Biochimica et Biophysica Acta, 692 (1982) 115–125
Elsevier Biomedical Press

BBA 71367

BASAL-LATERAL MEMBRANES FROM RABBIT RENAL CORTEX PREPARED ON A LARGE SCALE IN A ZONAL ROTOR

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(Received December 9th, 1981)
(Revised manuscript received May 26th, 1982)

Key words: Membrane fractionation; Basolateral membrane; Differential centrifugation; Zonal rotor; ($Na^+ + K^+$)-ATPase; Alkaline phosphatase; (Rabbit kidney)

Basal-lateral membranes from the renal cortex of the rabbit were isolated by sucrose gradient centrifugation in a zonal rotor which allows for a large-scale preparation of these membranes. A heterogeneous population of membranes (P_4) which contained 29% of the ($Na^+ + K^+$)-ATPase found in the homogenate of renal cortex was prepared by differential centrifugation. When pellet P_4 was subjected to centrifugation in a sucrose gradient the activity of ($Na^+ + K^+$)-ATPase, a marker for basal-lateral membranes, could be separated from enzymatic markers of other organelles. The specific activity of ($Na^+ + K^+$)-ATPase was enriched 12-fold at a density of 1.141 g/cm^3. Membranes (P_α) contained in the ($Na^+ + K^+$)-ATPase-rich fractions consisted primarily of closed vesicles which exhibited probenecid inhibitable transport of *p*-amino-hippurate. These membranes did not exhibit Na^+-dependent, phlorizin-inhibitable D-glucose transport. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins from P_α revealed at least six major protein bands with molecular weights of 91000, 81000, 73000, 65000, 47000 and 38000. A small fraction of total alkaline phosphatase found in the homogenate was found in pellet P_4. Membranes containing this alkaline phosphatase activity were distributed widely over the gradient, with peak activity found at a density of 1.141 g/cm^3. In contrast, when brush borders were subjected to gradient centrifugation under the same conditions as P_4, alkaline phosphatase was found in a narrow distribution, with peak activity at a density of 1.158 g/cm^3. The principle subcellular localization of the alkaline phosphatase found in P_4 could not be determined unambiguously from the data, but the activity did not seem to be primarily associated with classical brush borders.

Introduction

The fractionation of cellular membranes has resulted in a better understanding of the structure and function of plasma membranes [1–6]. Basal-lateral membranes of renal cortical cells have been somewhat difficult to isolate, although successful preparations from the rat and dog have been made with free flow electrophoresis [7], gradients of sucrose [8,9], and more recently with gradients of modified colloidal silica [10–12]. Except for the last method, these procedures have been cumbersome and lengthy or have resulted in rather low yields.

We wanted to develop a method to prepare, on a large scale, basal-lateral membranes from rabbit renal cortex. The method utilizes a zonal rotor, which because of its large volume, allows one to process much larger amounts of membranes than

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; SDS, sodium dodecyl sulfate.

can be processed easily by previously described techniques. High yields of basal-lateral membranes, identified by enzymatic content and transport properties, were obtained. An initial characterization of the proteins of the basal-lateral membranes was made by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Finally, evidence is presented which suggests that a small fraction of membrane bound alkaline phosphatase is probably not associated with classical brush borders.

Methods

All materials were of the highest grades commercially available. *p*-Nitrophenyl phosphate, disodium ATP, disodium succinate and sodium glucose 6-phosphate were obtained from Sigma. D-[1-^{3}H(N)]glucose, (15 Ci/mmol) and *p*-[*glycyl*-2-^{3}H]-aminohippuric acid (4.0 Ci/mmol) were obtained from New England Nuclear.

Preparation of membranes

Female New Zealand white rabbits, weighing about 2500 g were decapitated. Each kidney was perfused with 30 ml of iced buffer containing 50 mM mannitol/0.5 mM EDTA/2 mM Tris-HCl (pH 7.0) (buffer A). After the kidneys had blanched and increased in volume approx. 2–3-fold, they were excised and placed in iced buffer A. Once the kidneys had been removed from the rabbit, the renal tissue and subcellular fractions were kept at 4°C. The cortex was dissected from the rest of the kidney and homogenized in a volume of buffer A, equal in milliliters to 10-times the weight of the cortex in grams. This volume was used to resuspended other pellets described below. Homogenization was performed by an Omni-Mixer (Sorvall) for 10 min. Solid $MgCl_2$ was added to the homogenate to make a final concentration of 12 mM Mg^{2+} and the homogenate was stirred for 20 min. Then a series of differential centrifugations was carried out in an SS-34 rotor (Sorvall). The homogenate was centrifuged at 8000 rpm ($5900 \times g_{av}$) for 15 min. The resulting pellet, P_1, was resuspended in buffer containing 12 mM Mg^{2+} (as $MgCl_2$) and resuspended by 10 strokes of a Teflon-glass homogenizer having a clearance of 0.15 mm. The resuspended pellet was centrifuged at 3500 rpm ($1130 \times g_{av}$) for 15 min, and a pellet, P_2, was obtained. P_2 was resuspended in a manner similar to P_1, but in buffer containing 5.0 mM EDTA and no Mg^{2+}. Pellet P_2 was then stirred in the Omni-Mixer for 15 min at a low speed and then for 5 additional min at a high speed. This mixture was then centrifuged at 8200 rpm ($6204 \times g_{av}$) for 10 min. The supernatant, S_3, was centrifuged at 13000 rpm ($15600 \times g_{av}$) for 30 min. The pellet, P_4, was resuspended in a 9% solution of sucrose in preparation for centrifugation in a Ti-15 zonal rotor (Beckman Instruments).

