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PURIFICATION OF THE ($Na^+ + K^+$)-ADENOSINE TRIPHOSPHATASE FROM HUMAN RENAL TISSUE

J. MARK BRAUGHLER and CLINTON N. CORDER

University of Pittsburgh, School of Medicine, Department of Pharmacology and Medicine, Pittsburgh, Pa. 15261 (U.S.A.)

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Summary

($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was purified from human cadaver renal tissue and exhibited a linear reaction rate with time. 100 g of whole kidney would yield 1—3.5 mg protein with a specific activity of 50—200 mol · kg^{-1} · h^{-1} for ($Na^+ + K^+$)-ATPase. The preparation was completely inhibited by 100 μM ouabain with a K_i of 1.8 μM. K^+-dependent phosphatase increased during purification of ($Na^+ + K^+$)-ATPase to 7.8 mol · kg^{-1} · h^{-1}. There was no detectable Mg^{2+}-ATPase in the final preparation. Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis yielded three protein peaks of 117 000, 92 500, and 56 000 daltons. The peptide band corresponding to 92 500 daltons underwent an Na^+-dependent phosphorylation with [γ-^{32}P]-ATP. The band at 56 000 daltons stained for glycoprotein. The K_m for ATP was 0.38 mM and that for Mg^{2+} was 0.5 mM. The formation of ADP and inorganic phosphate from ATP was stoichiometric. The K_m for Na^+ in the presence of 20 mM K^+ was 16 mM and the K_m for K^+ in the presence of 100 mM Na^+ was 1.5 mM. The temperature optimum was 51°C and the pH optimum was 7.0.

($Na^+ + K^+$)-ATPase in whole homogenate, microsomes, and NaI-treated microsomes exhibited a slowing of reaction rate (non-linearity) with time such that the enzyme was inactive by 10—15 min of reaction. This non-linearity was eliminated during purification. The significance is discussed.

Introduction

($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is a membrane-bound enzyme complex which is believed to be responsible for the transport of Na^+ and K^+ across cell membranes. The renal medulla of the dog [1,2] and rabbit [3] as well as bovine brain [4] have been the primary mammalian sources of the pure [1—3] and partially pure [4] enzyme. The electric organ of

the electric eel [5] and the rectal gland of the dogfish shark [6] have proved to be excellent non-mammalian sources.

Studies of ($Na^+ + K^+$)-ATPase from human tissue have been carried out on microsomal preparations from kidney [7—9], heart [10] and erythrocytes [11]. In some of these studies the reaction rate was non-linear with assay time [8—10]. This laboratory has reported non-linearity of the enzyme in microsomal preparations from rat kidney [30,31]. Similar events were observed in microsomes prepared from human kidney. In order to facilitate the study of the factors responsible for this non-linearity, it was most desirable to isolate a preparation of ($Na^+ + K^+$)-ATPase which would exhibit a linear reaction rate with time.

Materials

Adenosine 5′-triphosphate (Tris and disodium salts), sodium adenosine 5′-diphosphate, sodium adenosine 5′-monophosphate, Tris/guanosine 5′-triphosphate, Tris/uridine 5′-triphosphate, sodium cytosine 5′-triphosphate, ouabain octahydrate, imidazole, Tris · HCl, Tris base, Tris/*p*-nitrophenyl phosphate, muscle phosphorylase, bovine serum albumin (crystalline lyophilized), dithiothreitol, 2-mercaptoethanol, Coomasie Blue, sodium dodecyl sulfate (SDS), and 2-amino-2-methyl-1,3-propanediol; Sigma Chemical Co. NaCl, KCl, $CaCl_2$, $MgCl_2$, disodium ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, NaI, sucrose, $(NH_4)_2SO_4$, bromphenol blue, glycerol, sodium phosphate, and sodium acetate; Fisher Scientific Co. Pyruvate kinase and lactate dehydrogenase; Boehringer. Acrylamide and bisacrylamide; Eastman Organic Chemicals. Mercaptomerin; Wyeth Laboratories. Furosemide; Hoechst. Chlorothiazide and ethacrynic acid; Merck Sharp and Dohme. Prostaglandins; Upjohn Company. [γ-^{32}P]Adenosine 5′-triphosphate; New England Nuclear.

Methods

Collection of tissue. Kidney was obtained at autopsy from subjects (ages 32—38 years) with normal function (serum creatinine $\leqslant$1.5 mg/100 ml) who had not received cardiac glycoside or diuretic therapy *. The delay from the time of death until the kidney was obtained was an average of 7 h (max. 12 h). Tissues were placed on ice and either used immediately or frozen at —90°C. There was no difference in ($Na^+ + K^+$)-ATPase prepared from fresh or frozen kidney.

Preparation of microsomes. All procedures were at 2°C unless stated otherwise. Kidneys which had been frozen were thawed in 100 mM Tris, pH 7.4, 0.33 M sucrose (Tris/sucrose). Extraneous fat, large vessels, and capsule were removed. The whole kidney was minced with scissors and homogenized 1 : 5 with Tris/sucrose in a Waring Blender at high speed for 40 s. This suspension was further homogenized in a glass homogenizer fitted with a Teflon pestle and then centrifuged at 10 000 × *g* for 20 min. The supernatant was saved and the

* Clearance from Committee on the Use of Human Subjects for Experimental Purposes, Department of Medicine, University of Pittsburgh School of Medicine, September 4, 1975.

pellet was rehomogenized in four volumes of Tris/sucrose and resedimented. The 10 000 × *g* supernatants were combined and centrifuged at 100 000 × *g* for 1 h. The 100 000 × *g* pellets were resuspended in three volumes of Tris/sucrose and centrifuged again at 100 000 × *g* for 1 h. This final precipitate was suspended in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA at a protein concentration of 10—14 mg/ml and frozen at —90° C.

