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

II. PREPARATION BY ZONAL CENTRIFUGATION OF HIGHLY ACTIVE $(Na^+ + K^+)$-ATPASE FROM THE OUTER MEDULLA OF RABBIT KIDNEYS

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SUMMARY

1. A method is described for the preparation by zonal centrifugation of highly active $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from the outer medulla of rabbit kidney.

2. Analysis of the sedimentation properties of $(Na^+ + K^+)$-ATPase shows that the enzyme activity is associated with particles of different size with a well-defined equilibrium density in sucrose gradients.

3. A large part of the $(Na^+ + K^+)$-ATPase in the tissue from the outer medulla can be collected by isopycnic-zonal centrifugation at a position in the sucrose gradient where the degree of contamination by other subcellular structures is relatively low. The specific activity of $(Na^+ + K^+)$-ATPase in the preparation obtained by isopycnic-zonal centrifugation is 900–1200 μmoles P_i per mg protein per h, and the yield is 1.5–2.5 mg protein per g tissue. The preparation is stable at 0°.

4. Further purification to a specific activity of $(Na^+ + K^+)$-ATPase of 1500 μmoles P_i per mg protein per h is achieved by rate-zonal centrifugation.

5. The purity of the preparation, calculated as the fraction of the total protein which consists of enzyme, is estimated to be 49 %.

6. The data suggest that the high purity of $(Na^+ + K^+)$-ATPase in the preparation from the outer medulla is due to the isolation of plasma membranes with a high density of enzyme sites per unit membrane area.

INTRODUCTION

The outer medulla of the rabbit and rat kidney is dominated by the broad ascending limb of the loop of Henle[1], which is known to have a large capacity for reabsorption of Na^+ against a steep electrochemical gradient[2]. Using material from this zone of rabbit kidneys, $(Na^+ + K^+)$-ATPase (ouabain sensitive; $(Mg^{2+} + Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3) was purified to a specific activity of 880–930 μmoles P_i per mg protein per h[3]. The procedure consisted in treatment of a microsomal fraction with deoxycholate and subsequent fractionation by differential centrifugation and sucrose gradient centrifugation in swinging bucket rotors.

In the present study, the possibility of using the zonal centrifugal technique was explored in an attempt to increase the yield of this preparation and to simplify the procedure. The sedimentation properties of $(Na^+ + K^+)$-ATPase in the zonal rotor were analysed, and it was investigated whether the microsomal fraction or the particulate fraction forms the best starting material for the preparation. Furthermore, the degree of contamination by other subcellular particles and the stability of $(Na^+ + K^+)$ ATPase in the preparation were determined.

It is shown that highly active preparations of $(Na^+ + K^+)$-ATPase can be obtained by an isopycnic-zonal centrifugation followed by a rate-zonal centrifugation in amounts sufficient for characterization and further purification.

EXPERIMENTAL

Tissue preparation

The tissue from the outer medulla was obtained by dissection of rabbit kidneys as described before[3,4]. For zonal gradient centrifugation two different preparations were used, the microsomal fraction and the particulate fraction. The microsomal fraction was prepared as before[4]. The particulate fraction[5] was obtained by centrifugation of 10 % (w/v) homogenates of tissue from the outer medulla in 0.25 M sucrose, 0.03 M histidine, pH 7.2 (20°), at 105000 × *g* for 60 min. The sediments were resuspended by homogenization in 1/3 of the original volume of sucrose–histidine and was stored in 5-ml aliquots for up to 2 weeks. Once thawed the aliquots were discarded.

Preparation of samples for zonal centrifugation

The microsomal fraction and the particulate fraction were incubated with deoxycholate under the conditions found optimal for demasking of all of the latent $(Na^+ + K^+)$-ATPase activity in the preparations[4]. The preparations were incubated for 45–60 min at 20° with 0.6 mg deoxycholate per ml, 2 mM EDTA, 25 mM imidazole, 3 % (w/v) sucrose, pH 7.0 (20°), at a protein concentration between 0.5 and 0.8 mg/ml. The incubation medium was used as a sample for zonal centrifugation.

Zonal gradient centrifugation

In most of the experiments a Beckmann L2-65B ultracentrifuge and a Titanium 14 rotor[6] with a total volume of 640 ml were used. The rotor was thermostated at 10° throughout the procedure. Introduction and recovery of gradient, sample and overlay, were done while the rotor was spinning at 3000 rev./min. Sucrose gradients of 450 ml were formed from light and heavy solutions of sucrose in 1 mM EDTA, 25 mM imidazole, pH 7.0 (20°), by a Beckmann Model 131 gradient pump and delivered into the rotor at a rate of 28 ml/min.

The gradient was followed by a cushion of 55 % (w/v) sucrose, 1 mM EDTA, 25 mM imidazole, pH 7.0 (20°). Sample and overlay (40–75 ml) were injected slowly with a syringe. After centrifugation, the gradient was displaced from the rotor by the gradient pump at a rate of 16 ml/min with 55 % (w/v) sucrose, 1 mM EDTA, 25 mM imidazole, pH 7.0 (20°), and 42 fractions of about 16 ml were collected in an LKB Ultrarac fraction collector. The samples were continuously monitored at 254 nm with an LKB Uvivord (5-mm cell). Density measurements were made with an Abbe, PCI refractometer at 20°. The exact volumes of the fractions were calculated from the

density and the difference in weight of the empty and full tubes. 1 mM EDTA was added to all solutions as a bacteriostatic agent[7] and in order to protect against contamination by metal cations. Digital computation of sedimentation coefficients and of the exact position of the fractions within the rotor was performed using the Gier computer and a modification in Algol of BISHOP's[8] programme.

In some experiments a Beckmann Ti-15 rotor[6] was used following the procedure described above except for modifications of volumes and pump rates to the larger volume of this rotor (1660 ml). Details are given in the legends.

