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# PURIFICATION AND CHARACTERISTICS OF ($Na^+$,$K^+$)-ATPase FROM CANINE KIDNEY BY ZONAL CENTRIFUGATION IN SUCROSE DENSITY GRADIENT

YUTARO HAYASHI, MAMIKO KIMIMURA, HARUO HOMAREDA and HIDEO MATSUI

*Department of Biochemistry, Kyorin University School of Medicine, Mitaka, Tokyo 181 (Japan)*

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## Summary

Microsomes were prepared from the outer medulla of canine kidney. Partially purified preparation of ($Na^+$,$K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) was obtained by solubilization of microsomes with sodium deoxycholate, and by precipitation with dilution. The deoxycholate-enzyme thus obtained was further purified by incubation with sodium dodecyl sulphate in the presence of ATP followed by a single zonal centrifugation in a sucrose density gradient by the method of Jørgensen [(1974) Biochim. Biophys. Acta 356, 36—52]. The ($Na^+$,$K^+$)-ATPase was purified to a specific activity of 1600—1800 $\mu$mol $P_i \cdot h^{-1} \cdot mg^{-1}$ protein. The yield was 20 mg per single centrifugation with a zonal rotor. Electron microscopy showed that the sectioned pellet of the purified enzyme contained exclusively membranous fragments in contrast with membranous vesicles of starting microsomes. Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed that almost all proteins were accounted for by two polypeptides with molecular weights of 105 000 and 58 000, and that the mass ratio of the large to the small polypeptide was 82 : 18.

## Introduction

To elucidate the relationship between structure and function of the $Na^+$,$K^+$-ATPase (ATP phosphohydrolase, EC 3.6.1.3), it is desirable to obtain highly purified and active preparation of the enzyme in high yield. Various approaches have been developed to purify the enzyme using detergents and chaotropic agents, singly or combinatively, such as deoxycholate [1—5], Lubrol [6,7], sodium dodecylsulphate [8,9] and NaI [10,11]. Highly purified preparations have been reported with membrane fractions from brain [7], kidney [3—5,8], rectal gland [12], electric organ [13] and nasal salt gland [9]. Mammalian

brain and kidney, because they are rich in the $Na^+,K^+$-ATPase and readily obtainable compared to rectal gland of dogfish and electric organ of electric eel, are the most routinely used sources. Microsomes, however, from brain were very heterogeneous [14,15], and the $Na^+,K^+$-ATPase preparation purified from it was extremely labile [7].

Kyte [4] purified the deoxycholate-activated microsomes from the canine renal outer medulla to a specific activity of 800 units per mg (units refer to μmol ATP hydrolyzed per h) by gel-filtration on agarose with a yield of 0.5 mg. Later, Lane et al. [5] obtained the purified $Na^+,K^+$-ATPase from the same tissue source with a specific activity of about 1500 units per mg with a yield of 16—18 mg per 100 g of the tissue. In their procedure, the partially purified enzyme (deoxycholate-enzyme), prepared by precipitation from the deoxycholate-solubilized microsomes with dilution, was further purified by ammonium sulphate fractionation in the presence of deoxycholate and cholate.

More recently, Jørgensen [8] showed a simplified purification procedure using outer medulla of rabbit kidney, in which microsomal fraction was purified to specific ATPase activities of 1900—2200 units per mg by incubation with sodium dodecyl sulphate and ATP, followed by a single zonal centrifugation in a sucrose density gradient.

In the present work, we report an improved purification procedure of the $Na^+,K^+$-ATPase from the outer medulla of canine kidney. The deoxycholate-enzyme prepared mainly by the method of Lane et al. [5] was treated with sodium dodecyl sulphate, then subjected to the zonal centrifugation according to the method of Jørgensen [8]. This combined method of deoxycholate-solubilization procedure and sodium dodecyl sulphate-zonal centrifugation provided a highly purified preparation with specific activity of 1600—1800 units per mg in yield of 15—25 mg per single centrifugation. The characteristics of the preparation are also described.

## Experimental

*Tissue source and isolation of microsomes*

Canine kidneys, provided by Heart Institute of Japan, Tokyo Women's Medical College, were packed in ice immediately after removal from anesthetized dogs and brought to the laboratory within 4—5 days thereafter, then stored at −20°C until used. The following procedure was performed at 0—4°C. Stocked kidneys were thawed in the preparation medium containing 0.25 M sucrose and 1 mM Tris/EDTA (pH 7.2 at 20°C) and the outer medulla removed. On an average, 4.7 g of the outer medulla was obtained from one kidney of about 40 g.