Deoxycholate treatment. The microsomes were thawed and prepared to give a final concentration of 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 0.6 mg/ml deoxycholate, and 0.5 mg/ml protein. The optimal protein to deoxycholate ratio for increasing the specific activity of $(Na^+ + K^+)$-ATPase from the human kidney was 0.5 mg protein/ml and 0.6 mg deoxycholate ml, as determined by the procedure of Jorgensen and Skou [12]. The microsomal suspension was incubated at 25° C for 30 min, then centrifuged at 5000 × *g* for 70 min. The 5000 × *g* supernatant was then centrifuged at 25 000 × *g* for 45 min. The resultant 25 000 × *g* pellet was suspended in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 0.25 M sucrose at an approximate protein concentration of 8 mg/ml. The original 5000 × *g* pellet was resuspended in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA and treated with deoxycholate and centrifuged identically to the microsomes. The pooled 25 000 × *g* pellets were combined and frozen at —90° C.

NaI treatment. The deoxycholate-treated microsomes were thawed and treated with NaI as described by Lane et al. [2] except for the following modifications. The final NaI suspension was centrifuged at 65 000 × *g* for 45 min. The resultant pellet was washed twice in 100 mM imidazole, pH 7.0, 1 mM disodium EDTA, and centrifuged again at 65 000 × *g* for 45 min. The final pellet was suspended in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 40 mM KCl, 400 mM NaCl, 0.25 M sucrose at a protein concentration of approx. 6 mg/ml and frozen at —90° C.

Deoxycholate solubilization-glycerol precipitation. The final NaI enzyme was solubilized with deoxycholate and precipitated with glycerol by the method described by Lane et al. [2]. The final glycerol precipitated enzyme, in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 40 mM KCl, 400 mM NaCl, 0.25 M sucrose at approx. 4 mg/ml protein, was frozen at —90° C.

Re-solubilization of glycerol enzyme. The glycerol-precipitated enzyme was thawed and adjusted to a final concentration of 4 mg protein/ml, 1 mM dithiothreitol and 1 mg deoxycholate per mg protein. This suspension was incubated at 2° C for 30 min, then centrifuged at 100 000 × *g* for 1 h.

Ammonium sulfate fractionation. The deoxycholate-solubilized enzyme was brought to 30% $(NH_4)_2SO_4$ by addition of a 100% saturated $(NH_4)_2SO_4$ solution at 2° C buffered to pH 7.0 with 25 mM imidazole, 1 mM disodium EDTA. This suspension was then centrifuged at 50 000 × *g* for 30 min. The supernatant was brought to 50% $(NH_4)_2SO_4$ and incubated with shaking at 2° C for 30 min. This suspension was centrifuged at 20 000 × *g* for 60 min. The resulting amorphous enzyme precipitate was dissolved in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 20% glycerol, dialyzed in the same buffer for 14 h, and centrifuged at 100 000 × *g* for 60 min. This pellet was resuspended in 25 mM imidazole, pH 7.0, 1 mM disodium EDTA, 0.25 M sucrose and frozen at —90° C.

Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis. Samples of enzyme were prepared for electrophoresis by incubation at 37°C for 60 min with 3% SDS, 10 mM sodium phosphate, pH 7.1, 1% 2-mercaptoethanol, 5% glycerol and 1—3 mg protein/ml. Samples (10—30 μl) containing 10—50 μg protein and 0.05% (w/v) bromphenol blue were layered on 6% acrylamide gels (6 × 65 mm) containing 0.1% SDS. The electrolyte buffer was 50 mM sodium phosphate, pH 7.1, 0.1% SDS. Electrophoresis was run at 10 mA per gel for 3—4 h at 21°C. The gels were fixed for 18 h in 10% acetic acid, 50% methanol and stained for 2 h with 0.1% Coomasie Blue in the fixing solution. Destaining was in 10% acetic acid, 50% methanol. Some gels, run with 50—150 μg protein, were also stained for glycoprotein with periodic acid-Schiff according to Zacharius et al. [13].

Phosphorylation of (Na^+ + K^+)-ATPase and sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis. Purified (Na^+ + K^+)-ATPase was phosphorylated by incubation of 100 μg of protein at 0°C with 50 mM Tris, pH 7.2, 0.1 mM [γ-^{32}P]ATP ($3 \cdot 10^9$ cpm), 6 mM $MgCl_2$ and 100 mM NaCl in a volume of 0.5 ml [6]. After 10 s at 0°C the reaction was stopped by addition of 0.2 ml of 40 mM sodium phosphate, pH 7.1, 20% glycerol, 12% SDS, 4% 2-mercaptoethanol [2]. This suspension was then layered on sodium dodecyl sulfate-polyacrylamide gels and electrophoresised as described. Non-specific ^{32}P incorporation was determined by replacing NaCl with 20 mM KCl in the reaction mixture. Following electrophoresis the gels were either sliced into sections of approx. 1.5 mM and assayed for radioactivity in a Packard liquid scintillation spectrometer, or stained as described.