Rate zonal ceutrifugation

Sucrose density gradients of 15 ml ranging from 10 to 30 % (w/v) sucrose were prepared using a mixing chamber[9] in the 1.6 cm × 10.1 cm tubes of the Beckmann SW 27.1 swinging bucket rotor. After centrifugation, the tubes were inserted in a holder and punctured horizontally by a needle near the bottom. The gradient was displaced upwards by 40 % (w/v) sucrose through an LKB Uvicord into the fraction collector (LKB Ultrarac).

Enzymic assays and other analyses

Assays for ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase (ouabain insensitive; ATP phosphohydrolase, EC 3.6.1.3) were done as described before [3,5]. NADH–cytochrome *c* reductase (NADH:cytochrome *c* oxidoreductase, EC 1.6.99.3) was assayed by the method of DALLNER[10] and cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) according to the method of WHARTON AND TZAGOLOFF[11]. K^+-nitrophenylphosphatase (K^+-dependent nitrophenyl phosphohydrolase, EC 3.1.3.1) activity was measured as described by ROBINSON[12]. RNA was determined by the orcinol method[13].

RESULTS

Initial fractionation

Table I shows the content of enzyme and of protein in the two preparations used as samples for the zonal rotor. Only the ATPase activity measured after incubation with deoxycholate is given. It is seen that the particulate fraction contained almost all of the ($Na^+ + K^+$)-ATPase activity in the homogenate, whereas 49 % of the protein in the homogenate was removed with the soluble phase or cell sap, which was devoid of ATPase activity. Accordingly, the specific activity of ($Na^+ + K^+$)-ATPase in the particulate fraction was nearly 2-fold higher than in the homogenate.

In the microsomal fraction the specific activity of ($Na^+ + K^+$)-ATPase was 5-fold higher than in the homogenate, but more than half (58 %) of the total activity of ($Na^+ + K^+$)-ATPase in the homogenate was lost during the differential centrifugation performed to obtain this preparation.

Sedimentation of ($Na^+ + K^+$)-ATPase in the zonal rotor

In order to evaluate the optimal conditions for preparation of ($Na^+ + K^+$)-ATPase in the zonal rotor, the equilibrium density and the sedimentation rate were determined for the particles with which the enzyme activity is associated.

The equilibrium density was determined in the experiment shown in Fig. 1. The

microsomal fraction was incubated with deoxycholate and subjected to zonal fractionation using a relatively large centrifugal force ($\omega^2 t = 10.4 \cdot 10^{10}$) and a sucrose gradient ranging from 1.05 to 1.17 g/ml. It is seen that a peak of ($Na^+ + K^+$)-ATPase was formed at a density of 1.14 g/ml. After this initial zonal centrifugation the three fractions with the highest activity of ($Na^+ + K^+$)-ATPase were diluted and centrifuged in an angle rotor. The pellets were resuspended and used as a sample in a prolonged

TABLE I

THE ($Na + K^+$)-ATPASE AND THE Mg^{2+}-ATPASE ACTIVITY AFTER INCUBATION WITH DEOXYCHOLATE OF THE PREPARATIONS FROM THE OUTER MEDULLA OF RABBIT KIDNEYS

Aliquots of the preparations corresponding to about 0.25 mg protein were incubated in 1 ml with 0.6 mg deoxycholate per ml, 2 mM EDTA, 25 mM imidazole, pH 7.0 (20°). After 30–35 min, 25 µl was transferred to test tubes containing 1 ml of 3 mM Mg^{2+}, 130 mM Na^+, 20 mM K^+, 3 mM ATP (Tris salt), 30 mM histidine, pH 7.5 (20°). After 10 min the reaction was stopped with 100 µl 50% trichloroacetic acid, and P_i was measured. ($Na^+ + K^+$)-ATPase was calculated as the difference in activity with and without 1 mM ouabain added to the test tubes. Mg^{2+}-ATPase is the activity in the presence of 1 mM ouabain. Mean values ± standard error of the means are given.

Prep.	*n*	*Protein (mg/g tissue)*	*ATPase*			
			($Na^+ + K^+$)- (μmoles P_i/mg protein per h)	*Mg^{2+}- (μmoles P_i/mg protein per h)*	*($Na^+ + K^+$)- (μmoles P_i/mg tissue per h)*	*Mg^{2+}- (μmoles P_i/mg tissue per h)*
Homogenate	5	116 ± 6	53 ± 6	56 ± 3	6.2 ± 0.6	6.5 ± 0.4
Particulate fraction	5	59 ± 2	95 ± 4	77 ± 4	5.6 ± 0.3	4.5 ± 0.5
Microsomal fraction	6	9.7 ± 0.3	268 ± 8	88 ± 4	2.6 ± 0.1	0.9 ± 0.1

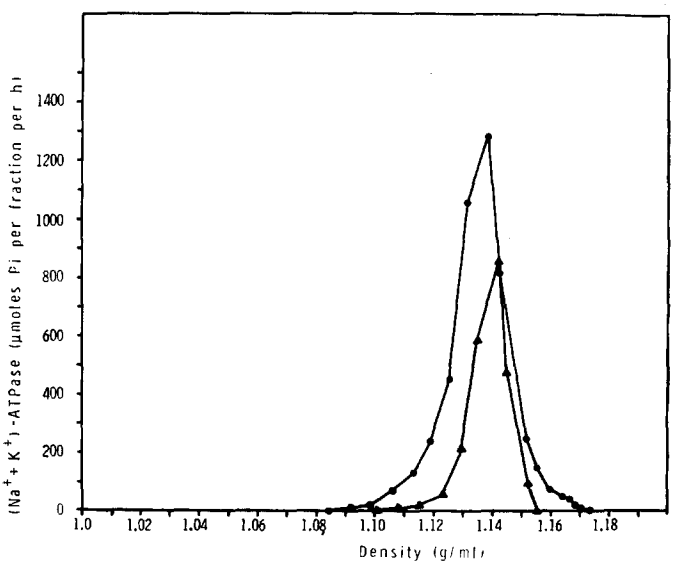

Fig. 1. Determination of the equilibrium density of ($Na^+ + K^+$)-ATPase in the sucrose gradients. ●—●, distribution of ($Na^+ + K^+$)-ATPase after zonal centrifugation of a microsomal fraction (16.8 mg protein) at 40 000 rev./min in the Ti-14 rotor for 90 min ($\omega^2 t = 10.4 \cdot 10^{10}$) using a sucrose gradient ranging from 15 to 45% (w/v). The three fractions with the highest activity of ($Na^+ + K^+$)-ATPase were diluted 3-fold and centrifuged for 90 min at 175 000 × *g* in an angle rotor. ▲—▲, distribution of ($Na^+ + K^+$)-ATPase after recentrifugation of the resuspended pellets at 40 000 rev./min for 360 min ($\omega^2 t = 38.8 \cdot 10^{10}$) in the Ti-14 rotor using the same sucrose gradient. Enzyme analysis as in Table I.