The isolated outer medulla was then minced with scissors and homogenized in 10 vols. of the preparation medium using a Polytron (Kinematica, PT 10) for approximately 100 s at a dial setting of 7. The homogenate was centrifuged at 10 000 rev./min in a Sorval GSA rotor for 35 min. The supernatant was filtered through 8 layers of cheesecloth and the pellet was homogenized again by Polytron in 5 vols. of the same preparation medium, then sedimented. The combined supernatants from the two centrifugations were spun at 25 000 rev./min in Beckman Type 35 rotor for 30 min. The resultant pellet was suspended

in approximately 0.7 ml of 0.25 M sucrose containing 25 mM imidazole · HCl (pH 7.1 at 20°C) and 1 mM Tris/EDTA per g of the starting material by homogenization with Teflon pestle homogenizer, frozen quickly in a solid $CO_2$/acetone mixture and stored at −20°C. On an average, 10 mg protein of microsomes were obtained from 1 g of outer medulla. The ouabain-sensitive ATPase activity of microsomes obtained varied between 34 and 147 units per mg with the duration of storage of kidney used. The kidneys stored for the longer period yielded microsomes with the higher activity. On the other hand, the ouabain-insensitive activity was constantly about 16—20 units per mg.

*Preparation of the deoxycholate-enzyme*

Deoxycholate treatment of the microsomes was done by the method of Lane et al. [5] with slight modifications. The microsomes (about 5.5 mg/ml) were incubated with 2.5—3.6 mg/ml of deoxycholate in 0.5 M NaCl containing 0.04 M KCl, 25 mM imidazole · HCl (pH 6.9 at 20°C), 1 mM Tris/EDTA and 10% sucrose at 0°C for 30 min, and then centrifuged at 40 000 rev./min for 30 min in a Beckman Type 42.1 rotor. Prior to the deoxycholate treatment, the optimal concentration of deoxycholate to solubilize and activate the microsomal enzyme were found in each preparation as shown in Fig. 1. Glycerol (final concn. 20%) was added to the clear supernatant. After incubation for 15 min in an ice bath, the mixture was diluted with an equal volume of 25 mM imidazole · HCl (pH 7.5 at 20°C) containing 1 mM Tris/EDTA and incubated for additional 45 min to precipitate the enzyme. The mixture was centrifuged at 35 000 rev./min for 100 min in a Beckman Type 35 rotor. The washed deoxycholate-enzyme was suspended in 1 mM Tris/EDTA (pH 7.2) to a final protein concentration of approximately 4 mg/ml above, and stocked at −80°C.

*Preparation of the dodecylsulphate-enzyme by zonal centrifugation*

The deoxycholate-enzyme was incubated for 30 min at 20°C at a protein concentration of 1.30—1.60 mg/ml with 0.45—0.70 mg/ml of dodecyl sulphate, 3 mM Tris/ATP, 2 mM Tris/EDTA and 50 mM imidazole · HCl (pH 7.5 at 20°C) as described by Jørgensen [8]. The optimal concentration of dodecyl sulphate to treat the enzyme was determined beforehand for each preparation of the deoxycholate-enzyme as shown in Fig. 2. The mixture (50 ml), and subsequently the overlay solution (50 ml of 25 mM imidazole · HCl containing 1 mM Tris/EDTA, pH 7.5), were injected by a syringe equipped with a density gradient fractionator (ISCO, Model 640) at a rate of 5 ml/min into a zonal rotor of Beckman Ti-14. A linear sucrose gradient was formed by a gradient pump (ISCO, Model 380) from 20 to 40% (w/v) sucrose in 25 mM imidazole · HCl (pH 7.2 at 20°C) and 1 mM Tris/EDTA, and delivered into the rotor at a rate of 20 ml/min prior to the injection of the sample. The rotor was centrifuged at 48 000 rev./min for 2 hrs by Beckman L5-65 ultracentrifuge. After centrifugation, 45 fractions of 15 ml were collected and analyzed for protein and ATPase activity. Concentration of sucrose were determined by refractive index measured with a Tsukasa refractiometer. The fractions containing the peak of $Na^+$,$K^+$-ATPase activity were diluted with 3.5 vols. of 25 mM imidazole · HCl (pH 7.5 at 20°C), 1 mM Tris/EDTA 3 mM Tris/ATP and were centrifuged for 100 min at 35 000 rev./min in a Beckman Type 35 rotor. The pellets were

washed twice by centrifugation (40 000 rev./min, 20 min), finally suspended in 1 mM Tris/EDTA (pH 7.2) to a protein concentration of approximately 3 mg/ml. The dodecyl sulphate-enzyme thus obtained was frozen quickly and stored at −80° C. The ATPase activity remained essentially unchanged for 10 days.