Assays. The ATPase assay, unless stated otherwise, was at 37°C with 0.05—0.1 mg protein/ml in 100 mM imidazole, pH 7.0, 6 mM ATP, 6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl and 0.02% bovine serum albumin. The reaction was started by the addition of enzyme into 1 ml of the pre-warmed assay mix. At 0, 1, 2, 4, 6, 8 min and other time intervals as necessary, 150-μl aliquots were transferred to a final concentration of 3% $HClO_4$ at 0°C. Zero time was the first aliquot taken immediately after addition of the enzyme. The $HClO_4$ precipitate was centrifuged at 200 × *g* for 10 min and the supernatant neutralized and assayed for inorganic phosphate according to Lowry and Lopez [14]. That activity referred to as Mg^{2+}-ATPase was that ATPase activity with 1 mM ouabain included in the assay system (Na^+ + K^+)-ATPase was determined as the difference between the total and Mg^{2+}-ATPase. In more purified preparations which were free of Mg^{2+}-ATPase, ouabain was omitted from the assay. The blank was the spontaneous phosphate evolution in an assay without enzyme. In the stoichiometric studies the neutralized supernatants were assayed for ATP, ADP, AMP, and inorganic phosphate by the hexokinase, pyruvate kinase, myokinase [15] and glycogen phosphorylase [16] methods, respectively.

The K^+-dependent phosphatase activity was determined with *p*-nitrophenolphosphate by the procedure of Susa and Lardy [17].

Protein was determined by the Lowry et al. method [18].

Enzyme activities are expressed as mol of liberated phosphate per kg of protein per unit time at 37°C ($mol \cdot kg^{-1} \cdot time^{-1}$).

For certain studies which required zero Na^+ or K^+ content, the enzyme was dialyzed prior to the assay against 1000 volumes of 25 mM imidazole, pH 7.0 and 1 mM EDTA (Na^+-free acid) for 2 h at 2°C.

TABLE I

PURIFICATION OF $(Na^+ + K^+)$-ATPase FROM HUMAN KIDNEY

This preparation represents 130 g of whole kidney.

Step	Volume (ml)	Total protein (mg)	Total Mg^{2+}-ATPase Specific activity ($mol \cdot kg^{-1} \cdot h^{-1}$)	Mg^{2+}-ATPase Specific activity ($mol \cdot kg^{-1} \cdot h^{-1}$)	$(Na^+ + K^+)$-ATPase Total ($mol \cdot h^{-1} \times 10^3$)	$(Na^+ + K^+)$-ATPase Specific activity ($mol \cdot kg^{-1} \cdot h^{-1}$)	K^+-dependent phosphatase Specific activity ($mol \cdot kg^{-1} \cdot h^{-1}$)
1. Microsomes	46	718	29	25	2.87	4	0.75
2. Deoxycholate-treated microsomes	34	306	40	18	6.75	22	4.9
3. NaI-treated microsomes	17	156	—	0	5.46	35	5.9
4. Glycerol-precipitated enzyme	7	51	—	0	3.57	70	7.5
5. $(NH_4)_2SO_4$ enzyme	1.1	2.3	—	0	0.166	72	7.8

Results

Purification of (Na^+ + K^+)-ATPase from human kidney. Each preparation of (Na^+ + K^+)-ATPase was from an individual subject (seven total). Table I shows the purification of (Na^+ + K^+)-ATPase in a typical preparation. 100 g of whole kidney yielded 25—40 mg of the glycerol-precipitated enzyme and 1—3.5 mg of the $(NH_4)_2SO_4$ enzyme. The specific activity of the glycerol-precipitated enzyme was 50—200 mol · kg^{-1} · h^{-1} depending upon the preparation, and the $(NH_4)_2SO_4$ enzyme was never more than 5% higher than the corresponding glycerol-precipitated enzyme. The specific activities found in whole human kidney are about one-sixth that reported for (Na^+ + K^+)-ATPase purified from the renal medulla of dog [1,2] and rabbit [3]. This preparation, however, represents (Na^+ + K^+)-ATPase from all areas of the kidney rather than the medulla exclusively. Human (Na^+ + K^+)-ATPase was also shown to decay in situ with time, even during cold perfusion [7]. Thus, various factors may have affected the final enzyme activity in the present study. The final enzyme was completely stable to storage at —90°C for 4 months and could be thawed and frozen 4—6 times without loss of activity.

The K^+-dependent phosphatase activity increased with the (Na^+ + K^+)-ATPase (Table I). This was also observed in preparations of (Na^+ + K^+)-ATPase from dog kidney [2], and bovine brain [4].

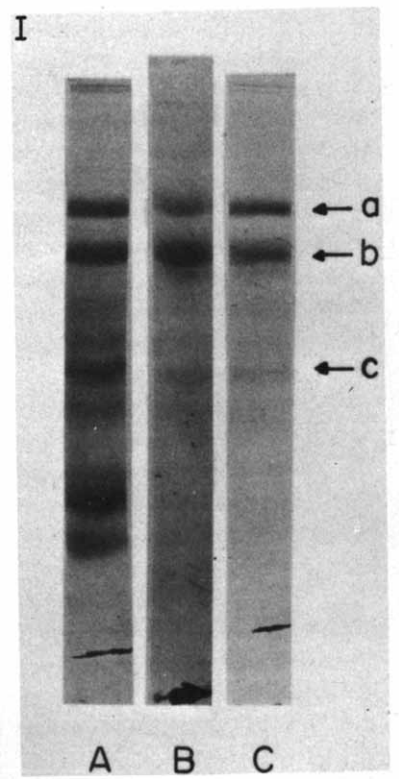


Fig. 1. For legend see opposite page.

