Reconstitution of Active Transport Catalyzed by the Purified Sodium and Potassium Ion-stimulated Adenosine Triphosphatase from Canine Renal Medulla*

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SUMMARY

Microsomal sodium and potassium ion-stimulated adenosine triphosphatase ((Na⁺ + K⁺)-ATPase) was prepared from canine renal medulla by the method of Kyte. The two polypeptides of the enzyme were co-purified to homogeneity by solubilization with sodium cholate in the presence of egg lecithin and by removal of contaminating protein by sedimentation.

This purified (Na⁺ + K⁺)-ATPase was reconstituted into lipid vesicles by slow removal of the cholate. A fraction of this enzyme was oriented in the vesicle membranes in such a way as to catalyze active uptake of 22Na⁺, dependent on externally added ATP and inhibitable by internally trapped cardiac glycosides, to a level 3-fold higher (60 mV) than the initial concentration of Na⁺ within the vesicles (20 mV).

Double label experiments with 4K⁺ and 22Na⁺ indicated that the ratio of K⁺ influx to Na⁺ uptake is far below the 2:3 ratio of K⁺ influx to Na⁺ efflux observed in nerve axons and erythrocyte ghosts. Parallel experiments employing 36Cl⁻ and 22Na⁺ demonstrated that 36Cl⁻ is co-transported along with 22Na⁺ in the amount necessary to maintain bulk electrical neutrality of charge transported across the membrane.

The mechanism of selective transport of Cl⁻ along with actively pumped Na⁺, rather than exchange of K⁺ for Na⁺, cannot be explained by the observation that the permeability of the vesicles to Cl⁻ is roughly 2-fold higher than that for K⁺. It appears that this reconstituted (Na⁺ + K⁺)-ATPase either is capable of pumping Cl⁻ as well as Na⁺ or is equipped with some specific mechanism for translocating Cl⁻ along with actively pumped Na⁺.
or erythrocytes (13), which show a 2.3 ratio of K+ influx to Na+ efflux; they are consistent with physiological studies of kidney tubules and are understandable in terms of the functional role played by the enzyme in the kidney.

**EXPERIMENTAL PROCEDURES**

**Materials**

Canine kidneys were a generous gift of the Cardiovascular Research Department of Massachusetts General Hospital. Sodium cholate was obtained from K & K Laboratories, Plainview, N. Y. l-Histidine, dioxodium and TriO ATP, strophanthin, ouabain, l-α-lecithin (type III-E from egg yolk), and sodium deoxycholate were purchased from Sigma. Sephadex G-50 (fine) was purchased from Pharmacia. All other chemicals were reagent grade. Standards for gel electrophoresis were: myosin, heavy chain (mol wt 220,000), David Brandon, Harvard University; bovine serum albumin (mol wt 67,000), Pentex; ovalbumin (mol wt 43,000), crystalline, Sigma; and β-lactoglobulin (mol wt 17,500), Calbiochem. Na+K+, Cl−, [H]cholate, [H]glucose, and [14C]glucose were all obtained from New England Nuclear.

**Methods**

**Enzyme Assay**

The assay used for determination of activity at all stages of purification of the enzyme prior to co-solubilization with phospholipid and reconstitution is that of Nakao et al. (14) as modified by Kyte (6). The assay used on the solubilized and reconstituted enzyme was a modification of Method D of Lindberg and Ernster (10). For determination of ATPase activity under optimal conditions, 10 to 50 μl of solubilized or reconstituted enzyme were incubated for 15 min at 37° + 0.5° in 0.5 ml of the incubation mixture of Kyte. The sample was quenched with 2.5 ml of 1.25% deoxycholate were purchased from Sigma. Sephadex G-50 (fine) was purchased from Pharmacia. All other chemicals were reagent grade. Standards for gel electrophoresis were: myosin, heavy chain (mol wt 220,000), David Brandon, Harvard University; bovine serum albumin (mol wt 67,000), Pentex; ovalbumin (mol wt 43,000), crystalline, Sigma; and β-lactoglobulin (mol wt 17,500), Calbiochem. Na+K+, Cl−, [H]cholate, [H]glucose, and [14C]glucose were all obtained from New England Nuclear.