400 ml of a 14% sucrose solution were loaded from the edge of the zonal rotor, followed by 150 ml of a 25% sucrose solution, followed by approx. 1200 ml of a 50% sucrose solution. 100 ml of a 9% sucrose solution were loaded from the center, followed by 15 ml of the membrane suspension, containing 300–400 mg of protein, followed in turn by 125 ml of a 9% sucrose overlay. Although the sucrose was loaded as a discontinuous gradient, the end result was a very reproducible linear sucrose gradient (see Results section). The gradient was centrifuged at 29000 rpm ($54000 \times g_{av}$) for 60 min. The rotor was unloaded by pumping a 60% sucrose solution from the edge. Fractions of 20 ml were collected.

Brush borders were prepared from rabbit renal cortex according to the method of Warnock and Yee [13].

Assays for enzymes and protein

Alkaline phosphatase (EC 3.1.3.1) was measured by a modification of the method of Bergmeyer [14,15]. Samples were preincubated for 1 h in buffer containing 50 mM mannitol/10 mM $ZnCl_2$/2 mM Tris-HCl (pH 7.0). $ZnCl_2$ was used to reverse potential inactivation of alkaline phosphatase by EDTA which was used in the buffers during the differential centrifugations. In preliminary experiments with enriched brush borders prepared from rabbits, 1.0 mM Zn^{2+} gave 84% reactivation of alkaline phosphatase in membranes exposed to EDTA. 0.1 ml of the preincubated sample was added to 0.9 ml reaction mix containing, in final concentration, 2 mM $MgCl_2$/10 mM *p*-nitrophenylphosphate/90 mM Tris-HCl (pH 9.5). The reaction was incubated for 10 min at 37°C and it was stopped by adding 2.0 ml 100

mM NaOH. Acid phosphatase (EC 3.1.3.2) was measured by a modification of the method of Bergmeyer [14]. 0.1 ml sample was added to 0.9 ml of reaction solution containing, in final concentration, 2.0 mg/ml bovine serum albumin/10 mM *p*-nitrophenylphosphate/25 mM sodium acetate (pH 5.0). The reaction was incubated for 15 min at 37°C and it was stopped by adding 2.0 ml 100 mM NaOH. The absorbance of *p*-nitrophenol was measured at 410 nm.

$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was measured by adding 0.3 ml sample to 0.2 ml reaction mix containing, in final concentration, 56 mM NaCl/2.2 mM $MgCl_2$/8.0 mM KCl/1.2 mM EGTA/1.0 mM NaN_3/8.0 mM Tris-HCl (pH 7.4)/4.0 mM disodium ATP. Each sample was assayed in the presence and absence of 2.0 mM ouabain. The reaction was stopped with 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid. This mixture was centrifuged at 3000 rpm for 10 min at 4°C in a Beckman TJ-6 centrifuge. Phosphate was measured in the supernatant according to the method of Ames [16]. 1.6 ml Ames reagent containing 6 parts 0.42% (w/v) ammonium molybdate in 1 N sulfuric acid and 1 part 10% (w/v) ascorbic acid, was added to 0.8 ml of the supernatant and incubated at 45°C for 5 min. Absorbance was read at 660 nm. The concentration of phosphate was determined by using a standard curve. The $(Na^+ + K^+)$-ATPase activity was taken as the ouabain-inhibitable generation of phosphate.

Succinate dehydrogenase (EC 1.3.99.1) was assayed according to the method of Singer [17]. 0.56 ml of sample was added to 0.31 ml of reaction mixture containing, in final concentration, 100 mM sodium succinate/200 mM potassium phosphate (pH 7.5)/100 mM potassium cyanide. This mixture was incubated at 30°C for 10 min. Then 0.03 ml of 0.05% (w/v) 2,6-dichlorophenolindophenol (DCIP)/100 mM potassium phosphate (pH 7.5)/0.10 ml of 0.33% (w/v) phenazine methosulfate were added. The final reaction was incubated at 30°C and the rate of decrease of absorbance at 600 nm was measured.

Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to Aronson and Touster [18]. A 0.05 ml sample was added to a 0.45 ml solution containing 22.2 mM sodium glucose 6-phosphate/19.4 mM histidine/1.2 mM EDTA/bovine serum albumin (10 mg/ml). The pH was adjusted to 6.5 with imidazole. The reaction was incubated for 30 min at 37°C and stopped with 1.5 ml 20% trichloroacetic acid. The mixture was centrifuged and phosphate was assayed in the supernatant as described above.

All enzymatic assays were linear with respect to concentration of enzyme and to time. A unit of activity of alkaline phosphatase and acid phosphatase is expressed as a nmol *p*-nitrophenol produced per min; a unit of activity of $(Na^+ + K^+)$-ATPase and glucose-6-phosphatase is expressed as a nmol P_i generated per min, and a unit of activity of succinate dehydrogenase is expressed as a nmol of DCIP reduced per min.

Protein was measured by the method of Lowry et al. [19]. Density was determined with a Bausch and Lomb refractometer.

Gel electrophoresis

SDS polyacrylamide gel electrophoresis of proteins from basal-lateral membranes was carried out in a slab gel (0.15 × 10 × 14 cm) by a modification of the method of Laemmli [20]. The separation gel contained 7.5% acrylamide and 0.18% bisacrylamide; the stacking gel contained 5% acrylamide and 0.11% bisacrylamide. Denaturation was accomplished by heating, in a boiling water bath, a mixture of membrane proteins and 1% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 5% (v/v) glycerol and 30 mM Tris-HCl (pH 6.7). About 150 μg of the denatured protein were electrophoresed at room temperature for 30 min at a constant voltage of 50 V and then at 80 V. The electrophoresis was stopped when the migration of the tracking dye, 0.05% (v/v) Bromphenol blue in 50% (v/v) glycerol, reached within 1 cm of the bottom of the gel. After electrophoresis, the gel was fixed and stained overnight with Coomassie blue as described by Weber and Osborn [21]. The gels were destained by several changes of a solution containing 7% (v/v) acetic acid and 10% (v/v) isopropanol.

Transport

Transport of *p*-aminohippurate and D-glucose was measured by the rapid filtration method [2]. Glucose transport was measured as previously reported [22]. The final concentration of glucose in

the reaction mixture was 0.47 mM. Transport of *p*-aminohippurate was started by combining 10 μl membrane, having a protein concentration of 24 mg/ml, with 50 μl reaction solution, containing in final concentration 0.1 mM *p*-aminohippurate/ 4.17 μCi/ml *p*-[^{3}H]aminohippurate/50 mM mannitol/5 mM $MgCl_2$/7 mM Tris-HCl (pH 7.0), in the presence and absence of 6.7 mM probenecid [23]. The reaction was carried out at 37°C and was stopped at each time point with 3 ml of an ice-cold solution of 50 mM mannitol/2 mM Tris-HCl (pH 7). Glass fiber filters, type A/E (Gelman Sciences), were used for *p*-aminohippurate transport studies. The uptakes of glucose and *p*-aminohippurate were determined by liquid scintillation in ACS (Amersham).

Electron microscopy

Membranes were fixed in 2% glutaraldehyde in buffered phosphate; post-fixed with 1% osmium tetroxide; and stained as described by Davison et al. [24].

Results

A heterogeneous subcellular fraction of renal cortical cells, pellet P_4, was obtained by differential centrifugation. Table I gives a detailed accounting of $(Na^+ + K^+)$-ATPase, a marker for basal-lateral membranes, and of alkaline phosphatase, which is associated mainly with brush borders. It has been known that divalent cations cause apparent aggregation of basal-lateral membranes, but not of brush borders [25,26]. Thus the first two centrifugations serve to separate $(Na^+ + K^+)$-ATPase from alkaline phosphatase such that pellet P_2 contains, relative to the homogenate, 70% of the total $(Na^+ + K^+)$-ATPase and only 10% of the total alkaline phosphatase. Pellet P_2 was then resuspended in buffered mannitol/5 mM EDTA to reverse the aggregating effects of Mg^{2+}. After P_2 had been treated with EDTA and centrifuged at $6204 \times g_{av}$ for 10 min, half of the $(Na^+ + K^+)$-ATPase activity found in P_2 was recovered in supernatant S_3. P_4, obtained by centrifuging S_3 at $15600 \times g_{av}$ for 30 min contained, with respect to the homogenate, 29% of $(Na^+ + K^+)$-ATPase, 16% acid phosphatase, 17% glucose-6-phosphatase, 8% succinate dehydrogenase, and 6% alkaline phosphatase (Table II). The recovery of total protein in pellet P_4 was 12% (Table I).