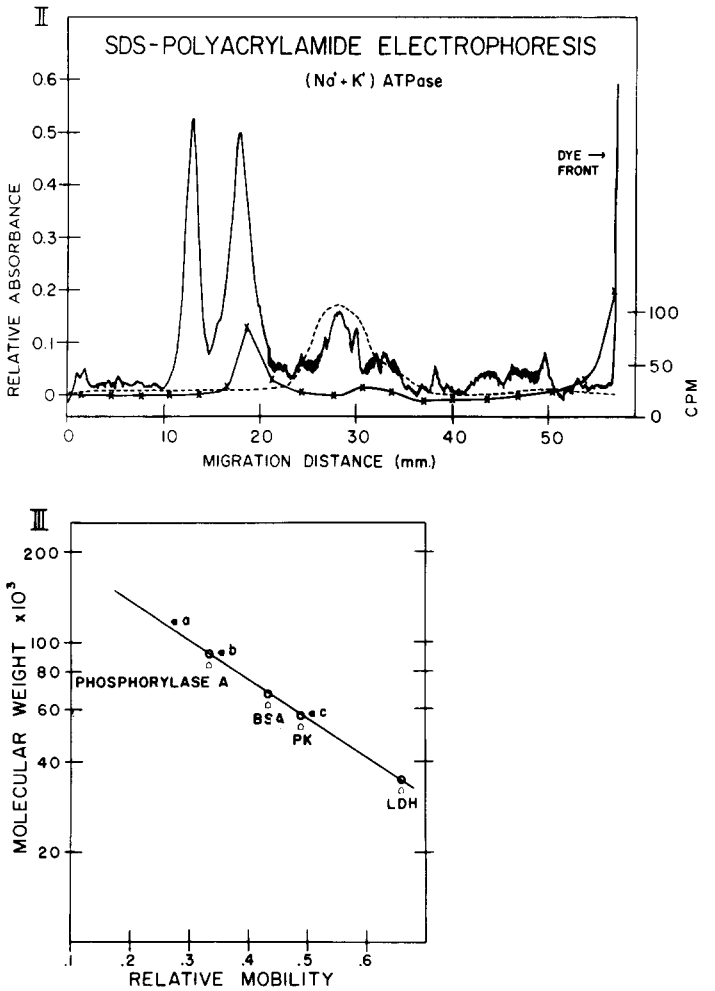


Fig. 1. Sodium dodecyl sulfate-polyacrylamide electrophoresis. (I) Sodium dodecyl sulfate-polyacrylamide gels were stained with Coomasie Blue as described in Methods. NaI-treated enzyme (A), glycerol-precipitated enzyme (B), $(NH_4)_2SO_4$ enzyme (C). The position of the tracking dye was marked with permanent India ink. (II) The $(NH_4)_2SO_4$ enzyme gel (C) was scanned at 560 nm with a Gilford spectrophotometer equipped with a linear transport gel scanner (———). The three major peaks correspond to bands a, b, c on the stained gels. The $(NH_4)_2SO_4$ enzyme was phosphorylated with [γ-^{32}P] ATP in the presence of Mg^{2+} and Na^+, electrophoresed and assayed for radioactivity as described in Methods (χ———χ). The $(NH_4)_2SO_4$ enzyme gel, stained with periodic acid-Schiff as described in Methods, was scanned at 560 nm (- - - - -). (III) Sodium dodecyl sulfate-polyacrylamide gels were calibrated with proteins of known molecular weight. Relative mobility was determined as the migration distance of protein versus migration distance of tracking dye. Phosphorylase *a*, 92 500 daltons; bovine serum albumin (BSA), 68 000 daltons; pyruvate kinase (PK), 57 000 daltons; lactate dehydrogenase (LDH), 35 000 daltons. (←), the relative mobilities of the three major bands on the $(NH_4)_2SO_4$ enzyme gel (C).

Sodium dodecyl sulfate-polyacrylamide electrophoresis of $(Na^+ + K^+)$*-ATPase.* Fig. 1 shows sodium dodecyl sulfate-polyacrylamide gel patterns of $(Na^+ + K^+)$-ATPase. A spectrophotometric scan of the $(NH_4)_2SO_4$ enzyme gel (C) reveals three peaks corresponding to 117 000, 92 500, and 56 000 daltons. The peak at 92 500 daltons was phosphorylated in the presence of ATP, Mg^{2+},

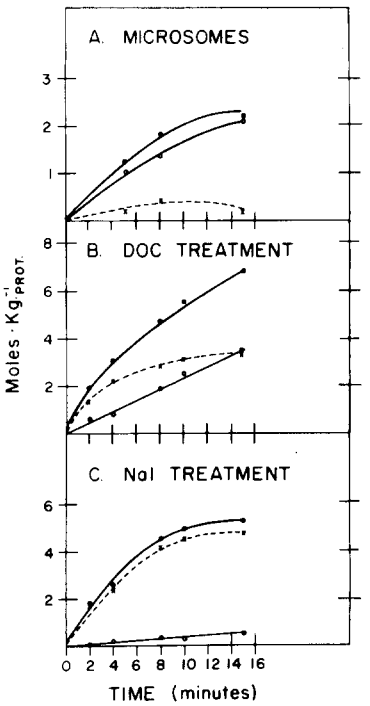


Fig. 2. Linearity of the $(Na^+ + K^+)$-ATPase reaction with time at early stages of purification. The enzyme was assayed at a protein concentration of 0.06 mg/ml as described in Methods. o———o, total ATPase; o———o, Mg^{2+}-ATPase; x- - - - -x, $(Na^+ + K^+)$-ATPase. DOC, deoxycholate.

and Na^+. Phosphorylation in the presence of Na^+ resulted in an incorporation of 80—100 cpm into this peptide band. When Na^+ was replaced with K^+ in the phosphorylation system, however, the radioactivity associated with this or any other band was no higher than backround (cpm with K^+ = 21—23; backround cpm = 20—23). This indicates that phosphorylation of the peptide band was Na^+-dependent. Gels stained for glycoprotein revealed a single band at the location of the 56 000 dalton polypeptide.