**Reconstitution of (Na+ + K+)-ATPase**

Salt-detergent-extracted microsomes were prepared by the method of Kyte (6). The resulting specific activity was 585 μmoles of Pi per mg per hour; this preparation was stored frozen in 0.5-ml aliquots at −70° as a 2.7-mg per ml suspension in 30 mM histidine, pH 6.8, 10 mM β-mercaptoethanol, 2 mM EDTA, and 0.25 mM sucrose and was stable for several months. l-α-Lecithin from egg yolk was obtained commercially and stored at −20° as a 10% (w/v) solution in decane under nitrogen. l-α-Lecithin solution (0.4 ml) was dried down under nitrogen and lyophilized for 2 hours to remove traces of solvent. This dried down lipid was immediately resuspended in 1.33 ml of a solution, on ice, containing 0.25 mM sucrose, 1.5 mM glucose, 10 mM β-mercaptoethanol, 30 mM histidine, pH 6.8, 120 mM KCl, 30 mM NaCl, 9 mM MgCl2, 30 mg per ml of phospholipid, and 30 mg per ml of sodium cholate. This suspension was mixed in a Vortex until optically clear and rechilled on ice. Thawed enzyme suspension (0.67 ml), on ice, was added to this solution, which was mixed in a Vortex and allowed to stand on ice for 20 min.

This cholate-solubilized preparation was spun down in a 2-ml polyallomer tube in a Spinco 30 rnr.4 NaCl, 9 mM MgCl2, 30 mg per ml of phospholipid, and 30 mM l-histidine, pH 6.8, 100 mM KCl, 20 mM NaCl, and 6 mM MgCl2. Dialysate was changed every 10 to 14 hours.

The resulting material is the reconstituted (Na+ + K+)-ATPase vesicles. For some of the double label 32P and 13Na experiments, an identical reconstitution procedure was used, except that the concentration of KCl was 20 mM and that of NaCl 30 mM in both the solubilized enzyme suspension and in the dialysate. The above procedure was on occasion scaled up by a factor of 2 or scaled down by a factor of 2 or 4, with no significant change in the properties of the resulting reconstituted vesicles.

Vesicles reconstituted in 100 mM K+ and in 20 mM K+ will be referred to in the rest of this section as high K+ buffer and low K+ buffer vesicles, respectively.

**Determination of Trapped Volume**

The ability of the reconstituted enzyme vesicles to trap glucose was measured by solubilization of the enzyme and removal of detergent in the presence of 1 mM [3H]- or [14C]-glucose, 2 × 10⁶ cpm per ml, in both the solubilization buffer and the dialysate. Free glucose was separated from trapped glucose at 4° on columns of Sephadex G-50 (fine), 9 mm in diameter and 18 cm in height, run at liquid head of 250 cm, giving a flow rate of 0.15 to 0.30 ml per min. Vesicles (0.5 ml) were added to the column; the elution buffer was identical in composition with the buffer employed in solubilization and dialysis, but with 1 mM cold glucose substituted for [3H]-glucose. Fractions (0.5 ml) were collected, A300 was read in a Zeiss PMQII spectrophotometer, and samples were counted on a Packard Tri-Carb liquid-scintillation spectrometer with 2-butanone-based BSA/lytium scintillator and counted on a Beckman LS-233 scintillation counter. By withdrawing aliquots of vesicles (4-ml initial volume) at various times during a 5-day dialysis period, trapped volume of glucose was measured as a function of time of dialysis.

Trapping of 3Na+ and 3Cl− was measured by means of a virtually identical procedure. 3Na+ fractions (1.0 ml) were collected and counted in a Picker Nuclear γ counter; 1.0-ml 3Cl− fractions were dissolved in 5 ml of Aquasol and counted on the Beckman LS-233. Due to the greater expense and radiation hazard of 3Na+ and 3Cl−, 0.5 ml portions of a 2-ml batch of the cholate-solubilized supernatant enzyme and lipid were separately dialyzed against 8- to 125-ml changes of buffer containing 50,000 cpm per ml of either 3Na+ or 3Cl−, i.e. the process of formation of vesicles by removal of detergent was scaled down by a factor of 4 with respect to the normal procedure, but was otherwise identical. The resulting values for trapped volume of 3Na+ and 3Cl− were compared with those determined by equilibrating vesicles formed in nonradioactive dialysis buffer with externally added radioactively labeled 3Na+ and 3Cl− by the method described below.

**Assay of Active and Passive Transport of 3Na+, 3K+, and 3Cl−**

3Na+ Transport—Aliquots (2 μl) of 3Na+, 100,000 cpm per μl, 20 mMCl per mmole in 0.1 M HCl, were added to 3-ml conical tubes. HCl and water were removed from each tube under a stream of nitrogen. The tubes were capped with Parafilm and placed on ice. Each individual transport measurement was performed as follows. Vesicles (125 μl) were added to the tube, on ice. For ATP-dependent 3Na+ uptake, 18 mM Tri-ATP in dialysis buffer, pH 6.8, was added to the vesicles to a final concentration of 3 mm. The solution was capped with Parafilm, mixed thoroughly in a Vortex, and immediately immersed in a shaking water bath at 37° ± 0.5° for a length of time ranging from 0 min to 60 min. At the end of the specified time period, the tube was immediately chilled on ice for 2 min. The entire contents of the tube was immediately run on a Sephadex G-50 (fine) column under conditions identical with those used in determination of trapped volume. Fractions (0.3 ml or 0.6 ml) were collected, the optical density read at 300 nm, and counted as described for the determination of trapped 3Na+ volume. Uptake of 3Na+ in the absence of Tri-ATP was measured precisely as above; 36 mm KCl, 2 mM β-mercaptoethanol, 30 mg per ml of phospholipid, and 30 mg per ml of sodium cholate. This suspension was mixed in a Vortex until optically clear and rechilled on ice. Thawed enzyme suspension (0.67 ml), on ice, was added to this solution, which was mixed in a Vortex and allowed to stand on ice for 20 min.