*Protein determination*

Protein was determined by the method of Lowry et al. [16]. A standard curve obtained for bovine serum albumin (dried over $P_2O_5$ in vacuo at 105° C for 40 h) was used for determination. When proteins were determined in the presence of sucrose, interferences by it were corrected by the use of standard protein curves determined at various sucrose concentrations.

*Assay of $Na^+$,$K^+$-ATPase activity and enzyme unit*

The reaction mixture contained, in a total volume of 0.9 ml, 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 1 mM Tris/EDTA, 50 mM imidazole · HCl buffer, 2 mM ATP and the enzyme. Ouabain (0.8 mM) was added as indicated. The reaction was carried out at 37° C and pH 7.3 (at 22° C) which was optimal for the ATPase activity of the dodecyl sulphate-enzyme. After preincubation for 4 min, the reaction was initiated by addition of appropriately diluted enzyme preparation and terminated by addition of 0.1 ml of 50% trichloroacetic acid. The liberated $P_i$ was determined by the method of Martin and Doty [17]. One unit of the ATPase activity was defined as 1 μmol $P_i$ liberated from ATP per h at 37° C.

*Sodium dodecyl sulphate-polyacrylamide gel electrophoresis*

This was done by the method of Fairbanks et al. [18], using gels containing 5.6% acrylamide and 0.21% bisacrylamide in 1% dodecyl sulphate (pH 7.4). The gels were stained with Coomasie Brilliant Blue, followed by destaining in 10% (v/v) acetic acid, according to the method of Fairbanks et al. [18], and scanned on a Gilford 2400 spectrophotometer at 560 nm. Gels to be scanned at 280 nm were treated in 25% (v/v), subsequently 10% (v/v) isopropanol containing 10% (v/v) acetic acid, followed by incubation in 10% (v/v) acetic acid in the same manner as staining and destaining processes. Periodic acid-Schiff staining were performed by the method of Zacharius et al. [19].

The electrophoresis for determination of molecular weight was performed with gels containing 5.4% acrylamide and 0.14% bisacrylamide as described by Weber and Osborn [20] with slight modifications [21].

*Electron microscopy*

After the centrifugations, the pellets of the dodecyl sulphate-enzyme and microsomes were prefixed with the cold aldehyde mixture [22] in 0.1 M phosphate buffer (pH 7.2) for 3 h, washed in the same buffer containing 8% sucrose, post-fixed with osmium, dehydrated in graded alcohols, and embedded in epon. Ultrathin sections were made, doubly stained with uranium and lead, and examined with a JEM 100 B electron microscope.

## Results

*Treatment of microsomes with deoxycholate.* The microsomes (final concentration 5.50 mg/ml) were solubilized with 3.58 mg/ml of deoxycholate at

varying concentrations of NaCl in 0.04 M KCl, 25 mM imidazole · HCl (pH 6.9 at 20° C), 1 mM Tris/EDTA, 10% sucrose at 0° C. The ATPase activity of the solubilized fraction separated by centrifugation was maximum at 0.5 M NaCl (data not shown). Fig. 1 shows that, at the fixed ionic strength of 0.5 M NaCl and 0.04 M KCl, 90% the microsomal protein were solubilized at the concentrations of deoxycholate higher than 2 mg/ml and that the ATPase activity of the supernatant was maximum at 3.0 mg/ml of deoxycholate. The optimal concentration of deoxycholate to solubilize and activate the $Na^+,K^+$-ATPase activity was varied in each preparation between 2.5 and 3.6 mg/ml.

*Treatment of the deoxycholate-enzyme with dodecyl sulphate in the presence of ATP.* Fig. 2 shows the curves for the inactivation of $Na^+,K^+$-ATPase of the deoxycholate-enzyme by dodecyl sulphate after incubation at various concentrations of the detergent. The concentration of dodecyl sulphate necessary to inactivate to 80% of the original $Na^+,K^+$-ATPase activity varied between 0.45 and 0.75 mg/ml.

These results were similar to those reported by Jørgensen [8] for the microsomes from rabbit kidney, although the activation of latent $Na^+,K^+$-ATPase at low concentrations of dodecyl sulphate was no longer observed in the deoxycholate-enzyme.