Linearity of the $(Na^+ + K^+)$-ATPase reaction at various stages of purification. Shown in Fig. 2 are examples of the ATPase reaction at the early stages of purification. In each case the $(Na^+ + K^+)$-ATPase activity is non-linear with time and is nearly zero after 8 min of incubation. Although the specific activity of the $(Na^+ + K^+)$-ATPase varied from preparation to preparation, this non-linearity of the reaction with assay time was a consistent finding in the microsomes, deoxycholate-treated, and NaI-treated enzymes. This laboratory has reported similar findings in rat kidney [30,31]. Because of the significance of the non-linearity, $(Na^+ + K^+)$-ATPase activity was routinely assayed at all steps of the purification by taking several time points. The specific activities in Table I are based upon the early part of the ATPase reaction which was the closest approximation of the initial enzymatic rate. This non-linearity of the ATPase reaction with time was not due to a limiting amount of substrate. Even if the activity is based upon the initial rate estimate of the NaI-treated enzyme

(Fig. 2C), only about 7% of the ATP available would have been hydrolyzed in 15 min.

Non-linearity was not a problem in more purified systems. Fig. 3 shows the (Na^+ + K^+)-ATPase reaction of a glycerol-precipitated enzyme from a typical preparation. The $(NH_4)_2SO_4$ enzyme was also linear with assay time (not shown).

One of the major aims of this study was to obtain a (Na^+ + K^+)-ATPase preparation which was free of the non-linear reaction character so that kinetic studies could be carried out on the human enzyme and to facilitate an investigation into the cause of the non-linearity. The glycerol-precipitated enzyme suited this purpose. Although the $(NH_4)_2SO_4$ enzyme was also linear, the yield of the glycerol-precipitated enzyme was far greater and there was only a minor difference in the specific activities. Despite the linearity of the glycerol-precipitated enzyme, all kinetic studies were based upon several time point determinations of activity to ensure that the reaction remained linear with changes in assay conditions.

Stoichiometry of the (Na^+ + K^+)-ATPase reaction. The utilization of ATP and the formation of ADP and inorganic phosphate by (Na^+ + K^+)-ATPase as a function of time was stoichiometric (Fig. 4). No AMP could be detected.

Linearity of (Na^+ + K^+)-ATPase with protein concentrations. (Na^+ + K^+)-ATPase activity was linear over the range of protein concentrations from 0.05 to 0.25 mg/ml (Fig. 5).

Effect of assay temperature and pH on (Na^+ + K^+)-ATPase activity. (Na^+ + K^+)-ATPase from the human kidney has a sharp temperature optimum around 51°C (Fig. 6) and a pH optimum around 7.0 (Fig. 7). Petterson and Schersten [9] reported a temperature optimum between 40 and 50°C for (Na^+ + K^+)-ATPase in a microsomal fraction prepared from human kidney tissue. The

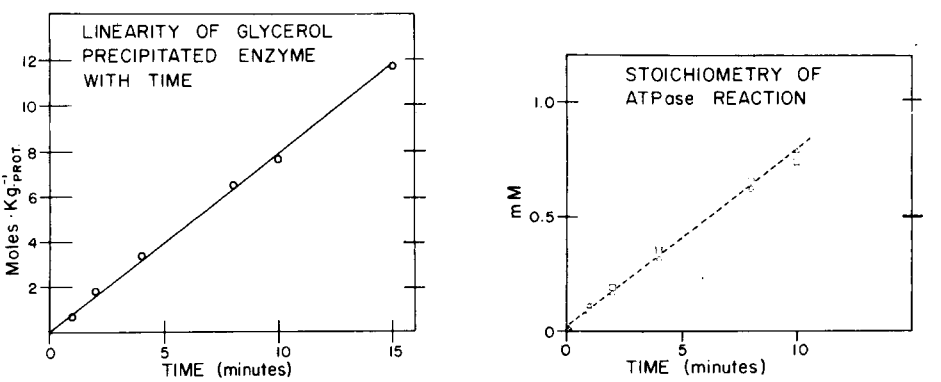
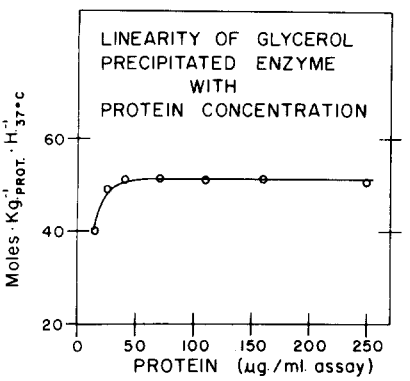


Fig. 3. Linearity of the glycerol-precipitated enzyme activity with time. A preparation of glycerol-precipitated enzyme was assayed at a protein concentration of 0.07 mg/ml. Each point represents the mean of three separate assays of the same preparation.

Fig. 4. Stoichiometry of the (Na^+ + K^+)-ATPase reaction with time. The standard ATPase reaction was run with the glycerol-precipitated enzyme at a protein concentration of 0.1 mg/ml. ATP, ADP, and inorganic phosphate levels were measured in aliquots removed from the assay system as described in Methods: □, ATP consumed; △, ADP produced; and ○, inorganic phosphate produced. Each point represents the mean of three separate determinations.



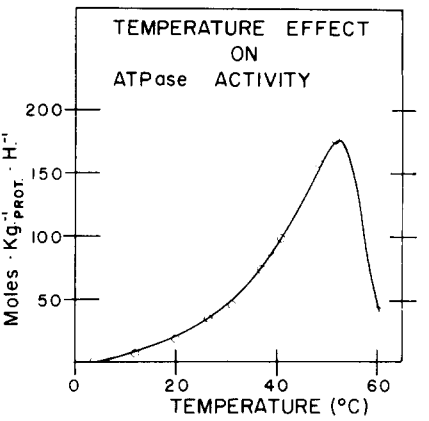


Fig. 5. Linearity of $(Na^+ + K^+)$-ATPase with varying protein concentration. The assay was run as described in Methods.