There was a 2-fold enrichment of $(Na^+ + K^+)$-ATPase in pellet P_4, compared to the homogenate. For alkaline phosphatase, acid phosphatase, glucose-6-phosphatase and succinate dehydrogenase, the specific activities in P_4 were respectively 0.5-, 1.4-, 1.7- and 0.5-times that of the homogenate. Thus, P_4 is a heterogeneous popu-

TABLE I

RECOVERIES OF ALKALINE PHOSPHATASE AND $(Na^+ + K^+)$-ATPase ACTIVITY

	Alkaline phosphatase activity recovered (%)	$(Na^+ + K^+)$-ATPase activity recovered (%)	Protein
Homogenate	100 [a]	100 [b]	100 [c]
S_1	35 ± 3 (4) [d]	2 ± 3 (11)	
P_1	47 ± 21 (4)	84 ± 10 (11)	
S_2	14 ± 5 (4)	2 ± 3 (11)	
P_2	10 ± 7 (4)	70 ± 12 (11)	
S_3	7 (2)	33 ± 7 (4)	
P_3	9 (2)	22 ± 12 (3)	
S_4	2 (2)	7 ± 3 (3)	
P_4	6 ± 1 (6)	29 ± 5 (7)	12%

[a] Activity, 289 ± 62 U/ml (mean ± S.D.).
[b] Activity, 714 ± 65 U/ml (mean ± S.D.).
[c] 6.47 mg/ml ± 0.55 (mean ± S.D.).
[d] Mean ± S.D. Number of experiments in parentheses.

TABLE II

RECOVERIES AND SPECIFIC ACTIVITIES OF ENZYMATIC MARKERS IN PELLET P_4 AND IN SUCROSE GRADIENT

	Recovery (%)		Specific activity (U/mg) for experiment depicted in Fig. 1		Mean enrichment [a] from 3 experiments
	P_4	Total gradient	Homogenate	Fraction 36	
$(Na^+ + K^+)$-ATPase	29 ± 5 (7) [b]	77 ± 11 (4)	74	1062	11.9 ± 2.6
Alkaline phosphatase	6 ± 1 (6)	64 ± 13 (3)	45	142	3.6 ± 1.5
Acid phosphatase	16 ± 2 (4)	53 ± 15 (4)	48	56	1.6 ± 0.4
Glucose-6-phosphatase	17 ± 6 (4)	84 ± 24 (4)	38	95	1.7 ± 0.7
Succinate dehydrogenase	8 ± 2 (4)	86 ± 39 (5)	60	81	1.3 ± 0.3

[a] In membranes at $\rho = 1.141$ g/cm^3.

[b] Mean ± S.D. Number of experiments in parentheses.

lation of organelles, or fragments of organelles, the major components of which are basal-lateral membranes, lysosomal membranes, and endoplasmic reticulum as evidenced by the recoveries of $(Na^+ + K^+)$-ATPase, acid phosphatase and glucose-6-phosphatase, respectively.

In order to process a large amount of pellet P_4 to obtain a partial purification of basal-lateral membranes, we attempted to resolve individual subcellular fractions on a sucrose gradient in a zonal rotor. Thus, P_4 was subjected to centrifugation in a linear sucrose gradient formed in a Ti-15 rotor. A sharp peak of $(Na^+ + K^+)$-ATPase activity was separated from the other major components of P_4 after centrifugation at 29000 rpm for 60 min (Fig. 1). Although there was some overlap, the membranes containing the $(Na^+ + K^+)$-ATPase were found at lower densities than fragments containing glucose-6-phosphatase and acid phosphatase (Fig. 1). The peak of the $(Na^+ + K^+)$-ATPase was found at a density of 1.141 g/cm^3 (fraction 36). The peak of glucose-6-phosphatase was found at a density of 1.169 g/cm^3. This distribution of enzymatic activity was exceedingly reproducible. The specific activities of these enzymes in fraction 36 for the experiment depicted in Fig. 1 are listed in table II. The specific activity of $(Na^+ + K^+)$-ATPase was 1062 U/mg, an enrichment of 14-fold (for the experiment pictured in Fig. 1) compared to the homogenate, and $(Na^+ + K^+)$-ATPase comprised 72% of the total Mg^{2+}-stimulated ATPase activity. This degree of purification was consistently found, as indicated by the mean enrichment in multiple preparations (Table II). Table II also lists the recovery of enzymatic activity from the entire gradient. There was good recovery of $(Na^+ + K^+)$-ATPase, alkaline phosphatase, glucose-6-phosphatase and succinate dehydrogenase. There was some loss of activity of acid phosphatase.

