Biochimica et Biophysica Acta, 356 (1974) 36–52

BBA 76681

PURIFICATION AND CHARACTERIZATION OF $(Na^+ + K^+)$-ATPase

III PURIFICATION FROM THE OUTER MEDULLA OF MAMMALIAN KIDNEY AFTER SELECTIVE REMOVAL OF MEMBRANE COMPONENTS BY SODIUM DODECYLSULPHATE

PETER LETH JØRGENSEN

Institute of Physiology, University of Aarhus, Aarhus (Denmark)

(Received January 31st, 1974)

SUMMARY

1. A procedure is presented that allows preparation of $(Na^+ + K^+)$-ATPase with specific activities of 32–37 units mg^{-1} protein (units refer to μmoles ATP hydrolysed per min) by incubation of a microsomal fraction with sodium dodecylsulphate and ATP followed by a single zonal centrifugation

2. In a technically simple version of this procedure preparations with specific activities of 20–26 units $\cdot mg^{-1}$ protein are obtained in quantities of 12–20 mg protein by a single centrifugation, in an angle rotor, of microsomal fractions from the outer medulla of rabbit, pig or sheep kidneys

3 Binding of ATP and ADP partially protects $(Na^+ + K^+)$-ATPase against inactivation by sodium dodecylsulphate In the incubation with sodium dodecylsulphate and ATP, 80 % of the protein in the microsomal fraction is solubilized whereas the $(Na^+ + K^+)$-ATPase remains bound to membrane fragments Tracer analysis shows that sodium dodecylsulphate forms less than 0 5 % of the mass of the purified preparations

4 Analysis of the protein composition by sodium dodecylsulphate gel electrophoresis and determination of the capacities for binding of ATP and ouabain and for sodium-dependent phosphorylation (Jørgensen, P. L (1974) Biochim Biophys Acta 356, 53–67 show that the procedures lead to a true purification of the enzyme

INTRODUCTION

All procedures of purification of $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3 6 1 3) consist of steps for separation of the plasma membranes from other cell organelles followed by steps for fractionation of the membrane components after treatments with detergents In one category of procedures the membrane components are fractionated after solubilization of the membranes by nonionic [1–3] or ionic [4–6] detergents In the procedures for purification of $(Na^+ + K^+)$-ATPase from the outer medulla of mammalian kidney developed in this laboratory [7–10] advantage

is taken of the firm association of the enzyme with the membrane. The enzyme is kept embedded in the bilayer, and other components are removed by mild treatment with sodium deoxycholate. In the present work this principle has been further exploited. After development of a procedure for incubation of the membranes with sodium dodecylsulphate in the presence of ATP it has been possible to isolate preparations with a $(Na^+ + K^+)$- ATPase activity of 32–37 units $\cdot$ mg^{-1} protein in a single isopycnic-zonal centrifugation of a microsomal fraction from the outer medulla of rabbit kidney. In a technically simple version of this procedure, only one centrifugation, in an angle rotor, of microsomal fractions from the outer medulla of rabbit, pig or sheep kidneys is required to prepare $(Na^+ + K^+)$-ATPase with activities of 20–26 units $\cdot$ mg^{-1} protein in quantities of 12–20 mg protein.

EXPERIMENTAL

Tissue preparation

All operations were carried out at 0–4 °C unless otherwise specified. The kidney were removed immediately after killing and bleeding the animals, and were stored in ice-cold 0.25 M sucrose, 0.03 M histidine, pH 7.2. Tissue from the dark red outer medulla was obtained by dissection [7, 9]. On average, the amount of tissue obtained from one kidney was 0.6 g for rabbit, 4 g for pigs and 5 g for sheep. The tissue was homogenized in 10 ml sucrose–histidine solution per g tissue with five strokes in a glass homogenizer with a tight fitting Teflon pestle rotated at 1000 rev./min. The homogenate was centrifuged at $6000 \times g$ for 15 min (e g. in Rotor SS 34 of the Sorwall RC-2B centrifuge). The sediment was resuspended by homogenization in the original volume of sucrose–histidine solution and centrifuged again at $6000 \times g$ for 15 min. The combined supernatants from the two centrifugations were centrifuged at $48000 \times g$ for 30 min The pellet, i e. the microsomal fraction, was resuspended in sucrose–histidine solution to a concentration of 5–6 mg protein per ml and was stored in 5–10 ml aliquots at −25 °C.

Purification procedure

After preparation of the microsomal fraction, the procedure consisted of two steps, incubation with sodium dodecylsulphate and density gradient centrifugation in a zonal rotor or in an angle rotor

Incubation with sodium dodecylsulphate

The microsomal fraction was incubated for 30 min at 20 °C at a protein concentration of 1.35–1.40 mg ml^{-1} with 0.58 mg sodium dodecylsulphate per ml, 3 mM ATP (disodium salt), 2 mM EDTA, 50 mM imidazole, pH 7.5 (20 °C). After mixing of the microsomal fraction with solutions of imidazole, EDTA and ATP, the incubation was started by addition, under continuous stirring, of the appropriate volume of a freshly prepared solution of sodium dodecylsulphate ($2 mg \cdot ml^{-1}$). The sequence of addition is important because part of the enzyme will be exposed to higher concentrations of sodium dodecylsulphate than intended, if the incubation is started by addition of the microsomal fraction. If protein concentrations other than 1.35–1.40 mg $\cdot$ ml^{-1} are used, the optimum concentration of sodium dodecylsulphate must be found as shown in Figs 6 and 10.

Density gradient centrifugation

(*a*) *Zonal rotor* Incubation of 50 ml of the medium above was initiated by addition of sodium dodecylsulphate 30 min prior to injection of the sample in the zonal rotor. Introduction and recovery of gradient sample and overlay were done while the rotor was spinning at 3000 rev /min. In the Ti-14 rotor (640 ml), linear sucrose gradients of 450 ml were formed by a gradient pump from 15 % (w/v) and 45 % (w/v) sucrose in 25 mM imidazole, 1 mM EDTA, pH 7 5 (20 °C) and delivered into the rotor at a rate of 28 ml · min^{-1}. The sample, 50 ml of the incubation medium and the overlay, 50 ml 25 mM imidazole, 1 mM EDTA, pH 7 5, were injected with a syringe The rotor was centrifuged at 48 000 rev /min for 120 min ($\omega^2 t = 2 \cdot 10^{11}$) After centrifugation, 40 fractions of 16 ml were collected and analyzed for protein and enzyme activity The fractions containing the peak of ($Na^+ + K^+$)-ATPase (Fig 7) were diluted from 16 to 35 ml with 25 mM imidazole, 1 mM EDTA, 3 mM ATP, pH 7.5 (20 °C) and were centrifuged for 3 h at 60 000 rev /min in an angle rotor The pellets were resuspended in 25 mM imidazole, 1 mM EDTA, pH 7 5 (20 °C) to a protein concentration of 1–2 mg per ml and were stored at 0 °C

