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SIDEDNESS OF THE EFFECTS OF SODIUM AND POTASSIUM IONS ON THE CONFORMATIONAL STATE OF THE SODIUM–POTASSIUM PUMP

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SUMMARY

1. (Na,K)ATPase from kidney membranes has been reconstituted into proteoliposomes following solubilization in cholate, by the freeze–thaw sonication procedure described by Kasahara & Hinkle (1977). The method is rapid and convenient.

2. Upon addition of ATP to the exterior medium the reconstituted vesicles sustain high rates of active ^{22}Na uptake and ^{86}Rb efflux with many properties similar to those of the Na/K pump in well characterized cells such as erythrocytes.

3. Observations on both active and passive transport of ^{22} Na and ^{86}Rb indicate that the vesicle population is heterogeneous; about 40 per cent contain Na/K pumps and the remainder seem to be plain lipid vesicles.

4. The major Na^+- or K^+-stabilized non-phosphorylated conformational forms of the (Na, K)ATPase, $E_1 . Na$ and $E_2 . (K)$ respectively, have been investigated in the proteoliposomes, with particular regard to sidedness of the actions of Na^+ and K^+.

5. Tryptic digestion of the vesicles reveals the Na^+- and K^+-stabilized conformations $E_1 . Na$ and $E_2 . (K)$ as characterized originally for purified (Na, K)ATPase (Jørgensen, 1975). Controlled trypsinolysis of $Tris^+$-loaded vesicles in a Na^+-or K^+-containing medium leads to typical biphasic (Na^+) or simple exponential (K^+) time courses respectively, for loss of ATP-dependent ^{22}Na uptake (assayed subsequent to the tryptic digestion in the presence of inophores valinomycin plus FCCP). Tryptic digestion of K^+- or Rb^+- or $Tris^+$-loaded vesicles suspended in a Na^+ medium results only in the biphasic ($E_1 . Na$) pattern of loss of ATP-dependent ^{22}Na uptake.

6. ATP-dependent ^{22}Na uptake and ^{86}Rb efflux are reduced by about the same extent following a short tryptic digestion in a Na^+-containing medium.

7. Vanadate ions inhibit ATP-dependent ^{22}Na uptake into the vesicles, at low concentrations ($K_{0.5} \sim 2 \times 10^{-7}$ M), following pre-incubation together with Mg^{2+} and K^+ ions. K^+ ions in the medium are effective, K^+ ions within the vesicle are not. Na^+ ions in the medium prevent inhibition by vanadate + Mg^{2+} but do not reverse inhibition in vesicles pre-incubated with vanadate, Mg^{2+} and K^+ ions.

8. The results show that the conformational forms $E_1 . Na$ and $E_2 . (K)$ are stabilized by Na^+ or K^+ ions respectively, bound to sites on the Na/K pump normally facing the cytoplasm. The significance of this finding is discussed in relation to the cation transport function of the pump.

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INTRODUCTION

The experiments in this paper have been designed primarily to test a hypothesis (Karlish, Yates & Glynn, 1978*a*) that Na^+ or K^+ ions stabilize the two major non-phosphorylated conformations of the (Na,K)ATPase, $E_1 . Na . E_2$ (or E_1 and $E_2 . (K)$ respectively, by combining competitively at cytoplasm oriented sites. The E_1 or $E_1 . Na$ conformations predominate in a Na^+- and K^+-free or Na^+-rich medium respectively and $E_2 . (K)$ is the enzyme form present in a K^+-rich medium. The $E_2 . (K)$ form contains occluded K^+ ions (Beaugé & Glynn, 1979) as proposed originally by Post, Hegyvary & Kume (1972). We have suggested (Karlish *et al.* 1978*a*) that the transitions between the E_1 and $E_2 . (K)$ states of the pump form part of the following reaction sequence of non-phosphorylated and phosphorylated intermediates which can account for the K^+ movements occurring in the normal Na/K exchange mode, in the reverse mode or in the K–K exchange mode of action of the Na/K pump (see Glynn & Karlish, 1975).

$$E_1 . Na . ATP \underset{Na^+_{in}}{\rightleftharpoons} E_1 . ATP \overset{K^+_{in}}{\rightleftharpoons} E_1 . K . ATP \underset{ATP}{\rightleftharpoons} E_2 . (K) \overset{P_i}{\rightleftharpoons} E_2P . K \underset{K^+_{out}}{\rightleftharpoons} E_2P$$

The E_1 conformations are shown as being bound with ATP which is probably the situation in the normal cellular environment, although of course with the isolated (Na,K)ATPase the $E_1 . Na$ to $E_2 . (K)$ transitions can be demonstrated in the absence of ATP. Also the Na^+-dependent half of the catalytic cycle is not shown here in full.

The roles of ATP in accelerating the transition of $E_2 . (K)$ to $E_1 . K$ and of phosphate in phosphorylating $E_2 . (K)$ to $E_2P . K$ can explain why the (K–K) exchange is accelerated by the presence of both ATP and P_i (Glynn, Hoffman & Lew, 1971; Simons, 1974) and why the normal Na–K exchange shows regulation by ATP in addition to the phosphorylating function (Glynn & Karlish, 1976). In experiments with inside-out vesicles from human red cell membranes, Blostein & Chu (1977) have shown that it is K^+ ions bound at high affinity extracellular sites which catalyse rapid hydrolysis of phosphoenzyme E_2P. Measurements of the rate of the transition $E_1 + K^+ \rightarrow E_2 . (K)$ using fluorescent probes showed that K^+ must combine with E_1 at low affinity sites ($K_{0.5} \sim 75$ mM) for which competing Na^+ has a high affinity ($K_{0.5} \sim 1$ mM) (Karlish *et al.* 1978*a*; Karlish, 1980). Therefore we suggested that these sites are at the cytoplasmic surface of the Na/K pump (Karlish *et al.* 1978*a*). In view of the indirect nature of this evidence for the cytoplasmic orientation of the K^+-binding sites of E_1, it seemed essential to obtain direct proof to the sidedness of the K^+ action on the conformational states.

The first step has been to prepare phospholipid vesicles containing functional (Na,K)ATPase. Several authors (Hilden & Hokin, 1975; Goldin, 1977; Racker & Fisher, 1975; Anner, Lane, Schwartz & Pitts, 1977) have reported that (Na, K)ATPase can readily be reconstituted into proteoliposomes by the 'cholate dialysis technique' described originally by Racker (1972). More recently Hokin & Dixon (1979) have applied the 'freeze–thaw sonication' procedure to both rectal gland and electric eel enzymes, and obtained a ten-fold improvement in efficiency. This method requires solubilization of the membrane protein with detergent prior to freezing with soyabean phospholipid, thawing and then sonication. The kidney (Na, K)ATPase in use in this

laboratory (see Jørgensen, 1974*a*) consists of membrane sheets embedded with mainly the one protein of interest. Therefore we have solubilized the protein with cholate as in the initial stages of the 'cholate dialysis procedure', and then reconstituted it into proteoliposomes by the freeze–thaw sonication technique.

Initially we intended to monitor the K^+-induced conformational change of the enzyme in the vesicles by making use of the formycin nucleotides (Karlish, Yates & Glynn, 1978*b*) intrinsic protein fluorescence (Karlish & Yates, 1978), or fluorescein labelled protein (Karlish, 1979, 1980) as described for the purified (Na,K)ATPase. However, preliminary experiments showed that K^+ ions did not significantly perturb the intrinsic protein fluorescence of the reconstituted vesicles. Since treatment with cholate is known to denature the ATPase (Hokin & Dixon, 1979) it then seemed preferable to adopt techniques which give information on the conformational states, through effects on the active transport capabilities of the vesicles, that is by definition on the functional Na/K pumps.

Tryptic digestion data provide convincing direct evidence for the existence and properties of the E_1 or $E_1.Na$ and $E_2.(K)$ conformations (Jørgensen, 1975). Controlled trypsinolysis of (Na,K)ATPase protein, in either Na^+- or K^+-rich media leads to production of different and distinct proteolytic fragments, and to disappearance of (Na,K)ATPase activity along a characteristic mono-exponential or biphasic time course respectively. As shown in this paper, controlled tryptic digestion of the reconstituted vesicles suspended in Na^+- or K^+-rich media results also in typical Na^+- or K^+-like patterns of loss of active Na^+ transport, suggesting that the same conformations are being detected. With the vesicles of course the sidedness is determined. An incidental finding described here concerns the relative effects of selective tryptic digestion on active Na^+ and Rb^+ transport in our vesicles. It was of interest to look at this in view of the report by Jørgensen & Anner (1979) that proteoliposomes reconstituted with a selectively trypsinized (Na,K)ATPase ('invalid' (Na,K)ATPase) sustained an ATP-dependent Na^+ influx which was significantly reduced relative to control but ATP-dependent Rb^+ efflux did not seem to be impaired.

Another tool which has proved useful for looking at the sidedness of Na^+ and K^+ actions is inhibition by vanadate. Binding of vanadate and consequent inhibition of (Na,K)ATPase are known to be stimulated by Mg^{2+} and K^+ ions, and to result in a stable complex of enzyme–Mg–K–vanadate (Cantley, Cantley & Josephson, 1978; Cantley, Josephson, Warner, Yanagisawa, Lecheve & Guidotti, 1977). We have shown recently, using both fluorescein labelled (Na,K)ATPase (Karlish, Beaugé & Glynn, 1979) or intrinsic tryptophan fluorescence (Jørgensen & Karlish, 1980) that vanadate stabilizes the species $E_2.(K)$–vanadate and prevents its reconversion to the E_1 state by added Na^+ (fluorescein signal) or ADP (tryptophan) respectively. Hence valuable information on the conformational states in the vesicles could be otained by looking at the K^+ and Mg^{2+} requirements for vanadate inhibition of the active Na^+ fluxes.

Throughout this paper sidedness effects of Na^+ and K^+ are described as cytoplasmic or extracellular in terms of the normal cellular orientation of the Na/K pumps. The reconstituted pumps may of course be arranged across the vesicle membrane in a random fashion; but only those pumps with cytoplasmic surface facing the vesicle

exterior (and extracellular surface facing the vesicle interior) will be activated by ATP added to the exterior medium, and thus detected in trypsin digestion or vanadate inhibition experiments.

