Purification of the Sodium- and Potassium-dependent Adenosine Triphosphatase from Canine Renal Medulla*

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SUMMARY

The (Na\(^+\) + K\(^+\)) adenosine triphosphatase was obtained from canine renal medulla and partially purified. A highly active, membrane-bound enzyme was prepared from the microsomal fraction of a homogenate. The enzyme was then transferred from this particulate state to the supernatant phase by treatment with sodium deoxycholate and, in this form, it was further purified by gel filtration. The final preparation had a high specific activity which was constant over a significant region of the elution profile. When this material was examined by electrophoresis in a sodium dodecyl sulfate solvent, more than 90% of the protein was accounted for by two polypeptide chains which were present in approximately equimolar amounts. The ratio of these two polypeptides remained constant in those fractions from the gel filtration which had the highest specific activity. The protein traveled as a single component in an acetic acid-urea electrophoresis system. These results suggest that a specific complex responsible for the (Na\(^+\) + K\(^+\)) adenosine triphosphatase activity has been isolated.

Several attempts to purify these supernatant enzymes have achieved only limited success (10-13). Recently, Towle and Copenhaver have described a preparation of the (Na\(^+\) + K\(^+\)) adenosine triphosphatase, in a supernatant form, whose specific activity is almost equivalent to those of the high activity particulate preparations (14).

A procedure for the preparation of a high specific activity, membrane-bound, (Na\(^+\) + K\(^+\)) adenosine triphosphatase from canine renal medulla is presented. The enzyme was subsequently transferred from the particulate to the supernatant phase with sodium deoxycholate in a high ionic strength solvent (15) and further purified by gel filtration in agarose under mild detergent conditions. The specific activity of the final preparation was greater than that of any supernatant (Na\(^+\) + K\(^+\)) adenosine triphosphatase yet reported and was at least as great as that of the high activity particulate preparations. When the purest preparation obtained was examined with polyacrylamide gel electrophoresis in a sodium dodecyl sulfate solvent system, it contained only two polypeptide chains, present in approximately equimolar amounts.

EXPERIMENTAL PROCEDURES

Materials

Fresh canine tissue was generously provided by the Cardiovascular Research Department at Massachusetts General Hospital. L-Histidine, sodium adenosine 5′-triphosphate, Tris adenosine 5′-triphosphate, strophanthinid, sodium deoxycholate, the acid form of ethylenediaminetetraacetic acid, and Tris base were obtained from Sigma. Sodium dodecyl sulfate was obtained from Fisher and recrystallized from 95% ethanol. Phosphatidy1-L-serine was obtained from Mann. Sepharose 2B, Blue Dextran 2000, and Sephadex G-100 were obtained from Pharmacia. Acrylamide and N,N′-methylene bisacrylamide, both of electrophoretic grade, were purchased from Eastman Organic Chemicals. Imidazole was purchased from Eastman, as well, and recrystallized from acetone. Bio-Gel A-5m was purchased from Calbiochem. All other chemicals used were reagent grade. All buffers were prepared by adding the un-ionized acid or base and adjusting the pH of the complete solution with either HCl or NaOH. Standards for gel electrophoresis and the Lowry assay, listed with their polypeptide molecular weights, were: bovine carbonic anhydrase (Mann), 29,000 (16); RNA-polymerase (Klaus Weber, Harvard University), α chain, 40,000 (17); pig heart fumarase (Calbiochem), 49,000 (16); yeast alcohol dehydrogenase (Worthington), 37,000 (16); bovine A-5m was purchased from Calbiochem. All other chemicals used were reagent grade. All buffers were prepared by adding the un-ionized acid or base and adjusting the pH of the complete solution with either HCl or NaOH. Standards for gel electrophoresis and the Lowry assay, listed with their polypeptide molecular weights, were: bovine carbonic anhydrase (Mann), 29,000 (16); RNA-polymerase (Klaus Weber, Harvard University), α chain, 40,000 (17); pig heart fumarase (Calbiochem), 49,000 (16); yeast alcohol dehydrogenase (Worthington), 37,000 (16); bovine

A sodium- and potassium-dependent adenosine triphosphatase, located in the plasma membranes of many eucaryotic cells, has been extensively studied since Skou identified its activity in crab nerve microsomes (1). Much evidence has been gathered to suggest that this catalytic activity is really the expression of that membrane-bound protein complex responsible for the coupled active transport of sodium and potassium (2-6). Several purified preparations of the enzyme, both particulate and supernatant, have been described. There are many published procedures for obtaining high specific activity (Na\(^+\) + K\(^+\)) adenosine triphosphatase in membrane-bound form (7-9). Nevertheless, there are absolute limits on the purity of a membrane bound protein because unrelated polypeptides remain in aggregation on the solid matrix of the particles. Although supernatant preparations of the enzyme can be obtained through the use of detergent, they lack the stability of the particulate preparations.