second zonal gradient centrifugation at high speed ($\omega^2 t = 38.8 \cdot 10^{10}$). It is seen that a peak of enzyme was found at almost the same density (1.137–1.142 g/ml) as after the first centrifugation. The apparent equilibrium density measured at the peak after the second centrifugation (1.142 g/ml) was used in the following calculation of equivalent sedimentation coefficients.

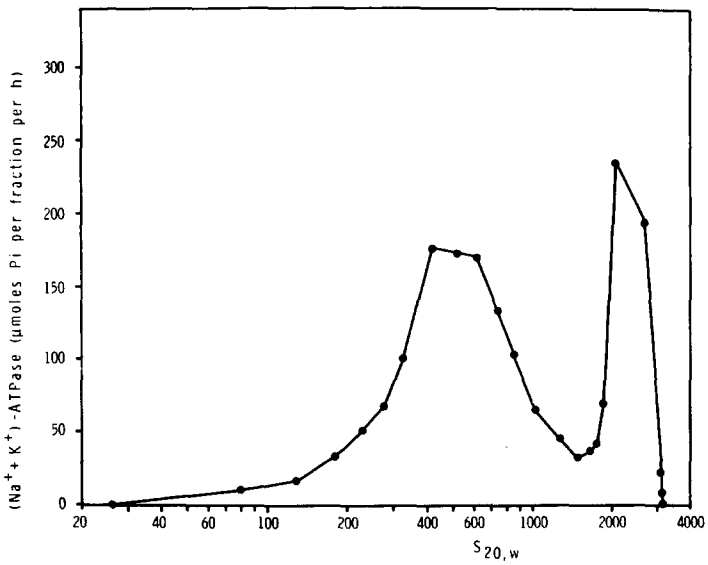

Fig. 2. Equivalent sedimentation coefficients of the particles with a high activity of ($Na^+ + K^+$)-ATPase. Particles recovered as described in Fig. 1 were used as sample in a second zonal centrifugation at 20000 rev./min for 60 min ($\omega^2 t = 16.9 \cdot 10^9$) using a gradient of lowered density (1.02–1.14 g/ml). Enzyme analysis as in Table I. Sedimentation coefficients, given in Svedberg units, were calculated as described under EXPERIMENTAL.

To determine the apparent sedimentation coefficient, the material recovered from another initial zonal gradient centrifugation was subjected to a second centrifugation in a less steep gradient and at a low total centrifugal force ($\omega^2 t = 16.9 \cdot 10^9$). After this centrifugation, the ($Na^+ + K^+$)-ATPase activity was found in two broad bands covering almost all fractions of the gradient. Fig. 2 shows the distribution of ($Na^+ + K^+$)-ATPase activity as related to the equivalent sedimentation coefficients[8], *i.e.* the sedimentation coefficient a particle with a density of 1.142 g/ml would have had if found in a given fraction. It is seen that the coefficients varied from $1 \cdot 10^2$ to $2 \cdot 10^3$ Svedberg units, suggesting a wide variation in the size of these particles.

Zonal fractionation of the microsomal fraction.

The particles with which the ($Na^+ + K^+$)-ATPase activity is associated thus had a well-defined equilibrium density in the zonal gradient, whereas their sedimentation rate varied greatly. Consequently, an isopycnic zonal gradient centrifugation was chosen as the first step in the fractionation of the microsomal fraction.

The sucrose gradient used for this fractionation and the position of the peak of ($Na^+ + K^+$)-ATPase in the zonal rotor after an isopycnic centrifugation is shown in Fig. 3. It is seen that the gradient was steep just below the sample zone and linear with

respect to rotor radius in the zone where the peak of $(Na^+ + K^+)$-ATPase was recovered.

Fig. 4 shows the distribution of ATPase activity and of protein on the fractions

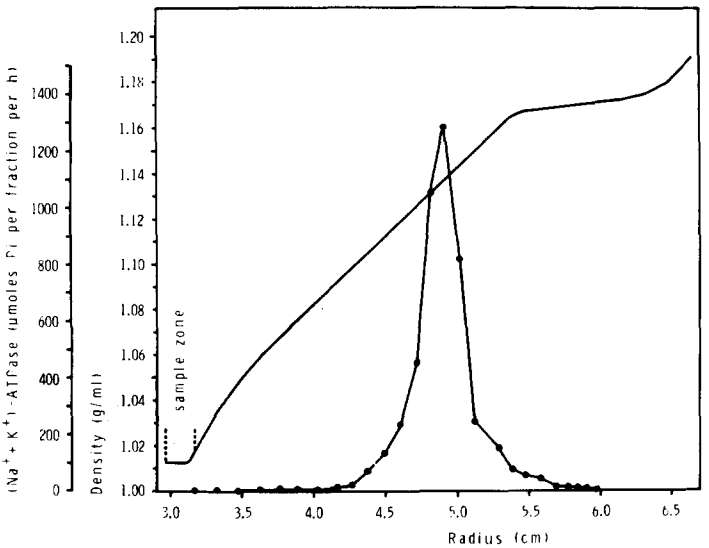

Fig. 3. The position of the sucrose gradient and of the peak of $(Na^+ + K^+)$-ATPase within the Ti-14 rotor after zonal centrifugation of a microsomal fraction (19.4 mg protein) at 40000 rev./min for 90 min ($\omega^2 t = 10.4 \cdot 10^{10}$). The preparation of the sample and the calculation of the exact position of the fractions within the rotor were done as described under EXPERIMENTAL. Enzyme analysis as in Table I.