Fig. 6. Effect of assay temperature on $(Na^+ + K^+)$-ATPase. The enzyme was assayed at a protein concentration of 0.07 mg/ml as described in Methods. The assay temperature of the assay was varied from 4 to 60°C in a water bath.

temperature optimum reported here is significantly higher than that reported by others for similarly purified $(Na^+ + K^+)$-ATPase [5,19]. A pH optimum of 7.0 correlates well with that found for purified $(Na^+ + K^+)$-ATPase from beef brain [19] and the dogfish shark rectal gland [20].

Effect of varying Mg^{2+} and ATP concentrations on $(Na^+ + K^+)$-ATPase activity. The effects of increasing ATP or Mg^{2+} concentrations on $(Na^+ + K^+)$-ATPase activity are shown in Fig. 8. The Michaelis constants for ATP and Mg^{2+} were 0.38 and 0.5 mM, respectively. The V for ATP and Mg^{2+} was at 4 mM. Activity remained constant for ATP up to 10 mM but fell off sharply for Mg^{2+} concentrations above 7 mM. The values are equivalent to those reported for the electric organ of the electric eel [5], dogfish shark rectal gland [20], and beef brain [19] $(Na^+ + K^+)$-ATPase preparations, but are smaller than those reported for crude microsomal preparations of $(Na^+ + K^+)$-ATPase from human kidney [7—9].

Effects of varying Na^+ and K^+ concentrations on $(Na^+ + K^+)$-ATPase activity. The Na^+ and K^+ concentrations required for half-maximal velocity of $(Na^+ + K^+)$-ATPase were 16 and 1.5 mM (Figs. 9 and 10), respectively. These are equivalent to the K_m values for Na^+ and K^+ for purified $(Na^+ + K^+)$-ATPase from the electric organ of the electric eel (determined with 20 mM K^+ or 120 mM Na^+) [5], bovine brain (determined with 20 mM K^+ or 100 mM Na^+) [19], and the rectal gland of the dogfish shark (determined with 20 mM K^+ or 130 mM Na^+) [20]. The K_m for Na^+ found for the purified human enzyme is similar to that (determined with 10 mM K^+) reported for less extensively purified microsomal $(Na^+ + K^+)$-ATPase from human kidney [8]. The K_m for K^+ (determined with 100 mM Na^+) is substantially lower [8]. Petterson and Schersten [9], however, reported a similar K_m for K^+ (determined with 106 mM Na^+) but a significantly higher K_m for Na^+ (determined with 6 mM K^+) than that found in this study.

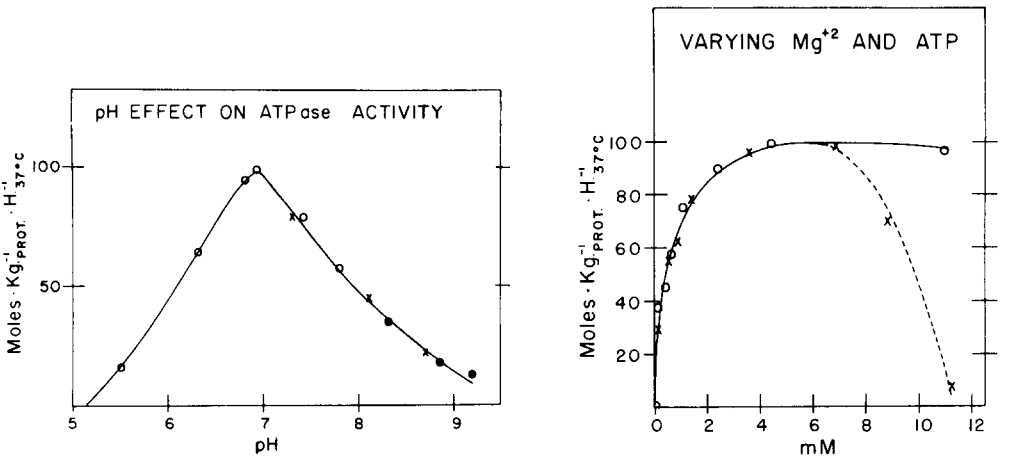


Fig. 7. Effect of pH on $(Na^+ + K^+)$-ATPase. The enzyme was assayed in the standard mixture at a protein concentration of 0.08 mg/ml. The assay buffers were 100 mM imidazole (○), 100 mM Tris (χ), or 100 mM 2-amino-2-methyl-1,3-propanediol [AMP_2] (●).

Fig. 8. Effect of varying Mg^{2+} and ATP concentration on $(Na^+ + K^+)$-ATPase activity. The assay system contained 0.07 mg protein/ml, 100 mM imidazole, pH 6.9, 100 mM NaCl, 20 mM KCl, 0.02% bovine serum albumin, and either 6 mM $MgCl_2$ with varying mM Tris/ATP (○———○), or 6 mM Tris/ATP with varying mM $MgCl_2$ (χ- - - - -χ). The system was not adjusted for small variations in the ionic strength.