This cholate-solubilized preparation was spun down in a 2-ml polyallomer tube in a Spinco 303376-2 ml adapter in a Spinco type 40 rotor at 35 K (100,000 × g) for 15 min. The supernatant was dialyzed for 4 days at 0° in Union Carbide 8 dialysis tubing (0.39 inch wide, 0.002 inch wall thickness) against 8 500-ml volumes of 0.25 mM sucrose, 1 mM glucose, 10 mM β-mercaptoethanol, 30 mM l-histidine, pH 6.8, 100 mM KCl, 20 mM NaCl, and 6 mM MgCl2. Dialysate was changed every 10 to 14 hours.

The resulting material is the reconstituted (Na+ + K+)-ATPase vesicles. For some of the double label 32P and 13Na experiments, an identical reconstitution procedure was used, except that the concentration of KCl was 20 mM and that of NaCl 30 mM in both the solubilized enzyme suspension and in the dialysate. The above procedure was on occasion scaled up by a factor of 2 or scaled down by a factor of 2 or 4, with no significant change in the properties of the resulting reconstituted vesicles.

Vesicles reconstituted in 100 mM K+ and in 20 mM K+ will be referred to in the rest of this section as high K+ buffer and low K+ buffer vesicles, respectively.
run simultaneously; 5-min fractions (1.0 ml) were collected automatically. The character of the curves of ATP-dependent versus passive $^{22}$Na uptake was independent of the order in which the points were taken.

**Double Label Measurements of $^{22}$Na and $^{36}$Cl$^-$ Transport—For simultaneous measurements of $Na^+$ and $K^+$ transport in low $K^+$ buffer vesicles, a radioactive buffer was prepared so that the final concentrations would be 30 mM $K^+$, 0.25 mM $Cl^-$, 0.01 mCi per ml; 0.25 mM sucrose; 1 mM glucose; 6 mM MgCl$_2$; 30 mM L-histidine, pH 6.8; and 10 mM $\beta$-mercaptoethanol. For determinations of ATP-dependent and passive Na$^+$ and K$^+$ transport, 125 $\mu$l of low K$^+$ buffer vesicles were added on ice to a 3-ml conical tube containing 100 $\mu$l of radioactive buffer and 46 $\mu$l of 18 mM Tris-ATP or 36 mM sucrose. Samples were incubated and subjected to Sephadex chromatography as in the single label $^{22}$Na experiments.

All fractions and standards were counted on a Picker Nuclear $\gamma$ counter within 10 hours of the calibration time for the isotopic specifications of the $K^+$. Because $K^+$ has a half-life of 12.5 hours and that of $^{22}$Na is 2.6 years, the fractions and standards were counted again after being stored for 8 days; during the storage period $K^+$ present had gone through almost 16 half-lives and thus constituted a negligible fraction of the remaining $\gamma$ emissions. The remaining counts in each fraction, which constituted the $^{22}$Na$^+$ distribution among the fractions, were subtracted from the original counts in each fraction, allowing one to independently resolve transport of $K^+$ from that of $Na^+$.

**Parallel Determinations of $^{22}$Na$^+$ and $^{36}$Cl$^-$ Transport—Low K$^+$ buffer was prepared, 0.03 mCi per ml in $Cl^-$. Including the $Cl^-$ buffer was prepared, 0.03 mCi per ml in $Na^+$. Parallel measurements of ATP-dependent and passive transport of Na$^+$ and Cl$^-$ were performed on 100 mM K$^+$ buffer, the experiment was performed as above, but with high K$^+$ buffer employed as the vesicle reconstitution buffer, the radioactive buffer, and in the Tris-ATP and control solutions.

**Inhibition of ATP-dependent $^{22}$Na$^+$ Uptake by Trapped and Reconstituted (Na$^+$ + K$^+$)-ATPase—Enzyme suspensions (10$^6$ cpm per ml) were slowly removed from the enzyme and lipid by dialysis against detergent-free buffer containing sucrose to stabilize the enzyme, $\beta$-mercaptoethanol to inhibit oxidation, KCl and NaCl, the ionic constituents to be trapped in the vesicles, and on occasion $[^{36}Cl^-]$ or $[^{1}H]$glucose to be used as a marker in determination of trapped volume. The composition of the buffer employed was identical with that used by Racker et al. to reconstitute proton-translocating cytochrome oxidase (19, 20) and the Ca$^2+$-ATPase (21); cholate was slowly removed from the enzyme and lipid by dialysis, ensuring that the concentrations of solutes trapped inside the vesicles were equal to the concentrations of those components in the dialysate.