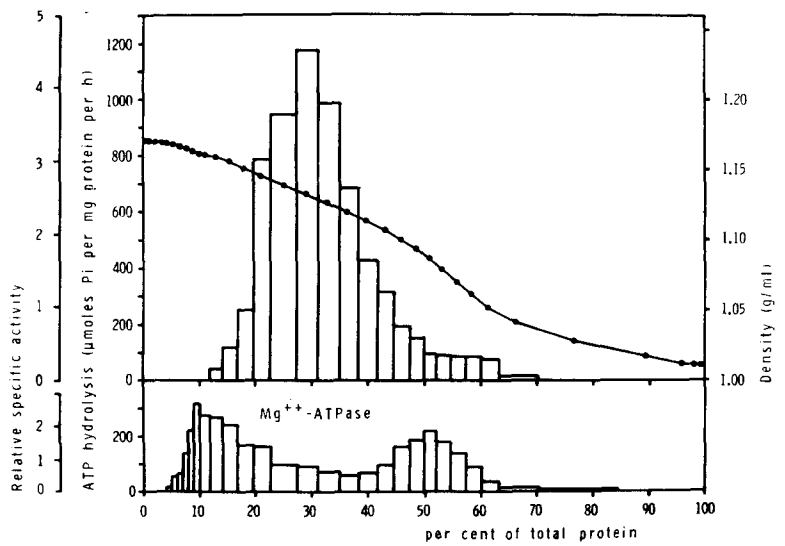

Fig. 4. Distribution of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities in fractions of the microsomal fraction obtained by zonal centrifugation as in Fig. 1. The sample contained 29.9 mg of microsomal protein and was prepared as described under EXPERIMENTAL. The plot is made in the manner adapted by DE DUVE *et al.*[14]. Relative protein content of fractions is shown on the absicssa. Specific activity of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase and the relative specific activity, *i.e.* the ratio between the specific activity of the fractions and the specific activity of the sample, are shown on the ordinate. Enzyme analysis as in Table I. ●—●, density of the fractions.

obtained after fractionation of the microsomal fraction as in Fig. 3. It is seen that a major part of the ($Na^+ + K^+$)-ATPase activity was recovered in three fractions with densities from 1.13 to 1.14 g/ml. The specific activity of ($Na^+ + K^+$)-ATPase in these fractions was between 940 and 1150 μmoles P_i per mg protein per h and about 4-fold higher than in the microsomal fraction, whereas the specific activity of Mg^{2+}-ATPase was the same as in the microsomal fraction.

Table II shows the results of experiments performed to test the capacity of the Ti-14 zonal rotor for separation of the ($Na^+ + K^+$)-ATPase activity. The rotor was loaded with increasing amounts of microsomal protein and the recovery and specific activity of ($Na^+ + K^+$)-ATPase in the three fractions at the peak were measured. Furthermore, the width at half height of the peak of enzyme was recorded. It is seen that both the specific activity and the recovery of ($Na^+ + K^+$)-ATPase became lower and that the peak of enzyme became wider as the amount of protein in the sample was increased. However, even with a sample of 55 mg protein, 50 % of the ($Na^+ + K^+$)-

TABLE II

THE INFLUENCE OF THE SIZE OF THE SAMPLE ON THE RECOVERY OF ENZYME AND PROTEIN IN THE THREE FRACTIONS WITH THE HIGHEST ACTIVITY OF ($Na^+ + K^+$)-ATPASE AFTER ZONAL CENTRIFUGATION OF THE MICROSOMAL FRACTION

The samples were prepared as described under EXPERIMENTAL and enzyme analysis was performed as in Table I. The sucrose gradients and the centrifugation were as in Fig. 3. dr denotes the width at half height of the peak of ($Na^+ + K^+$)-ATPase.

Sample		*Fractions at peak of ($Na^+ + K^+$)-ATPase activity*				
Protein (mg)	*Vol. (ml)*	*Protein mg*	*Recovery %*	*($Na^+ + K^+$)-ATPase μmoles P_i/mg protein per h*	*Recovery %*	*dr cm*
16.9	40	2.38	14.1	1219 ± 77 (3)	62.8	0.33
28.8	40	4.95	17.2	1049 ± 119 (3)	60.2	0.42
54.7	75	7.96	14.5	999 ± 50 (3)	50.0	0.60

TABLE III

THE RECOVERY OF ENZYME AND PROTEIN IN THE FRACTIONS AT THE PEAK OF ($Na^+ + K^+$)-ATPASE AFTER ZONAL CENTRIFUGATION OF THE MICROSOMAL FRACTION IN THE Ti-15 ZONAL ROTOR

A sucrose gradient of 1200 ml ranging from 15 to 45% (w/v) and an overlay of 100 ml were used. After centrifugation for 120 min at 35000 rev./min ($\omega^2 t = 10.3 \cdot 10^{10}$), the gradient was displaced from the rotor at a rate of 40 ml/min and 40 fractions af about 40 ml were collected. Enzyme analysis as in Table I.

Sample		*Fractions at peak of ($Na^+ + K^+$)-ATPase activity*			
Protein (mg)	*Vol. (ml)*	*Protein (mg)*	*Recovery (%)*	*($Na^+ + K^+$)-ATPase (μmoles P_i/mg protein per h)*	*Recovery (%)*
82.8	150	10.75	13.0	1035 ± 50	56.0
134.1	200	16.0	11.9	916 ± 34	54.2

ATPase activity could be recovered with a specific activity of about 1000 μmoles P_i per mg protein.

The output of the preparation in a single centrifugation could be increased from 5–8 mg protein to 10–16 mg protein when the Ti-14 zonal rotor (649 ml) was replaced by the larger Ti-15 rotor (1666 ml) (*cf.* Tables II and III). The specific activity and the recovery of $(Na^+ + K^+)$-ATPase in the fractions at the peak were nearly the same for the two rotors.