Effect of inhibitors on $(Na^+ + K^+)$-ATPase. The glycerol-precipitated enzyme in all preparations was over 99% inhibited by ouabain. Total inhibition of $(Na^+ + K^+)$-ATPase occurred at ouabain concentrations above 0.1 mM (Fig. 11). The concentraton of ouabain required for 50% inhibition (K_i) was 1.8 μM. This is slightly higher than values of 1.0 μM for bovine brain [20], 1.2 μM for the

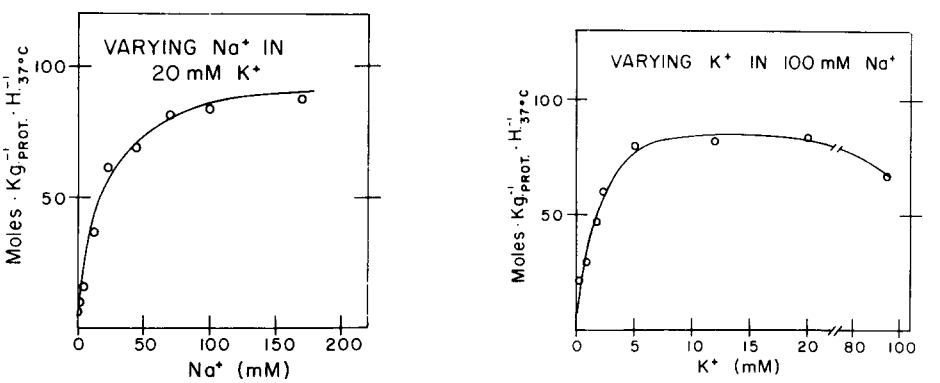


Fig. 9. Effect of varying Na^+ concentration on $(Na^+ + K^+)$-ATPase. The assay system contained 0.08 mg protein/ml, 100 mM imidazole, pH 6.9, 6 mM Tris/ATP, 6 mM $MgCl_2$, 20 mM KCl, 0.02% bovine serum albumin. The Na^+ concentration was varied as shown without adjustment for changes in ionic strength.

Fig. 10. Effect of varying K^+ concentration on $(Na^+ + K^+)$-ATPase. The assay system contained 0.07 mg protein/ml, 100 mM imidazole, pH 6.9, 6 mM Tris/ATP, 6 mM $MgCl_2$, 100 mM NaCl, 0.02% bovine serum albumin. The K^+ concentration was varied as shown without adjustment for changes in ionic strength.

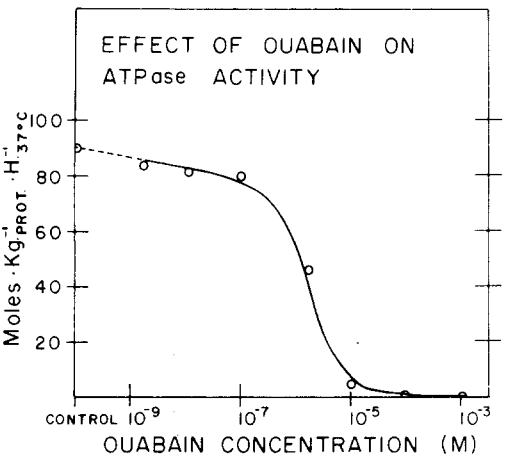


Fig. 11. Effect of ouabain concentration on $(Na^+ + K^+)$-ATPase. The assay system was as described in Methods with 0.07 mg protein/ml. The reaction was started by addition of enzyme to the prewarmed reaction mixture.

electric organ of the electric eel [5], and 0.62 μM for the rectal gland of the dogfish shark. K_i values for ouabain determined with crude microsomal preparations of $(Na^+ + K^+)$-ATPase from human kidney range from 0.1 to 4.2 μM [7–9].

The effects of various inhibitors were tested by adding varying concentrations to the ATPase assay prior to the addition of enzyme. K_i values were determined from plots of enzyme activity versus inhibitor concentration. The mercurial, mercaptomerin, at a concentration of 14.7 mM caused a maximal inhibition of 83% with a K_i of 0.2 mM. Chlorothiazide (19.2 mM), caused a 100% inhibition of the enzyme with a K_i of 7 mM. Ethacrynic acid at 4 mM, caused an 83% inhibition with a K_i of 2.2 mM. Furosemide (4 mM) only resulted in a 36% inhibition of enzyme activity. Ca^{2+} added as the chloride salt to the assay caused a greater than 90% inhibition of $(Na^+ + K^+)$-ATPase with a K_i of 0.7 mM. Prostaglandin E_2 in a concentration of 63.5 μg/ml (0.18 mM) caused no depression of activity.

TABLE II

NUCLEOTIDE SPECIFICITY OF HUMAN $(Na^+ + K^+)$-ATPase

The glycerol-precipitated enzyme was assayed at a protein concentration of 0.07 mg/ml. The assay system contained 100 mM imidazole, pH 6.9, 6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 0.02% bovine serum albumin. The various nucleotides were added in a concentration of 6 mM as: Tris/ATP, sodium CTP, Tris/GTP, Tris/UTP, sodium ADP, sodium AMP.

Substrate (6 mM)	mol · $kg^{-1}_{protein}$ · $h^{-1}_{37°C}$
ATP	86
CTP	25
GTP	5
UTP	0
ADP	0
AMP	0

Nucleotide specificity of (Na^+ + K^+)-ATPase. Phosphate liberated in the presence of nucleotides other than ATP was minimal (Table II). With 6 mM CTP or GTP as substrates, (Na^+ + K^+)-ATPase activity of the glycerol-precipitated enzyme was 29 and 2.8%, respectively, of that with 6 mM ATP as substrate. UTP, ADP, and AMP gave no activity. The findings with ADP and AMP confirm the stoichiometry of the (Na^+ + K^+)-ATPase reaction (Fig. 4).

Discussion

The results presented here concern the purification of (Na^+ + K^+)-ATPase from human renal tissue. This is the first report on the extensive purification of the enzyme from a human source. A major reason for carrying out this study was to eliminate the non-linearity of ATP hydrolysis with reaction time. The purified (Na^+ + K^+)-ATPase reaction was linear with assay time. This suggests that there are factors which control ATPase activity and can be eliminated by purification of the (Na^+ + K^+)-ATPase.