**Removal of [H]$\beta$-Cholate by Dialysis—Supernatant enzyme (2 ml) in L-$\alpha$-lecithin was prepared as described, but in the presence of $[^{1}H]$cholate, 10$^6$ cpm per ml. High $K^+$ buffer was formed in the presence of 10$^{-4}$ M strophanthin in high K$^+$ buffer containing 1% dimethylformamide as a carrier for strophanthin. The procedure for solubilization and reconstitution of the enzyme was otherwise identical with that described above. ATP-dependent and passive $^{22}$Na$^+$ uptake was determined as described, with 10$^{-4}$M strophanthin in 1% dimethylformamide present in the high K$^+$ buffer containing 18 mM Tris-ATP or 36 mM sucrose added to 125 $\mu$l of reconstituted vesicles before incubation at 37$^\circ$.

**Formulation of Phospholipid Vesicles without (Na$^+$ + K$^+$)-ATPase—Vesicles containing the enzyme were formed by a technique akin to that used by Racker et al. to reconstitute proton-translocating cytochrome oxidase (19, 20) and the Ca$^2+$-ATPase (21); cholate was slowly removed from the enzyme and lipid by dialysis, ensuring that the concentrations of solutes trapped inside the vesicles were equal to the concentrations of those components in the dialysate.

**RESULTS—Sealed phospholipid vesicles can incorporate solubilized, purified (Na$^+$ + K$^+$)-ATPase oriented in the vesicle membranes in such a way that active transport is catalyzed by this enzyme and can be quantitated. Further experiments determined the orientation of the enzyme's glycoside inhibitor-binding site with respect to its site for ATP hydrolysis and defined the direction, magnitude, and stoichiometry of ATP-dependent transport of Na$^+$, $Cl^-$, and K$^+$.

**Protein Determinations—Protein was determined by the method of Lowry as discussed by Bailey (18). Interference by phospholipid present in solubilized or reconstituted (Na$^+$ + K$^+$)-ATPase was corrected for by adding to all blanks and standards an equivalent amount of solubilized phospholipid or reconstituted phospholipid vesicles prepared in the absence of added enzyme by an otherwise identical procedure. Interference by components of the buffer containing the sample was corrected for by adding an equivalent volume of the same buffer to all blanks and standards.
Fig. 1. Time course of increase in trapped volume and of detergent removal during dialysis. a, trapped volume for glucose of reconstituted vesicles as a function of dialysis time. Aliquots (0.5 ml) of a 4-ml batch of cholate-solubilized phospholipid were removed at the indicated time intervals. Trapped volume for [14C]glucose was determined as described in the text. b, removal of cholate from cholate-solubilized phospholipid as a function of dialysis time. Aliquots (10 μl) of a 2-ml batch of phospholipid solubilized in the presence of [3H]cholate were removed and counted. Percentage of cholate remaining was determined as described in the text.

Fig. 2. Separation of trapped from free [14C]glucose on Sephadex G-50. Total counts per min added to column, 10,800. Recovery of total counts per min from both peaks, 97%.

(FIG. 2), 1.8% of the total volume of buffer is trapped in the vesicles after 4 days of dialysis. This corresponds to 0.9 ml of buffer per g of phospholipid.

The process of co-solubilization of the salt-detergent-extracted microsomes with detergent in the presence of added phospholipid, followed by high speed sedimentation of the partially clarified suspension, results in further purification of the enzyme. Sodium dodecyl sulfate acrylamide gel electrophoresis (Fig. 3) of the supernatant reveals that most of the material in the microsomes not present as the polypeptides of apparent molecular weights of 84,000 and 57,000, as characterized by Kyte (6), is removed by sedimentation. Thus it appears that the bulk of the impurities in the salt-detergent-extracted microsomes are not solubilized by cholate at the ionic strength employed and are pelleted at 100,000 × g.

The two major polypeptides of 84,000 and 57,000, which remain in the supernatant, account for greater than 90% of the A185 present in scans of the Coomassie blue-stained gels of the supernatant enzyme, as opposed to 60% of this absorbance in the initial salt-detergent-extracted microsomes. The degree of purification of these two polypeptides is hence similar by the criterion of sodium dodecyl sulfate gel electrophoresis to that obtained by Kyte in his original purification steps (6). No observable changes in the polypeptide composition of the preparation due to proteolysis or other factors occur during the process of vesicle formation (Fig. 3e). Based on recovery of these two polypeptides from the supernatant, the yield of enzyme is estimated to be 80%.