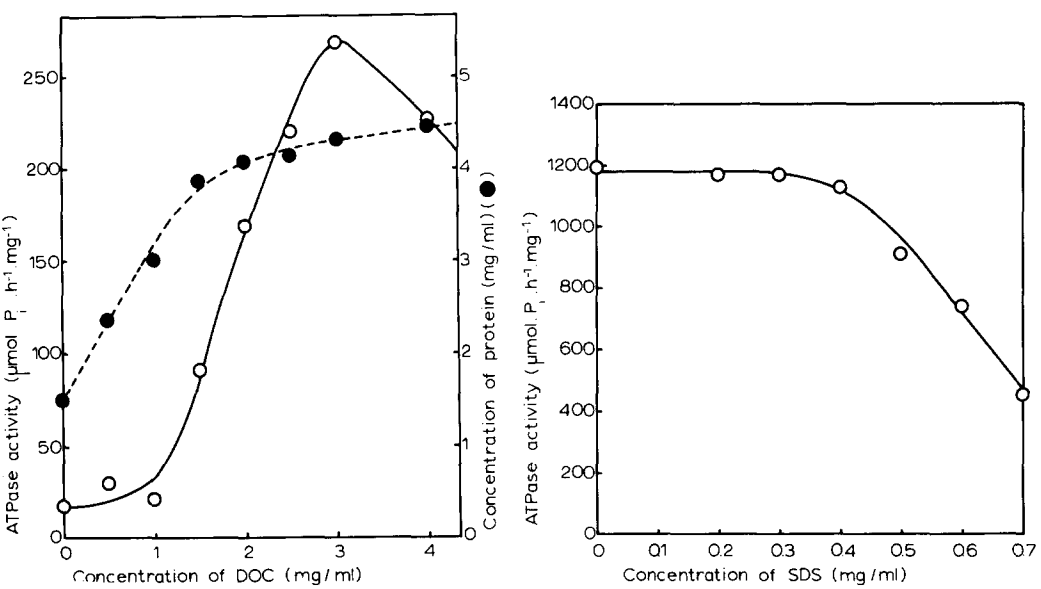


**Fig. 1. Specific ATPase activity and concentration of microsomal protein solubilized with sodium deoxycholate (DOC). Microsomes (5.32 mg/ml) were incubated with various concentrations of DOC in 0.5 M NaCl, 0.04 M KCl, 1 mM Tris/EDTA, 10% sucrose and 25 mM imidazole · HCl (pH 6.9 at 20°C) at 0°C for 30 min and then subjected to centrifugation at 40 000 rev./min for 30 min. Immediately, the supernatants were diluted 5-fold with chilled 1 mM Tris/EDTA (pH 7.2), and specific ATPase activities (○) and protein concentrations (●) were measured.**

**Fig. 2. Inactivation of $Na^+,K^+$-ATPase activity of the deoxycholate-enzyme by incubation with various concentrations of sodium dodecyl sulphate (SDS). The deoxycholate-enzyme (1.34 mg/ml) was treated with the concentrations of sodium dodecyl sulphate (SDS) shown on the abcissa in 3 mM Tris/ATP, 2 mM Tris/EDTA and 0.05 M imidazole · HCl (pH 7.5 at 25°C) at 20°C for 1 h. Aliquots of 10 μl were transferred to test tubes containing 0.89 ml of reaction mixture for assay of ATPase.**

*Zonal centrifugation of the deoxycholate-enzyme after incubation with dodecyl sulphate.* When the deoxycholate-enzyme was incubated with sodium dodecyl sulphate, and subjected to the zonal centrifugation, the $Na^+,K^+$-ATPase activity was recovered in the two peaks at sucrose solution densities of 1.12 and 1.13 g/ml (Fig. 3). In regard to the specific activity of ATPase and the dodecyl sulphate gel electrophoresis, the two peaks were not different from each other, so they were collected together and referred to as dodecyl sulphate-enzyme. After it has been collected and washed by sedimentation, the dodecyl sulphate-enzyme had an ouabain-sensitive activity of 1610 units per mg protein and an ouabain-insensitive activity of 2.85 units per mg. The yield of this enzyme preparation was 15.1 mg per a single zonal centrifugation and per 439 mg of microsomes as starting material (Table I). On average, the yield was 20 mg per single centrifugation.

*Electron microscopy of microsomes and the dodecyl sulphate-enzyme.* By electron microscopy the microsomal fractions contained mostly vesicular smooth membranous fragments (Fig. 4A). A few mitochondria and dense bodies were, however, occasionally found among the membranes. On the other hand, the dodecyl sulphate-enzyme revealed primarily triple-layered smooth membranes of uniform size, which formed singly curved or cup-shaped lines and stacked in lamellar arrangement (Fig. 4B and C). The findings obtained here are essentially similar to that of the $Na^+,K^+$-ATPase purified from the rabbit kidney observed by Jørgensen [23,24].