(b) *Angle rotor* 80 ml of the medium for incubation with sodium dodecylsulphate were prepared Discontinuous density gradients of 25 ml were made in the 2.5 cm × 9 cm tubes of the Ti-60 Beckmann fixed-angle rotor The gradients consisted of three successive layers of sucrose 12 5 ml of 29 4 %, 7 5 ml of 15 %, and 5 ml of 10% (w/v) sucrose in 25 mM imidazole, 1 mM EDTA, pH 7 5 (20 °C) Portions of the sample of 10 ml were layered on each of the gradients in the eight tubes of the angle rotor and were centrifuged at 60 000 rev /min for 90 min ($\omega^2 t = 2\,4\ \ 10^{11}$) at 0–4 °C After centrifugation the gradients were removed with a pipette and the eight pellets were resuspended by homogenization in 25 mM imidazole, 1 mM EDTA, pH 7.5 (20 °C) to a protein concentration of about 2 mg ml^{-1} and were stored at 0 °C

Protein

This was determined by the method of Lowry et al. [11] after precipitation and two washes with 5 % trichloroacetic acid at 0–4 °C As standards for this method bovine serum albumin and samples of the enzyme preparations that had been washed three times by centrifugation in 10 mM acetate buffer, pH 7 5 (20 °C) and analyzed for protein nitrogen by the micro-Kjeldahl method [12] were used

Other enzyme assays and analyses for phospholipid and cholesterol were done by standard procedures as described before [8, 9].

Dodecylsulphate–polyacrylamide gel electrophoresis

This was done as described by Weber and Osborn [13] with minor modifications. Gels containing 7 5 % acrylamide (T % [14]) and 1 8 % bisacrylamide (C % [14]) were prepared in 15 cm long glass tubes with an inner diameter of 5 mm The enzyme preparation was dissolved in 2 % sodium dodecylsulphate, 1 % 2-mercaptoethanol, 1 mM EDTA, 10 mM sodium phosphate, pH 7 0 (20 °C) to a protein concentration of 1–2 mg/ml. The mixture was heated in a boiling water bath for 3 min 15–40 μg protein was applied per gel. Electrophoresis was performed at a current of 8 mA per gel in an apparatus in which the glass tubes were thermostated with water at 20 °C until the marker dye, bromphenol blue, had moved 9 cm through the gel

(5–6 h). The gels were stained with Coomassie brilliant blue [13] The molecular weights of the polypeptide chains were determined by calibration against known standards: β-galactosidase (M_r = 130 000), serum albumin, dimer and monomer, (M_r = 68 000), leucine aminopeptidase (M_r = 53 000), lactate dehydrogenase (M_r = 36 000), and cytochrome *c* (M_r = 11 700) [13]

RESULTS

The procedures for purification of $(Na^+ + K^+)$-ATPase from the outer medulla of rabbit kidney are summarized in Fig 1. In the procedure described before [9], preparations with a specific activity of 25 units · mg^{-1} protein were obtained by isopycnic-zonal and rate-zonal centrifugations (Steps A and B, Fig. 1) after incubation of the microsomal fraction with deoxycholate in a concentration which gave maximum activation of latent $(Na^+ + K^+)$-ATPase [8]. In the present procedure (Step C), the microsomal fraction was incubated with sodium dodecylsulphate in the presence of ATP and preparations with a specific activity of 32–37 units · mg^{-1} protein were isolated in a single isopycnic-zonal centrifugation.

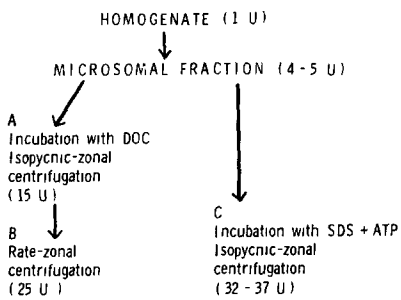

Fig. 1. A summary of the procedures for purification of $(Na^+ + K^+)$-ATPase from the outer medulla of rabbit kidney. Steps A and B were described before [7–10] Step C is described in the present work. The specific activity of $(Na^+ + K^+)$-ATPase after each step is given in parentheses U = μmoles P_i $min^{-1} \cdot mg^{-1}$ protein SDS, sodium dodecylsulphate. DOC, deoxycholate

Incubation with sodium dodecylsulphate

The present purification procedure was developed after a study of the effects of sodium dodecylsulphate on the preparation obtained by Step B in Fig 1. The aim was to see whether extraneous protein could be removed by sodium dodecylsulphate from the membranes with which $(Na^+ + K^+)$-ATPase is associated without damaging the enzyme.

It is seen from Fig 2 that incubation with increasing concentrations of sodium dodecylsulphate caused a gradual decrease in the activity of $(Na^+ + K^+)$-ATPase in the preparation obtained by Step B in Fig 1. Addition of 3 mM ATP partially protected the enzyme against this inactivation by sodium dodecylsulphate. The concentration of sodium dodecylsulphate necessary to give 50 % inactivation was 56 % higher in the presence of 3 mM ATP than in its absence. Fig. 3 shows that this protective effect of ATP became apparent at low concentrations (0.1 mM) of ATP and that ADP had the same effect as ATP. ITP, AMP and CTP had practically no

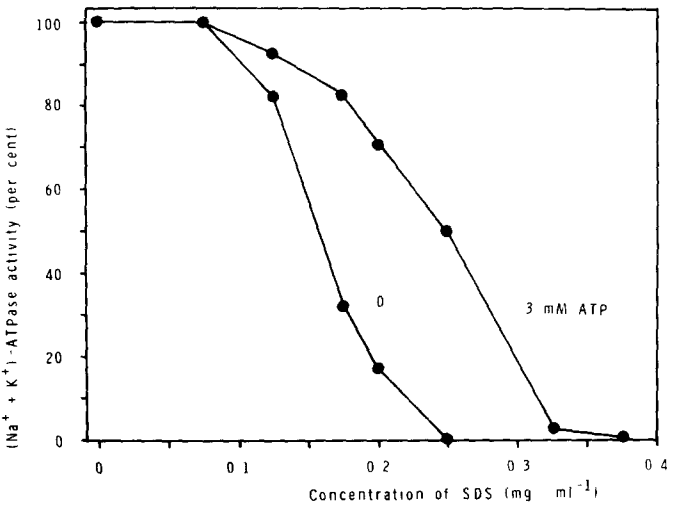

Fig 2 The effect of incubation with sodium dodecylsulphate, in the presence and in the absence of ATP, on the activity of $(Na^+ + K^+)$-ATPase in a preparation (22 units mg^{-1} protein) obtained by Step B in Fig 1. Aliquots containing 29 μg of protein were incubated at 20 °C in 0 1 ml with the concentrations of sodium dodecylsulphate indicated on the abscissa and 2 mM EDTA, 50 mM imidazole, pH 7 5 (20 °C) with and without 3 mM ATP (disodium salt) After 1 h, 10-μl portions were transferred to test tubes containing 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, 3 mM ATP (Tris salt), 30 mM histidine, pH 7 5 (37 °C) and where appropriate 1 mM ouabain in a total volume of 1 ml After 5 min at 37 °C the reaction was stopped with 100 μl of 50 % trichloroacetic acid and P_i was measured as before [9] $(Na^+ + K^+)$-ATPase activity was taken to be the difference in activity with and without ouabain SDS, sodium dodecylsulphate