The first two sections of the results describe the preparation and some transport properties of the reconstituted proteoliposomes and the third and fourth sections describe their use in tryptic digestion and vanadate inhibition experiments.

METHODS

(Na, K)ATPase was prepared from pig kidney red outer medulla by the simpler of the two procedures described by Jørgensen (1974*a*). The membranes were suspended in a medium containing imidazole, 25 mM, pH 7·5; EDTA, 1 mM and sucrose 250 mM at a protein concentration of 1–2 mg per ml., and stored at −20 °C. Before use about 1 ml. of the suspension was dialysed overnight against 1 l. ice-cold imidazole, 25 mM, pH 7·0; EDTA, 1 mM.

(Na, K)ATPase activity and protein were assayed as in Jørgensen (1974*b*). (Na, K)ATPase activity was 17–22 units per mg protein and ouabain-insensitive ATPase was usually not detectable.

Reconstitution procedure

Crude soyabean lecithin was suspended in imidazole, 25 mM, pH 7·0; EDTA, 1 mM, usually at a concentration of 40 mg per ml., vortexed for about 5 min and then sonicated on a Bransonic 12 bath sonicator until the suspension was translucent (about 1–2 min). 20% cholic acid neutralized to pH 7·4 with NaOH was purified from crude cholic acid as described by Kagawa & Racker (1971). Cholate was added to a suspension of (Na, K)ATPase (1–2 mg per ml.) at 0 °C to give the required ratio of cholate to protein (usually 5–6 mg cholate per mg protein see Fig. 2*A*). When desired the undissolved protein was removed by centrifugation on a Beckman Airfuge for 10 min at an air pressure of 20 p.s.i. (about 120,000 *g*). Usually the cholate–protein mixture was not centrifuged but was mixed immediately in 20 ml. Pyrex glass tubes with an ice-cold suspension of soyabean lecithin (40 mg/ml.) and salts, inhibitors or isotopes etc. to be incorporated into the vesicles. The mixture was vortexed for 5 sec, frozen in liquid air and was then allowed to thaw at room temperature. The suspension, now highly turbid, was sonicated for 30–60 sec on the Bransonic 12 bath sonicator, until a partial clearing was observed. It was not possible to standardize exactly this procedure for the effectiveness of sonication depended both on the volume of the suspension, the shape of the tube, depth of water in the sonicator bath, etc.

Transport assays

In order to remove external isotopes and free cholate, and to exchange the external medium for one of choice, the following procedure was carried out, after the sonication step (Penefsky, 1977). Disposable syringes (volume 1 or 5 ml.) were filled with a boiled de-aerated suspension of Sephadex G-50, which was then washed with 5–6 column volumes of the solution of choice. The syringes were placed in conical plastic centrifuge tubes which were centrifuged for 3 min at 300–400 *g* on an International Centrifuge, thus forming columns of Sephadex G-50 within the syringes. The vesicle suspension (about 100 μl. per ml. of column) was applied to the columns, and the syringes centrifuged again. The vesicles pass through the Sephadex columns, suspended now in the column wash medium. Protein determinations showed that all of the vesicle protein passes through the column, but sometimes the volume of suspension obtained was less than applied. If this occurred, the volume of the vesicle suspension was retored to its original size with the Sephadex wash medium. Control experiments using solutions of ^{22}Na or ^{86}Rb showed the Sephadex columns to be about 99·9% effective at removing cations in the original suspension.

All transport assays were run at room temperature, 20–24 °C. Assays were initiated by rapid dilution of vesicles into the appropriate reaction mixtures, usually with and without ATP. At the times chosen, aliquots were removed to determine the radioactivity in the vesicles. If ^{22}Na uptake was to be followed, ^{22}Na was added to the external medium. If ^{86}Rb efflux was to be followed, ^{86}Rb was incorporated into the vesicles and the external isotope removed prior to assay by the Sephadex G-50 centrifugation method. The separation of vesicles from the medium in the transport assays was performed on 5–6 cm columns of Dowex-50 X8 (Tris-form) poured in Pasteur pipettes fitted

with glass wool plugs (as described by Gasko, Knowles, Shertzor, Soulina & Racker, 1976). After pouring, each column was washed with 1 ml. sucrose, 250 mM plus bovine serum albumin 25 mg/ml. and stored at 4 °C. Just before the assay, columns were washed with a further 1 ml. of the same ice-cold solution. Up to 100 μl. of the vesicle suspension, with total salt concentration of 150–200 mM was applied per Dowex column, and the vesicles were eluted into counting vials by addition of 1·5 ml. ice-cold 250 mM sucrose solution. Application of 100 μl. 150 mM-NaCl + ^{22}Na or 150 mM-RbCl + ^{86}Rb showed that the Dowex columns let through only 0·03 or 0·01 per cent of applied ^{22}Na or ^{86}Rb respectively. Comparison of the turbidity of the eluted volume with that of vesicles added directly to 1·5 ml. sucrose showed that essentially all the vesicles pass through the column. Excellent reproducibility of isotope content in replicate samples of vesicles incubated with ^{22}Na or ^{86}Rb confirmed that conclusion. Elution time was about 30 sec. ^{22}Na was estimated by its γ-emission and ^{86}Rb by Cerenkov radiation. All transport assays were run with duplicate samples. Rates of ATP-dependent ^{22}Na uptake were obtained from the difference in uptake with and without ATP. From the fraction of total isotope take up and the Na^+ concentrations, absolute rates of Na^+ transport were calculated. Absolute rates of ^{86}Rb efflux were calculated in vesicles loaded with ^{86}Rb during their formation, from the isotope efflux and initial specific activity of the suspension (i.e. before removal of exterior isotope on the Sephadex columns). Errors in flux measurements refer to S.E. of mean. Vesicle protein was determined as described by Jørgensen (1974*b*), following solubilization of the vesicles in 2 % deoxycholate.

Source of materials

^{22}Na and ^{86}Rb were obtained from the Radiochemical Centre, Amersham, U.K. Crystalline disodium ATP from Boehringer was freed from sodium by passage through Dowex-50 X8 columns and was neutralized with Tris base. Dowex-50 X8 50–100 mesh and ammonium orthovanadate were obtained from Merck. Ouabain, soyabean phospholipid (P5638), trypsin, Type III, soyabean tryptic inhibitor, valinomycin and Sephadex G-50–40 were purchased from Sigma. FCCP *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone was a gift from Professor M. Avron. All other materials were of analytical grade.

RESULTS

Vesicle preparation

Fig. 1 shows raw data for uptake of ^{22}Na at 20 °C into K^+-loaded vesicles incubated in a medium containing NaCl + ^{22}Na and $MgCl_2$, with or without ATP. Four separate estimations of protein in vesicles prepared in optimal conditions lead to a figure of $1{\cdot}55 \pm 0{\cdot}19$ mg of ATPase protein per 10 μl. vesicles and another $1{\cdot}36 \pm 0{\cdot}07$ μg of protein is contributed by the soyabean phospholipid itself. The initial rate of ATP-dependent ^{22}Na uptake in Fig. 1 is 1600 n-mole per min per mg ATPase protein. This is five to twentyfold higher than for the kidney enzyme reconstituted by the cholate dialysis technique (Goldin, 1977; Racker & Fisher, 1975; Anner, Lane, Schwartz & Pitts, 1977) and two to threefold higher than reported for vesicles prepared by the freeze–thaw sonication procedure (Hokin & Dixon, 1979). The other important feature in Fig. 1 is the relatively low passive permeability to ^{22}Na, and thus a high ratio of ATP-dependent to -independent ^{22}Na flux.

Four major factors have been found to be responsible for the high efficiency of the reconstitution.

First the ratio of cholate to protein in the initial solubilization step showed a definite optimum (Fig. 2*A*) of 5·5–8·0 mg per mg protein. At the routinely used ratio of 5·5 mg cholate per mg ATPase about 40 % of the protein is solubilized. Generally, however, separation of the solubilized protein from intact membranes was not performed, for reconstitution of active transport was improved by about 30 % if the ice-cold membrane–cholate mixture was added to the lipid immediately and then

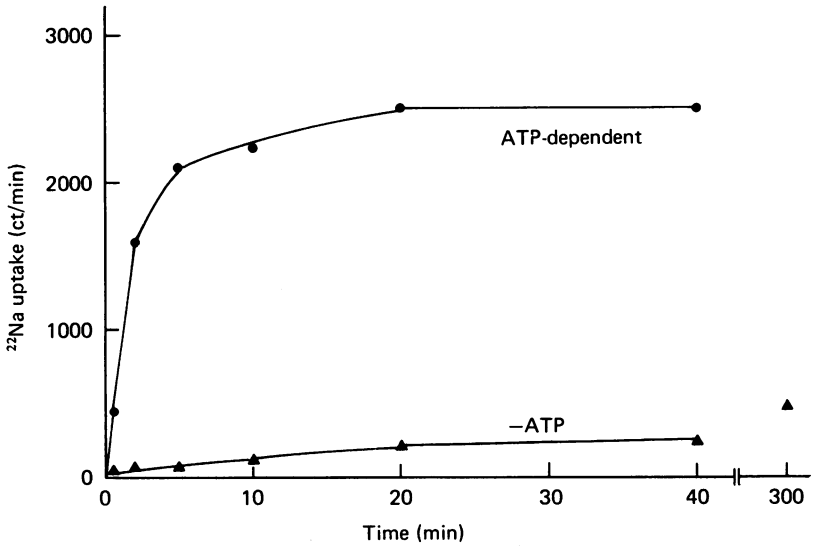

Fig. 1. Time course of ATP-dependent and -independent ^{22}Na uptake into reconstituted proteoliposomes. 550 μl. of proteoliposome suspension was prepared in a medium containing KCl, 200 mM, from lipid vesicles (16 mg) and the soluble supernatant obtained by centrifuging in the Airfuge a mixture of enzyme (0·5 mg) and cholate (2·6 mg). The vesicles were then centrifuged on a Sephadex G-50 column equilibrated with Tris HCl, 190 mM, pH 7·0; NaCl, 10 mM and $MgCl_2$, 3 mM. For the assay 200 μl. of proteoliposome suspension was added at room temperature to 300 μl. of a reaction medium containing Tris HCl, 190 mM, pH 7·0; NaCl, 10 mM (+ ^{22}Na); $MgCl_2$, 3 mM, with or without ATP (Tris), 6 mM. At the times indicated duplicate samples of 40 μl. (i.e. containing 16 μl. of the original vesicle suspension) were transferred to Dowex columns. Total radioactivity was also measured on a 40 μl. sample of suspension.

frozen quickly and thawed. It was however apparent that it is cholate-solubilized protein which becomes incorporated into the proteoliposomes (as in Fig. 1). The ATP-independent ^{22}Na flux was not greatly affected by the cholate concentration.