The average specific activity of the glycerol-precipitated enzyme in six separate preparations was 132 ± 63 mol · $kg^{-1}_{protein}$ · h^{-1} (mean ± S.D.). This represents an 18—71-fold purification based on the starting specific activity of the corresponding microsomes (4.2 ± 2 mol · $kg^{-1}_{protein}$ · h^{-1}). Specific activities of 800—2160 have been reported for the purified enzyme from the renal medulla of the dog [1,2] and rabbit [3]. These activities represent a 16—26-fold purification over the specific activity in the microsomes from these sources. Therefore, although the specific activity of the purified human enzyme is about one-sixth that reported for renal (Na^+ + K^+)-ATPase from other species [1—3], the fold purification is as good or better than that reported by others.

Sodium dodecyl sulfate-polyacrylamide electrophoresis yields three major polypeptide fragments of 117 000, 92 500, and 56 000 daltons. The two smaller polypeptides have masses equivalent to the two subunits of (Na^+ + K^+)-ATPase from other systems [1—6]. The finding of a heavily staining large polypeptide fragment corresponding to 117 000 daltons on sodium dodecyl sulfate-polyacrylamide gels run at pH 7.0 is similar to a polypeptide of 114 000 daltons found on SDS gels run at pH 9.0 by Kyte [1]. Kyte, however, did not find this fragment on gels electrophoresed at pH 7.0. The possibility that the 117 000 dalton polypeptide associated with the human enzyme is an artifact of sodium dodecyl sulfate-polyacrylamide gels run at pH 7.0 cannot be ruled out. The chances that it was due to post-mortem autolysis were not ruled out, but it has been noted in preparations from six kidneys with varying anoxic times from 5 min to 7 h. Giotta [21] has reported that the large polypeptide of (Na^+ + K^+)-ATPase can be cross-linked to form dimers, trimers and tetramers. In the present study only a dimer of the 56 000 dalton peptide could produce a band in the region of the 117 000 daltons. This is unlikely however, since the 117 000 dalton peptide did not stain for glycoprotein whereas the 56 000 dalton peptide did. The possibility that the absence of this large polypeptide band on sodium dodecyl sulfate-polyacrylamide gels of other purified preparations of (Na^+ + K^+)-ATPase [1—6,22,23] is an artifact cannot be ruled out either. To date, reconstitution of the subunits of (Na^+ + K^+)-ATPase into an active complex has not been obtained and it is not known precisely which poly-

peptide bands on an sodium dodecyl sulfate-polyacrylamide gel are required for activity. The only tentative conclusion which can be made is that the 84 000—95 000 dalton polypeptide, which can be phosphorylated, is probably the catalytic subunit of $(Na^+ + K^+)$-ATPase.

The human enzyme appears to have similar kinetic parameters to purified $(Na^+ + K^+)$-ATPase from other sources [1—6] except in two areas. The $(Na^+ + K^+)$-ATPase from human renal tissue has a temperature optimum 10°C higher than that reported for other preparations, from the electric organ of the electric eel [5] and the rectal gland of the dogfish shark [20]. In addition, the human enzyme is particularly sensitive to the Mg^{2+} concentration in the assay (Fig. 8). In the presence of 6 mM ATP, there is a sharp fall in $(Na^+ + K^+)$-ATPase activity at Mg^{2+} concentrations above 7 mM. Concentrations of Mg^{2+} as high as 15 mM do not inhibit pure $(Na^+ + K^+)$-ATPase from the electric organ of *Electrophorus electricus* [5].

Tulloch et al. [8] and Post et al. [11] reported that the crude human enzyme fron renal tissue and erythrocytes, respectively, can hydrolyze ADP at a rate of 16 and 50% of the rate with ATP as substrate. The enzyme purified in this study could not use ADP as a substrate.

The non-linear reaction rate of less purified microsomal $(Na^+ + K^+)$-ATPase has been reported by us [30,31] for the rat kidney enzyme and by others for microsomal $(Na^+ + K^+)$-ATPase from human myocardium [10] and human renal tissue [8,9]. The significance of this non-linearity becomes apparent when kinetic parameters determined on a crude enzyme preparation are compared with those obtained with a more purified enzyme. The K_m values for ATP, Mg^{2+}, Na^+ and K^+ and the K_i for ouabain reported by Nechay et al. [7], Tulloch et al. [8], and Pettersson and Schersten [9] for less pure microsomal preparations of the enzyme from human kidney differ dramatically from those reported in the present study. Even a cross comparison of their findings reveals striking differences in kinetic parameters between the three studies. A probable cause for these discrepancies is the differences in assay times at which the enzyme activity was determined. Each based their results on a single time point determination of activity. Assay times of 10 min [7], 20 min [8], and 30 min [9] would allow for appreciable changes in activity to occur as the result of non-linearity in the crude preparations.

The idea that other proteins or enzyme systems can modify $(Na^+ + K^+)$-ATPase activity has been proposed by others [24—28]. A possible relationship between cyclic AMP-dependent endogenous protein kinase and $(Na^+ + K^+)$-ATPase has been suggested. Endogenous protein kinase activity was associated with crude preparations of beef brain and dog kidney, but not with the highly purified enzyme [26]. This laboratory has recently reported preliminary evidence that cyclic AMP-dependent protein kinase in purified human kidney $(Na^+ + K^+)$-ATPase is nearly absent [29]. The relationship of kinase and other factors to the linearity of the reaction remains to be elucidated and are under investigation in this laboratory.

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