Recovery of enzymatic activity, however, is much lower. The solubilization of the enzyme inactivates its ouabain-sensitive ATPase activity (Table I); a 15-fold decrease in specific activity is observed. Removal of detergent by dialysis results in a 5-fold recovery of activity, bringing the specific activity back to 35% of the initial value and bringing the total activity recovered to 18% of the starting value.

In order to study the stoichiometry of ion uptake under conditions that would permit more sensitive measurement of ATP-dependent transport of Cl⁻ and K⁺, salt-detergent-extracted microsomes were co-solubilized with phospholipid and reconstituted into vesicles in low K⁺ buffer (20 mM K⁺, 30 mM Na⁺) by an otherwise identical procedure. The properties of the resulting vesicles were similar to the high K⁺ buffer vesicles in all respects except that protein and enzyme activity remaining in the 100,000 × g supernatant and associated with the vesicles was 30% lower. The trapped volume of these vesicles for glucose was 0.8 ml per g of phospholipid; their concentration of
protein was 0.32 mg per ml of vesicles, with similar ATPase specific activity (225 μmoles of Pi per mg per hour).

The absence of any changes in the sodium dodecyl sulfate gel pattern of the preparation during dialysis, the time course of glucose-trapping ability that closely parallels removal of detergent, the reproducibility of the system, and the conditions of dialysis (0' in the presence of 10 mM β-mercaptoethanol) all make unlikely the possibility that bacterial growth occurs in an amount substantial enough (several grams of cells per liter) to produce artifacts in the glucose trapping and in the transport measurements described below.

**Active Transport of Na^+ Catalyzed by Purified, Reconstituted (Na^+ + K^+)-ATPase**—The enzyme was initially reconstituted in a buffer system (100 mM KCl and 20 mM in NaCl) with an ionic composition similar to that of the interior of most eucaryotic cells. Na^+, carrier-free so as not to change the external concentration of Na^+, was externally added to the reconstituted vesicles. In addition, Tris-ATP was externally added to a final concentration of 3 mM in 6 mM MgCl₂. The control was done in an identical fashion, with sucrose added instead of Tris-ATP in an osmolarity equal to that of the Tris-ATP it replaced. No net gradient of Na^+ was established by these additions, so that any transport of Na^+ into the vesicles would either be passive isotope exchange or active pumping of Na^+. After 30 min of incubation at 37°C, a 3-fold ATP-dependent stimulation of Na^+ uptake was reproducibly observed over the passive isotope exchange amount that occurs when the isotope is passively equilibrated with Na^+. After 30 min of incubation at 37°C, a 3-fold ATP-dependent stimulation of Na^+ uptake was reproducibly observed over the passive isotope exchange amount that occurs when the isotope is passively equilibrated with Na^+.

The time course of the diffusion exchange of radioactive Na^+ which occurs in the absence of ATP was measured (Fig. 5, Curve a). Isotope exchange in reconstituted enzyme vesicles reaches equilibrium after about 30 min at 37°C, 0.27% of the total counts per min added. Incubation of the vesicles in the presence of externally added ATP (Fig. 5, Curve c) results in an accumulation of Na^+ to a level at the end of 60 min which is greater than 3 times the amount that occurs when the isotope is passively equilibrated with the vesicles; 0.88% of the total Na^+ is incorporated. This indicates that the average concentration of Na^+ within the vesicles is (0.88/0.27) × 20 mM = 65 mM at the end of 60 min, assuming the initial intravesicular Na^+ is the same as that of the medium. Thus a greater than 3-fold concentration of Na^+ within the vesicles has been observed. Na^+ carried by penetra-

### Table I

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<th>Preparation</th>
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<th>Specific Activity</th>
<th>Total ATP-sensitive Activity</th>
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<td>0.92</td>
<td>207</td>
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*Assay method was that of Kyte (6), with phosphomolybdate extracted into organic phase before reduction as described under "Methods."

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** ATP-stimulated uptake of Na^+. Vesicles were incubated in the absence or in the presence of ATP and run in parallel on Sephadex G-50, as described in the text. Optical density at 300 nm of vesicles with 3 mM ATP (■—■), and without 3 mM ATP (□—□). Percentage of total Na^+ counts per min in each fraction with 3 mM ATP (△—△), and without 3 mM ATP (○—○). Counts per min added to each column, 150,000.

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** Time course of ATP-stimulated uptake of Na^+. Each point represents the total counts per min minus background eluting with optical density at 300 nm on Sephadex G-50, as shown in Fig. 4. Curve a (■—■), reconstituted (Na^+ + K^+)-ATPase incubated without ATP. Curve b, vesicles formed without enzyme incubated in the presence of (○—○), or in the absence of (□—□), ATP. Curve c (△—△), reconstituted (Na^+ + K^+)-ATPase incubated with ATP.