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Enzyme Assay

The assay was adapted from that used by Nakao et al. (18). Incubation mixtures routinely contained 30 mm l-histidine, pH 6.8; 100 mm NaCl; 20 mm KCl; 6 mm MgCl₂; 10 mm 2-mercaptoethanol; 3 mm Na₃ATP; 0.06 mg per ml of phosphatidyl-L-serine; and an appropriate amount of enzyme in a final volume of 1.00 ml. The incubation medium was brought to 37 °C for 15 min. The reaction was usually initiated by adding enzyme in 25- or 50-μl samples. After 15 min the reaction was quenched by the addition of 1.50 ml of 0.417% ammonium molybdate, 0.833 N H₂SO₄. Phosphate was then determined by the procedure of Leloir and Cardini (19). An incubation mixture from which enzyme had been omitted was used as a blank to correct for time-dependent evolution of phosphate in the strong acid.

In order to correct for nonspecific, Mg²⁺-stimulated adenosine triphosphatase, samples were assayed in the presence of 0.1 mm strophantidin by adding 10 μl of a 10 mm solution in N,N-dimethylformamide to selected tubes.

It has been reported that phosphatidyl-L-serine enhances enzyme activity (14, 20). This was the case with canine renal microsomes, which had been previously run on sodium dodecyl sulfate. The enzyme activity was increased by 50% in the presence of phosphatidyl-L-serine, and an appropriate amount of enzyme in a final volume of 1.00 ml. The incubation medium was brought to 37 °C for 15 min. The reaction was usually initiated by adding enzyme in 25- or 50-μl samples. After 15 min the reaction was quenched by the addition of 1.50 ml of 0.417% ammonium molybdate, 0.833 N H₂SO₄. Phosphate was then determined by the procedure of Leloir and Cardini (19). An incubation mixture from which enzyme had been omitted was used as a blank to correct for time-dependent evolution of phosphate in the strong acid.

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It has been reported that phosphatidyl-L-serine enhances enzyme activity (14, 20). This was the case with canine renal medulla preparations as well, the requirement for this supplement increasing as the enzyme was purified (Table I). The phospholipid was included routinely in the assay at a concentration yielding maximum stimulation. Preliminary incubation of enzyme with phosphatidyl-L-serine did not appreciably increase its effect.

Protein Determinations

Protein concentrations were routinely determined by the method of Lowry as discussed by Bailey (21), precipitating the protein with trichloroacetic acid to avoid interference and correcting for protein soluble in the trichloroacetic acid. When protein concentrations were too small to be accurately measured by this procedure, amino acid analysis was used, including norleucine as an internal standard.

Polyacrylamide Gel Electrophoresis

Three types of electrophoretic systems were used. Polyacrylamide gels in 0.1% sodium dodecyl sulfate (22) were prepared as described by Weber and Osborn (16). Only gels which contained one-half the normal amount of cross-linker (0.11% methylene bisacrylamide, final concentration) were used. This sodium dodecyl sulfate method was also used at high pH, buffering both the polymerization reaction and the reservoirs at pH 9.0 with 0.1 m sodium borate. Although a high pH gel method has been reported by Laemmli (23) his technique differs significantly from the borate system described here. Samples were prepared in both cases by adding, in order, buffer, 2-mercaptoethanol, and sodium dodecyl sulfate to a final concentration of 0.1 m, 5% and 3.5%, respectively, and then brought immediately to 100°C in a boiling water bath to prevent proteolytic degradation during denaturation (24). The gels shift pH 7.0 were run at 40 volts; those at pH 9.0, 60 volts. The pH 9.0 gels routinely contained a discontinuity about 5 mm from the top of the gel where turbid material collected during the electrophoresis. It was assumed that sieving did not occur above this point and it was usually taken as the top of the gel in calculating mobilities.

In order to examine quantitatively the separated polypeptide chains from sodium dodecyl sulfate gels, stained protein bands were hydrolyzed directly for amino acid analysis. The desired region of the gel was excised with a razor blade and added to a hydrolysis tube containing 2 to 3 ml of 6 N HCl, 0.1% mercaptoethanol, and the tube was thoroughly evacuated, sealed, and incubated at 110°C for 24 hours. The hydrolysate was then refrigerated overnight and precipitated acrylate removed by centrifugation. The sample was evaporated, taken up in citrate buffer (pH 2.2), and directly analyzed for acidic and neutral amino acids with a Beckman-Spinco model 120C amino acid analyzer. Bovine serum albumin, hemoglobin, and phosphorylase a, which had been previously run on sodium dodecyl sulfate polyacrylamide gels and stained, were used as standards. The amino acid compositions obtained agreed with those published for these proteins except in the case of glycine and serine, whose values were too high. When vacant polyacrylamide gel was hydrolyzed, significant amounts of glycine and serine were present. Therefore, only the following amino acids were measured: aspartic acid, threonine, glutamic acid, proline, alanine, valine, leucine, isoleucine, tyrosine, and phenylalanine. Norleucine was included as an internal standard in all hydrolysis tubes and its recovery was always greater than 90%.

Protein was eluted from sodium dodecyl sulfate gels (pH 9.0) in the following manner. The regions containing each polypeptide were excised with a razor blade from unstained gels and were then hydrolyzed as described above. The amino acid compositions obtained agreed with those published for these proteins except in the case of glycine and serine, whose values were too high. When vacant polyacrylamide gel was hydrolyzed, significant amounts of glycine and serine were present. Therefore, only the following amino acids were measured: aspartic acid, threonine, glutamic acid, proline, alanine, valine, leucine, isoleucine, tyrosine, and phenylalanine. Norleucine was included as an internal standard in all hydrolysis tubes and its recovery was always greater than 90%.