*Polypeptide chains involved in the dodecyl sulphate-enzyme.* Dodecyl sulphate-polyacrylamide gel stained with Coomasie Brilliant Blue showed that

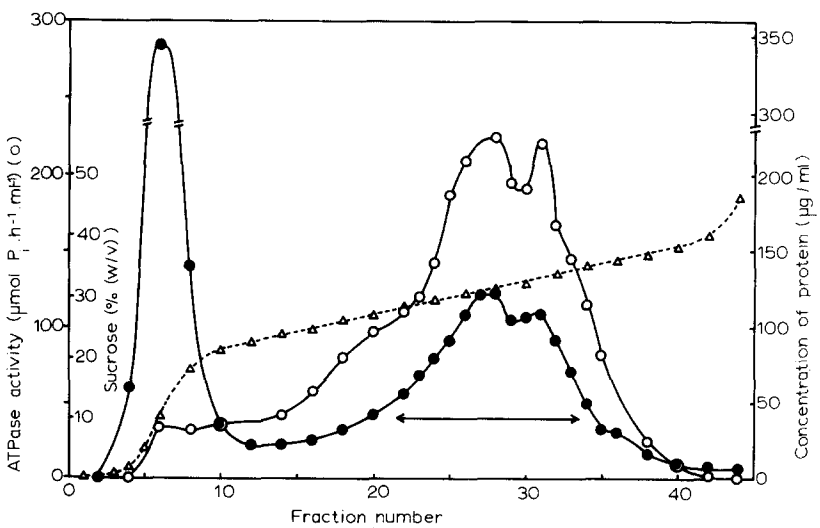


Fig. 3. Purification of $Na^+,K^+$-ATPase by zonal centrifugation of the deoxycholate-enzyme treated with sodium dodecyl sulphate (SDS). The deoxycholate-enzyme (1.49 mg/ml) was incubated with 0.54 mg/ml SDS at 20°C for 30 min in the same medium as described in the legend for Fig. 2, and 50 ml of the mixture was injected into a zonal rotor of Ti-14 and centrifuged at 48 000 rev./min for 2 h. After the centrifugation, 45 fractions of 15 ml each were collected and analyzed for protein (●), ATPase activity (○) and sucrose concentration (△). The fractions indicated by the symbol: ←——→ were collected as the SDS-enzyme.