Although $(Na^+ + K^+)$-ATPase showed the most striking increase of specific activity on the gradient, there also was an enrichment in alkaline phosphatase at a density of 1.141 g/cm^3. The profile of

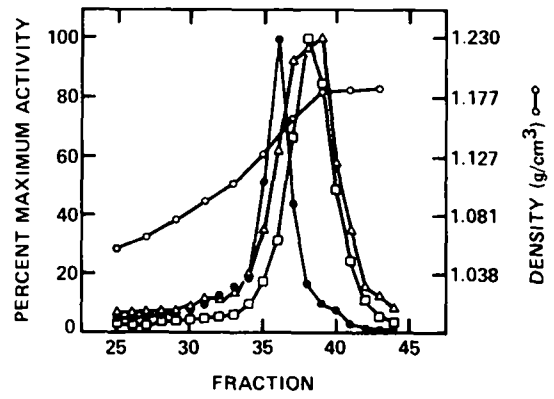

Fig. 1. Enzyme profiles from sucrose gradient in zonal rotor. Activities of membrane bound enzymes: $(Na^+ + K^+)$-ATPase (●), acid phosphatase (△), glucose-6-phosphatase (□). Density (○).

alkaline phosphatase activity from the same gradient depicted in Fig. 1 is seen in Fig. 2. At lower densities the distribution of alkaline phosphatase from pellet P_4 coincided with that of $(Na^+ + K^+)$-ATPase, but there was a broad shoulder of activity at higher densities. We then asked whether brush borders would distribute along the sucrose gradient in the same pattern as membranes of P_4 which contained alkaline phosphatase. As seen in Fig. 2, the distribution on the gradient of cell fragments from P_4 containing alkaline phosphatase was clearly different from the distribution of alkaline phosphatase when brush border membranes were centrifuged under the same conditions. The peak of alkaline phosphatase activity from brush borders was found at a density of 1.158 g/cm^3, compared to 1.141 g/cm^3, the density at which the major peak of alkaline phosphatase from P_4 was found. Also, the distribution of alkaline phosphatase from brush borders was narrower than the distribution of the enzyme associated with P_4.

Since the data on the distribution of alkaline phosphatase in density gradients indicated that the alkaline phosphatase in P_4 was not mainly associated with classical brush borders, we sought further corroboration of the absence of brush borders in the gradient's fractions by testing for the presence of sodium stimulated glucose transport, a function of brush borders [2–6]. We were particularly interested in demonstrating the absence of brush borders in fractions 31–36 (Fig. 1 and Fig. 2),

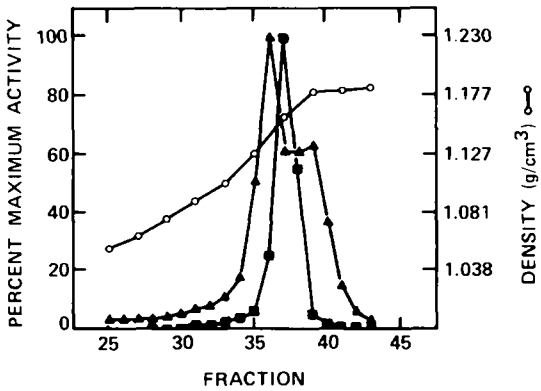

Fig. 2. Activities of alkaline phosphatase. Activity bound to membranes of P_4 wich partially overlapped the distribution of basal-lateral membranes (▲); activity bound to brush-border membranes (■). Density (○).

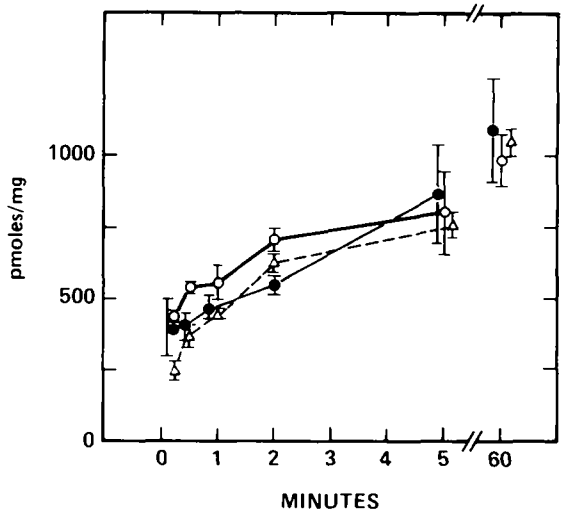

Fig. 3. Transport of D-glucose in basal-lateral membranes isolated by gradient centrifugation. Gradient fractions 31–36 were pooled and membranes were collected by centrifugation. D-Glucose uptake as a function of time was measured in the presence of an initial concentration gradient of 70 mM NaCl (○) or KCl (●), or an initial gradient of 70 mM NaCl in the presence of 0.2 mM phlorizin (△).