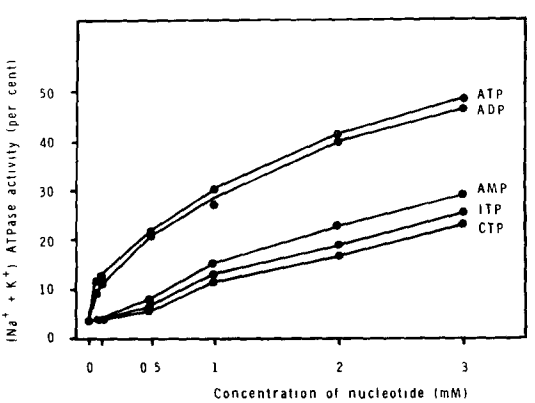

Fig 3 The effect of ATP, ADP, AMP, ITP and CTP on the inactivation of $(Na^+ + K^+)$-ATPase by incubation with sodium dodecylsulphate. Aliquots of the preparation used for Fig 2 containing 20 μg of protein were incubated at 20 °C in 0 1 ml with 0 2 mg sodium dodecylsulphate per ml, 2 mM EDTA, 50 mM imidazole, pH 7 5 (20 °C) and increasing concentrations of the sodium salt of the nucleotides After 1 h, 10-μl portions were transferred to test tubes for measurements of $(Na^+ + K^+)$-ATPase activity as described in Fig 2

protective effect at concentrations lower than 0.5 mM. It is not surprising that ATP and ADP had nearly the same effect because part of the ATP added to the medium would be hydrolyzed in the prolonged incubation with a high enzyme concentration at 20 °C The ATP binding site of $(Na^+ + K^+)$-ATPase has a relatively high affinity for ADP, whereas the affinity of this site for AMP, ITP and CTP is low [15]. It seems likely, therefore, that it is the binding of ATP or ADP to the substrate site that protects the enzyme against inactivation by sodium dodecylsulphate. The fact that 2 mM EDTA was present in all incubation media excludes the possibility that the protective effect of ADP and ATP is due to chelation of metal ions

All incubations with sodium dodecylsulphate were done at pH 7.5 (20 °C) Changes in the pH had a profound influence on the results. At pH 7 0 and 6 5, the concentrations of sodium dodecylsulphate necessary for 50 % inactivation of the enzyme was 20 % and 50 % lower than at pH 7 5 (not shown) Examination of the time course of the inactivation of $(Na^+ + K^+)$-ATPase showed that there was a rapid initial inactivation followed by a slower process in which the rate of inactivation was 5–10 % per hour (not shown)

Separation of the proteins solubilized by sodium dodecylsulphate from the membrane bound enzyme was achieved by density gradient centrifugations. The $(Na^+ + K^+)$-ATPase activity was not solubilized by sodium dodecylsulphate under these conditions and was rapidly sedimented to its equilibrium density in the sucrose gradients (1 12–1 15 $g \cdot ml^{-1}$) whereas the sedimentation rate of the solubilized

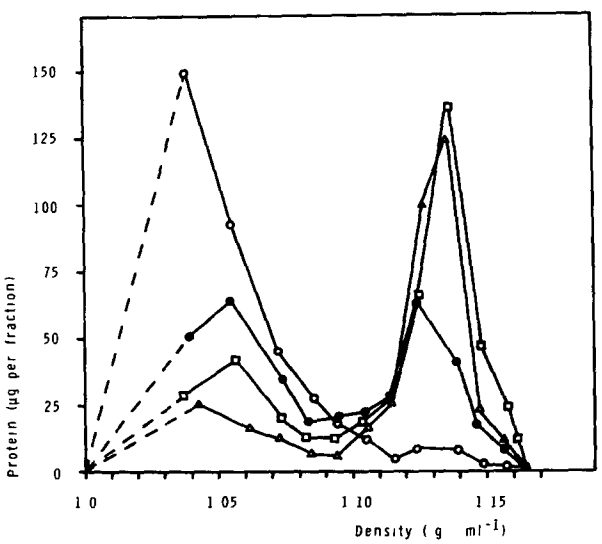

Fig 4 The effect of incubation with sodium dodecylsulphate on the distribution of proteins after density gradient centrifugation of the preparation obtained by Step B in Fig. 1 Aliquots containing 0 5 mg protein were incubated at 20 °C in 4 ml with 2 mM EDTA, 3 mM ATP, 50 mM imidazole, pH 7 5 (20 °C), and the following concentrations of sodium dodecylsulphate 0 1 (△—△), 0 15 (□—□), 0 25 (●—●) and 0 5 (○—○) mg ml^{-1}. After incubation for 1 h, the media were layered on top of sucrose gradients of 14 ml ranging from 15 to 45 % (w/v) sucrose in 1 mM EDTA, 25 mM imidazole, pH 7.5 (20 °C) prepared in the 1 6 cm × 10.1 cm tubes of the Beckmann SW 27.1 rotor After centrifugation for 4 h at 27 000 rev /min, the fractions were collected [8] and analyzed for protein as described under Experimental

proteins was much lower Fig 4 shows the distribution of the protein, after density gradient centrifugation at a large centrifugal force, of preparations treated with increasing concentrations of sodium dodecylsulphate. After treatment with a low concentration of sodium dodecylsulphate that did not cause inactivation of $(Na^+ + K^+)$-ATPase, most of the protein was recovered along with the enzyme in fractions with densities of 1 12–1 15 g · ml^{-1} After treatment with a concentration that inactivated the enzyme completely, nearly all of the protein was solubilized and remained in the sample zone of the gradient At intermediate concentrations, the distribution of protein between the two bands was dependent on the ratio of sodium dodecylsulphate to protein in the incubation medium

By balancing between this solubilization of protein and the inactivation of enzyme an optimum ratio of sodium dodecylsulphate to protein for separation of enzyme and protein was found Fig 5 shows the distribution of enzyme and protein in a sucrose gradient of a preparation of $(Na^+ + K^+)$-ATPase (22 units mg^{-1} protein) treated with an optimum concentration of sodium dodecylsulphate, 1 1 mg sodium dodecylsulphate per mg protein, in the presence of 3 mM ATP After this incubation the activity in the sample had decreased to 86 % of the original activity. After centrifugation, 34 % of the protein was solubilized and remained in the fractions at the top of the tube The $(Na^+ + K^+)$-ATPase activity was recovered in a peak at 1 10–1 13 g ml^{-1} with a specific activity of 34 units mg^{-1} protein In a series of similar experiments preparations with specific activities of 33–36 units mg^{-1} protein were obtained In an attempt to circumvent Steps A