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Purification of (Na$^+$ + K$^+$) Adenosine Triphosphatase

**Step 1: Microsomes**—This and the following step are a modification of the method of Jørgensen and Skou (7). All procedures were carried out at 4°C unless otherwise noted. The kidneys were removed from 10 mongrel dogs immediately following death by exsanguination, and the red inner medulla was carefully isolated and minced with scalpels. This yielded 150 g of tissue. The material was forced through 0.5 mm × 4 cm slits in a 3 cm deep steel block (27). The paste obtained was suspended in 1.5 liters of 30 mM histidine, pH 7.1-0.25 M sucrose-1 mM EDTA and homogenized in a Teflon pestle homogenizer. The homogenate was spun at 7,500 rpm in a Sorvall SS-34 rotor (7,000 × g) for 15 min, and the supernatant saved. The pellet was homogenized again in one-half the original volume of buffer and sedimented. The two supernatants were combined and spun at 24,000 rpm in a Spinco L-30 rotor (15,000 × g) for 30 min. The supernatants were poured off and discarded. The pellet contained two layers, an upper white or pink layer and a lower tan, clay-like layer. Only the upper layer was resuspended in fresh buffer by homogenization. This suspension was resedimented at 50,000 × g and the same sorting procedure repeated. The resuspended material was chalky white, and it was frozen in acetone-Dry Ice.

**Step 2: Deoxycholate Treatment**—The microsomes were added to a solution which had been prepared so that the final concentrations would be 25 mM imidazole, pH 7.0; 0.6 mg per ml of sodium deoxycholate; 2 mM EDTA; 0.25 mg per ml of protein. This mixture stood at room temperature for 30 min. It was then spun at 7,500 rpm in a Sorvall SS-34 rotor (7,000 × g) for 70 min at 2°C. The supernatant was decanted and spun at 16,250 rpm in a Spinco L-14 rotor (78,000 × g) for 45 min at 2°C. The pellets were washed once in 25 mM imidazole, pH 7.0-2 mM EDTA-10 mM 2-mercaptoethanol, and finally resuspended in 25 mM histidine, pH 6.8-10 mM 2-mercaptoethanol-2 mM EDTA-0.25 M sucrose. The final volume was 5.3 ml. This was divided into 1-ml aliquots and frozen in acetone-Dry Ice.

**Step 3: Potassium Iodide Treatment**—The 25,000 × g pellet was resuspended in 25 mM imidazole, pH 7.0-2 mM EDTA-10 mM 2-mercaptoethanol. The 5,600 × g pellet was resuspended in the small amount of supernatant fluid which was left in each centrifuge tube after the bulk of the supernatant had been collected (final volume ~ 250 ml) and protein concentration was determined roughly by optical density at 280 nm. This suspension was then treated precisely as the initial microsome suspension. The 25,000 × g pellet suspensions from these two cycles were combined.

**Step 4: Deoxycholate-Salt Wash**—The potassium iodide-extracted material was added to a solution which had been prepared so that the final concentrations would be 25 mM Tris, pH 7.5; 2 mM EDTA; 10 mM 2-mercaptoethanol; 160 mM KCl; 33 mM NaCl; 0.6 mg per ml of sodium deoxycholate; 0.25 mg per ml of protein. This mixture stood at room temperature for 30 min. The suspension was then sedimented at 30,000 rpm in a Spinco L-30 rotor (78,000 × g) for 45 min at 2°C. The pellets were washed once in 25 mM imidazole, pH 7.0-2 mM EDTA-10 mM 2-mercaptoethanol, and finally resuspended in 25 mM histidine, pH 6.8-10 mM 2-mercaptoethanol-2 mM EDTA-0.25 M sucrose. The final volume was 5.3 ml. This was divided into 1-ml aliquots and frozen in acetone-Dry Ice.

**Step 5: Supernatant Enzyme**—For the following steps, aliquots of 1.15 ml of the detergent-salt-extracted material were used in order to cater to the dimensions of the final column. The resuspended protein was added to a solution, on ice, which had been prepared so that the final concentrations would be 25 mM Tris, pH 7.5; 2 mM EDTA; 10 mM 2-mercaptoethanol, 267 mM KCl; 40 mM NaCl; 6 mg per ml of sodium deoxycholate; 2.5 mg per ml of protein. Glycerol was added to make an 18% solution and the mixture was immediately spun at 38,000 rpm in a Spinco L-40 rotor (94,000 × g) for 30 min at 2°C. The supernatant was carefully collected, passed through a 0.45 μm Millipore filter, and diluted with an equal volume of 30 mM imidazole, pH 6.8-40 mM NaCl-1 mM EDTA-10 mM 2-mercaptoethanol. This material was layered over a 50% glycerol cushion and spun at 33,000 rpm in a Spinco L-SW50 rotor (100,000 × g) for 45 min at 2°C. The tubes were punctured at the bottom and the contents divided into fractions. All fractions containing the turbid material which had gathered at the interface were discarded. The transparent upper fractions were pooled and utilized immediately.