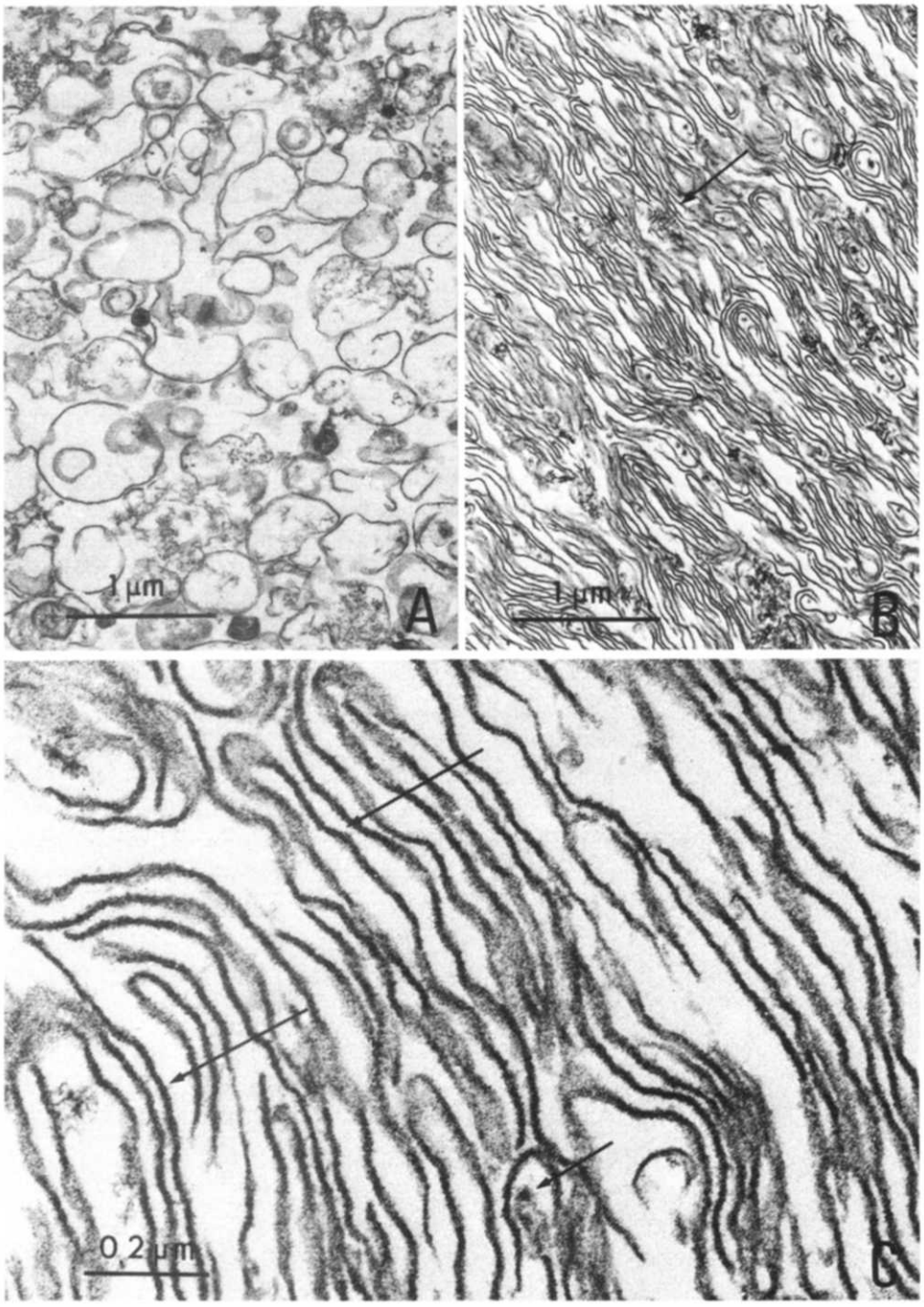


Fig. 4. Electron micrographs of sectioned pellets of the microsomal fraction (A) and the dodecyl sulphate-enzyme (B and C). A. Microsomal fractions. Smooth membranous vesicles of various size are predominantly found. A few mitochondria and dense bodies are scattered among them. Magnification: X20 000. B, Dodecyl sulphate-enzyme. Smooth membranes, singly curved or cup-shaped are conspicuous and stacked in lamellar arrangement. Particulated structures (arrow) occasionally found, may be aggregated materials of membranous fragments. Magnification: X20 000. C, Dodecyl sulphate-enzyme. Smooth membranes in lamellar arrangement at higher magnification. The triple-layered structure (long arrows) is observed. Particulated structures as seen in B reveal dotted membrane fragments (short arrow) which tend to aggregate at the end of curved lines. Magnification X84 000.

TABLE I

PURIFICATION OF $Na^+,K^+$-ATPase FROM OUTER MEDULLA OF CANINE KIDNEY USING A ZONAL ROTOR IN THE FINAL STEP

Purification procedures were described under Experimental. The dodecyl sulphate-enzyme was prepared by the single zonal centrifugation which was the same experiment described in Fig. 3.

| Step | Protein (mg) | Specific ATPase activity ($\mu$mol $P_i \cdot h^{-1} \cdot mg^{-1}$ protein) | | Total ATPase activity ($\mu$mol $P_i \cdot h^{-1}$) | |
|---|---|---|---|---|---|
| | | Ouabain-sensitive | Ouabain-insensitive | Ouabain-sensitive | Ouabain-insensitive |
| Microsomes | 439 | 101 | 15.8 | 44 300 | 6940 |
| Deoxycholate-enzyme | 70.9 | 1170 | 6.98 | 83 000 | 495 |
| Dodecyl sulphate-enzyme | 15.1 | 1610 | 2.85 | 24 300 | 43.0 |

almost all the polypeptide components of the dodecyl sulphate-enzyme were accounted for by only the two polypeptide chains with molecular weights of 58 000 and 105 000 (Fig. 5). The densitometer tracing scanned at 280 nm for the unstained gel showed essentially the same profile as that scanned at 560 nm for the stained gel (Fig. 5). The former chain, referred to as the small chain, was positive to periodic acid-Schiff staining. The latter chain, referred to as the