The presence of pump ligands such as ATP, Mg^{2+}, Na^+ or K^+ ions in the cholate solubilization stage did not affect the efficiency of reconstitution, although K^+ ions were of course required at the freeze–thaw step in order to produce K^+-loaded vesicles (see also Table 2).

Solubilization of the (Na, K)ATPase in deoxycholate or Triton-X100 gave very poor reconstitution of active transport.

The second important factor was the ratio of the soyabean phospholipid to ATPase, which showed (Fig. 2*B*) a rather sharp optimum of ATP-dependent to -independent ^{22}Na uptake at about 40 mg lipid per mg of total protein, (or 100 mg lipid per mg of cholate-solubilized protein). This is similar to that reported for freeze–thaw reconstitution of electric eel and shark rectal gland (Na, K)ATPase (Hokin & Dixon, 1979).

A brief sonication (30–60 sec) of the lipid–ATPase mixture after thawing, improved the rate of ATP-dependent ^{22}Na uptake by about threefold compared to a non-sonicated suspension, without affecting significantly the passive ^{22}Na uptake (not

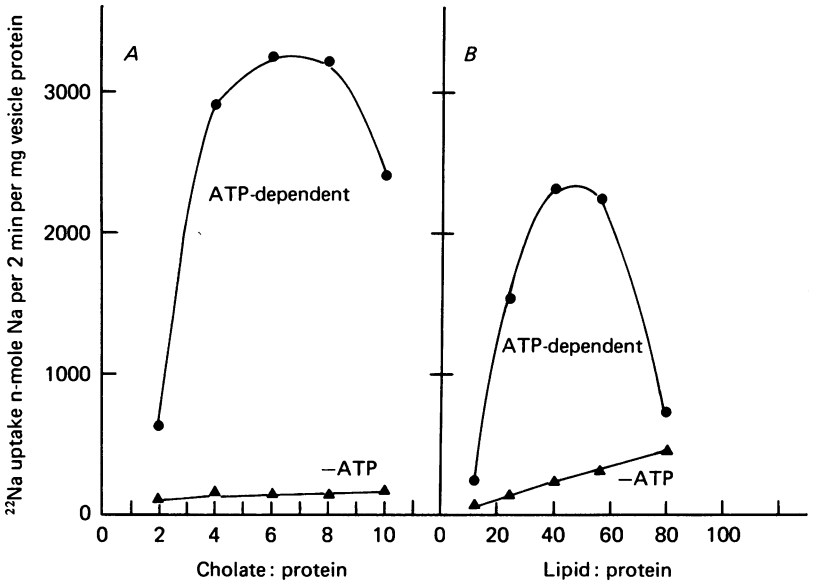

Fig. 2. Optimal ratios of cholate:protein and lipid:protein. *A*, five sets of 160 μl. of proteoliposomes were prepared in a medium containing KCl, 200 mM, from lipid vesicles (4 mg) and enzyme (0·1 mg) plus cholate, 0·2, 0·4, 0·6, 0·8 or 1·0 mg as indicated. The vesicles were centrifuged on five columns of Sephadex equilibrated with Tris HCl, 190 mM, pH 7·0; NaCl, 10 mM and $MgCl_2$, 3 mM. For assay 20 μl. of vesicles were incubated for 2 min in duplicate with 20 μl. of the same reaction mixture as in Fig. 1. *B*, five sets of 130 μl. of proteoliposomes were prepared in a medium containing KCl 200 mM, from lipid vesicles, 0·6, 1·2, 2, 2·8 or 4 mg respectively, and enzyme (0·05 mg) plus cholate (0·28 mg). Other procedures as in *A*.

shown). Although it was not easy to standardize the sonication procedure, in practice a partial clarification of the thawed suspension was sufficient. The vesicle suspension could be clarified to near transparency by sonication for 2–3 min but without improvement of reconstitution.

Finally, the efficiency of reconstitution was increased roughly twofold, when the pH of the medium for enzyme solubilization and freeze–thawing was 6·5–7·0 rather than at the more standard pH range 7·5–8·0.

Active Na^+ and Rb^+ transport

We assumed that the pumps may be incorporated into the vesicle in an un-oriented fashion but only those pumps with their ATP-binding sites facing the exterior should be activated by the impermeant substrate ATP. Such pumps should also characteristically be activated by internal K^+ ions, and inhibited by ouabain when incorporated into the vesicles but not when present outside. These features are seen very clearly in Tables 1 and 2 for the ATP-dependent ^{22}Na uptake. The dependence on internal K^+ is also demonstrated indirectly from the experiments of Table 3 and Fig. 7 in which the ionophores valinomycin and FCCP have been used to ensure a constant internal concentration of K^+, added initially to the external medium. When the external Na^+ ion concentration was varied, the ATP-dependent ^{22}Na uptake into

TABLE 1. Dependence of ATP-dependent ^{22}Na uptake on K^+ ions within the proteoliposome (n-mole Na^+ uptake per 2 min per mg vesicle protein)

Type of vesicles	Passive	ATP-dependent
Tris HCl-loaded (200 mM)	348+18	406±87
NaCl-loaded (200 mM)	361±20	529±146
KCl-loaded (200 mM)	310±30	3148±151

Three sets of 150 μl. of vesicles were prepared as in Fig. 2*A* in media containing Tris HCl, 200 mM; NaCl, 200 mM or KCl, 200 mM. The assay was as in Fig. 2*A*.

TABLE 2. Inhibition of ATP-dependent ^{22}Na uptake by ouabain incorporated into the proteoliposomes (Na^+ uptake n-mole per 2 min per mg vesicle protein)

Condition	Passive	ATP-dependent
(*a*) Control vesicles	145±20	2688±135
(*b*) +External ouabain 0·5 mM	193±23	2483±67
(*c*) Vesicles containing ouabain 0·5 mM	155±15	181±16
(*d*) Vesicles containing ouabain 0·5 mM +external ouabain 0·5 mM	181±8	194±12

Two sets of vesicles were prepared as in Fig. 2*A*, one set containing 0·5 mM-ouabain. The vesicles were assayed as in Fig. 2*A* except that, where indicated, the assay medium contained also 0·5 mM-ouabain.

K^+-containing vesicles, was activated along a sigmoid curve, Fig. 3*A*. This could be fitted well to an equation of the form

$$v = V.\left(\frac{S}{K+S}\right)^n$$

where $n = 2$ or 3, $K = 1{\cdot}41$ mM or 0·76 mM respectively and $V = 2805$ n-mole Na^+ per mg vesicle protein per 2 min. (Fig. 3*B*). As expected, with $n = 1$ the plot of $[Na^+]/V^{1/n}$ versus $[Na^+]$ deviated upwards from a straight line at the lowest Na^+ concentrations.

In Fig. 4, the active and passive influxes of ^{22}Na into vesicles prepared to contain 50 mM-RbCl have been compared with ^{86}Rb efflux from identically prepared vesicles containing also ^{86}Rb, but suspended in a reaction mixture lacking ^{22}Na. Rb^+ ions are known to act like K^+ ions at the external aspect of the Na- pump. The ratios of the cumulative ATP-dependent ^{22}Na efflux to cumulative ATP-dependent ^{86}Rb efflux were 1·40, 1·37, 1·68 and 1·40 at each time point respectively, with an average of 1·48±0·07. However, the estimates of the true initial rates of both the ATP-dependent ^{22}Na uptake and particularly ^{86}Rb efflux in these vesicles was uncertain, for linearity was not maintained for more than about 0·5 min. This was obviously due in part to rapid depletion of the vesicular Rb^+ content (and possibly to accumulation of Na^+, see Fig. 7). In vesicles containing initially 200 mM-KCl the linear rate of active Na^+ uptake was sustained for about 2 min (see Fig. 1).

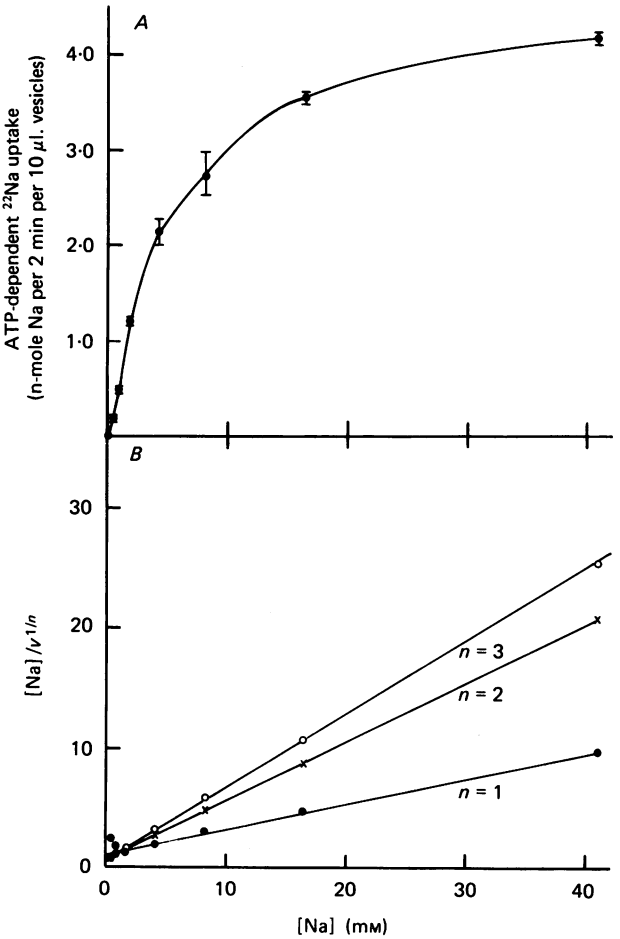

Fig. 3. Dependence of active Na^+ uptake on Na^+ concentration. 300 μl. of proteoliposomes containing 100 mM, KCl were prepared from lipid vesicles (7 mg) and enzyme (0·18 mg) plus cholate (0·9 mg), and the exterior solution was exchanged on a Sephadex column equilibrated with Tris HCl, 100 mM, pH 7·0. To aliquots of 40 μl. of the vesicles was added 70 μl. of a mixture of Tris HCl + NaCl (total concentration 100 mM), adjusted so that in the final assay there was 0·41, 0·82, 1·63, 4·1, 8·2, 16·3 and 41 mM, NaCl respectively. The assay was started by adding 25 μl. of this vesicle suspension to 10 μl. of a reaction mixture containing Tris HCl, 100 mM; $MgCl_2$, 10 mM; ^{22}Na (150,00 ct/min) with or without ATP (Tris) 10 mM, and incubated for 2 min at room temperature (final $MgCl_2$ and ATP concentrations were 3·75 mM).