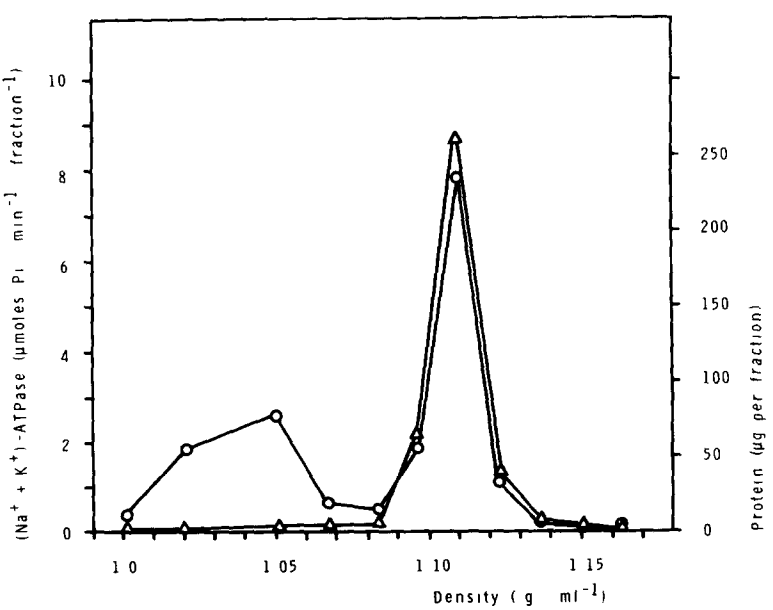

Fig 5 The distribution of protein (○—○) and of $(Na^+ + K^+)$-ATPase (△—△) after density gradient centrifugation of the preparation obtained by Step B in Fig 1 An aliquot containing 500 µg of enzyme protein was incubated at 20 °C with 0 15 mg sodium dodecylsulphate per ml, 2 mM EDTA, 3 mM ATP, 50 mM imidazole, pH 7 5 (20 °C) in a total colume of 5 ml After incubation for 1 h the medium was layered on top of the gradient and centrifuged as in Fig 4

and B (Fig. 1) in this purification procedure, it was found that incubation of the microsomal fraction with optimum concentrations of sodium dodecylsulphate and 3 mM ATP followed by a zonal centrifugation also gave preparations with specific activities in this range.

Incubation of the microsomal fraction with sodium dodecylsulphate

Fig. 6 shows curves for the inactivation of $(Na^+ + K^+)$-ATPase by sodium dodecylsulphate after incubation at various concentrations of microsomal protein. The dotted line indicates the activation of latent $(Na^+ + K^+)$-ATPase at low concentrations of sodium dodecylsulphate [8]. The concentration of sodium dodecylsulphate necessary to give a certain inactivation of $(Na^+ + K^+)$-ATPase, e g. to 80 % or 50 % of the maximum activity, was not linearly related to the protein concentration. The optimum ratio of sodium dodecylsulphate to protein for separation of $(Na^+ + K^+)$-ATPase and protein must therefore be determined for each concentration of microsomal protein. In centrifugation experiments similar to those in Figs 4 and 5 it was found that incubation at a protein concentration of 1.40 mg · ml^{-1} with 0.57–0.59 mg sodium dodecylsulphate per ml gave an optimum separation of enzyme and protein in the preparation from rabbit kidney (cf. Fig. 10). This concentration of sodium dodecylsulphate was 2 6-fold higher than the concentration necessary for maximum activation of latent enzyme activity in the preparation It is seen from Fig 6 that at this relatively high concentration of sodium dodecylsulphate

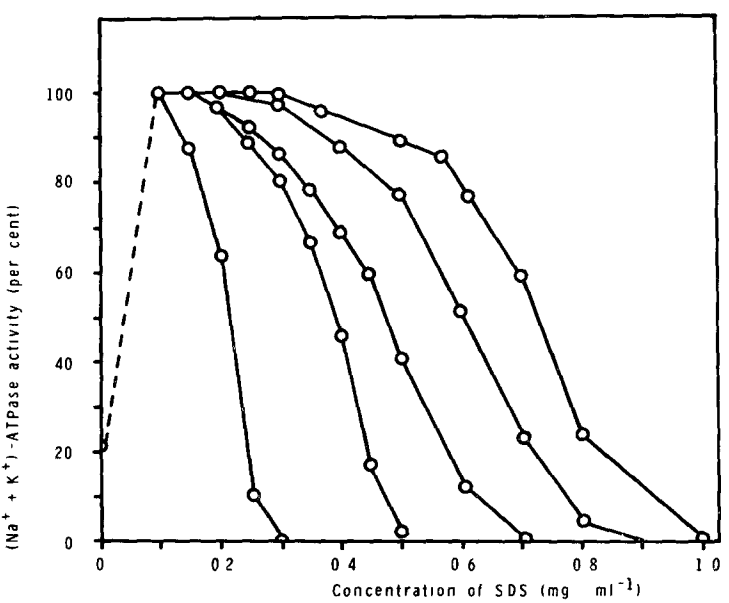

Fig 6 The influence of the concentration of microsomal protein on the inactivation of $(Na^+ + K^+)$-ATPase by incubation with sodium dodecylsulphate Aliquots of a microsomal fraction from the outer medulla of rabbit kidney containing (curves from left to right) 0.16, 0 44, 0 62, 0 94 and 1 41 mg protein were incubated at 20 °C in 1 ml with the concentrations of sodium dodecylsulphate shown on the abcissa and 2 mM EDTA, 3 mM ATP, 50 mM imidazole, pH 7.5 (20 °C) Afterwards, 45 min aliquots of 10 μl were transferred to test tubes for assay of $(Na^+ + K^+)$-ATPase as in Fig. 2. SDS, sodium dodecylsulphate

the activity of $(Na^+ + K^+)$-ATPase was still 85–90 % of the maximum activity in the microsomal fraction For preparations from pig and sheep kidney, the optimum concentration was slightly lower, 0 55–0 57 mg sodium dodecylsulphate per ml, for a protein concentration of 1 35–1 40 mg ml^{-1}

Zonal centrifugation of the microsomal fraction

After zonal centrifugation at a large total centrifugal force of a microsomal fraction incubated with the optimum concentration of sodium dodecylsulphate, the $(Na^+ + K^+)$-ATPase activity was recovered in a peak at densities of 1 12–1 14 g ml^{-1} (Fig 7). The Mg^{2+}-ATPase activity formed a separate peak at 1 06–1 08 g ml^{-1}, and this activity was absent in most of the fractions at the peak of $(Na^+ + K^+)$-ATPase. About 80 % of the protein in the sample was recovered in fractions with densities below 1 08 g ml^{-1} near the sample zone of the rotor Only small amounts of protein were found in fractions peripheral to the peak of $(Na^+ + K^+)$-ATPase