Zonal fractionation of the particulate fraction

During the preparation of the microsomal fraction, about half of the $(Na^+ + K^+)$-ATPase activity in the homogenate was lost (*cf.* Table I). This is readily explained by the large variation in the sedimentation coefficient of the particles with which the enzyme activity is associated (*cf.* Fig. 2) as the differential centrifugation separates mainly by differences in sedimentation rate. An attempt was therefore made to prepare the enzyme by isopycnic-zonal centrifugation of the particulate fraction which contained almost all of the $(Na^+ + K^+)$-ATPase activity in the homogenate. The results of the experiments with the particulate fraction are shown in Fig. 5 and Table IV.

It is seen from Fig. 5 that after zonal centrifugation of the particulate fraction a sharp peak of $(Na^+ + K^+)$-ATPase was formed with a position in the gradient similar to the peak found after centrifugation of the microsomal fraction.

Table IV shows the combined results of two experiments in which the three fractions at the peak of $(Na^+ + K^+)$-ATPase had been collected after the zonal centrifu-

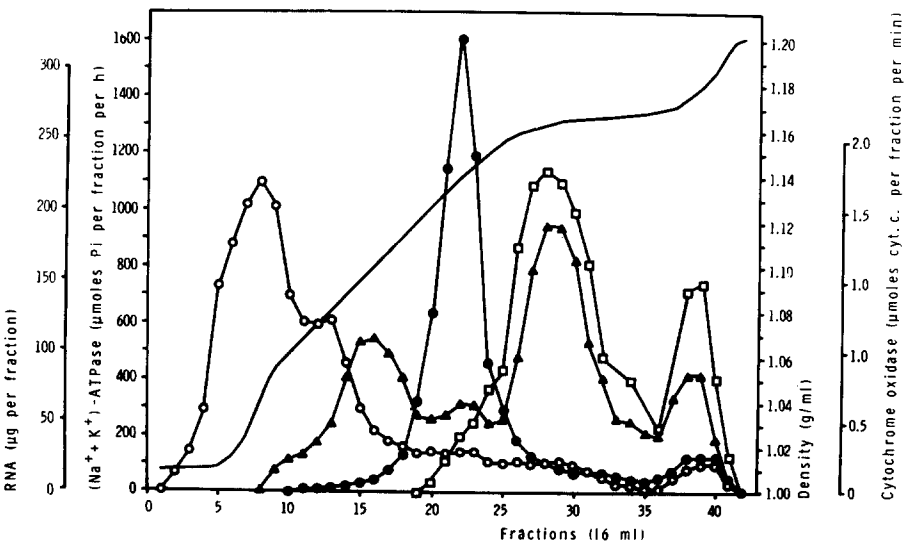

Fig. 5. The distribution of $(Na^+ + K^+)$-ATPase (●—●), RNA (○—○), NADH–cytochrome *c* reductase (▲—▲), and cytochrome oxidase (□—□) in fractions obtained by zonal centrifugation of the particulate fraction. ———, density of the fractions. The sample (80 ml) contained 72.2 mg of protein and was prepared as described under EXPERIMENTAL from 1.23 g of medullary tissue. Zonal centrifugation was performed as in Table IV. Assay for $(Na^+ + K^+)$-ATPase was as in Table I, and determination of RNA and other enzyme activities were done as described under EXPERIMENTAL.

TABLE IV

THE RECOVERY OF ENZYME AND PROTEIN IN THE THREE FRACTIONS WITH THE HIGHEST ACTIVITY OF ($Na^+ + K^+$)-ATPASE AFTER ZONAL CENTRIFUGATION OF THE PARTICULATE FRACTION

The combined results of two experiments are shown. The samples were prepared as described under EXPERIMENTAL and zonal centrifugation performed as in Fig. 1. Enzyme analysis as in Table I.

	Protein (mg)	*Recovery (%)*	*($Na^+ + K^+$)-ATPase (μmoles P_i/mg protein per h)*	*Recovery (%)*	*Mg^{2+}-ATPase (μmoles P_i/mg protein per h)*	*Recovery (%)*
Particulate fraction	206.1	100	92 (2)	100	84 ± (2)	100
Fractions at peak	8.8	4.3	1014 ± 33 (6)	47.1	98 ± 9 (6)	5.0

TABLE V

THE RECOVERY OF ENZYME AND PROTEIN BY SEDIMENTATION IN AN ANGLE ROTOR OF FRACTIONS AT THE PEAK OF ($Na^+ + K^+$)-ATPASE AFTER ZONAL GRADIENT CENTRIFUGATION OF THE MICROSOMAL FRACTION.

The fractions were diluted 3-fold with 25 mM imidazole, 1 mM EDTA, pH 7.0 (20°) and were centrifuged for 60 min at 175000 × *g* in an angle rotor. The pellets were resuspended in 1/10 of the original volume and stored at 0° in a Colotherm (Struers). Enzyme analysis as in Table I.

	Protein (mg)	*Recovery (%)*	*($Na^+ + K^+$)-ATPase (μmoles P_i/mg protein per h)*	*Recovery (%)*	*Mg^{2+}-ATPase (μmoles P_i/mg protein per h)*	*Recovery (%)*
Fractions at peak	18.0	100	995 ± 41 (12)	100	80 ± 7 (12)	100
Sediment of fractions at peak	14.0	78.1	976 ± 51 (12)	76.0	64 ± 13 (12)	69.2

gation of the particulate fraction. It is seen that 47 % of the total activity of ($Na^+ + K^+$)-ATPase activity is recovered with a specific activity similar to that obtained with the microsomal fraction. It is also seen that the relative specific activity, *i.e.* the ratio between the specific activity of ($Na^+ + K^+$)-ATPase in the fractions at the peak and the specific activity in the sample, was much higher with the particulate fraction (11.0) than with the microsomal fraction (3.9) (*cf.* Fig. 4).

The stability of ($Na^+ + K^+$)-ATPase after zonal fractionation

In the fractions from the sucrose gradient, the enzyme was unstable, and the activity of ($Na^+ + K^+$)-ATPase fell by 5–10 % per day, probably due to denaturation at the low protein concentrations.