Also, no more than 30–40 % of the total ^{86}Rb trapped initially in the vesicles can be extruded in the presence of ATP; this high background level of isotope is another major reason for the unreliability of the estimates of initial rates of ATP-dependent ^{86}Rb efflux. The phenomenon could imply that 60–70 % of the vesicle population either contain no sodium pumps or contain pumps with their ATP binding sites oriented to the interior. These possibilities have been tested in the following manner (Fig. 5). Unpublished work of S. Karlish and W. D. Stein has shown that the reconstituted vesicles sustain a rapid Rb–Rb exchange in the presence of ATP,

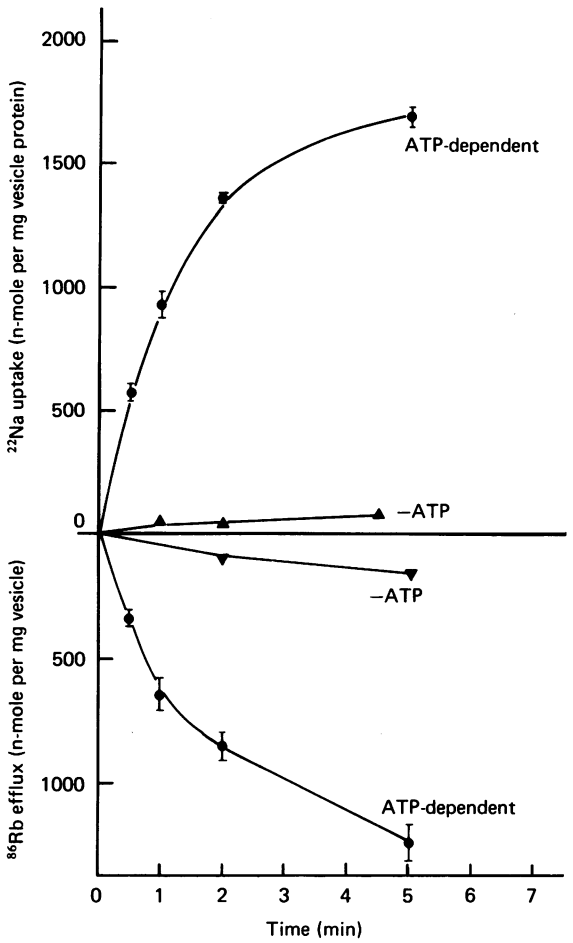

Fig. 4. Coupling of ATP-dependent ^{22}Na uptake to ATP-dependent ^{86}Rb efflux. Two sets of 300 μl. of vesicles were prepared in a medium containing RbCl, 50 mM and Tris HCl, 100 mM pH 7·0 (with or without ^{86}Rb ~ 1 μCi), from azolectin vesicles (6 mg) and enzyme (0·16 mg) plus cholate (0·9 mg), and were then centrifuged on Sephadex G-50 columns equilibrated with Tris HCl, 140 mM, pH 7·0 and NaCl, 10 mM. For ^{22}Na uptake assay 150 μl. of the ^{86}Rb-free vesicles were mixed at room temperature with 150 μl. of reaction mixture containing Tris HCl, 140 mM; NaCl, 10 mM (+^{22}Na); $MgCl_2$, 6 mM with or without ATP (Tris) 6 mM. For ^{86}Rb efflux, 150 μl. of the ^{86}Rb-containing vesicles were mixed with 150 μl. of the same reaction mixtures, but lacking ^{22}Na. At the times indicated duplicate 25 μl. samples were transferred to Dowex columns. The initial ^{86}Rb content of the vesicles was estimated within 10 sec of mixing, on the ATP-free samples. The initial specific activity of the ^{86}Rb, was also measured, prior to the Sephadex step.

orthophosphate and Mg^{2+} ions. This Rb–Rb exchange has been exploited to load with ^{86}Rb specifically those vesicles that have their pump ATP binding sites facing the exterior (and hence avoid incorporating ^{86}Rb into all of the vesicles during their formation). After a short incubation of vesicles containing 100 mM-RbCl but no isotope, in an exchange medium containing ATP, P_i, Mg^{2+}, 2 mM-Rb^+ and ^{86}Rb, the vesicles are separated from the exchange medium on short columns of Sephadex G-50.

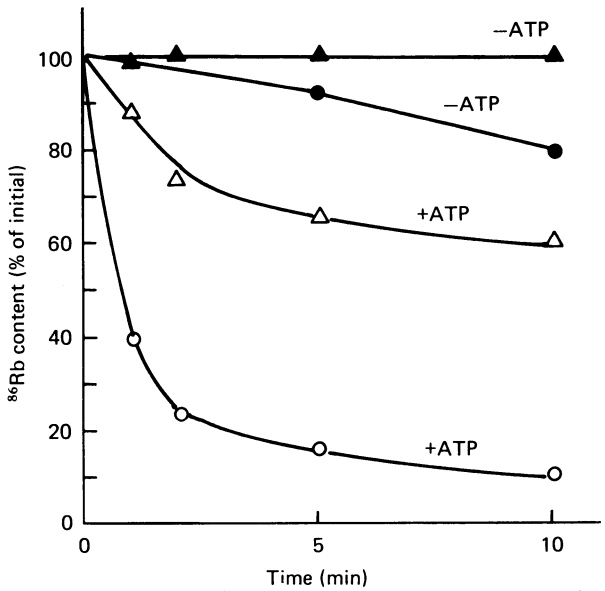

Fig. 5. Active and passive ^{86}Rb efflux from vesicles loaded with ^{86}Rb in two different ways. Two sets of 300 μl. of vesicles were prepared in a medium containing RbCl, 100 mM (with or without ^{86}Rb ~ 1 μCi); Tris HCl, 50 mM as in Fig. 4, and the exterior medium was exchanged on Sephadex columns for one containing only Tris HCl, 150 mM, pH 7·0. The set containing ^{86}Rb was kept on ice. The other set was incubated at room temperature for 5 min with a solution containing Tris HCl, 140 mM, pH 7·0; phosphate (Tris), 10 mM; ATP (Tris), 2 mM, RbCl 2 mM + ^{86}Rb 1 μCi; $MgCl_2$, 5 mM. Then both sets of vesicles were recentrifuged on fresh columns of Sephadex equilibrated with Tris HCl 150 mM pH 7·0. The assay was initiated by adding at 22 °C, 100 μl. of vesicles to 300 μl. of reaction mixture containing Tris HCl, 80 mM; NaCl, 60 mM; $MgCl_2$, 3 mM with or without ATP (Tris), 4 mM. The initial radioactivity was estimated within 10 sec of mixing on 30 μl. samples from the reaction mixture lacking ATP, and duplicate samples of 30 μl. were transferred to the Dowex columns at the times indicated. It was not possible to calculate the absolute rate of Rb^+ efflux in samples loaded by Rb–Rb exchange for internal ^{86}Rb specific activity was not known with certainty. ▲, △ ^{86}Rb loading during vesicle formation; ●, ○ ^{86}Rb loading by Rb–Rb exchange.

Upon addition of ATP, Na^+ and Mg^{2+} to the vesicles at room temperature, 90 % of the ^{86}Rb trapped during the exchange, was rapidly extruded from the vesicles. By contrast in vesicles loaded with Rb^+ 100 mM + ^{86}Rb in the conventional way, no more than 40 % of the isotope could be pumped out (Fig. 5). The experiment shows:

(1) The vesicle population is heterogeneous (see also Fig. 6).

(2) Rates of ATP-dependent ^{86}Rb efflux measured at say 1 min in conventionally loaded vesicles are inaccurate and certainly underestimate the initial rates for over 60 % of ^{86}Rb has been extruded.

(3) The passive ^{86}Rb permeability of the selectively loaded vesicles appears to be higher than that of the total population.

Subsequently selectively loaded vesicles have proved invaluable for measuring accurately small differences in initial rates of ATP driven ^{86}Rb extrusion (see Fig. 10).