In tracer studies with sodium dodecyl[^{35}S]sulphate (Fig 8) nearly all of the detergent was found in the fractions containing the solubilized protein at a concentration of 0 6–1 2 mg sodium dodecylsulphate per mg protein The concentration in the fractions at the peak of $(Na^+ + K^+)$-ATPase was low, 38 ± 7 μg sodium dodecylsulphate per mg protein After sedimentation and washing of the enzyme preparation, this value was reduced to 9 ± 4 μg per mg protein This is less than 0 5 % of the mass since the ratio of protein to lipid in the preparation is close to 1 (Table II)

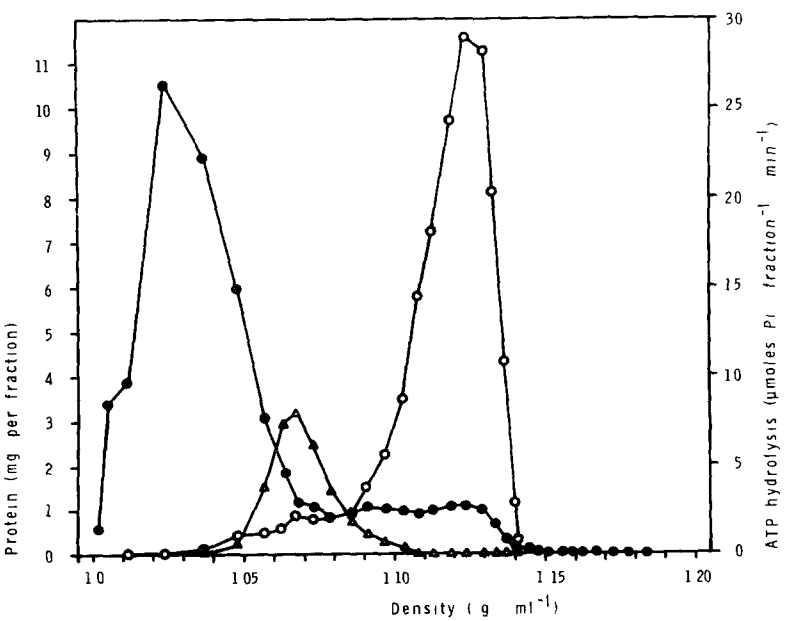

Fig 7 The distribution of protein (●—●), $(Na^+ + K^+)$-ATPase (○—○) and Mg^{2+}-ATPase (△—△) after isopycnic-zonal centrifugation in the Ti-14 zonal rotor of a microsomal fraction from the outer medulla of rabbit kidney. 69 mg of microsomal protein were incubated for 45 min at 20 C with 0 57 mg sodium dodecylsulphate per ml, 2 mM EDTA, 3 mM ATP, 50 mM imidazole, pH 7 5 (20 °C) in a total volume of 50 ml The incubation medium was injected as a sample in the zonal rotor and centrifuged as described under Experimental

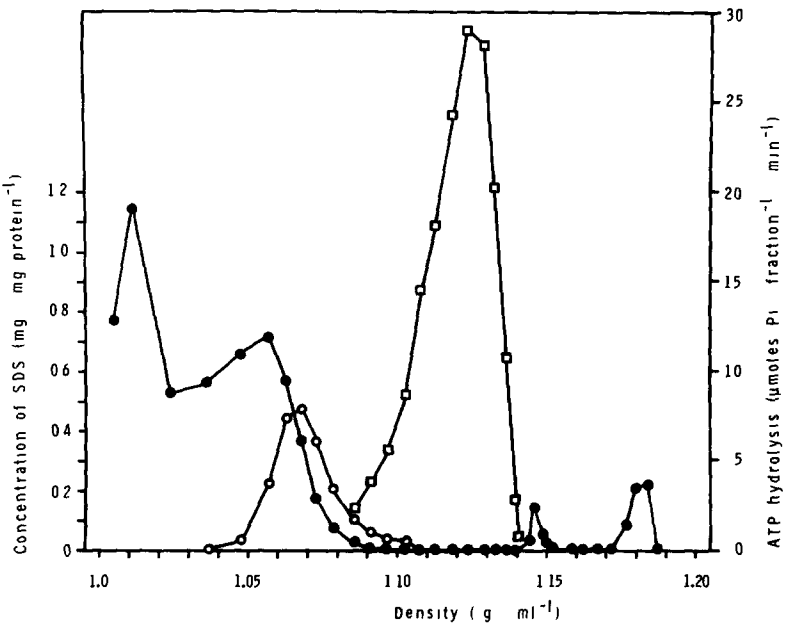

Fig 8. The distribution of sodium dodecyl[^{35}S]sulphate (●—●), Mg^{2+}-ATPase (○—○) and ($Na^+ + K^+$)-ATPase (□—□) after zonal gradient centrifugation of a microsomal fraction from the outer medulla of rabbit kidney as in Fig. 7 but with sodium dodecyl[^{35}S]sulphate added to the medium for incubation with sodium dodecylsulphate and ATP After centrifugation, 25 -μl portions of the fractions were transferred to counting vials and counted in a Packard Tricarb scintillation counter with an internal standard for correction of quenching SDS, sodium dodecylsulphate

The ($Na^+ + K^+$)-ATPase was recovered from the fractions at the peak after dilution and centrifugation at high speed in an angle rotor and the sediments were resuspended to a protein concentration of 1–2 mg · ml^{-1}. Fig. 9 shows the specific activities of ($Na^+ + K^+$)-ATPase in the resuspended sediments of the fractions collected in all zonal centrifugations performed up to date of microsomal fractions incubated with sodium dodecylsulphate. The first three experiments were performed during the development of the technique The volume of the samples and the concentrations of protein and sodium dodecylsulphate were varied to find the optimum conditions. In these experiments the specific activities were lower than later because the incubations with sodium dodecylsulphate were initiated by addition of dilute suspensions of the microsomal fractions resulting in exposure of part of the enzyme to higher concentrations of sodium dodecylsulphate than intended In the following experiments (Nos 3–6), specific activities of 35–40 units mg^{-1} protein were regularly obtained In experiment Nos 7–9, the activities were lower due to problems with the cooling system of the ultracentrifuge. Later (Nos 10–14) the specific activities in the best fractions were 32–37 units · mg^{-1} protein. These fractions were collected from the top and the peripheral part of the peak of ($Na^+ + K^+$)-ATPase in the gradient, whereas the activities were lower in the fractions from the central part of the peak (cf. Fig 7). The amount of protein per sediment was 0 5–1 0 mg. On average, the sum of the protein in the sediments was 4 4±0.3 (n = 14) mg per centrifugation and

