\,Rb_2}{Rb_2+K_2^{Rb}}, \quad \epsilon = \frac{e\,K_1^{Rb}}{Rb_1+K_1^{Rb}}, \quad \phi = \frac{f\,K_2^{Rb}}{Rb_2+K_2^{Rb}}.$$

For net ^{86}Rb flux from side 1 to side 2, $Rb_2 = 0$.

Hence $\delta = 0$, $\phi = f$

$$\underset{1\to 2}{V_{max}} = E_{Total} \cdot \frac{acf}{f(a+b)+c(a+f)}, \qquad (A\ 1)$$

$$K_m^{Rb} = K_1^{Rb} . \frac{(e+f)(b+c)}{f(a+b)+c(a+f)}. \quad (A\ 2)$$

For unidirectional ^{86}Rb uptake from side 1 to side 2 into a Rb concentration effectively infinite, $Rb_2 = \infty$.

Hence $\delta = d$, $\phi = 0$

and the unidirectional flux in this case has

$$V_{max,\ exch.} = E_{Total} \frac{abcd}{(b+c)(db+ac+ad)}, \quad (A\ 3)$$

$$K_m^{Rb}{}_{,\ exch.} = K_1^{Rb} . \frac{b(e+d)+e(c+d)}{db+a(c+d)}. \quad (A\ 4)$$

We thank Mrs R. Goldshlegger for excellent technical assistance.

REFERENCES

Avron, M. (1961). Photophosphorylation as a tool for synthesis of specifically labelled nucleotides. *Analyt. Biochem.* **2**, 535–543.

Beauge, L. A. & Glynn, I. M. (1979). Occlusion of K ions in the unphosphorylated Na pump. *Nature, Lond.* **280**, 510–512.

Beauge, L. A. & Glynn, I. M. (1980). The equilibrium between different conformations of the unphosphorylated sodium pump: effects of ATP and of potassium ions, and their relevance to potassium transport. *J. Physiol.* **299**, 367–383.

Blostein, R. (1970). Sodium activated adenosine triphosphatase activity of the erythrocyte membrane. *J. biol. Chem.* **245**, 270–275.

Cantley, L. C., Cantley, L. G. & Josephson, L. (1978). A characterization of vanadate interactions with (Na, K)ATPase. *J. biol. Chem.* **253**, 7361–7368.

Cantley, L. C., Resh, M. & Guidotti, G. (1978). Vanadate inhibits the red-cell $(Na^+ + K^+)$ATPase from the cytoplasmic side. *Nature, Lond.* **272**, 552–554.

Cha, S. (1968). A simple method for derivation of rate equations for enzyme-catalysed reactions under the rapid equilibrium assumption or combined assumptions of equilibrium and steady-state. *J. biol. Chem.* **243**, 820–825.

Glynn, I. M. & Karlish, S. J. D. (1975). The sodium pump. *A. Rev. Physiol.* **37**, 13–55.

Glynn, I. M. & Karlish, S. J. D. (1976). ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of intracellular ATP and extracellular sodium. *J. Physiol.* **256**, 465–496.

Glynn, I. M., Karlish, S. J. D. & Yates, D. W. (1979). The use of formycin nucleotides to investigate the mechanism of $(Na^+ + K^+)$ATPase. In *(Na, K)ATPase Structure and Kinetics*, ed. Skou, J. C. & Norby, J. G., pp. 101–113. London: Academic Press.

Glynn, I. M., Lew, V. L. & Luthi, U. (1970). Reversal of the potassium entry mechanism in red cells, with and without reversal of the entire pump cycle. *J. Physiol.* **207**, 371–391.

Glynn, I. M. & Richards, D. E. (1980). Factors affecting the release of occluded rubidium ions from the sodium pump. *J. Physiol.* **308**, 58*P*.

Glynn, I. M. & Richards, D. E. (1982). In *Proceedings of Third International Conference on (Na, K)ATPase*. Yale University, Aug. 1981 (in the Press).

Jorgensen, P. L. (1974*a*). Purification and characterization of $(Na^+ + K^+)$ATPase. VII. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim. biophys. Acta.* **356**, 36–52.

Jorgensen, P. L. (1974*b*). Isolation of $(Na^+ + K^+)$ATPase. *Meth. Enzym.* **32**, 277–290.

Jorgensen, P. L. (1975). Purification and characterization of $(Na^+ + K^+)$ATPase. VIII. Differential tryptic modification of catalytic functions of the purified enzyme in the presence of NaCl and KCl. *Biochim. biophys. Acta* **466**, 97–108.

JORGENSEN, P. L. & KARLISH, S. J. D. (1980). Defective conformational response in a selectively trypsinized $(Na^+ + K^+)$ATPase studied with tryptophan fluorescence. *Biochim. biophys. Acta* **597**, 305–317.

KARLISH, S. J. D. (1980). Characterization of conformational changes in (Na, K)ATPase labelled with fluorescein at the active site. *J. Bioenerg. Biomembranes* **12**, 111–136.

KARLISH, S. J. D., LIEB, W. R. & STEIN, W. D. (1982). Combined effects of ATP and phosphate on Rb–Rb exchange mediated by Na–K-ATPase reconstituted into phospholipid vesicles. *J. Physiol.* **328**, 333–350.

KARLISH, S. J. D. & PICK, U. (1981). Sidedness of the effects of sodium and potassium ions on the conformational state of the sodium-potassium pump. *J. Physiol.* **312**, 505–529.

KARLISH, S. J. D. & STEIN, W. D. (1982). Effects of either ATP or phosphate on passive rubidium fluxes mediated by Na–K-ATPase reconstituted into phospholipid vesicles. *J. Physiol.* **328**, 317–331.

KARLISH, S. J. D. & YATES, D. W. (1978). Tryptophan fluorescence of $(Na^+ + K^+)$ATPase as a tool for study of the enzyme mechanism. *Biochim. biophys. Acta* **527**, 111–130.

KARLISH, S. J. D., YATES, D. W. & GLYNN, I. M. (1978). Conformational transitions between Na^+-bound and K^+-bound forms of $(Na^+ + K^+)$ATPase studied with formycin nucleotides. *Biochim. biophys. Acta* **525**, 252–264.

KING, E. L. & ALTMAN, C. (1956). A schematic method of deriving the rate laws for enzyme catalyzed reactions. *J. phys. Chem.* **60**, 1375–1379.

MATSUI, H., HAYASHI, Y., HOMAREDA, H. & KIMIMURA, M. (1977). Ouabain-sensitive ^{42}K binding to Na^+, K^+-ATPase purified from canine kidney outer medulla. *Biochem. biophys. Res. Commun.* **75**, 373–380.

MURPHY, J. & RILEY, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica. chim. Acta* **27**, 31–36.

POST, R. L., HEGEVARY, C. & KUME, S. (1972). Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. *J. biol. Chem.* **247**, 6530–6540.

REPKE, K. R. H. & DITTRICH, F. (1979). Sub-unit–sub-unit interaction: determinant of reactivity and co-operativity of Na, K-ATPase. In *(Na, K)ATPase – Structure and Kinetics*, ed. SKOU, J. C. & NORBY, J. G., pp. 487–500. London: Academic Press.

SIMONS, T. J. B. (1974). Potassium–potassium exchange catalysed by the sodium pump in human red cells. *J. Physiol.* **237**, 123–155.

SKOU, J. C. & ESMAN, M. (1980). Effects of ATP and protons on the Na:K selectivity of the $(Na^+ + K^+)$ATPase studied by ligand effects on intrinsic and extrinsic fluorescence. *Biochim. biophys. Acta* **601**, 386–403.