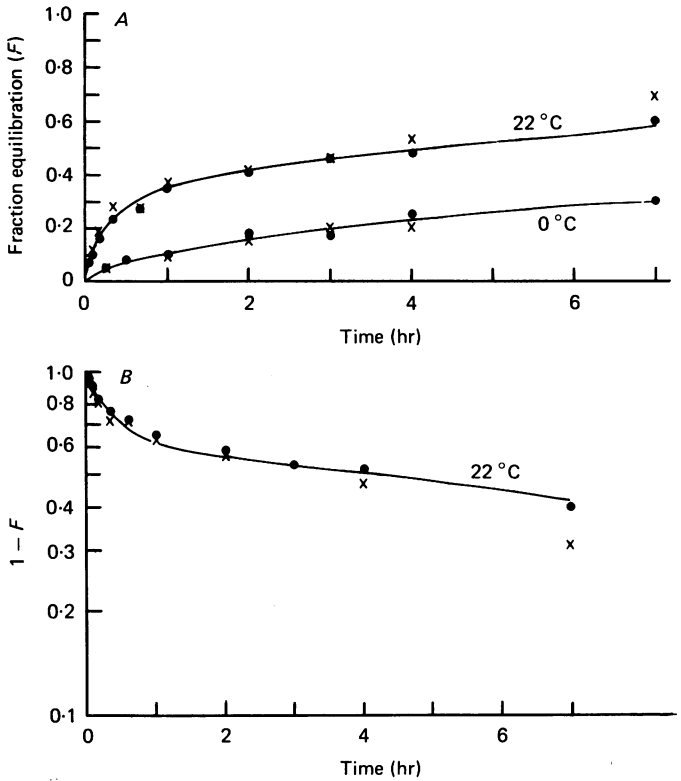

Fig. 6. Passive permeation of ^{86}Rb and ^{22}Na. 1·2 ml. of proteoliposomes was prepared in a medium containing Tris HCl, 150 mM, pH 7·0, from lipid vesicles (22·65 mg) and enzyme (0·55 mg) plus cholate (3 mg), followed by centrifugation on Sephadex G-50 equilibrated with Tris HCl, 150 mM. 300 μl. was added to 1 ml. of Tris HCl, 140 mM, pH 7·0 + NaCl, 10 mM (+ ^{22}Na) or to 1 ml. of Tris HCl, 140 mM + RbCl, 10 mM (+ ^{86}Rb), either at 22 °C or at 0 °C. Duplicate samples of 50 μl. were removed to Dowex columns at the times indicated. 50 μl. of the reaction mixtures diluted by a factor 1·3 were also applied in triplicate to columns and eluted with 1·5 ml. sucrose, and this background radioactivity was subtracted from that in the vesicles. At isotopic equilibration, assumed to be complete at 48 hr, and 22 °C, the vesicles contained 0·57 % of the total ^{22}Na or ^{86}Rb radioactivity (from which figure a value of 1·2 μl. internal volume per mg lipid was calculated). The fractional equilibration F was calculated from the radioactivity in the vesicles at any time divided by that at infinite time. ^{22}Na, ●; ^{86}Rb, ×.

Passive Na^+ and Rb^+ permeability

^{22}Na or ^{86}Rb added externally, permeated passively into the reconstituted vesicles at the same rate, both at room temperature and at 0 °C, Fig. 6*A*. At 22 °C the permeation was clearly biphasic; the line drawn through the semilogarithmic plot of Fig. 6*B* fits the equation $1-F = 0{\cdot}35\,e^{-0{\cdot}055t} + 0{\cdot}65\,e^{-0{\cdot}001t}$ where t is the time in minutes. In another experiment the amplitudes of the two phases were about 0·4 and 0·6 and the two rate constants were 0·035 min^{-1} and 0·0015 min^{-1} respectively. For comparison the half-time for ^{22}Na permeation at 22 °C into lipid vesicles, prepared as in Fig. 6 but without the (Na, K)ATPase was about 7 hr (i.e. rate constant 0·00165 min^{-1}), (not shown). Therefore it seems reasonable to assume that

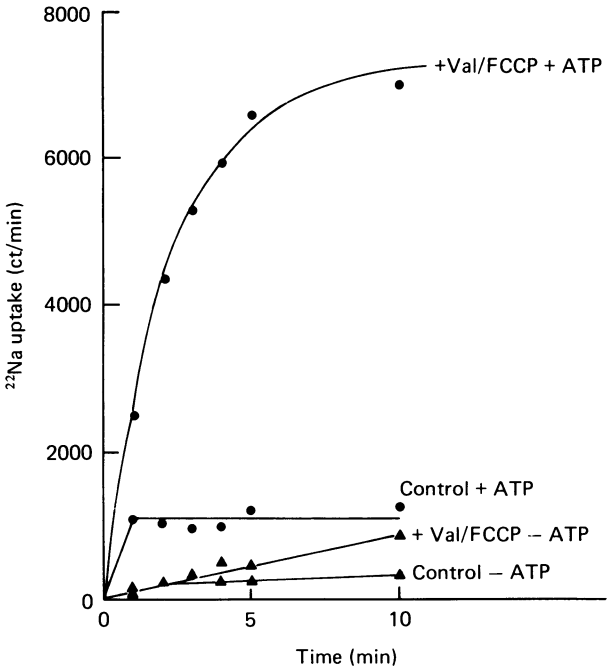

Fig. 7. ATP-dependent ^{22}Na uptake measured in the presence of valinomycin plus FCCP. 400 μl. of proteoliposomes were prepared in a medium containing 100 mM Tris HCl and 50 mM-Tris acetate, pH 7·0, from lipid vesicles (8 mg) and enzyme (0·2 mg) plus cholate 1 mg and centrifuged on a Sephadex column equilibrated with Tris HCl, 100 mM + Tris acetate, 50 mM. 200 μl. of vesicles were added to two sets of 200 μl. of a solution containing NaCl, 60 mM; KCl, 20 mM; Tris HCl, 70 mM, and then 4 μl. ethanol or 4 μl. of an ethanolic solution of valinomycin (Val.; 40 μM) plus FCCP (40 μM) (final 0·8 mM of each ionophore) was added. After 20 min at 0 °C the treated and untreated vesicle suspensions were divided, warmed to room temperature and 200 μl. mixed with 50 μl. of a reaction medium containing NaCl, 30 mM (+ ^{22}Na); KCl, 10 mM; Tris HCl, 110 mM, pH 7·0; $MgCl_2$, 15 mM, with or without ATP (Tris) 15 mM. At the times indicated aliquots were removed to the Dowex columns.

(Na, K)ATPase has been incorporated into 35–40 % of the population (the more permeable vesicles), while the remaining 60–65 % are plain lipid vesicles.

The other striking feature of Fig. 6*A* is very large drop in rate of permeation at 0 °C (half-time of about 14 hr).

Tryptic digestion of reconstituted vesicles

In the experiments of Figs. 8 and 9 the sensitivity of the reconstituted pumps to tryptic digestion was tested with vesicles containing either K^+ or $Tris^+$ ions and suspended in Na^+ or K^+ media. This involved first controlled proteolytic digestion and then assay of ATP-dependent ^{22}Na uptake. It was therefore essential to introduce the K^+ necessary for activating the ATP-dependent ^{22}Na uptake, into the vesicles after treatment with trypsin. This was achieved by suspending the vesicles in a medium with both Na^+ and K^+ and treating with a combination of the K^+-selective ionophore valinomycin and H^+-ionophore FCCP. In Table 3 ATP-dependent and passive ^{22}Na uptake are compared for either K^+ or $Tris^+$-loaded vesicles, untreated or treated

TABLE 3. Active and passive Na^+ fluxes in reconstituted proteoliposomes treated with ionophores (n-mole Na^+ uptake per mg vesicle protein per 2 min ± S.E. of mean)

	Control		+ Valinomycin		+ FCCP		+ Valinomycin + FCCP	
Type of vesicles	Passive	ATP-dependent	Passive	ATP-dependent	Passive	ATP-dependent	Passive	ATP-dependent
KCl-loaded	871 ± 122	3516 ± 174	1148 ± 39	3000 ± 226	839 ± 11	3677 ± 477	761 ± 5	3194 ± 483
Tris HCl-loaded	406 ± 65	548 ± 71	438 ± 548	2065 ± 122	477 ± 16	587 ± 26	935 ± 40	2870 ± 361

Two sets of 400 μl. of proteoliposomes were prepared in media containing either Tris HCl, 200 mM, pH 7·0 or KCl, 200 mM, from lipid vesicles (8 mg) and enzyme (0·2 mg) plus cholate (1 mg). The vesicles were then centrifuged on Sephadex columns equilibrated with NaCl, 100 mM; KCl, 10 mM and Tris acetate, 50 mM, pH 7·0. Each set was divided into equal volumes of 100 μl., and 1 μl. ethanol, or ethanolic solutions of valinomycin (20 μM), FCCP (20 μM) or valinomycin plus FCCP (20 μM) was added as appropriate (final concentrations 0·2 μM). For assay 20 μl. of vesicle suspension was incubated for 2 min with 20 μl. of a reaction mixture containing NaCl, 100 mM ($+^{22}Na$); KCl, 10 mM; Tris acetate, 50 mM, pH 7·0; $MgCl_2$, 6 mM, with or without ATP (Tris), 6 mM.

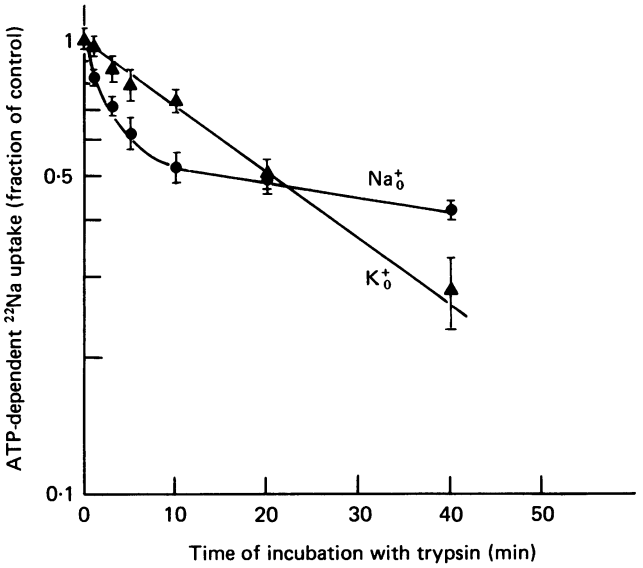

Fig. 8. Inactivation of ATP-dependent ^{22}Na uptake by controlled tryptic digestion in a Na^+- or K^+-containing medium. 1·7 ml. of proteoliposomes were prepared in a medium containing Tris HCl, 150 mM, pH 7·0, from lipid vesicles (16 mg) and enzyme (0·4 mg) plus cholate (2 mg) and centrifuged on Sephadex G-50 columns equilibrated with Tris HCl, 150 mM, pH 7·0. The suspension was warmed to room temperature, divided, and NaCl (1 M) or KCl (1 M) added to a final concentration of 9 mM. 100 μl. of each suspension (control samples) was removed into 300 μl. of an ice-cold medium containing NaCl, 40 mM; KCl, 12·5 mM; Tris HCl, 110 mM, pH 7·0, soya bean tryptic inhibitor, 1 μg and trypsin, 0·35 μg. To the remaining 750 μl., 3 μg of trypsin (130 μl. of 100 μg/ml.) was added and 100 μl. was removed at the times indicated into 300 μl. of the ice-cold medium containing the tryptic inhibitor, 1 μg. At the end of incubation with trypsin, 5 μl. of an ethanolic solution of valinomycin (40 μM) and FCCP (40 μM) was added to each sample (final concentration of ionophores 0·5 μM) and left on ice for about 30 min. For assay of active Na^+ transport, 80 μl. of vesicles were added at room temperature to 20 μl. of reaction medium containing NaCl, 30 mM (+ ^{22}Na); KCl, 10 mM; Tris HCl, 100 mM; $MgCl_2$, 15 mM, with or without ATP (Tris), 15 mM, and incubated for 2 min prior to transfer to Dowex columns. ▲, K^+ medium; ●, Na^+ medium.