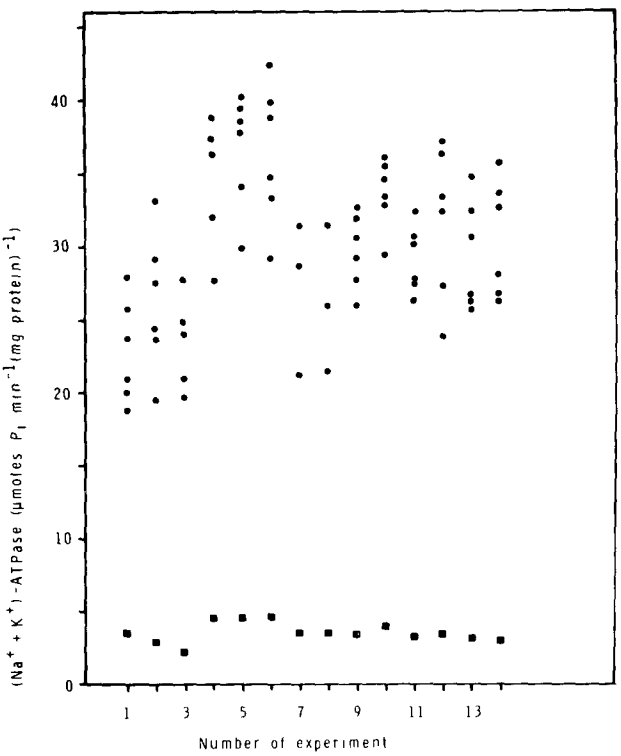

Fig 9 The specific activity of $(Na^+ + K^+)$-ATPase in the samples after incubation with sodium dodecylsulphate and ATP (■) and in the resuspended sediments of the fractions at the peak of $(Na^+ + K^+)$-ATPase (●) after zonal centrifugation of the microsomal fraction as in Fig 7 Recovery of the enzyme from the fractions as described under Experimental

50–60 % of the amount of $(Na^+ + K^+)$-ATPase in the microsomal fractions were recovered in the sediments Mg^{2+}-ATPase activity was not detectable in the sediments from 10 of the 14 experiments, in the remaining sediments the activity was lower than 0 3 units mg^{-1} protein

It is apparent from Fig 7 that the maximum equilibrium density of the enzyme particles was 1 14 g ml^{-1} The equivalent sedimentation coefficients [16] of these particles, calculated as before [9] from the distribution of the enzyme after two rate-zonal centrifugations at different centrifugal forces, were evenly distributed around values of 400–500 Svedberg units. These coefficients were used to calculate the minimum centrifugal force necessary to sediment the particles in an angle rotor It was not possible to increase the specific activity further by rate-zonal centrifugations

A rapid purification procedure

Most procedures for purification of $(Na^+ + K^+)$-ATPase are technically complicated and time consuming. In developing a more simple version of the present procedure angle rotors were used since the resolution in isopycnic centrifugations in this kind of rotor is superior to that in swinging bucket rotors [17] It was shown in Fig 7 that only small amounts of protein were found in the fractions peripheral to the

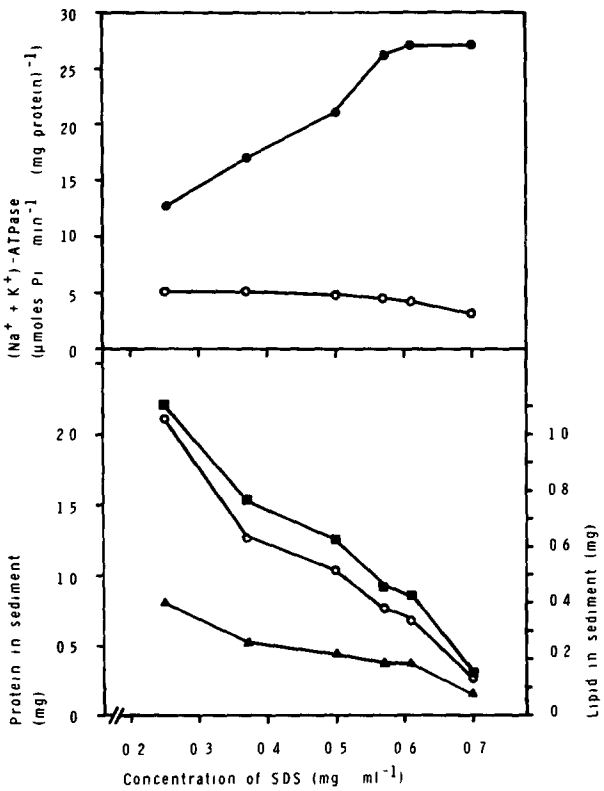

Fig 10 Determination of the optimum concentration of sodium dodecylsulphate for the rapid purification procedure Six aliquots of a microsomal fraction from the outer medulla of rabbit kidney containing 6 3 mg protein were incubated with increasing concentrations of sodium dodecylsulphate in 4 5 ml of the medium used in Fig. 7. The activity of $(Na^+ + K^+)$-ATPase (○—○) was measured after incubation for 45 min at 20 °C and the media were layered on six gradients and centrifuged in the Ti-60 angle rotor as described under Experimental. After resuspension of the sediments, the activity of $(Na^+ + K^+)$-ATPase (●—●) and the contents of protein (○—○), phospholipid (■—■), and cholesterol (▲—▲) were determined as described under Experimental. SDS, sodium dodecylsulphate,

peak of $(Na^+ + K^+)$-ATPase after the isopycnic-zonal centrifugation of the microsomal fraction treated with sodium dodecylsulphate. It was therefore an obvious modification to collect the enzyme as a pellet in the tubes of an angle rotor after sedimentation through a gradient designed to prevent sedimentation of the Mg^{2+}-ATPase and of the solubilized proteins

Fig. 10 shows the relationship between the concentration of sodium dodecylsulphate in the incubation medium and the specific activity of $(Na^+ + K^+)$-ATPase in the sample and in the sediment after centrifugation of the microsomal fraction in this gradient. It is seen that the specific activity in the sediments was increased with the concentration of sodium dodecylsulphate in the sample until a plateau was reached at 27 units · mg^{-1} protein. The best recovery of enzyme with this specific activity was obtained after incubation of the microsomal fraction with 0.58 mg sodium dodecylsulphate per ml at a protein concentration of 1 4 mg · ml^{-1}. From

TABLE I

THE SPECIFIC ACTIVITY AND THE RECOVERY OF $(Na^+ + K^+)$-ATPase AFTER CENTRIFUGATION IN AN ANGLE ROTOR OF MICROSOMAL FRACTIONS FROM THE OUTER MEDULLA OF RABBIT, PIG AND SHEEP KIDNEYS

Purification procedure as described under Experimental Mean values ± S E are given with the number of preparations in brackets The combined results of six and eight consecutive preparations from rabbit and pig kidney are shown For sheep kidney the average values of two preparations obtained in one centrifugation are given

Preparation	Protein (mg)	$(Na^+ + K^+)$-ATPase (μmoles P_i min^{-1} mg^{-1} protein)	Recovery (%)	Mg^{2+}-ATPase (μmoles P_i min^{-1} mg^{-1} protein)
Rabbit kidney				
Samples	344	4 2 ± 0 1 (6)		0 8 ± 0 1 (6)
Sediments	37 4	25 8 ± 1 3 (6)	67	0 5 ± 0 2 (6)
Pig kidney				
Samples	1321	3 0 ± 0 2 (8)		0 4 ± 0 04 (8)
Sediments	173	20 9 ± 0 6 (8)	91	0 3 ± 0.1 (8)
Sheep kidney				
Samples	12 6	2 6 (2)		0 04 (2)
Sediments	1 4	19 0 (2)	81	0

the lower part of Fig 10 it is seen that the amounts of phospholipid and protein in the sediment were reduced almost in parallel whereas less cholesterol was removed from the membranes along with the protein In consequence, the molar ratio of cholesterol to phospholipid was increased from 0 53 in the microsomal fraction to 0 76–0 90 in the sediments with the highest activity of $(Na^+ + K^+)$-ATPase.