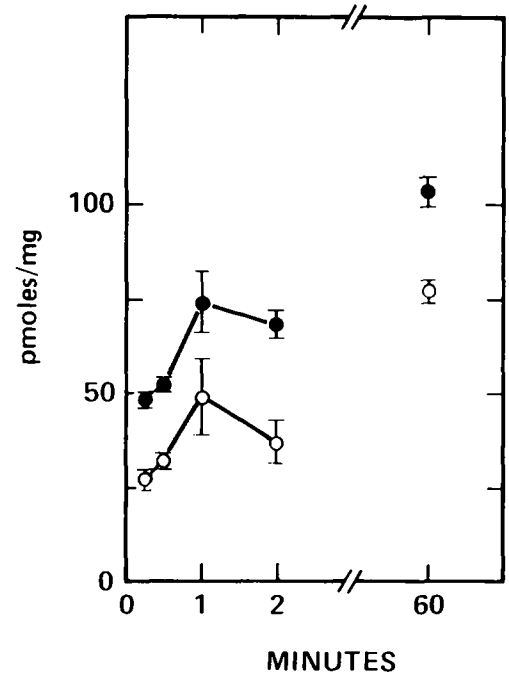

Fig. 4. Transport of *p*-aminohippurate in basal-lateral membranes isolated by gradient centrifugation. Membranes in gradient fractons 31–36 were used. The uptake of *p*-aminohippurate was measured in the presence of an initial concentration gradient of 5 mM $MgCl_2$ in the presence (○) and absence (●) of 6.7 mM probenecid.

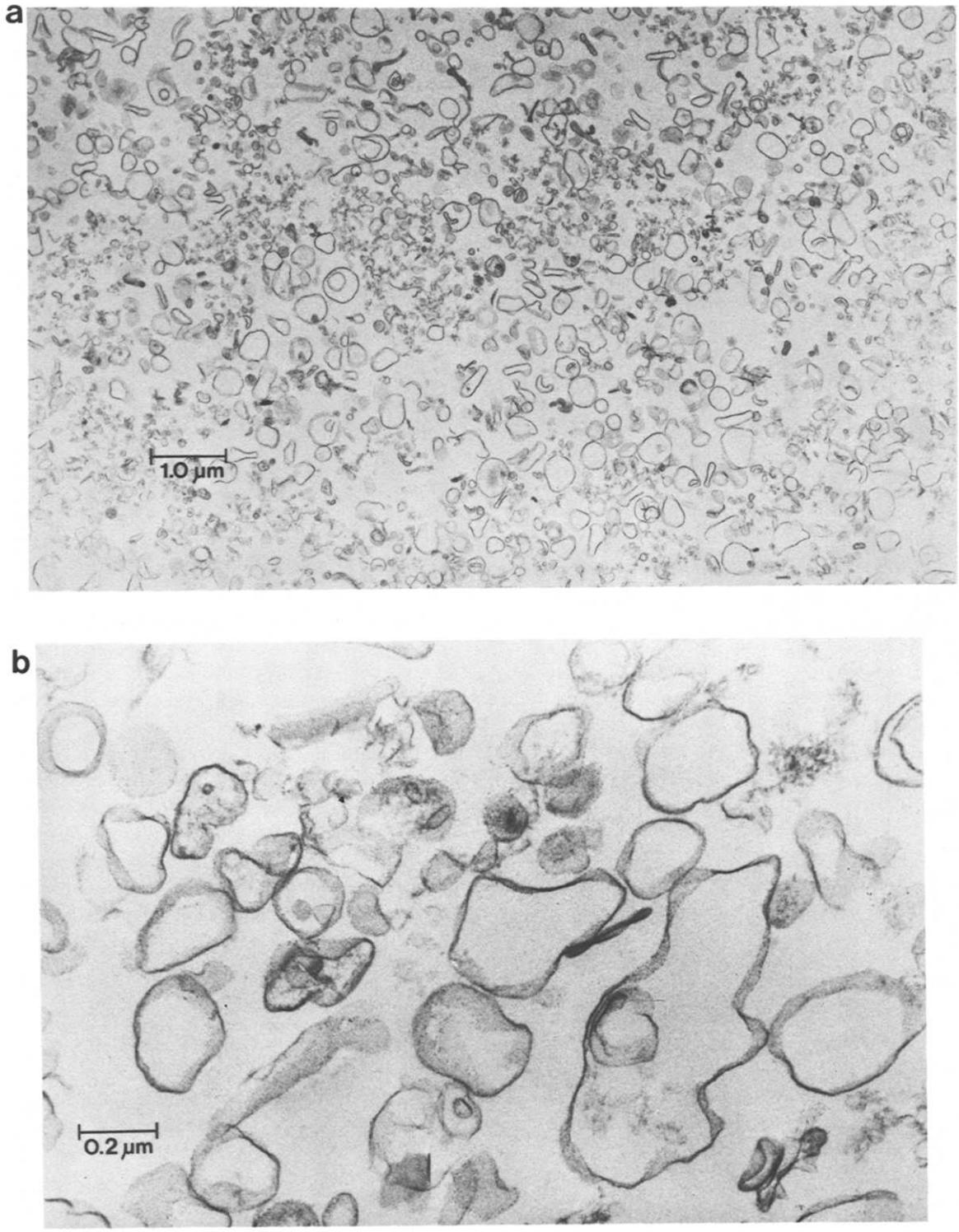

Fig. 5. Electron micrographs of membranous vesicles. Membranes in pooled gradient fractions 31–36 were collected by centrifugation. Magnifications of (a) 12850× and (b) 64240×.