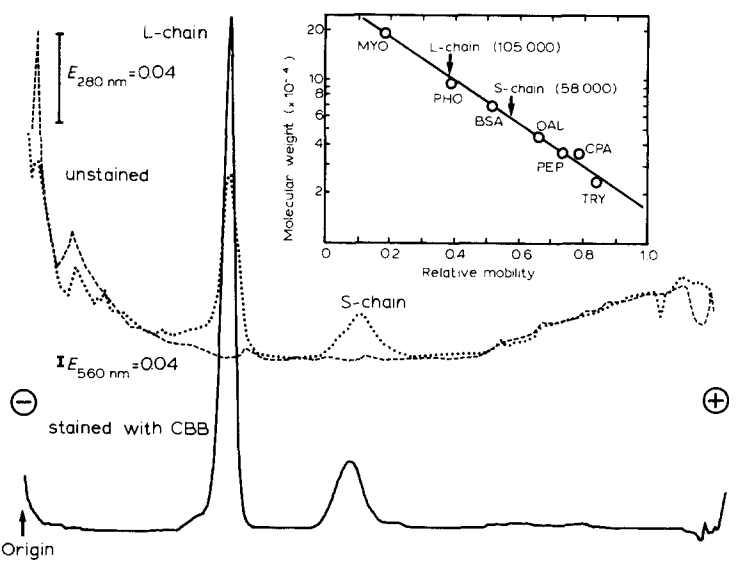


Fig. 5. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of the final purified $Na^+,K^+$-ATPase preparation, the SDS-enzyme. The gel (———; 27 $\mu$g applied) stained with Coomassie Brilliant Blue (CBB) was scanned with the spectrophotometer with a slit aperture of 0.05 X 2.36 mm in a cuvette of 5 mm light path at 560 nm. The unstained gels (· · · · ·; 6.4 $\mu$g - - - - -; 0 $\mu$g) were scanned with a slit aperture of 0.20 X 2.36 mm at 280 nm. Inset is standard curve for determination of molecular weights of the polypeptide chain involved in the enzyme by electrophoresis. Relative mobilities of protein bands were determined by using Bromphenol Blue as a tracking dye. Standard proteins were heavy chain of myosin (MYO, molecular weight 190 000), phosphorylase A (PHO, 94 000), bovine serum albumin (BSA, 67 000), ovalbumin (OAL, 45 000) Pepsin (PEP, 35 000), carboxypeptidase A (CPA 34 600) and trypsin (TRY, 23 500). L-chain, the large chain; S-chain, the small chain.

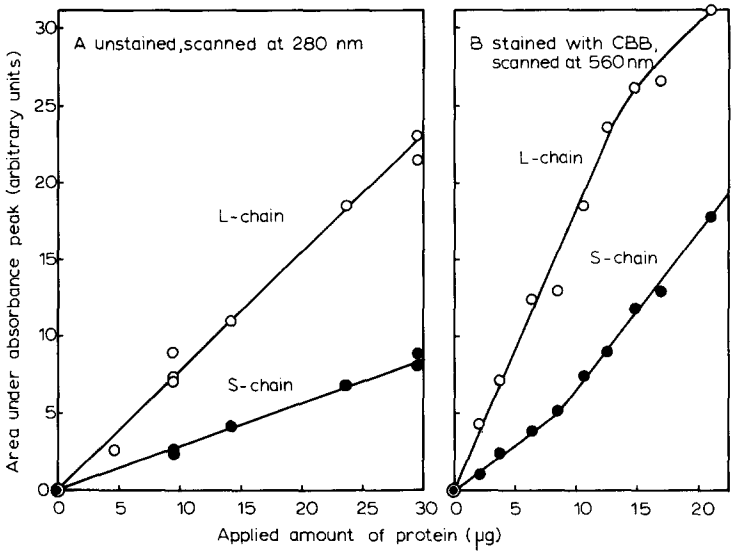


Fig. 6. Relation between amounts of the dodecyl sulphate-enzyme applied and areas under absorbance peak of the two polypeptide components separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The electrophoreses were carried out as described in Fig. 5. (A) The unstained gels were scanned at 280 nm. (B) The gels stained with Coomassie Brilliant Blue (CBB) were scanned at 560 nm. The areas were determined by weighing of the peaks cut out from charts scanned. L-chain, the large chain; S-chain, the small chain.

large chain, was not detectable by this procedure. Another periodic acid-Schiff positive band was found a little behind the tracking dye. This component was supposed to be glycolipid, because it was neither stained by the dye nor showed any absorbance at 280 nm.

*Mass ratio of the large to the small polypeptide chain in the dodecyl sulphate-enzyme.* To estimate the mass ratio of the two polypeptide chains, various amounts of the enzyme were subjected to dodecyl sulphate-gel electrophoresis, and gels stained with and without Coomasie Brilliant Blue were scanned at 560 nm and 280 nm, respectively. In the densitometry at 280 nm, areas of absorbance peak corresponding to each polypeptide chain against amounts of the enzyme applied gave straight lines over almost all ranges investigated (Fig. 6A). Area ratio of the large to the small chain was estimated to be 0.73 : 0.27 from the slopes of the two lines. The ratio of the extinction coefficients at 280 nm of the large chain to the small chain was calculated to be 0.60 : 1.0 from the tyrosyl and tryptophanyl contents reported by Kyte [25] following the methods of Wetlaufer [26]. The mass ratio of the large to the small chain was, therefore, estimated to be 0.82 : 0.18.