Tracer studies with sodium dodecyl[^{35}S]sulphate gave a distribution similar to that observed after zonal centrifugation (Fig 8) Only 1 5 % of the detergent was recovered in the sediment at an average concentration of 42 μg sodium dodecylsulphate per mg protein This value was reduced to 9 μg per mg protein by washing of the preparation without reduction of the enzyme activity

In Table I are shown the results of routine preparations by this procedure of enzyme from rabbit and pig kidney and of an experiment with material from sheep kidney It is seen that the specific activity of $(Na^+ + K^+)$-ATPase was higher in the preparations from rabbit kidney than in preparations from pig and sheep kidney In all preparations the Mg^{2+}-ATPase activity was low or absent The output of the preparation in a single centrifugation was 12–20 mg protein when sample volumes of 10–15 ml containing 14–21 mg of microsomal protein per tube of 35 ml were employed The yield of $(Na^+ + K^+)$-ATPase in the preparation from rabbit kidney was 27 units mg^{-1} tissue or 23 % of the total amount of enzyme in the tissue

The properties of the preparations

In Table II, some enzymic and chemical properties of the two preparations are summarized and compared with analysis on the microsomal fractions It is seen that the K^+-dependent *p*-nitrophenylphosphatase activity was enriched in parallel with the $(Na^+ + K^+)$-ATPase activity The weight ratio of protein to the sum of cholesterol

TABLE II

SUMMARY OF ENZYMIC AND CHEMICAL CHARACTERISTICS OF THE PREPARATIONS FROM THE OUTER MEDULLA OF RABBIT KIDNEY

Enzyme assays and lipid analysis as described in Fig 2 and under Experimental The unit for enzyme activities is μmoles substrate min^{-1} mg^{-1} protein The unit for cholesterol and phospholipid is μg mg^{-1} protein.

	Microsomal fraction	Preparation from angle rotor	Preparation from zonal rotor
$(Na^+ + K^+)$-ATPase	4 4	24 7	36 1
Mg^{2+}-ATPase	1 5	0 3	0
K^+-*p*-nitrophenylphosphatase	0 8	2 6	4 6
Adenylate kinase	0 24	0 04	0
Cholesterol	135	248	304
Phospholipid	523	599	730
Yield (mg protein per g tissue)	9 7	1 1	0.63

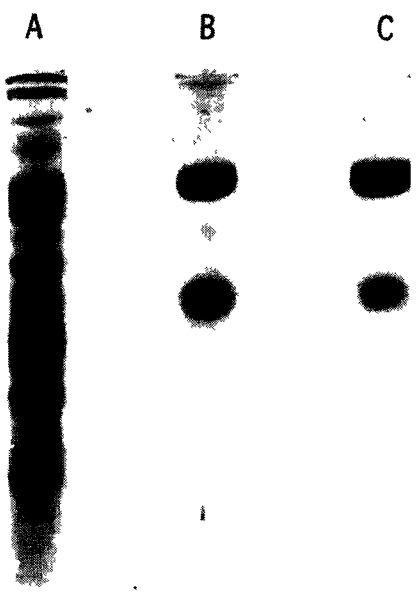

Fig. 11. Sodium dodecylsulphate–polyacrylamide gel electrophoresis of preparations from the outer medulla of rabbit kidney. (A), a microsomal fraction, (B), a preparation with a specific activity of 26 units mg^{-1} protein obtained by centrifugation in the angle rotor, (C), a preparation with a specific activity of 37 units mg^{-1} protein prepared by zonal centrifugation as in Fig 7 The electrophoresis was done as described under Experimental

and phospholipid in the preparation was close to 1 In the purification procedure more phospholipid than cholesterol was removed along with the protein (cf Fig 11) resulting in a high cholesterol to phospholipid molar ratio in the purified preparations

Polyacrylamide gel electrophoresis in sodium dodecylsulphate of the preparations shows that the microsomal fraction contained a multitude of protein components (Fig 11A) In the preparation obtained by centrifugation in the angle rotor (Fig 11B) some of these were visible along with two prominent bands. In the most pure preparation (Fig. 11C) only two bands were seen In a series of gels of similar preparations, the average molecular weights of the two bands were $96\,000 \pm 1000$ (S E , $n = 26$) and $56\,900 \pm 600$ (S E , $n = 26$) as calibrated against known standards [13] As shown repeatedly [2, 3, 6, 18, 19], it was found that the large chain carried the phosphate incorporated from ATP in the pressure of Mg^{2+} and Na^+ (not shown)

The stability of the preparations

When stored at 0 °C the activity of $(Na^+ + K^+)$-ATPase remained unchanged for 2–3 weeks After storage for 7 weeks a decrease in activity to 85–90 % of the original activity was observed in three batches of enzyme

DISCUSSION

The present purification procedure represents a considerable improvement of the previous procedure [8, 9] The specific activity of $(Na^+ + K^+)$-ATPase, 32–37 units $\cdot$ mg^{-1} protein, is higher and other ATP hydrolyzing enzymes are effectively separated from the enzyme The procedure is less time consuming as only one zonal centrifugation is required and the output per preparation is higher than that of the previous procedure [9] In the rapid version of the procedure the specific activity is 30 % lower than after centrifugation in the zonal rotor, due to the difference in resolution of the centrifugal systems However, the output after a single centrifugation of the microsomal fraction in the angle rotor was 12–20 mg protein and the specific activity of 20–26 units $\cdot$ mg^{-1} protein compares well with that obtained after much more complicated procedures. Lane et al [6], for example, used five purification steps and eight centrifugations to isolate identical amounts of protein with a specific activity of 26 units mg^{-1} protein from the microsomal fraction of the outer medulla of canine kidney In the procedure by Hokin et al [2], membrane fractions from the rectal gland of the dogfish are solubilized by Lubrol. After several purification steps, 15–20 mg protein with an $(Na^+ + K^+)$-ATPase activity of 20–25 units mg^{-1} protein are obtained in one preparative run In an alternative fractionation procedure by Nakao et al [3], pig brain membranes are solubilized by Lubrol at a low ionic strength and fractionated by ion exchange chromatography Small amounts of protein (0 1–0.3 mg) are eluted with an $(Na^+ + K^+)$-ATPase activity varying from 8 to 117 units mg^{-1} protein due to lability of the enzyme. The specific activity is the highest reported so far, but difficulties with the analysis of the protein were reported The preparation isolated by Kyte [5] from the outer medulla of canine kidney is claimed to be a 90–100 % pure preparation of $(Na^+ + K^+)$-ATPase [5, 20] It has a specific activity of 10–13 units mg^{-1} protein and is isolated in quantities less than 1 mg after gradient centrifugation and gel filtration of membranes solubilized by deoxycholate at a high salt concentration Grisham and Barnett [21] slightly modified our