**Step 6: Sepharose 2B Column**—The supernatant enzyme was added directly to a column (43 × 2.3 cm) of Sepharose 2B which had been equilibrated in the cold with 25 mM Tris, pH 7.5-1 mM EDTA-10 mM 2-mercaptoethanol-0.4 M KCl-40 mM NaCl-0.5 mg per ml of sodium deoxycholate-20% glycerol. The column was run at 19 ml per hour and fractions were collected over 15-min intervals. The effluent was assayed for enzyme activity within 16 hours of sample addition. Protein was determined by the Lowry procedure, and organic phosphate was measured as described by Ames and Dubin (29). In order to correct for inorganic phosphate, samples were assayed without ashing. The results are presented in Fig. 1.

The final stage of the purification appears in Table I. Also listed is the specific activity of the Mg$^{2+}$ adenosine triphos-
sodium deoxycholate on the membrane-bound proteins was utilized to remove proteins from the particulate phase (Step 4). The strong ionic strength dependence of the effect of sodium deoxycholate (Fig. 2). The enzyme goes into the supernatant phase in a well defined range of ionic strength. This behavior can be used to advantage during purification as undesirable proteins can be extracted from the membrane at 0.29 M ionic strength, in the experiment represented, and can be left behind in the pellet at 0.45 M ionic strength. A titration curve of this type, however, must be determined for each purification since the inflection points are a function of the protein preparation and the source of the sodium deoxycholate. The differences between the ionic strengths in Fig. 2 and those used in the outlined enzyme preparation will attest to this fact. Although only potassium chloride was used to vary ionic strength, though only potassium chloride was used to vary ionic strength, though only potassium chloride was used to vary ionic strength, though only potassium chloride was used to vary ionic strength.

**Yield of Enzyme Activity**—At each step in the purification the material which was to be discarded was assayed for activity. When the total activity in all fractions from the first deoxycholate treatment was determined, there were 98,800 units present. This represented a 4.1-fold increase during the first deoxycholate treatment (7). The supernatant of the detergent-salt treatment contained 6,380 units of which about 30 to 40% could have been sedimented at higher forces than those used. This would have increased the yield at this step but was impractical because of the large volumes involved. The total activity observed in the pellet and the supernatant after the first 94,000 x g centrifugation following detergent treatment (Step 5) varied but was usually 35 to 40% of the total activity added to the tube. The pellet generally accounted for only about 5 to 10% of the recovered activity. When supernatant enzyme (Step 5) was passed through the Sepharose 2B column, 70% of the added activity was recovered (Fig. 1).

**Stability of Enzyme**—At all stages in the purification procedure up to the solubilization with sodium deoxycholate, the enzyme remained active for at least 2 weeks at 4° in 0.25 M sucrose and could be stored for months in the frozen state without loss of activity. It was necessary to include the sucrose in all preparations which were frozen in order to retain complete activity.

The (Na+ + K+) adenosine triphosphatase which had been rendered soluble by treatment with detergent could be dialyzed against 30 mM histidine, pH 7.1-0.25 M sucrose-1.0 mM EDTA-10 mM 2-mercaptoethanol for several days. The activity actually increased slightly during this treatment and was then stable for at least a week. The enzyme could be frozen and stored in this state. The final material from the Sepharose column could be concentrated at least 5 fold by vacuum dialysis against column buffer with a 20% loss of activity. It was necessary to add glycerol to a concentration of 20% to all preparations of soluble enzyme or the activity was lost completely over several hours (14). Glycerol added to the assay mixture was inhibitory. The soluble enzyme seemed stable in the assay mixture at the low concentrations used since the liberation of phosphate remained a linear function of time.

**Deoxycholate-Salt Interactions**—The ionic strength of the medium has a marked effect on the ability of sodium deoxycholate to interact with the membrane (15). The distribution of (Na+ + K+) adenosine triphosphatase between supernatant and pellet can be followed as a function of ionic strength in the presence of sodium deoxycholate (Fig. 2). The enzyme goes into the supernatant phase in a well defined range of ionic strength. This behavior can be used to advantage during purification as undesirable proteins can be extracted from the membrane at 0.29 M ionic strength, in the experiment represented, and can be left behind in the pellet at 0.45 M ionic strength. A titration curve of this type, however, must be determined for each purification since the inflection points are a function of the protein preparation added and the source of the sodium deoxycholate. The differences between the ionic strengths in Fig. 2 and those used in the outlined enzyme preparation will attest to this fact. Although only potassium chloride was used to vary ionic strength, it has been reported that other salts are as effective (15).

**Protein to Detergent Ratio**—It has been shown that there is an optimal ratio of detergent to protein at which the maximum activity of a membrane enzyme can be transferred to a high speed supernatant (12, 13, 15, 30). The same observation was made in these studies. As shown in Fig. 3, there is a definite ratio of sodium deoxycholate to protein at which the maximum yield of activity remaining in the 100,000 x g supernatant is achieved. If either sodium deoxycholate or protein is held at constant concentration and the other concentration is varied, the same detergent to protein ratio is most effective. Although the specific

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**Fig. 2. Effect of ionic strength on the interaction of deoxycholate with the enzyme.** Potassium iodide-extracted microsomes (0.097 mg) were added to solutions which had been prepared so that final concentrations would be 30 mm imidazole, pH 7.0; 2 mM EDTA; 35 mM NaCl; 10 mM 2-mercaptoethanol; and 0.6 mg per ml of sodium deoxycholate, and the final volume would be 0.150 ml. The ionic strength was varied by adding different amounts of 1.60 M KCl to the initial solution. The final mixtures stood at room temperature for 30 min and were then spun at 98,000 rpm in a SpincO L-40 rotor (94,000 x g) for 30 min at 2°. Supernatants were decanted and assayed directly for total activity (O-O). Pellets were reuspended in 0.50 ml of 30 mm imidazole, pH 6.8; 2 mM EDTA; 10 mM 2-mercaptoethanol; 0.35 M sucrose, and assayed for total activity after about 3 hours (n-n).