The enzyme activity in the fractions at the peak was recovered in the sediment when the fractions were diluted and centrifuged in an angle rotor for 1 h at 175000 × *g*. It is seen from Table V that the specific activity of ($Na^+ + K^+$)-ATPase in the sediments resuspended in buffer without sucrose was the same as in the fractions from the sucrose gradient.

The enzyme in the resuspended sediments was stable when stored at 0°. Fig. 6 shows that only a minor loss of ($Na^+ + K^+$)-ATPase was observed during storage for 2 months, while the activity of Mg^{2+}-ATPase fell to about half its original level during the first month.

The content of other enzymes and of RNA in the preparations

Fig. 5 shows that the peak of $(Na^+ + K^+)$-ATPase formed after the isopycnic-zonal gradient centrifugation of the particulate fraction was recovered in fractions with a relatively low content of RNA, NADH–cytochrome *c* reductase and cytochrome oxidase. A major part of the RNA in the particulate fraction was found in the fractions near the sample zone (Fig. 5). To the left of the peak of $(Na^+ + K^+)$-ATPase in Fig. 5 a band of NADH–cytochrome *c* reductase was found, suggesting the presence of endoplasmic reticulum. At higher densities, bands of cytochrome oxidase and of NADH cytochrome *c* reductase most likely of mitochondrial origin, are seen.

It is seen from Table VI that the specific activities of cytochrome oxidase and NADH–cytochrome *c* reductase in the fractions at the peak of $(Na^+ + K^+)$-ATPase and in the sediment of these fractions were nearly the same as the activities in the samples.

TABLE VI

THE ACTIVITY OF CYTOCHROME OXIDASE AND NADH–CYTOCHROME *c* REDUCTASE AND THE RNA: PROTEIN RATIO OF THE PREPARATIONS OBTAINED BY ZONAL CENTRIFUGATION

The preparations from the microsomal fraction were obtained as in Table III and the preparations from the particulate fraction as in Table IV. Enzyme assays and RNA analysis as described under EXPERIMENTAL. Mean values ± standard error of the means are given.

	Activity (*μmoles cytochrome c/mg protein per min*)		*RNA* (*μg/mg protein*)
	Cytochrome oxidase	*NADH–cytochrome c reductase*	
Microsomal fraction	0.10 ± 0.01 (5)	0.30 ± 0.01 (3)	79 ± 9 (5)
Fractions at peak	0.13 ± 0.01 (5)	0.31 ± 0.02 (5)	36 ± 6 (5)
Sediment of fractions at peak	0.13 ± 0.01 (6)	0.33 ± 0.04 (5)	31 ± 4 (5)
Particulate fractions	0.25 ± 0.07 (9)	0.25 ± 0.05 (4)	66 ± 10 (6)
Fractions at peak	0.24 ± 0.07 (9)	0.42 ± 0.05 (6)	37 ± 7 (6)
Sediment of fractions at peak	0.10 ± 0.05 (5)	0.23 ± 0.02 (3)	22 ± 3 (7)

The K^+-dependent nitrophenylphosphatase activity was distributed in the density gradient in the same manner as $(Na^+ + K^+)$-ATPase. Fig. 7 shows that there was a linear relationship between the specific activity of the two enzymes in the fractions from the zonal gradient. The specific activity of K^+-nitrophenylphosphatase in the fractions at the peak was between 150 and 180 *μ*moles nitrophenyl per mg protein per h and the ratio $(Na^+ + K^+)$-ATPase/K^+-nitrophenylphosphatase was 6.6 ± 0.1 ($n = 13$).

Further separation of $(Na^+ + K^+)$-ATPase by rate-zonal centrifugation

The preparation obtained by the isopycnic-zonal gradient centrifugation was contaminated with mitochondria as indicated by the presence of cytochrome oxidase (*cf.* Table V). An attempt was therefore made to separate the mitochondrial fragments and the particles containing the $(Na^+ + K^+)$-ATPase on the basis of differences in their sedimentation rates.

The centrifugal force necessary to sediment particles with sedimentation coefficients ($s_{20,w}$) higher than 3000 in the gradient shown in Fig. 8 was calculated[15]. In a series of centrifugations at forces higher and lower than this calculated centrifugal force, the conditions used for the experiment in Fig. 8 were found to give the best separation.