with the ionophores. The untreated Tris HCl loaded vesicles of course sustained very little active Na^+ uptake (column 2, line 2). Addition of valinomycin alone caused a large increase of ATP-dependent ^{22}Na transport but this was still somewhat lower than the maximal rate in K^+-loaded vesicles (column 4). With the combination of valinomycin and FCCP the same high rate of active Na^+ transport was observed as in K^+ loaded vesicle (column 8), although FCCP itself was quite ineffective (column 6) (see Discussion for the probable explanation). The combination of FCCP and valinomycin appeared to raise somewhat the passive Na^+ permeation, but this did not seriously affect the subsequent measurements of interest. The passive Na^+ permeation was also higher in K^+ loaded vesicles, particularly in the presence of valinomycin (compare top and bottom rows), presumably due to formation of an outside-positive K^+ diffusion potential. Maximal rates of active Na^+ uptake are also observed with media containing 30 mM-Na^+ + 10 mM-K^+, and routinely this is

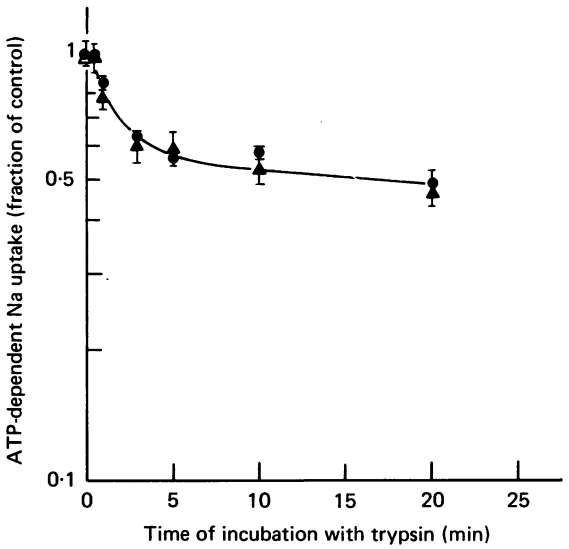

Fig. 9. Inactivation of ATP-dependent ^{22}Na uptake by controlled tryptic digestion of proteoliposomes loaded with $Tris^+$ or K^+ ions and suspended in a Na^+-containing medium. Two sets of 300 μl. of proteoliposomes were prepared in media containing either Tris HCl, 150 mM, pH 7·0 or Tris HCl, 140 mM + KCl, 10 mM, from lipid vesicles (6 mg) and enzyme (0·15 mg) plus cholate (0·8 mg), and were then centrifuged on Sephadex columns equilibrated with Tris acetate, 50 mM + Tris HCl, 100 mM. NaCl was added to a final concentration of 10 mM. 50 μl. of each type of proteoliposome (control samples) was removed into 150 μl. of an ice-cold medium containing NaCl, 30 mM; KCl, 12·5 mM; Tris HCl, 100 mM; soya bean tryptic inhibitor, 1 μg, and trypsin, 0·3 μg. 3 μg of trypsin was added to 250 μl. of the vesicles and at the times indicated 50 μl. was removed into the medium containing tryptic inhibitor. 5 μl. of valinomycin plus FCCP solution was added (final concentration 0·2 μM). For the assay (2 min) duplicate samples of 40 μl. of vesicle suspension at room temperature was added to 20 μl. of reaction medium containing NaCl, 30 mM; KCl, 10 mM; Tris HCl, 100 mM pH 7·0; $MgCl_2$, 9 mM, with or without ATP (Tris) 9 mM. ●, K^+ loaded; ▲, $Tris^+$ loaded.

preferable to 100 mM-NaCl (as in Table 3) for the passive ^{22}Na leak is much reduced relative to the fully saturated active Na^+ uptake.

Fig. 7 shows the time course of active and passive Na^+ uptake suspended in the optimal medium, with or without the ionophore combination. The untreated vesicles sustain a small ATP-dependent ^{22}Na uptake of short duration which is attributable to leakage of a small amount of K^+ into the vesicles prior to addition of the ATP, and then rapid ejection. The very rapid ATP-dependent ^{22}Na uptake in the ionophore treated vesicles suggests that the K^+ is indeed leaking inwards and balances the active K^+ extrusion. The drop in the initial rate after 2 min may be due to accumulation of high concentrations of Na^+ inside the vesicles. In subsequent experiments using this technique, fluxes have been measured for 2 min. (Acetate ions were present in the medium of this and the trypsin experiments in Figs. 8 and 9, but acetate was subsequently found to be unnecessary and was omitted.)

Fig. 8 shows that when $Tris^+$-loaded vesicles are exposed to controlled tryptic digestion together with either 10 mM-NaCl or -KCl, and the active Na^+ transport is subsequently assayed using the valinomycin/FCCP method, the activity is inactivated

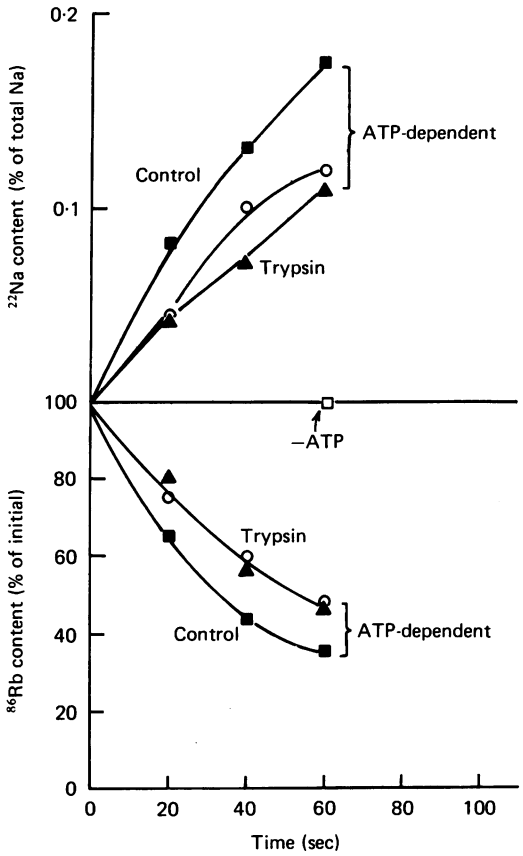

Fig. 10. Active ^{22}Na and ^{86}Rb fluxes in vesicles digested with trypsin in a Na^+-containing medium. 700 μl. of proteoliposome suspension was prepared in a medium containing RbCl, 100 mM and Tris HCl, 50 mM, pH 7·0, from lipid vesicles (12 mg) and enzyme (0·29 mg) plus cholate (1·3 mg), and were then centrifuged on Sephadex columns equilibrated with Tris HCl, 150 mM, pH 7·0. The preparation was warmed to room temperature and divided into equal portions. To each set ATP (Tris), 1 mM; phosphate (Tris), 5 mM; $MgCl_2$, 3 mM and RbCl, 2 mM were added. ^{86}Rb ($\sim$ 1 μCi) was added to one portion only. After 5 min the two sets of vesicles were then recentrifuged on Sephadex columns equilibrated with Tris HCl, 150 mM, pH 7·0 and CDTA (Tris), 1 mM. NaCl was added to a concentration of 50 mM. To 110 μl. of each set was added a mixture of 10 μl. of trypsin (100 μg/ml.) and 10 μl. soya bean tryptic inhibitor (400 μg/ml.) (control samples). To 110 μl. of duplicate samples from each set, 10 μl. of trypsin (100 μg/ml.) was added, and after 2 or 4 min of incubation the digestion was terminated with 10 μl. of tryptic inhibitor (400 μg./ml.) For the assay, 60 μl. of vesicle suspension was mixed at room temperature with 250 μl. of reaction medium containing NaCl, 50 mM (with ^{22}Na for those vesicles not loaded with ^{86}Rb, and lacking ^{22}Na for the ^{86}Rb-loaded vesicles); Tris HCl, 100 mM, pH 7·0; $MgCl_2$, 4 mM, with or without ATP (Tris), 4 mM. At the times indicated duplicate samples of 40 μl. were applied to the Dowex columns. The initial ^{86}Rb content of the isotope-loaded vesicles was obtained from the ATP free sample. Since there was no measurable loss of ^{86}Rb over 60 sec in the ATP-free sample, only one time point has been included in the Figure. Also the ATP-independent ^{22}Na uptake over 60 sec was very small and is not shown. ■, Control; ▲, 2 min; ○, 4 min of tryptic digestion.

along quite distinct and different time courses for the NaCl or KCl medium respectively. The typical pattern of a simple exponential loss of activity in the K^+ medium and the biphasic rate of loss in the Na^+ medium is essentially similar to effects of trypsin on (Na, K)ATPase activity of the purified kidney enzyme (Jørgensen, 1975). We conclude that the Na- or K-form of the protein is being induced or stabilized by Na^+ or K^+ binding to what is naturally the cytoplasmic face of the pump, and the trypsin senses the different structures of the reconstituted pumps. Higher concentrations of trypsin increased the total rate of loss of activity but the difference of rates in the Na^+ and K^+ medium was always clearly detectable even at the shortest times of trypsinolysis tested (0·5 min). In the experiment of Fig. 8 the exponential in the K^+ medium is represented by $A_t = A_0\ (1-e^{-0.03t})$ and the continuous line through the points for the Na^+ medium is the curve $A_t = A_0\ (0{\cdot}45\,e^{-0.4t} + 0{\cdot}55\,e^{-0.007t})$, where A_0 and A_t are the activities at time 0 or t min. In several experiments (see also Fig. 9) the amplitude of the two phases of inactivation in the Na^+-medium were about 0·4 and 0·6 respectively, which is very close to that observed for the purified (Na, K)ATPase. The ratio of the fast to slow rate constants for inactivation in the Na^+-medium could not be estimated very accurately and in different experiments varied between 57 and 127 at 20 °C. Tryptic digestion at 37 °C (experiment not shown) also gave rise to the distinct K and Na patterns, the latter having also amplitudes of fast and slow phase of 0·4 and 0·6 respectively, but the calculated ratio of fast to slow rate constants of about 14 was clearly lower than at 20 °C, and is similar to the figure reported for the (Na, K)ATPase activity (Jørgensen & Peterson, 1979).