**RESULTS**

**Purification of (Na+ + K+) Adenosine Triphosphatase**

The purification of the (Na+ + K+) adenosine triphosphatase was approached with the following strategy. The particulate nature of the starting material permitted the sorting of the microsomal fragments on the basis of their size (Steps 1 and 2). It also permitted the extraction of contaminating proteins with potassium iodide and sodium deoxycholate while the enzyme itself remained bound to the solid matrix of the membrane (Steps 2 to 4). The strong ionic strength dependence of the effect of sodium deoxycholate on the membrane-bound proteins was utilized to remove proteins from the particulate phase (Step 4).

The enzyme could then be separated, under controlled conditions of ionic strength and sodium deoxycholate concentration (Step 5), from material that sedimented at 94,000 x g over 30 min. This supernatant enzyme was finally chromatographed on agarose.

The distribution of organic phosphate on the Sepharose 2B column (Fig. 1) was also measured. It traveled mainly in the inclusion volume of the column. The concentration of organic phosphate in Fractions 16 to 27 was too low to be measured accurately. However, preliminary measurements suggest that there are between 0.5 and 0.7 pmole of organic phosphorus per mg of protein in this region. Therefore, it is quite possible that the high apparent molecular weight of this material may be caused by a vesicular structure.

**Yield of Enzyme Activity**—At each step in the purification the material which was to be discarded was assayed for activity. When the total activity in all fractions from the first deoxycholate treatment was determined, there were 98,800 units present. This represented a 4.1-fold increase during the first deoxycholate treatment (7). The supernatant of the detergent-salt treatment contained 6,380 units of which about 30 to 40% could have been sedimented at higher forces than those used. This would have increased the yield at this step but was impractical because of the large volumes involved. The total activity observed in the pellet and the supernatant after the first 94,000 x g centrifugation following detergent treatment (Step 5) varied but was usually 35 to 40% of the total activity added to the tube. The pellet generally accounted for only about 5 to 10% of the recovered activity. When supernatant enzyme (Step 5) was passed through the Sepharose 2B column, 70% of the added activity was recovered (Fig. 1).
activities of the membrane preparations used in the two experiments illustrated (Fig. 3) differed by a factor of 4, the same fraction of the added activity was transferred to the high speed supernatant in each case. The preparation which had an initial specific activity of 475 pmoles of Pi mg⁻¹ hour⁻¹ (Fig. 3A) was much less sensitive to detergent concentration than the preparation that had a specific activity of 115 pmoles of Pi mg⁻¹ hour⁻¹. In the former case, the protein which was extracted was a mixture of only two polypeptide chains and was therefore relatively homogeneous. MacLennan has used the differential sensitivity of membrane-bound proteins to deoxycholate in order to purify the Ca²⁺ adenosine triphosphatase from sarcoplasmic reticulum (31). The ratio of detergent to protein routinely used was 2.4 as it was generally safer to add a slight excess of protein. The yields in the two experiments shown (Fig. 3) were slightly lower than those observed when glycerol was employed. As long as protein and detergent were varied at constant ratio, it was possible to obtain (Na⁺ + K⁺) adenosine triphosphatase, which would not sediment at 100,000 × g, in a solution of any desired concentration. The specific activities of a 0.10 mg per ml of preparation and a 1.0 mg per ml of preparation, as long as they were prepared from the same membranes, were indistinguishable.

**Aggregation State of Enzyme**—The (Na⁺ + K⁺) adenosine triphosphatase which was eluted from the Sepharose 2B column and which had the greatest specific activity was apparently of high molecular weight. It traveled with a smaller distribution coefficient than Blue Dextran 2000. When the concentration of sodium deoxycholate in the column buffer was varied, the mean distribution coefficient of the protein added to the column changed (Fig. 4). In the two experiments illustrated (in this case agarose 5m was used to indicate this particular effect more clearly), identical enzyme preparations were added to the same column and eluted at the same rate. The only difference between the two experiments was the column detergent concentration. In the column containing 1.0 mg per ml (Fig. 4B) of sodium deoxycholate the protein added had a distribution coefficient which indicates that it is in a much lower molecular weight form than the protein eluted from the column containing 0.5 mg per ml of sodium deoxycholate (Fig. 4A). However, the (Na⁺ + K⁺) adenosine triphosphatase activity was present mainly in the void volume in each case. The overall yield of activity from the former column was only 25% of that from the latter column which was nearly quantitative and the specific activity of the material in the void volume decreased 2-fold. Enzyme which had eluted in the middle region of a Sepharose 2B column and which corresponded to the purest fraction (Fig. 1) could be pooled and concentrated 5-fold by vacuum dialysis against column buffer. When this preparation was added back to the same Sepharose 2B column and run under identical circumstances, all of the (Na⁺ + K⁺) adenosine triphosphatase activity and all of the protein traveled in the void volume.