since the enrichment of $(Na^+ + K^+)$-ATPase indicated that the membranes in these fractions would be useful to study the characteristics of basal-lateral membranes. Fractions 31–36 were pooled, diluted 1:4, and centrifuged at 13000 rpm for 30 min in an SS-34 rotor. The membranes in the resulting pellet (P_α) were used to study the transport of D-glucose. In the presence of an initial 70 mM Na^+ gradient, uptake at 1 and 2 min equaled 559 pmol/mg membrane protein and 712 pmol/mg, respectively (Fig. 3), The uptakes in the presence of either 0.2 mM phlorizin or an initial 70 mM gradient of potassium were virtually the same as the uptake in the presence of sodium. Also, there was no overshoot of glucose uptake in the presence of sodium. Thus the membranes in fractions 31–36 associated with a 3.5-fold enrichment of alkaline phosphatase did not exhibit transport characteristics of brush borders.

The transport of *p*-aminohippurate was studied to test the conclusion that the membranous material in fractions 31–36 originated mostly from the basal-lateral membrane. These vesicles exhibited probenecid inhibitable transport of *p*-aminohippurate, a function of basal-lateral membranes [27,28] (Fig. 4). When vesicles were incubated in 0.1 mM *p*-aminohippurate, the uptakes at 0.25 and 1 min were 47 pmol/mg protein and 75 pmol/mg, respectively. In the presence of 6.7 mM probenecid, the uptakes at 0.25 and 1 min were 19 and 26 pmol/mg. Thus there was clear inhibition of *p*-aminohippurate transport by probenecid.

The mean glucose space of the vesicles determined in six separate experiments was 1.06 ± 0.25 μl (S.E.) per mg membrane protein. In order to examine the leakiness of the vesicles, and to determine that the uptake of *p*-aminohippurate and glucose reflected transport into the intravesicular space, vesicles were added to reaction buffers containing increasing concentration of sucrose, ranging from 0.1 to 1.0 M. Uptake of *p*-aminohippurate or glucose was measured after an incubation of 120 and 60 min, respectively. The uptake declined linearly as a function of the reciprocal of the incubation media's osmolarity. Thus the vesicles appear to restrict the entry of sucrose. The total binding of *p*-aminohippurate was 22.5 pmol/mg protein. This was determined by extrapolating the line plotting *p*-aminohippurate uptake at 120 min against the reciprocal of the buffer's osmolarity to $osM^{-1} = 0$. The probenecid inhibitable binding was 6 pmol/mg at equilibrium.

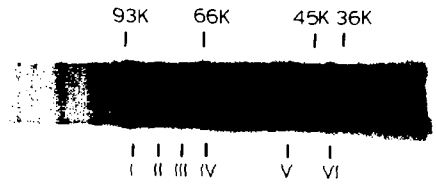

Fig. 6. SDS-polyacrylamide gel electrophoresis of proteins from basal-lateal membranes. 150 μg protein were electrophoresed in 7.5% acrylamide. Molecular weights of the major protein bands were as follows: I, 91000; II, 81000; III, 73000; IV, 65000; V, 47000; VI, 38000. Protein standards used were phosphorylase *b* (93000), bovine serum albumin (66000), ovalbumin (45000), and pepsin (36000).

Electron micrographs of membranes in P_α are shown in Fig. 5. At a magnification of 14600 (Fig. 5a) a population of membranous vesicles and some amorphous material were seen. Higher magnification (Fig. 5b) revealed that the vesicles appear generally as closed structures, approx. 0.3 μm in diameter. There are no other recognizable organelles.

The apparent molecular weights of the proteins from the basal-lateral membranes as determined by electrophoretic migration on SDS-polyacrylamide gels are shown in Fig. 6. There are numerous bands of proteins along the whole range of molecular weights resolved by the gels. There were at least six major bands, with molecular weights of 91000, 81000, 73000, 65000, 47000 and 38000.

Discussion

We have prepared a subcellular fraction from rabbit renal cortex which has enzymatic, morphologic and transporting characteristics of basal-lateral membranes. A principle feature of the method is the use of the zonal rotor. Because of the rotor's large volume, a single, easily fractionated gradient can be employed in place of multiple small gradients which are typically used in standard gradient techniques to prepare membranes. Thus basal-lateral membranes can be effi-

ciently isolated from large quantities of a relatively crude subcellular fraction.