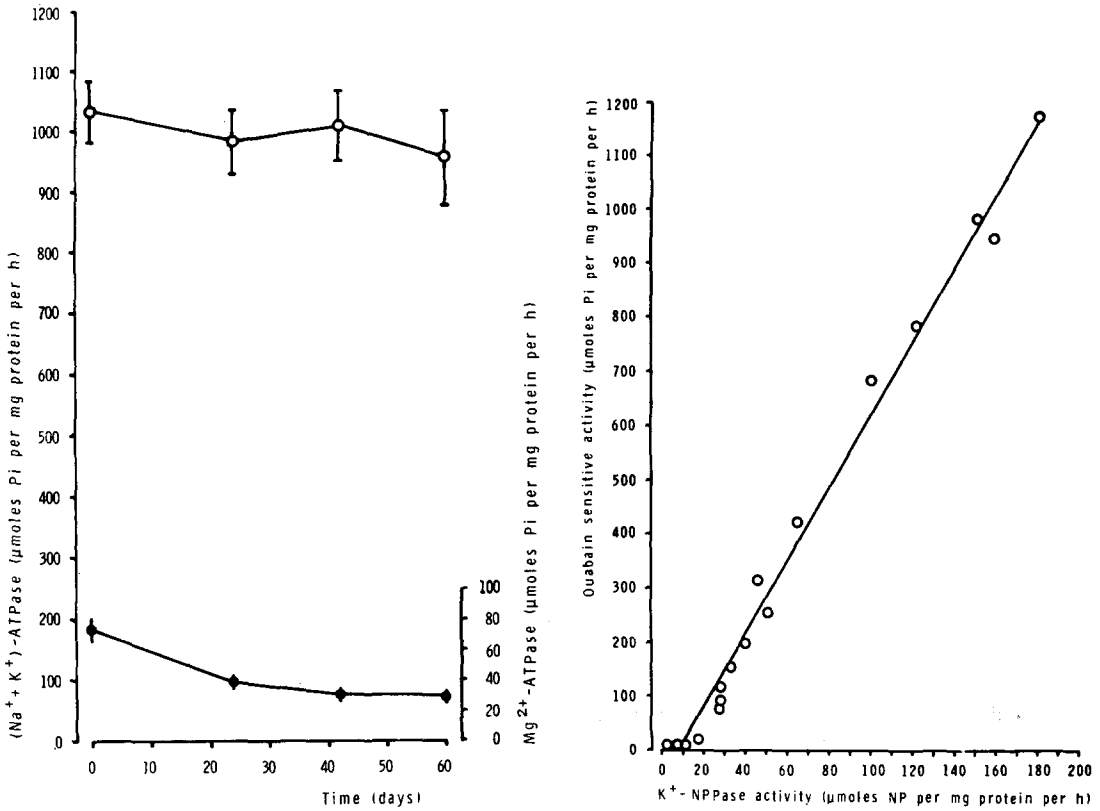

Fig. 6. The stability of ($Na^+ + K^+$)-ATPase (○—○) and of Mg^{2+}-ATPase (●—●) in the resuspended sediments of fractions at the peak of ($Na^+ + K^+$)-ATPase after zonal centrifugation of the particulate fraction. Preparation of samples and zonal gradient centrifugation were as in Table IV. Four fractions at the peak of ($Na^+ + K^+$)-ATPase were diluted and sedimented as in Table III. The pellets were resuspended in 25 mM imidazole, 1 mM EDTA, pH 7.0 (20°), to a protein concentration of 1 mg/ml and were stored at 0° in a Colotherm. At intervals of 2–3 weeks the preparations were assayed for ATPase activity as in Table I. Mean values ± standard error of the means of the four preparations are given.

Fig. 7. The relationship between the activities of ($Na^+ + K^+$)-ATPase and K^+-nitrophenylphosphatase (K^+-NPPase) in the fractions collected after zonal centrifugation of the microsomal fraction as in Fig. 4.

It is seen from Fig. 8 that most of the cytochrome oxidase activity was found in the pellet after this centrifugation. About 66% of the ($Na^+ + K^+$)-ATPase activity remained in the upper part of the tube. The specific activity of ($Na^+ + K^+$)-ATPase in these fractions was about 1500 µmoles P_i per mg protein per h, which is 1.5 times as

high as in the sample used for the centrifugation. The specific activity of Mg^{2+}-ATPase in the fractions was 50–60 μmoles P_i per mg protein per h corresponding to a ratio between ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase of 25–30. A loss of about 33 % of the ($Na^+ + K^+$)-ATPase in the sediment and in fractions with a lower specific activity could not be avoided.

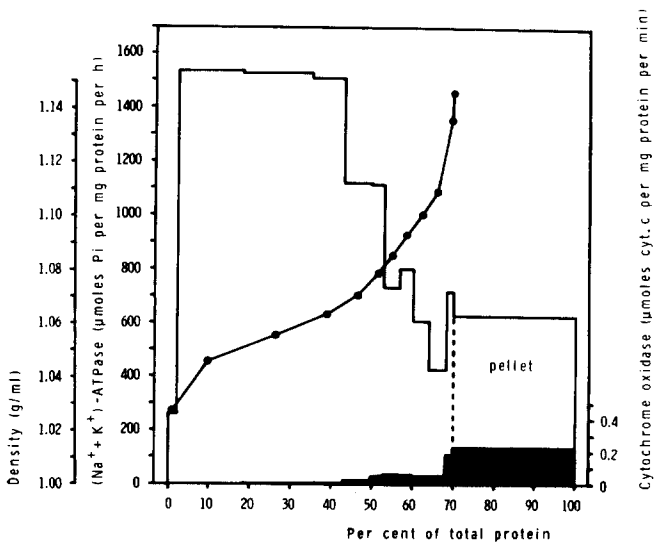

Fig. 8. Separation of ($Na^+ + K^+$)-ATPase (white columns) and cytochrome oxidase (black columns) by rate-zonal centrifugation. ●—●, density of the fractions. The sample contained 1.5 mg protein and was prepared as in Table V by sedimentation of fractions at the peak of ($Na^+ + K^+$)-ATPase after zonal centrifugation of the microsomal fraction. The sucrose gradient was formed as described under EXPERIMENTAL and centrifuged for 30 min at 13000 rev./min at 10° in the SW 27.1 swinging bucket rotor (Beckmann). Assay for ($Na^+ + K^+$)-ATPase as in Table I and assay for cytochrome oxidase as described under EXPERIMENTAL.

DISCUSSION

The results show that relatively large amounts of a preparation of ($Na^+ + K^+$)-ATPase with a specific activity of 1000–1200 μmoles P_i per mg protein per h can be obtained by a single isopycnic-zonal centrifugation of the microsomal fraction or of the particulate fraction from the outer medulla of rabbit kidneys. Further purification to a specific activity of 1500 μmoles P_i per mg protein per h is achieved by rate-zonal centrifugation.

The specific activity of ($Na^+ + K^+$)-ATPase in these preparations is 2–4 times as high as previously reported for preparations from other tissues such as brain[16–18], kidney cortex[19], salivary glands[20], and the electroplax of eels[21,22].

The yield and the specific activity of ($Na^+ + K^+$)-ATPase is higher than in the preparation obtained by fractionation of the microsomal fraction from the outer medulla of rabbit kidney by differential centrifugation and sucrose gradient centrifugation in swinging bucket rotors[3]. These differences are due to the better resolution and the larger capacity of the zonal centrifugal system used in the present study.