Asymmetric inhibition of active Na^+ uptake by strophanthidin.
and ouabain was demonstrated (Table II). Strophanthidin or ouabain added to the outside of the vesicles is ineffective in inhibiting the observed active transport; 90% of the ATP-dependent $^{22}$Na$^+$ incorporation is unaffected by $10^{-6}$ M strophanthidin or $3 \times 10^{-4}$ M ouabain. However, the above concentrations of strophanthidin or ouabain trapped within the vesicles during dialysis were effective in inhibiting all of the observed active transport. The possibility that these glycosides, when present in the dialysis medium, interfere with proper reconstitution of the enzyme seems unlikely but must be acknowledged.

The above evidence consistently points to the conclusion that a portion of the purified (Na$^+$ + K$^+$)-ATPase has been incorporated into vesicles in such a way as to hydrolyze ATP at the exterior surface, bind cardiac glycoside at the interior surface, and actively pump Na$^+$ into the vesicles.

**Stoichiometry of Active Transport of Reconstituted (Na$^+$ + K$^+$)-ATPase**

**Coupling of Active Transport of Na$^+$ to Hydrolysis of ATP—A substantial fraction of the ATPase activity of reconstituted**

enzyme vesicles is insensitive to the action of externally added strophanthidin, when these vesicles are assayed under precisely the same conditions as those used to observe active transport of Na$^+$. It is apparently this fraction of ATPase activity which is responsible for the observed active transport of Na$^+$. Of the ATPase activity determined under these conditions, 22% is strophanthidin-insensitive (Fig. 6). The linearity of ATP hydrolysis between 10 and 60 min of incubation precludes the possibility that a significant portion of this external glycoside-insensitive ATPase activity is due to lack of complete glycoside binding to accessible sites. This assay revealed no measurable ATPase activity in vesicles formed in the presence of glycoside. Less than 2% of the ATPase activity of the initial salt-detergent-extracted microsomes is strophanthidin-insensitive. No strophanthidin-insensitive ATPase activity could be detected when the incubation medium of Kyte was used. This medium gives hyp-osmotic conditions that would cause vesicle lysis and allow penetration of glycoside.

After 10 min of incubation of vesicles of reconstituted (Na$^+$ + K$^+$)-ATPase, the net ATP-dependent transport of Na$^+$ is 0.152 pmole per mg of protein in the absence of strophanthidin and 0.131 pmole per mg in the presence of this externally added strophanthidin (calculated from data in Table II). This difference is experimentally insignificant, based on the ATPase activity observed with the same preparation of vesicles for the same time period under identical incubation conditions (Fig. 6); 0.071 sodium ion is transported per ATP hydrolyzed in the absence of strophanthidin.

External addition of strophanthidin markedly improves the stoichiometry; 0.30 sodium ion is actively transported per ATP hydrolyzed. This is so because the ATPase activity which is inhibited by externally added strophanthidin is almost exclusively not ATPase activity associated with the active transport of Na$^+$ in this in vitro system. The presence of this type of ATPase activity suggests that a substantial fraction of the enzyme is oriented in such a way that both the ATP-hydrolyzing and cardiac glycoside-binding sites are accessible to these molecules when added externally. Enzyme so oriented does not actively transport Na$^+$ into the vesicles.

**Coupling of K$^+$ and Cl$^-$ Transport to Active Pumping of Na$^+$—**

There is an apparent obligatory coupling of transport of 2 potassium ions into the cytoplasm of axons and red cells for every 3 sodium ions actively extruded by the (Na$^+$ + K$^+$)-ATPase in the plasma membrane of these cells (11, 12, 23). The (Na$^+$ + K$^+$)-ATPase, purified from canine renal medulla, has in common with the enzymes in red cells and nerves a requirement for K$^+$ for maximal ATPase activity (24).

Based on this K$^+$ requirement and the similarity of the polypeptide composition of this preparation to that of the two major polypeptides of the preparation isolated from brain (6-8), one might expect a similar coupling of active transport of K$^+$ to that of Na$^+$. In the in vitro system described here, where one is observing active transport by that fraction of (Na$^+$ + K$^+$)-ATPase which is accessible to externally added ATP and oriented in vesicles “inside out” with respect to its in vivo orientation, 2 K$^+$ ions would be extruded for every 3 Na$^+$ ions actively pumped into the vesicles. To test this hypothesis, double label experiments were performed to simultaneously measure the time course of both passive and active transport of Na$^+$ and K$^+$.