In a complementary experiment (Fig. 9) the influence of internal K^+ ions was determined by looking at the tryptic inactivation of active Na^+ transport in vesicles containing Tris HCl without or with 10 mM-KCl and suspended in a medium with 10 mM-NaCl. Points from the two types of vesicle are superimposable on the same Na-pattern curve. In three other experiments, vesicles containing either 150 mM-KCl or 100 mM-RbCl, and suspended in a 10 mM-NaCl medium, also gave rise to the typical Na pattern of digestion (0, 2 and 4 min time points of tryptic digestion from one experiment are included in Fig. 10).

In view of Jørgensen & Anner's (1979) report that vesicles reconstituted with (Na, K)ATPase digested partially with trypsin in a Na^+-rich medium sustain a normal active Rb^+ efflux, it was of special interest to compare active Na^+ and Rb^+ fluxes in vesicles subjected to a short trypsinolysis in the Na^+ medium as in Fig. 8 or 9. In four different experiments with vesicles loaded conventionally with ^{86}Rb during their formation, and then trypsinized to the stable form it was not possible to decide whether ATP-dependent ^{86}Rb efflux was unaltered or reduced. The measurement of the initial rates, and hence of small differences, was too inaccurate. However, using vesicles loaded specifically with ^{86}Rb by Rb–Rb exchange, prior to trypsinization this problem has been overcome (Fig. 10). Compared to the controls, in the trypsinized vesicles the active Na^+ influx and Rb^+ efflux were reduced 45 and 35% respectively at the earliest timepoint of the assay, 20 sec. At 40 and 60 sec the reduction of Rb^+ flux was still clearly discernible but the extent of inhibition was lower than at 20 sec due to the greater non-linearity of the flux in the control vesicles. As expected the rates of Na^+ and Rb^+ fluxes were essentially the same after 2 and 4 min of tryptic digestion in the Na^+-medium.

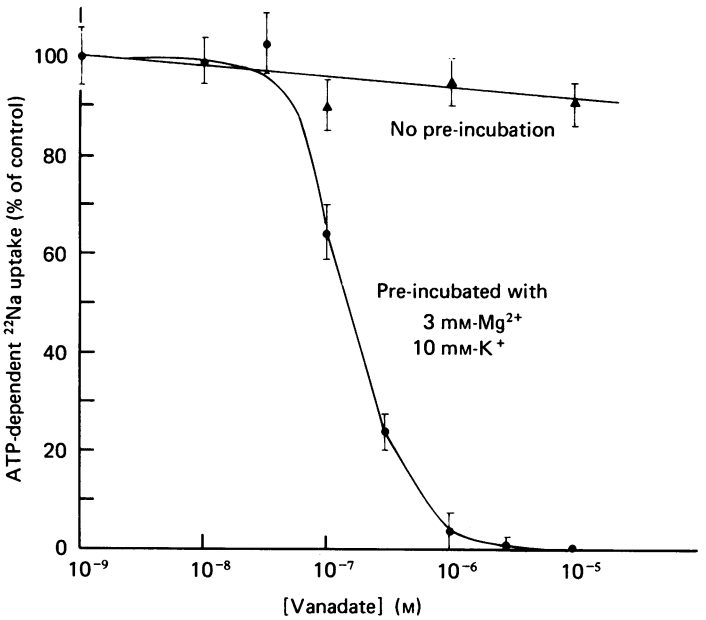

Fig. 11. Inhibition of ATP-dependent ^{22}Na uptake by vanadate ions. 400 μl. of proteoliposomes were prepared in a medium containing Tris HCl, 150 mM, pH 7·0, from lipid vesicles (7 mg) and enzyme (0·17 mg) plus cholate (0·9 mg), and were centrifuged on a Sephadex column equilibrated with Tris HCl, 150 mM, pH 7·0. To 300 μl. of vesicles was added KCl, 10 mM and $MgCl_2$, 3 mM. This preparation was divided into seven sets of 40 μl. 4 μl. of ammonium orthovanadate was added to give final concentrations between 0–10^{-5} M. After 15 min incubation at room temperature, 60 μl. of a solution containing NaCl, 50 mM; KCl, 10 mM; Tris HCl, 90 mM and $MgCl_2$, 3 mM was added and then 2 μl. of a 20 μM ethanolic solution of valinomycin + FCCP. 100 μl. of the non-pre-incubated vesicles was mixed with 100 μl. of a medium containing NaCl, 60 mM; KCl, 20 mM; $MgCl_2$, 6 mM and Tris HCl, 70 mM, and then valinomycin + FCCP was added. The assay was run by mixing aliquots in duplicate with an equal volume of reaction mixture containing NaCl, 30 mM (+^{22}Na); KCl, 10 mM; $MgCl_2$, 3 mM, and Tris HCl, 110 mM, with or without ATP (Tris), 6 mM. For non-preincubated vesicles ammonium orthovanadate was added to the reaction mixture at twice the desired final concentration.

Inhibition by vanadate

In order to look at the sidedness of the effects of K^+ ions on vanadate inhibition of active Na^+ pumping, it was necessary first to establish optimal conditions for inhibition. Fig. 11 shows that in Tris HCl loaded vesicles pre-incubated with vanadate and externally added Mg^{2+} and K^+ ions and then treated with valinomycin and FCCP in a Na^+- plus K^+-containing medium, as described above, inhibition was apparent at very low vanadate concentrations ($K_{0.5} \sim 2 \times 10^{-7}$ M). By contrast addition of up to 10 μM-vanadate together with the reaction mixture containing 3 mM-ATP, led to little or no inhibition. Antagonism between ATP or Na^+ and vanadate is well documented (Cantley, Cantley & Josephson, 1978) but the effect seemed to be more total in our system than has been observed before. Although in the incubation conditions of Fig. 11 K^+ was added externally, the vanadate was still present during the subsequent ionophore treatment and could therefore have combined with the pump under the influence of internal K^+.

TABLE 4. Inhibition of ATP-dependent ^{22}Na uptake by vanadate is enhanced by pre-incubation with K^+ in the exterior medium (ATP-dependent Na^+ uptake, n-mole Na^+ per mg vesicle protein per 2 min)

External cations Mg^{2+} 3 mM, $Tris^+$ 100 mM	Tris HCl-loaded vesicles		KCl-loaded vesicles	
	Control	Vanadate	Control	Vanadate
+(*a*) $Tris^+$ 50 mM	4237±85	2914±26	2979±66	1722±101
+(*b*) Na^+ 50 mM	4230±233	4069±219	2683±121	3315±27
+(*c*) K^+ 50 mM	3882±90	290±28	3457±231	451±219

Two sets of 300 μl. of proteoliposomes were prepared in media containing either Tris HCl, 150 mM, pH 7·0, or KCl, 150 mM, from lipid vesicles (7 mg) and enzyme (0·17 mg) plus cholate (0·9 mg), and were centrifuged on Sephadex columns equilibrated with Tris HCl, 150 mM, pH 7·0. Each set of vesicles was divided into equal portions of 100 μl., to which was added 100 μl. of three different solutions: (*a*) Tris HCl, 150 mM; $MgCl_2$, 6 mM (*b*) NaCl, 100 mM; Tris HCl, 50 mM; $MgCl_2$, 6 mM or (*c*) KCl, 100 mM; Tris HCl, 50 mM, pH 7·0; $MgCl_2$, 6 mM. Each set of six samples were divided again into two equal portions of 100 μl. and to one of these was added orthovanadate (Tris salt) to a final concentration of 1 μM. After 10 min incubation at *0 °C*, the vesicle suspensions were recentrifuged on 12 × 1 ml. columns of Sephadex G-50 equilibrated at 0 °C with Tris HCl, 150 mM, pH 7·0. To each sample of 100 μl. at 0 °C, 100 μl. of a solution containing NaCl, 60 mM; KCl, 20 mM; Tris HCl, 70 mM, pH 7·0 was added and then 4 μl. of a valinomycin + FCCP solution to give a final ionophore concentration of 0·4 μM. After 20–30 min incubation at 0 °C, the transport assays were done. Duplicate samples of 40 μl. were incubated for 2 min at room temperature with 20 μl. of reaction mixture containing NaCl, 30 mM (+^{22}Na); KCl, 10 mM; Tris HCl, 110 mM; $MgCl_2$, 6 mM, with or without ATP (Tris), 9 mM.

The enzyme–K–vanadate complex is known to be rather stable (Cantley, Cantley & Josephson, 1978; Karlish *et al.* 1979), so it seemed possible first to combine the inhibitor with the pumps in defined conditions of external or internal vesicle cations, and then to remove the vanadate before treating with ionophores and assaying active Na^+ transport. A 'memory' experiment of this sort with Tris HCl or KCl-loaded vesicles is shown in Table 4. The pre-incubation with 1 μM-vanadate was at 0 °C in order to preclude the possibility of significant K^+ permeation (see Fig. 7). Separation on Sephadex and much of the period of ionophore treatment were also done at 0 °C but the assay was at room temperature. With vesicles loaded initially with Tris HCl, exterior K^+ was fully effective in producing inhibited pumps. In the exterior $Tris^+$ medium, 1 μM vanadate gave some inhibition (31%) but exterior Na^+ totally prevented inhibition. With KCl-loaded vesicles the relative effects of exterior $Tris^+$, Na^+ and K^+ were essentially similar to those in the $Tris^+$-loaded vesicles although the absolute rates of Na^+ uptake were somewhat lower. In particular 150 mM-K^+ inside the vesicles affected very little the degree of pseudo-irreversible inhibition by vanadate in the exterior $Tris^+$ medium (42%) – the small increase may reflect less than perfect separation between vesicles and their formation medium containing 150 mM-K^+ (a 99·9% efficiency of the Sephadex columns would still leave 150 μM-K^+ in the exterior medium).