In the second step of the present procedure, the isopycnic-zonal centrifugation, the specific activity of ($Na^+ + K^+$)-ATPase is increased to a level 18–22 times as high as in the homogenate both when the microsomal fraction and the particulate fraction are used as starting material. A major part of the ($Na^+ + K^+$)-ATPase activity in the tissue from the outer medulla seems to be associated with particles having a well-defined equilibrium density in sucrose gradients (1.135–1.145 g/ml). These particles can be collected by isopycnic banding at a position in the gradient where the contamination by other subcellular particles is moderate. A similar distribution of ($Na^+ + K^+$)-ATPase in sucrose gradients has been found after isopycnic-zonal centrifugation of preparations from Ehrlich cells[23] and brain[24]. COTMAN *et al.*[24] used an isopycnic-zonal centrifugation for preparations of nerve-end membranes with a specific activity of ($Na^+ + K^+$)-ATPase which was 7 times as high as in homogenates of brain tissue.

The output of the preparation in a single isopycnic-zonal centrifugation is not limited by the band capacity (Table II; *cf.* ANDERSON[25]) but by the volume of the sample, because the concentration of protein in the sample must be kept below 1 mg/ml to obtain an optimum activation by deoxycholate[4]. The maximum output in a single centrifugation is therefore two to three times as high for the microsomal fraction as for the particulate fraction in agreement with the ratio between the specific activities of ($Na^+ + K^+$)-ATPase in the two preparations (Table I). The yield of the highly active preparation is lower for the microsomal fraction (1.5 μmoles P_i per mg tissue per h) than for the particulate fraction (2.5 μmoles P_i per mg tissue per h). A larger amount of tissue is therefore required when the microsomal fraction is used as starting material. However, in our experience the time required for dissection of this extra amount of tissue is less than the time used for an extra zonal centrifugation. At present, we therefore use the microsomal fraction as starting material in our routine preparations.

The analysis of the sedimentation properties shows that the ($Na^+ + K^+$)-ATPase in the highly active preparations is associated with particles of different size. Electron microscopy has shown that these particles have the morphological characteristics of plasma membranes[26]. The high activity of ($Na^+ + K^+$)-ATPase in these preparations can be due either to a high molecular activity of the enzyme or to a high density of enzyme sites per unit membrane area. The first possibility is unlikely because the molecular activity is within the range reported before for this enzyme system and because the number of enzyme sites increases in parallel with the specific activity during the preparation[4]. The data therefore suggest that plasma membranes with a high density of ($Na^+ + K^+$)-ATPase or Na^+ pump sites per unit membrane area have been isolated.

The purity of the preparation can be calculated as the fraction of the total protein which consists of enzyme. For the preparation with a specific activity of ($Na^+ + K^+$)-ATPase of 1500 μmoles P_i per mg protein per h, the purity is 49 % if a molecular weight of 250000 (ref. 27) and a molecular activity of 12850 min^{-1} (ref. 4) are used in the calculation. Higher estimates of the molecular weight of ($Na^+ + K^+$)-ATPase are available, *e.g.* 500000 (ref. 28). If this figure is used in the calculation, the purity is 98 %. Such a high value seems unreasonable in view of the nature of the preparation. The present results therefore suggest that the molecular weight of ($Na^+ + K^+$)-ATPase is lower than 500000.

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REFERENCES

1 D. McFARLANE, *J. Pathol. Bacteriol.*, 52 (1941) 17.
2 C. W. GOTTSCHALK, *Physiologist*,4 (1961) 35.
3 P. L. JØRGENSEN AND J. C. SKOU, *Biochem. Biophys. Res. Commun.*, 37 (1969) 39.
4 P. L. JØRGENSEN AND J. C. SKOU, *Biochim. Biophys. Acta*, 233 (1971) 366.
5 P. L. JØRGENSEN, *Biochim. Biophys. Acta*, 192 (1969) 326.
6 N. G. ANDERSON, D. A. WATERS, W. D. FISHER, G. B. CLINE, C. E. NUNLEY, L. H. ELROD AND C. T. RANKIN, *Anal. Biochem.*, 21 (1967) 235.
7 A. M. EL-BADRY AND J. A. BASSHAM, *Biochim. Biophys. Acta*, 197 (1970) 308.
8 B. S. BISHOP, in N. G. ANDERSON, *The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis, J. Natl. Cancer Inst. Monograph*, 21 (1966) 175.
9 R. J. BRITTEN AND B. B. ROBERTIS, *Science*, 131 (1960) 32.
10 G. DALLNER, *Acta Pathol. Microbiol. Scand Suppl.*, 166 (1963).
11 D. C. WHARTON AND A. TZAGOLOFF, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 245.
12 J. D. ROBINSON, *Biochemistry*, 8 (1969) 1348.
13 W. C. SCHNEIDER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 680.
14 C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
15 C. DE DUVE, J. BERTHET AND H. BEAUFAY, *Progr. Biophys.*, 9 (1959) 325.
16 I. C. SKOU, *Biochim. Biophys. Acta*, 58 (1962) 314.
17 W. SCHONER, C. VON ILBERG, R. KRAMER AND W. SEUBERT, *European J. Biochem.*, 1 (1967) 234.
18 A. YODA AND L. E. HOKIN, *Biochem. Biophys. Res. Commun.*, 40 (1970) 880.
19 D. W. TOWLE AND J. H. COPENHAVER, *Biochim. Biophys. Acta*, 203 (1970) 124.
20 A. SCHWARTZ AND C. A. MOORE, *Am. J. Physiol.*, 214 (1968) 1163.
21 R. W. ALBERS, G. J. KOVAL AND G. J. SIEGEL, *Mol. Pharmacol.*, 4 (1968) 324.
22 R. W. ALBERS, *Ann. Rev. Biochem.*, 36 (1967) 727.
23 D. F. H. WALLACH AND V. B. KAMAT, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 721.
24 C. COTMAN, H. R. MAHLER AND N. G. ANDERSON, *Biochim. Biophys. Acta*, 163 (1968) 272.
25 N. G. ANDERSON, *Quart. Rev. Biophys.*, 1 (1968) 217.
26 P. L. JØRGENSEN AND A. B. MAUNSBACH, in preparation.
27 G. R. KEPNER AND R. I. MACEY, *Biochim. Biophys. Acta*, 163 (1968) 188.
28 N. MIZUNO, K. NAGANO, T. NAKAO, Y. TASHIMA, M. FUJITA AND M. NAKAO, *Biochim. Biophys. Acta*, 168 (1968) 311.