**Polyacrylamide Gel Electrophoresis**—When protein from any of the Fractions 10 to 27 (Fig. 1) was examined by electrophoresis...
in sodium dodecyl sulfate at pH 9.0, three polypeptide components accounted for more than 90% of the protein. A representative gel is presented in Fig. 5B. Similarly denatured proteins of known molecular weight were run in corresponding gels, and the mobilities of all polypeptides were determined. A standard curve was constructed from these results (Fig. 6) (16, 22). The masses obtained from this curve for the three major polypeptides present in the (Na⁺ + K⁺) adenosine triphosphatase preparation are 114,000, 84,000, and 57,000 daltons, respectively (Fig. 6).

Samples of each polypeptide chain, obtained by slicing entire stained bands directly out of the gel, were analyzed for amino acids. The total mass of each polypeptide chain on each gel was approximated by summing the masses of all of the measured amino acids present in each band. From the masses obtained and the approximate molecular weight, an approximate molar ratio of the polypeptides could be calculated. The average molar ratio from two gels, which contained the entire contents of Fractions 21 and 26, respectively (Fig. 1), was 0.19 mole of 114,000-dalton polypeptide to 1.00 mole of 84,000-dalton polypeptide to 0.91 mole of 57,000-dalton polypeptide.

The material which has a mobility corresponding to that of a peptide of 114,000 daltons on sodium dodecyl sulfate gels run at pH 9.0 is an artifact of the electrophoretic system. It is only observed on sodium dodecyl sulfate gels run at pH 9.0, and never on those run at pH 7.0 (Fig. 5C). It is observed when other preparations of membrane proteins are submitted to electrophoresis at this pH (Fig. 5A). When either the 84,000- or the 57,000-dalton polypeptide was electrophoretically purified and then rerun in the pH 9.0 system the artifact was again present (Fig. 7). To perform this experiment a sample of the enzyme from Step 5 of the purification was used. A series of six identical gels was run. One was stained and from the other five, pieces I and II were cut. Corresponding segments were combined and the protein eluted as described under "Methods." Protein from Region I and II was resubmitted to electrophoresis and the results are presented in Fig. 7, B and C, respectively, along with the mobilities of each polypeptide. As shown, when protein, which had been eluted from a region of the gel containing only 57,000-dalton polypeptide, was rerun, appreciable material of a mobility identical with the 114,000-dalton polypeptide appeared (Fig. 7C). However, when higher molecular weight material was rerun, although the 114,000- and 84,000-dalton polypeptides were present (Fig. 7B), little or no 57,000-dalton polypeptide was observed. Very little material of a mobility corresponding to the 57,000-dalton polypeptide was present in the gel on which the 57,000-dalton material had been rerun (Fig. 7C). This indicated that the cutting had been accurate. However, as material of this mass was not completely absent, the possibility that 57,000- and 84,000-dalton materials are interconvertible has not been ruled out.

Sodium dodecyl sulfate gels, run at pH 9.0, of samples from various regions of the Sepharose 2B column (Fig. 1) were scanned on a recording spectrophotometer. The areas of the optical density peaks corresponding to each band on the stained gel were calculated. The ratios between the area of the 84,000-dalton band and that of the 57,000-dalton band from five separate polyacrylamide gels of selected fractions from the column are presented in Table II. As shown, the ratio between the total optical density peak for the 84,000-dalton band and that for the 57,000-dalton band varied from 0.5 to 1.0.

FIG. 5. Polyacrylamide gel electrophoresis of (Na⁺ + K⁺) adenosine triphosphatase. A, sodium dodecyl sulfate polyacrylamide gel, run at pH 9.0, of microsomal material (Step 1). B, sodium dodecyl sulfate polyacrylamide gel, run at pH 9.0, of entire Fraction 24 (Fig. 1). C, sodium dodecyl sulfate polyacrylamide gel, run at pH 7.0, of pooled Fractions 16 to 27 (Fig. 1). D, polyacrylamide gel run in acetic acid-urea by the method of Widnell and Unkeless (26) of supernatant enzyme (Step 5, Table I).

FIG. 6. Standard curve for determination of molecular weights by sodium dodecyl sulfate electrophoresis at pH 9.0. The standards were dissolved in 0.1 M NaBO₃, pH 9.0; 4% 2-mercaptoethanol at concentrations of 0.2 to 0.3 mg per ml. Sodium dodecyl sulfate (20% solution) was added to a final concentration of 4%. The sample was immediately heated to 100°C for 2 min and then incubated at 45°C for 2 hours. A sample of Fractions 16 to 27 (Fig. 1, Table I), which had been dialyzed against dilute phosphate buffer, pH 7.0, and concentrated with dry Sephadex G-100, was prepared in the same manner. Mobilities for standards were determined by direct measurement of stained gels. The arrows indicate the mobilities of the major (Na⁺ + K⁺) adenosine triphosphatase polypeptides. The labeling is the same as that on Fig. 6B.
densities of the two protein bands did not change throughout the region of highest specific activity. The molar ratios between the two major polypeptides, which were determined by amino acid analysis of polyacrylamide gels of Fractions 21 and 26, did not differ significantly from each other.