Beaugé, Cavieres, Glynn & Grantham (1980) have produced evidence recently that Na^+ ions external to red cells antagonize vanadate inhibition of ouabain-sensitive fluxes. In a similar 'memory' experiment to that in Table 4 we have compared vesicles containing Tris HCl 150 mM or Tris HCl 110 + NaCl 40 mM. In both cases pre-incubation with 1 μM-vandate, Mg^{2+} and exterior (i.e. 'cytoplasmic') K^+ ions

produced seriously inhibited active Na^+ transport (70 and 80% respectively) but pre-incubation with vanadate and exterior Na^+ ions showed very little inhibition (5 and 20% respectively).

DISCUSSION

The reconstitution procedure

The freeze–thaw sonication method described by Hokin & Dixon (1979) and in this paper is superior to the cholate dialysis technique both in terms of observed rates of active transport, and time saved. In the dialysis technique, cholate is in prolonged contact with the protein, a minimum of 16 hr (Goldin, 1977), and it seems possible that more extensive denaturation occurs than in the present method in which the detergent is removed within 15 min of the initial solubilization. In most important respects our procedure is similar to that of Hokin & Dixon (1979) but unlike these authors we have used sonicated suspensions of crude soyabean azolectin, without taking special care to purify or deaerate or add traces of ether to the lipid. Also other factors such as inclusion of Na^+ ions in the freeze–thaw medium or the precise conditions of subsequent sonication did not seem to be critical. The kidney (Na, K)ATPase appears over-all to be less sensitive to adverse effects of the manipulations than shark rectal gland or electric eel enzyme, and this may account for the higher rates of active Na^+ and Rb^+ transport than observed by Hokin & Dixon (1979).

Cation transport in the vesicles

Classical properties of the Na/K pump observed in the reconstituted vesicles are:

1. Dependence of active Na^+ uptake on presence of internal K^+ or Rb^+ ions. (This is shown directly in Table 1 and indirectly by the ionophore experiments of Table 3 and Fig. 7.) The success of valinomycin and FCCP in sustaining ATP-dependent ^{22}Na uptake suggests strongly that the active K^+ extrusion is being balanced by the ionophore-mediated leak. The failure of valinomycin alone to sustain a maximal rate of pumping (Table 3, column 4) implies that net entry of K^+ into the vesicles down its concentration gradient is rate limited, probably by a relatively low permeability to the major anion Cl^- which does not effectively short-circuit the inside-positive K^+-diffusion potential. Addition of the H^+ ionophore should permit collapse of such a membrane potential by rapid counter movements of H^+. Similar effects of valinomycin, plus FCCP, have been observed in red cells (Harris & Pressman, 1967) and explained similarly.

2. Coupling of ATP-dependent ^{22}Na uptake to ^{86}Rb extrusion with a transport ratio of about 1·5 Na^+ ions per Rb^+ ion (Fig. 4). The minimal Na^+ concentration gradient maintained by the active Na/K exchange can be estimated from experiments like that of Fig. 1 in which the ATP-dependent accumulation in the steady state is about five times the ^{22}Na content of the vesicles after full passive equilibration. Since in addition only 30–40% of the vesicles seem to contain active pumps (Fig. 6), the real internal Na^+ concentration must be about 2·5–3 times higher than measured. Therefore, the Na concentration ratio at the steady state will be not five but twelve to fifteenfold, which is in the physiological range.

3. Sigmoid dependence of active Na^+ uptake on Na^+ concentration ($n = 2$ or 3) (Fig. 3).

4. Inhibition by ouabain at the extracellular face of the pump, (i.e. inside the vesicles) (Table 2).

5. Rb–Rb exchange. Unpublished experiments (S. Karlish and W. D. Stein) have shown that in the presence of ATP, orthophosphate and Mg^{2+}, an exchange of internal for external Rb^+ occurs. The process shows many properties in common with the ouabain-sensitive K–K exchange studied in red cells, including a maximal rate which is about 20% of the maximal Na/K exchange (Glynn, Lew & Luthi, 1970; Simons, 1974). This work will be the subject of a subsequent publication (S. Karlish and W. D. Stein, manuscript in preparation).

6. Vanadate inhibition: see below.

7. Tryptic digestion: see below.

Sidedness of effects of Na^+ and K^+

Tryptic digestion

1. Trypsin was effective from the cytoplasmic surface of the reconstituted pumps, as found also in human red cells (Giotta, 1975).

2. Taken together the two trypsin experiments (Figs. 8 and 9) make it virtually certain that Na^+ and K^+ or Rb^+ act at the cytoplasmic surface (vesicle exterior) in stabilizing the alternate conformations, and are not effective at the extracellular surface (vesicle interior) alone. The experiment of Fig. 8 was performed before it was realized that the vesicle population is heterogeneous, and the protein-containing vesicles are more permeable to Na^+, Rb^+ and presumably K^+ ions than the population as a whole. It is likely that by the end of trypsinolysis, i.e. 40 min, an appreciable fraction of the K^+ ion had equilibrated within the vesicles. But it is unlikely that it is the internal K^+ which produced the K pattern of inactivation because from 0·5 min of trypsinolysis the loss of activity fell on a single exponential. If one assumes for K^+, as for Rb^+, a half-time of equilibration of about 15 min, at 0·5 min one could expect there to be no more than 0·23 mM-K^+ ion inside. This is about a tenfold lower concentration of K^+ than is necessary to sustain the conformational change as monitored by the tryptic digestion. Nevertheless in view of the slight doubt concerning the penetration of Rb^+ or K^+ ions into the vesicles within, say, 1 min at room temperature, one might argue that in the conditions of Fig. 8 low concentrations of K^+ or Rb^+ are necessary at both surfaces in order to produce the K pattern of digestion. The experiment with vanadate, Table 4, performed at 0 °C, makes that unlikely.

2. The finding in Fig. 10 that the active Rb^+ efflux is reduced by the short trypsinolysis to about the same extent at the active Na^+ uptake is inconsistent with the report (Jørgensen & Anner, 1979) that 'invalid' (Na, K)ATPase catalyses a normal Rb^+ extrusion. One might propose that the trypsinized pumps in our vesicles are not equivalent to the 'invalid' (Na, K)ATPase, or suggest that the difference lies in the fact that Jørgensen & Anner (1979) were reconstituting an already trypsinized enzyme while we have digested normal pumps after reconstitution. But it seems more likely that the discrepancy is due to the difficulty of the measurement of the active Rb^+ efflux from a population of vesicles loaded unselectively with ^{86}Rb.

Jørgensen (1977) has studied in detail the properties of the 'invalid' (Na, K)ATPase, which is the product of the first proteolytic split in a Na^+ medium. This enzyme has

about 60% of original (Na, K)ATPase activity. Its abnormal functional properties are consistent with a shift in the conformational equilibrium between the E_1 and E_2 forms in the direction of E_1 (Jørgensen & Klodos, 1978), a conclusion which has been verified directly by monitoring protein fluorescence signals (Jørgensen & Karlish, 1980).

Vanadate inhibition

1. The magnitude of inhibitory concentrations, a requirement for both Mg^{2+} and K^+ ions, and antagonism by Na^+ or ATP all parallel observations with isolated (Na, K)ATPase (Cantley, Cantley & Josephson, 1978; Cantley *et al.* 1977). In an unpublished experiment it has also been found that vesicles prepared with vanadate (1 μM) inside and pre-incubated with Mg^{2+} and K^+ do not show inhibited ATP-dependent ^{22}Na uptake. Thus, vanadate inhibits the reconstituted pumps from their 'cytoplasmic' face as found also in human red cells (Cantley, Resh & Guidotti, 1978).

2. The success of the 'memory' experiment of Table 4 indicates that the formation of complex $E_2 . (K)$–vanadate is largely irreversible at 0 °C. One can conclude that K^+ bound to the cytoplasmic face of the non-phosphorylated Na/K pump causes tight vanadate binding and inhibition. By contrast K^+ at the external surface (vesicle interior) does not appear to help the vanadate inhibition in the conditions of these experiments. Also Na^+ ions at the cytoplasmic surface (vesicle exterior) prevent the vanadate from binding, but addition of Na^+ after vanadate has been bound tightly in the presence of K^+ does not reverse the inhibition. In the human red cell (Beaugé & Glynn, 1978) and squid nerve (Beaugé & Di Polo, 1979*a*), vanadate inhibition of active Na^+ or Rb^+ transport requires the presence of *external* K^+ or Rb^+ ions. This does not contradict our result, for of course the conditions are not comparable. In whole cells the Na/K pumps are active and there will be interconversions between all of the enzyme forms involved in the ATPase turnover cycle. $E_2 . (K)$ formation will occur, and thus tight binding of vanadate, following dephosphorylation stimulated by K^+ bound to external sites on active Na/K pumps (see scheme in Introduction). However puzzling, aspects of vanadate inhibition in red cells are a requirement for higher concentrations of K^+ than are necessary to saturate active uptake (Beaugé, 1979), and apparent antagonism by external Na^+ ions (Beaugé *et al.* 1980). The second of these features is not observed with our vesicles in the conditions of the 'memory' experiments described above; but the role of high extracellular K^+ concentrations remains as yet unexplained.

Conclusion

The trypsin digestion and vanadate inhibition experiments provide strong and independent evidence that K^+ ions stabilize the $E_2 . (K)$ conformation following binding to cytoplasmic site(s). Indirect evidence, leading to the same conclusion comes from experiments to look at ATPase and *p*-nitrophenol phosphatase activities in inside-out vesicles prepared from red cell membranes (Blostein & Chu, 1977; Blostein, 1979; Blostein, Pershadsingh, Drapeau & Chu, 1979) and from observations on active Na^+ fluxes in dialysed squid axons (Beaugé & Di Polo, 1979*b*). The conclusion supports the biochemical scheme proposed previously (Karlish *et al.* 1978*a*) to explain K^+ movements sustained by the Na/K pump (see Introduction) and provide a working hypothesis on which to base further investigation of cation fluxes mediated by the Na/K pump.

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