On the other hand, when the stained gels were scanned at 560 nm, a linear relationship between the area and the enzyme amount was obtained in regard to the large chain, whereas the plot was not linear in regard to the small chain (Fig. 6B).

## Discussion

The ouabain-sensitive activity of the microsomes was highly increased by the deoxycholate treatment (Table I). The ATPase activity of the deoxycholate-

enzyme was no longer activated by a low concentration of dodecyl sulphate (Fig. 2). These results seem to suggest that full unmasking of the latent $Na^+,K^+$-ATPase is accomplished at the step of treatment with deoxycholate and that the much activation of ATPase activity at the deoxycholate treatment is attributed to the unmasking.

Electron micrographs (Fig. 4) showed that microsomal fraction mainly consisted of vesicular memebranes whereas the dodecyl sulphate-enzyme was membranous fragments. These findings suggest that the microsomal vesicles might be degraded to small fragments by the apparent solubilization with deoxycholate and that the fragments could not be reassembled any longer to the vesicles by dilution of deoxycholate. Therefore, the unmasking of the active site for $Na^+,K^+$-ATPase might be caused by the fragmentation of the microsomal vesicles, so that substrate could become easily accessible to the active site located in the inner side of the microsomal membrane [28].

As judged from dodecyl sulphate-gel electrophoresis, about 80% of the polypeptide components in the deoxycholate-enzyme was accounted by the large chain and the small chain (data not shown), and therefore the highly effective stage for purification seemed to be the deoxycholate treatment. Lane et al. [5] showed that the further purified preparation of $Na^+,K^+$-ATPase with activity of 1600 units per mg was obtainable by the ammonium sulphate fractionation of the deoxycholate-enzyme in the presence of deoxycholate and cholate. We, however, unfortunately could not succeed in obtaining more purified enzyme than the deoxycholate-enzyme by their method, because the enzyme would be very susceptible to deoxycholate and cholate. On the other hand the deoxycholate-enzyme could be further purified by the dodecyl sulphate treatment and subsequent zonal centrifugation according to the method of Jørgensen [8]. The two peaks of $Na^+,K^+$-ATPase appeared in the sedimentation profile (Fig. 3). However, significant difference in specific ATPase activity and dodecyl sulphate-gel electrophoresis was not observed between these two fractions. Therefore, the difference in density of these enzyme may be due to the different content and composition of lipids bound to the enzyme.

Using 70—75 mg of the deoxycholate-enzyme, a yield of 20 mg per a single centrifugation with a zonal rotor was obtained as finally purified enzyme, the dodecyl sulphate-enzyme, with activity of 1600 units per mg. This yield and activity of $Na^+,K^+$-ATPase preparation from canine kidney was well comparable to those obtained using an angle rotor from rabbit kidney by Jørgensen [8]. He also showed [8] a preparation with the higher activity of 2100 units per mg in a yield of 4.4 mg per zonal centrifugation.

It was shown by dodecyl sulphate-gel electrophoresis that almost all polypeptides of the dodecyl sulphate-enzyme were accounted for by the large & small chains with mol. wts. of 105 000 and 58 000, respectively. The ratios in the area of the absorbance peak estimated by electrophoresis (Fig. 6A) and with extinction coefficient at 280 nm indicated the mass ratio of the large chain to the small chain to be 0.82 : 0.18. This mass ratio (4.6) was higher than the values reported of 2.8 [27], 2.6 [9], 2.3 [12], 1.9 [5] and 1.7 [25]. When the molecular weights described above are adopted, the molar ratio of the large to the small chain is calculated to be about one mole of the small chain to two

moles of the large chain. However, the molecular weights of the two polypeptide chains, involved in the $Na^+,K^+$-ATPase purified from mammalian kidney were reported to be in range 84 000—139 000 for the large chain and 35 000—62 000 for the small chain [4,5,8,9,25]. Since the small chain is thought to be glycoprotein, the molecular weight of 58 000 by sodium dodecyl sulphate electrophoresis may probably be overestimated [29]. As recently reported [30], the large chain tends to form high molecular weight aggregates, and thus the molecular weight obtained by other techniques may be overestimated when compared with the value obtained by dodecyl sulphate-electrophoresis. If the true molecular weights were 105 000 for the large chain, and 35 000 for the small chain estimated by gel-filtration in guanidine · HCl [25], the mass ratio of 0.82 : 0.18 would give a molar ratio of 0.7 small chains per one large chain. Therefore, until their true molecular weights, especially for the small chain, have been described, the stoichiometric relation between them cannot be concluded, i.e. whether the molar ratio of the large chain to the small chain is 2 : 1 or 1 : 1, or neither.

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