The same type of analysis was performed on sodium dodecyl sulfate gels run at pH 7.0. In this case two distinct components were observed (Fig. 5C). The standard curve from this system is less precise than that from the pH 9.0 gels as it is nonlinear in this region (16). However, the approximate masses of the two components are 90,000 daltons (mobility slightly less than muscle phosphorylase a) and 57,000 daltons (between bovine serum albumin and fumarase). The approximate molar ratio from one gel, based on amino acid analysis, was 1.00 mole of 90,000-dalton polypeptide to 0.91 mole of 57,000-dalton polypeptide.

To establish the identity or nonidentity of bands observed in gel electrophoresis, a statistical analysis (32) was performed. The results are presented in Table III. Cook et al. (32) calculated variances of the order of 0.001 when duplicates of the same protein were compared. In this case it is clear that the polypeptides which have the same apparent molecular weight in each system are identical and that they are distinct from the other major polypeptide present.

Samples of supernatant (Na+ + K+) adenosine triphosphatase (Step 5) displayed almost the same electrophoretic patterns as the enzyme which had been chromatographed on Sepharose 2B (Fig. 7A). When 0.15 mg of this material was submitted to electrophoresis in the acidic urea system described by Widnell and Unkeless (26), only one major protein component was resolved (Fig. 5D). The soluble preparation of (Na+ + K+) adenosine triphosphatase of the highest purity obtained previously (14) separated into two protein components in this system. One of these two components appears to have approximately the same mobility as that observed in my experiments.

### DISCUSSION

The results which have been presented concern the general problem of the purification of particulate enzymes through the use of detergents and the specific issue of the (Na+ + K+) adenosine triphosphatase. Sodium deoxycholate proved to be very useful because of its relatively mild detergent properties and the ionic strength dependence of its interactions with the bound proteins. As a result of this second property the membrane could be used as solid matrix, much like an ion exchange resin-protein complex. The enzyme remained bound to this matrix and could be separated with it while other proteins were eluted. Finally, the (Na+ + K+) adenosine triphosphatase could be separated under rigidly defined conditions from protein that sedimented at 94,000 x g over 30 min.

The requirement for a precise detergent to protein ratio has been discussed previously (12, 13, 15, 30). This result can be very easily explained if it is realized that the majority of the detergent added is bound directly to the protein and lipid present.
Unless the free concentration of detergent is small compared to the bound concentration, the optimal protein to detergent ratio would be a function of the concentration of protein. However, the ratio does not change as protein concentration is varied over an order of magnitude. If most of the detergent binds to added protein and lipid, then it is reasonable that there will be a point at which bound detergent is sufficient to disperse the particulate material but insufficient to affect those constraints which hold the protein in its active conformation.

The overall yield of activity during the purification procedure presented in this paper is very low (Table I). When yields of this magnitude are observed there is always a danger that the final product represents a minor component of the total activity present in the original material. Any discussion of this particular point becomes more difficult when the total activity recovered in all the fractions from a purification step increases several-fold or when the total recovery is not quantitative. To provide the most pessimistic estimate, the yield was considered only from Step 2. The salt-detergent-extracted microsomes contain 75% of the total activity which can be accounted for in all fractions to this point. When they are treated with sodium deoxycholate at high ionic strength, 95% of the activity remaining in the tube is in the supernatant after 100,000 x g centrifugation. This supernatant material, even before the removal of the remaining turbidity in a process which causes much activity to be discarded, is almost indistinguishable from the material from Fractions 16 to 27 of the Sepharose column (Table I) when it is examined by sodium dodecyl sulfate gel electrophoresis. These results suggest that the final enzyme preparation contains a representative sample of that protein complex responsible for the majority of the (Na\(^+\) + K\(^+\)) adenosine triphosphatase of the original membranes. It must be stressed that the supernatant preparation of the enzyme which can be derived from the salt-detergent microsomes by extraction with sodium deoxycholate at high ionic strength contains 30 to 40% of the total activity added with the microsomes. This material has an electrophoretic pattern in sodium dodecyl sulfate almost indistinguishable from that of the purest enzyme. The enzyme can be used for most studies at this stage.