As in the single label $^{22}$Na$^+$ measurements the radioactive isotopes, $^{22}$Na$^+$ and $^{22}$K$^+$, were added to the incubation medium so that an isotopic gradient of these ions was established, but the actual concentrations of Na$^+$ and K$^+$ were initially the same
In concentration of Na+ should be accompanied by a significant decrease in K+; thus if Na+ influx to K+ efflux, one would expect a decrease in K+ concentration of about 13 to 20 mM. In this situation, the observed decrease in K+ concentration would be about 13 to 20% of the equilibrium level of incorporation. However, no such decrease was observed.

To obtain more sensitive measurements of the coupling of K+ efflux to Na+ influx, an experiment of identical design was performed on vesicles reconstituted in 20 mM K+ and 30 mM Na+, in an incubation medium of the same external concentrations. Again, vesicles pre-equilibrated with the externally added isotope exhibited no significant ATP-dependent efflux of K+ to accompany an approximately 2-fold ATP-dependent increase in Na+ above the equilibrium level (Fig. 8a). In this case, the initial ionic concentrations in the vesicles and incubation medium were such that the ATP-dependent decrease in Na+ above the equilibrium level (Fig. 8a) was observed. The observed equilibrium level of incorporation of externally added Na+ and K+ in the absence of ATP agrees well with trapped volume determinations for these ions when Na+ and K+ were trapped inside the vesicles by co-solubilizing the enzyme with phospholipid and forming vesicles in the presence of Na+ and K+ as in the procedure used to determine trapped volume of glucose. Trapped volume for Na+ was 0.140 ml per g of phospholipid. A value of 0.115 ml per g was obtained by equilibrating a batch of vesicles formed at the same time from the same batch of solubilized material, with externally added Na+ as described. These values were, respectively, 0.135 ml per g and 0.125 ml per g for Na+ and K+. The agreement between these two numbers was within the limits of the reproducibility of trapped volume determinations obtained on two separate occasions by the external equilibration method. This confirms that, at equilibrium, external addition of isotope to the vesicles does not produce isotopic gradients of these ions, and that any significant decrease in Na+ above the equilibrium level is indeed active transport and not ATP-dependent Na+ exchange.

It thus appears that, in this in vitro system, the Na+ + K+-ATPase transports close to 1 Cl- ion against its concentration gradient for every Na+ actively transported, and that active extrusion of K+ out of the vesicles is almost negligible.

**DISCUSSION**

Experiments done on red blood cells and squid axons have clearly demonstrated that the (Na+ + K+)-ATPase is oriented in vivo in an asymmetric fashion. Whittam and Ager (25, 26)
have shown that the site on this enzyme in the red blood cell which hydrolyzes ATP is on the interior surface of the cell. In both red blood cells (13) and squid axons (11, 12) it is known that for pumping to occur, Na+ is required within the cell and K+ without, and that the fluxes of these ions appear to be coupled in a 3:2 Na+:K+ ratio. More recent results indicate that the stoichiometry of the ion fluxes may vary under different conditions (29) and from tissue to tissue (27). Finally, evidence suggests that in squid axon (30), cardiac glycosides inhibit the enzyme from the outside of the cell and are ineffective when present within the axoplasm.

One has no reason to assume that, in reconstituted vesicles, the enzyme is uniformly oriented in the manner exhibited in vivo. On the contrary, one might expect that a certain fraction of the reconstituted active transport system would be oriented backwards, i.e. with the ATP-hydrolyzing site on the outside of the "cell" (vesicle), and the site for strophanthidin inhibition on the inside (hence inaccessible to the action of strophanthidin added externally). The experiments described herein were designed to detect active transport by this fraction of reconstituted enzyme; in this case, active transport of Na+ would be directed into the vesicle and extrusion of K+ out of the vesicle when ATP is added externally.

The results obtained clearly show ATP-dependent active transport of Na+ and its inhibition by ouabain and strophanthidin trapped within the vesicles during dialysis. The direction of the observed active transport and the sidedness of its inhibition by cardiac glycosides with respect to its site for ATP hydrolysis is consistent with the properties of the (Na+ + K+)-ATPase in nerves and red blood cells. However, the behavior of the reconstituted, purified (Na+ + K+)-ATPase differs from these active transport systems in two respects; the active transport of Na+ is less efficiently coupled to ATP hydrolysis than in these other tissues (31), and, rather than exhibiting an active flux of K+ opposite to the active flux of Na+ in a ratio of 2:3, close to 1 Cl- ion is translocated in the same direction for every Na+ ion actively pumped.

There are several explanations for the apparent inefficiency of coupling of Na+ active transport to the hydrolysis of ATP. One is that much of the (Na+ + K+)-ATPase in this preparation is not associated with sealed vesicles. This is supported by the fact that much of the (Na+ + K+)-ATPase activity is inhibitable by externally added glycosides, without significant effect on active Na+ uptake; (Na+ + K+)-ATPase oriented in sealed vesicles and hydrolyzing externally added ATP would be inaccessible to inhibition by externally added glycoside.