previous procedure [9] and isolated the enzyme in high yield from the outer medulla of sheep kidney with an activity of 18 units · mg^{-1} protein. This is close to the activity in the preparation from sheep kidney obtained by the rapid version of the present procedure (Table I). The relative purity of the preparations mentioned above is estimated later [22] on the basis of the available criteria.

After the incubation with sodium dodecylsulphate and ATP, as much as 80 % of the protein in the microsomal fraction is solubilized and remains in the sample zone after the zonal centrifugation In incubations with deoxycholate, the fraction of solubilized protein could not be increased to more than 45 % of the protein in the microsomal fraction without inactivation of enzyme [8]. Deoxycholate is not bound to proteins in significant amounts [23], and this may explain why much less protein is removed from the membranes by deoxycholate than by sodium dodecylsulphate. It is known that sodium dodecylsulphate disrupts membranes by binding firmly to their proteins (for ref., see ref. 20). The process does however not consist of a random disaggregation [24] The different membrane proteins are solubilized in various rates and evidence for a selective solubilization of membrane proteins by sodium dodecylsulphate has been presented [25]. It is conceivable that the proteins removed from the membranes in the incubation with sodium dodecylsulphate are relatively loosely attached, whereas $(Na^+ + K^+)$-ATPase remains embedded in the bilayer because it is more firmly associated with the membrane structure and because ATP to some degree prevents binding of sodium dodecylsulphate to its polypeptide chains. It is in agreement with this hypothesis that the enzyme remains bound to rapidly sedimenting membrane fragments throughout the purification procedure and that only small amounts of detergent are bound to these membranes. Nearly all of the sodium dodecylsulphate added to the incubation medium is recovered in the fractions containing the solubilized proteins and the detergent forms less than 0.5 % of the mass of the purified preparation

This content of detergent is very low in comparison with that found in preparations isolated after solubilization of the enzyme In the preparation by Nakao et al [3], the content of Lubrol is 5–13 times that of the protein [26]. Lubrol forms 16–30 % of the total mass of the preparation purified by Hokin et al [2, 27]. This means that special precautions must be taken in work with the solubilized preparations because Lubrol interferes both with the enzyme assay and with the analysis of phosphate and protein [28]. The significance of the presence of detergents in the preparations for the catalytic function of $(Na^+ + K^+)$-ATPase is discussed later [22].

ACKNOWLEDGEMENTS

I wish to thank Mrs Janne Petersen and Mrs Karen Siesing for excellent technical assistance, Mr Hilmar Hald for collecting kidneys at the slaughterhouse and Mr Ivan Bogner for careful maintenance of the centrifugal equipment. The study was supported by grants from Carlsbergfondet and Aarhus Universitets Forskningsfond.

REFERENCES

1 Swanson, P D , Bradford, H. F and McIlwain, H (1964) Biochem J 92, 235–247
2 Hokin, L E , Dahl, J L , Deupree, J P , Dixon, J F , Hackney, J F and Perdue, J F (1973) J Biol Chem 248, 2593–2605
3 Nakao, T , Nakao, M , Nagai, F , Kawai, K , Fujihara, Y , Hara, Y and Fujita, M (1973) J Biochem (Tokyo) 73, 781–791
4 Towle, D W and Copenhaver, J H (1970) Biochim Biophys Acta 203, 124–132
5 Kyte, J (1971) J Biol Chem 246, 4157–4165
6 Lane, L K , Copenhaver, J H , Lindenmayer, G E and Schwartz, A (1973) J Biol Chem. 248, 7197–7200
7 Jørgensen, P L and Skou, J C (1969) Biochem Biophys Res Commun 37, 39–46
8 Jørgensen, P L and Skou, J C (1971) Biochim Biophys Acta 233, 366–380
9 Jørgensen, P L , Skou, J C and Solomonson, L P (1971) Biochim Biophys Acta 233, 381–394
10 Jørgensen, P L (1972) in Role of Membranes in Secretory Processes (Bolis, L , Keynes, R. D and Wilbrands, W , eds), pp 247–255, North-Holland, Amsterdam
11 Lowry, O H , Rosebrough. N J , Farr, A L and Randall, R J (1951) J Biol Chem 193. 265–275
12 Ballentine, R (1957) in Methods in Enzymology (Colowick, S P and Kaplan, N O , eds), Vol 3, pp 984–995, Academic Press, New York
13 Weber, K and Osborn, M (1969) J Biol Chem 244, 4406–4412
14 Shuster, L (1971) in Methods in Enzymology (Jacoby, W B , ed), Vol 22, pp 412–433, Academic Press, New York
15 Jensen, J and Nørby, J G (1971) Biochim Biophys Acta 233, 395–403
16 Bishop, B S (1966) in The Development of Zonal Centrifuges and Ancillary Systems (Anderson, J G , ed), Natl Cancer Inst Monograph, Vol 21, pp 175–188, Natl Cancer Inst , Bethesda, Md
17 Johnson, C , Attridge, T and Smith, H (1973) Biochim Biophys Acta 317, 219–230
18 Kyte, J (1971) Biochem Biophys Res Commun 43, 1259–1265
19 Collins, R C and Albers, R W (1972) J Neurochem 19, 1209–1213
20 Guidotti, G (1972) Annu Rev Biochem 41, 731–752
21 Grisham, C M and Barnett, R E (1972) Biochim Biophys Acta 266, 613–624
22 Jørgensen, P L (1974), Biochim Biophys Acta 356, 53–67
23 Makino, S , Reynolds, J A and Tanford, C (1973) J Biol Chem 248, 4926–4932
24 Razin, S (1972) Biochim Biophys Acta 265, 241–296
25 Ne'eman, Z , Kahane, I and Razin, S (1971) Biochim Biophys Acta 249, 169–176
26 Kawai, K , Nakao, M , Nakao, T and Fujita, M. (1973) J Biochem (Tokyo) 73, 979–991
27 Uesugi, S , Dulak, N C , Dixon, J F , Hexum, T D , Dahl, J L , Perdue, J F and Hokin, L E (1971) J Biol Chem 246, 531–543
28 Nakao, T , Nakao, M , Mizuno, N , Komatsu, Y and Fujita, M (1973) J Biochem (Tokyo) 73, 609–619