The elution volume of the most active enzyme on the Sepharose 2B column was less than that of Blue Dextran 2000 (Fig. 1). It is difficult to calibrate a column with known standards of such a high molecular weight under possible denaturing conditions. Both Towle and Copenhagen (14) and Fitzpatrick et al. (34) obtained preparations of (Na\(^+\) + K\(^+\)) adenosine triphosphatase which were of apparently high molecular weight. In those experiments, the enzyme remained in the void volume during agarose chromatography. The results of Medzhybrozhy, Kline, and Hokin (30) and Uesugi et al. (28) suggest that under certain conditions the (Na\(^+\) + K\(^+\)) adenosine triphosphatase can exist in a form whose molecular weight is 6 x 10\(^4\) and remain active. However, experiments in which the mean molecular weight of the protein was varied (Fig. 4, A and B) suggest that the (Na\(^+\) + K\(^+\)) adenosine triphosphatase examined here must exist in higher molecular weight forms, possibly vesicular, to remain active. There are several other explanations of these results. The detergent may be binding to the active site or denaturing the protein as it increases the apparent molecular weight. In order to make a judgment concerning the homogeneity of the purified material, an inventory of the polypeptides present was essential. The properties of sodium dodecyl sulfate are ideally suited for the assessment of the polypeptide composition of a protein sample (16, 35-39). When the purest preparation of enzyme obtained in these studies was submitted to electrophoresis in this solvent at pH 7.0, only two distinct polypeptides were observed and they were present in approximately equimolar amounts (Fig. 5C). When the material was submitted to electrophoresis at pH 9.0, three polypeptides were observed (Fig. 5B). However, since the 114,000-dalton component observed under these conditions is an artifact, these results show that greater than 90% of the protein in Fractions 16 to 27 (Fig. 1) is present as two distinct polypeptides with masses of 57,000 and 84,000 daltons. The sum of these values (molecular weight 141,000), together with the specific activity of 800 µmoles of Pi (mg of protein)\(^{-1}\) hour\(^{-1}\) for the final preparation of enzyme, give a value of 1900 min\(^{-1}\) as the minimum value of the turnover number of the enzyme.\(^3\)

These results can be compared with previous estimates of both the turnover number and the molecular weight of the enzyme (40, 41). There are several estimates of the turnover number based on the results of studies of the phosphorylated intermediate (40, 42). These estimates vary between 3,300 and 10,000 min\(^{-1}\) which seems to indicate that the enzyme preparation described here is only 20 to 60% pure. The most pessimistic explanation for the disparity is that the (Na\(^+\) + K\(^+\)) adenosine triphosphatase is neither of the polypeptide chains described here. However, these estimates of the purity of the enzyme are probably low ones for several reasons. First of all, the values for the turnover numbers quoted above (3,300 to 10,000 min\(^{-1}\)) are necessarily only upper limits of the true turnover number.\(^4\) In the second place, the value of 1900 min\(^{-1}\) obtained here is a minimum estimate. If there were two asymmetric units per active molecule of enzyme, the turnover number would be 3800 min\(^{-1}\). Many enzymes have more polypeptide chains than active sites (45-48). Finally, the value of 800 µmoles of P\(_i\) (mg of protein)\(^{-1}\) hour\(^{-1}\) obtained for the supernatant enzyme preparation may not be directly comparable to the specific activity of the enzyme in the normal environment of the membrane.

An estimate of the molecular weight of the (Na\(^+\) + K\(^+\)) adenosine triphosphatase has been obtained by Kopner and Macey (49) by electron targeting of various preparations of membranes.

*2 Even if only one of the two polypeptides present in the final preparation is the enzyme, the turnover number would still be 1900 min\(^{-1}\).

*3 The turnover number of 3,300 min\(^{-1}\) is the most reliable estimate since it was calculated from experiments which were intended to determine systematically the maximum value of phosphorylated sites (42). This value was obtained with saturating concentrations of ATP and in the absence of phospholipid supplement. The value of 1300 min\(^{-1}\) was also obtained with saturating concentrations of substrate but in the presence of phospholipid.

*4 Any hydrolysis of the acyl phosphate intermediate during isolation or the use of phosphorylation conditions which do not maximize incorporation leads to an overestimate of the turnover number. Even if the maximum number of phosphorylation sites has been accurately estimated, this does not rule out the existence of a significant equilibrium between dephosphoenzyme and phosphoenzyme even at saturated levels. If the equilibrium constant between the enzyme substrate complex (E-ATP) and the covalent intermediate (E-P) does not completely favor the latter, the concentration of the covalent intermediate is less than the concentration of enzyme in all of its forms (43, 44) (E-P)/E_{out} < 1, where E_{out} = E-ATP + E-P).
The value for the enzyme in situ is 190,000 to 300,000 which corresponds to the sum of the individual molecular weights of all the components (including lipids) involved in the enzyme unit. A value of 190,000 to 300,000 for the functional enzyme unit is compatible with a value of 141,000 for the minimal molecular weight of the protein part of the enzyme.

The results presented in this paper suggest that the purest (Na\(^+\) + K\(^+\)) adenosine triphosphatase preparation obtained consists of a specific molecular complex responsible for the enzymatic reaction. The specific activity of the final material is one of the highest yet observed (Table I). The specific activity is constant throughout all regions of the Sepharose 2B elution profile which do not overlap either the void volume or the inclusion volume positions (Fig. 1). The protein travels as a single component in one electrophoretic system (Fig. 5D). More than 90\% of the protein in Fractions 16 to 27 from the Sepharose 2B column (Fig. 1, Table I) is accounted for by two unique polypeptide chains present in approximately equimolar ratio. That these two polypeptides are present in the same ratio in selected fractions from the Sepharose 2B column (Table II) suggests that they are in specific aggregation. At the present time there is no conclusive evidence to decide whether the two polypeptides present are subunits of the (Na\(^+\) + K\(^+\)) adenosine triphosphatase or are functionally unrelated to each other.

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*Similar studies with radiation inactivation, performed by NAKAO et al. (50), have indicated that the mass of the phosphorylated intermediate is 60% of that of the entire enzyme and have been interpreted as evidence for the presence of more than one subunit in the enzyme. It is striking that the 84,000-dalton polypeptide comprises 59% of the mass of the sum of the two polypeptide chains.