However, even when glycoside is added externally to inhibit this fraction of the enzyme, the stoichiometry is still only 0.5 Na+ ion transported per ATP hydrolyzed. Much of the remaining inefficiency can be explained by the fact that there is a 6-fold discrepancy between the ability of the vesicles to trap glucose and their ability to trap monovalent ions. This discrepancy is due to the higher permeability of the vesicles to these ions than to glucose. To minimize back diffusion of Na+ it was necessary to run the Sephadex G-50 columns at a high pressure head and at 4°. Most of the trapped Na+ leaked out of vesicles passed on columns at much lower flow rates or at room temperature (or both); this was not the case for trapped glucose. Thus, assuming that the enzyme distributes itself impartially among these vesicles, a maximum of only one-sixth of the total active transport of Na+ can be resolved by this gel filtration technique. By increasing the phospholipid to protein ratio and using faster flowing Sephadex columns we have recently been able to increase the stoichiometry 3-fold, bringing the ratio of Na+ transported to ATP hydrolyzed close to 1.0. Further attempts to optimize our ability to resolve active transport are under way. It can be mathematically shown that this back diffusion on the Sephadex columns will affect the total but not the relative (ATP-dependent versus passive) ion transport observed. Our recent unpublished studies confirm this.

The active transport of Na+ into the vesicles by the (Na+ + K+)-ATPase must be accompanied by sufficient co-translocation of anions or countertranslocation of cations (or both) to maintain bulk electrical neutrality of transport across the vesicle membrane. If the only ion actively transported by this enzyme is Na+, the ratio of the K+ and Cl- permeabilities of the vesicle membrane will determine the relative fluxes of these two species required to neutralize the influx of positive charge.

It can be shown that

\[ \frac{P_{K^+}}{P_{Cl^-}} = \frac{t_{1/2 \, K^+}}{t_{1/2 \, Cl^-}} \]

where \( t_{1/2 \, Cl^-} \) and \( t_{1/2 \, K^+} \) are the times required for isotope exchange of Cl- and K+ to reach one-half of their equilibrium values. From Fig. 8b, \( P_{K^+}/P_{Cl^-} \) is 0.36.

The differential form of the Goldman-Hodgkin-Katz equation (32) describes the fluxes of ions across a membrane in response to electrical and chemical gradients. One can assume that the reconstituted enzyme only pumps Na+ and that Cl- and K+ distribute themselves passively according to their relative permeabilities to the vesicle membrane. In this case, integration of the Goldman-Hodgkin-Katz equation for Cl- and K+, insertion of the applicable boundary conditions, and computer analysis of the appropriate form of the integrated equations reveal that the observed 2.0-fold increase in Na+ due to active transport would, under the conditions of the experiment, be accompanied by a 25 to 18% decrease in K+ and a 34 to 36% increase in concentration of Cl-.

In actuality, what is observed is a 40% increase in Cl- and only a 6.5% decrease in K+, less than one-third of the predicted value. This result suggests that the enzyme selectively and actively translocates Cl- along with Na+ rather than letting K+ and Cl- passively distribute themselves according to their permeabilities to the vesicle membrane. The experimental variability of this system, based on analysis of duplicates and the reproducibility of the equilibrium curves, is ±5% within a single experiment. Thus, the above conclusion is a viable but tentative one.

However, it is certain that the stoichiometry of K+ counter to Na+ by the reconstituted enzyme is much lower than 2:3, as discussed previously. The fact that the reconstituted enzyme exhibits no significant pumping of K+ may be because the enzyme is purified or reconstituted in such a way that its "K+ pump" portion is missing or inoperative. Or, the enzyme in its native state in canine renal medulla may in fact not pump K+ counter to Na+. Physiological experiments performed on isolated canine nephrons shed some light on this matter but do not completely clarify it.

This enzyme is isolated from renal medulla. Renal medulla contains the ascending loop of Henle, the part of the nephron that is responsible for active reabsorption of NaCl and water (33). It is this portion of renal medulla that makes it a rich source of (Na+ + K+)-ATPase (23). Ouabain inhibits renal ATPase activity and reabsorption of NaCl (34, 35). Furthermore, micropuncture studies on isolated single nephrons reveal that not only is the flux of K+ counter to that of Na+ much lower than 2:3, but that K+ is actively reabsorbed as well, travelling
in the same direction as Na\textsuperscript{+} (36). Whether Cl\textsuperscript{-} reabsorption is passive or active is currently debated in the literature (27, 28); direct electrophysiological studies on the ascending loop of Henle have not yet been performed.

What is apparently agreed upon by physiologists is that the tissue from which the enzyme is isolated is specialized for reabsorption of NaCl and that this reabsorptive process is ouabain-sensitive. Hence the behavior of the reconstituted enzyme is consistent with what is known about the function it catalyzes.

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