The initial step of this preparation, consisting of mixing the homogenate of renal cortex with 12 mM Mg^{2+}, is based on the knowledge that in the presence of divalent cations basal-lateral membranes can be precipitated at rather low centrifugal forces whereas brush borders cannot be precipitated [9,25,26]. This step is a modification of the method used by Kinsella et al. in a preparation of basal-lateral membranes from the dog [9]. Thus, almost all of the $(Na^+ + K^+)$-ATPase was recovered in the moderate-speed pellet, P_1, and the low-speed pellet, P_2, whereas the activity of alkaline phosphatase was about equally divided between each pellet and its respective supernatant. The recovery of $(Na^+ + K^+)$-ATPase in supernatant S_3 indicates that the effect of Mg^{2+} on basal-lateral membranes was partially reversed when the membranes were exposed to EDTA, which presumably chelated Mg^{2+}. For example, when membranes of P_1, exposed to 12 mM Mg^{2+}, were centrifuged at only $1130 \times g$ for 15 min almost all the $(Na^+ + K^+)$-ATPase was found in the resulting pellet, P_2. When P_2 was exposed to 5 mM EDTA, resuspended and centrifuged at $6204 \times g$, about 50% of the $(Na^- + K^+)$-ATPase activity contained in P_2 was found in the supernatant, S_3. In fact, S_3 had to be centrifuged at $15600 \times g$ for 30 min in order to obtain greater than 90% recovery of the $(Na^+ + K^+)$-ATPase activity in P_4.

The recovery in P_4 of about 30% of the total $(Na^+ + K^+)$-ATPase present in the homogenate, and the recovery of almost all this activity in the sucrose gradient makes it very likely that P_4 and P_α, the membranes harvested from the gradient, contain membranes which are representative of a major membrane fraction. The major contaminants of P_4 seem to be lysosomal and microsomal (endoplasmic reticulum) fragments, as indicated by the recoveries of acid phosphatase and glucose-6-phosphatase, respectively. The sucrose gradient was used to separate a large proportion of the membranes containing $(Na^+ + K^+)$-ATPase from membranes containing these other enzymes. Although there was some overlap of the distribution of $(Na^+ + K^+)$-ATPase with acid phosphatase and glucose-6-phosphatase (Fig. 1), part of the distribution of $(Na^+ + K^+)$-ATPase was relatively free of these enzymes. Thus it was possible to pool some fractions of the gradient which were enriched for $(Na^+ + K^+)$-ATPase and which did not include a large amount of these other contaminants. Electron micrographs of P_α did not reveal any structurally intact organelles other than membranous vesicles, confirming the lack of major contamination from lysosomes. The morphology of the vesicles prepared by us is similar to that shown by others [7–9].

The basal-lateral membranes of the rabbit exhibited transport characteristics similar to those found in other species [2,15,27,28]. These membranes contain a transport system for *p*-aminohippurate which is inhibited by probenecid. The uptake of D-glucose in the membranes of P_α was minimally inhibited by phlorizin, although occasionally D-glucose uptake into membranes from P_α was inhibited in the range of 25% by phlorizin. Also, D-glucose transport in P_α was not stimulated by a gradient of Na^+. The failure of Na^+ to stimulate the transport of D-glucose and the failure of phlorizin to inhibit the transport of D-glucose are consistent with P_α being composed of mostly basal-lateral membranes [2,15,29].

The combination of enzymatic, morphologic and physiologic data indicated that pellet P_α was mainly comprised of basal-lateral membranes. The cellular origin of the alkaline phosphatase, which was present in pellet P_4 and which co-sedimented in large part with $(Na^+ + K^+)$-ATPase in the sucrose gradient, cannot be definitely determined from the data. However, when partially purified brush borders were centrifuged in like manner to P_4, the distribution of alkaline phosphatase contained in these brush borders was clearly different from the distribution of the alkaline phosphatase found in P_4 (Fig. 2). This suggests that the small amount of alkaline phosphatase in P_4 is not bound to classical brush borders. It is possible that the shoulder of alkaline phosphatase activity in fractions 37–39 (Fig. 2), obtained by centrifuging P_4 on the gradient, contains some brush borders; however, the alkaline phosphatase found at lower densities, which overlaps the $(Na^+ + K^+)$-ATPase activity, is probably not of brush border origin. This is consistent with the findings of others that alkaline phosphatase is not found exclusively in brush borders [30,31]. Also, the lack of Na^+-stimulated

transport activity on the fractions enriched for $(Na^+ + K^+)$-ATPase and the lack of microvilli in the electron micrographs of P_α strongly indicate that brush borders are not a major contaminant of P_α and that the alkaline phosphatase in P_α is from some other unidenitified source. It should be emphasized, however, that the alkaline phosphatase in P_α represents less than 5% of the total cellular alkaline phosphatase. Interestingly, in a preparation of basal-lateral membranes from the dog [9] and rat [11,12], there was a 2.5–3-fold enrichment of alkaline phosphatase in the basal-lateral fraction despite an enrichment on $(Na^+ + K^+)$-ATPase of about 20-fold [9,11,12].

From Figs. 1 and 2 it can be seen that basal-lateral membranes defined by the presence of $(Na^+ + K^+)$-ATPase [7] have a slightly lower density than brush borders defined by the presence of alkaline phosphatase [7]. This is similar to renal membranes from the rat [8,10–12,15]. In gradients of either modified colloidal silica [10,15] or sucrose [8], $(Na^+ + K^+)$-ATPase was found in membranes having a lower density than membranes containing alkaline phosphatase. the density in sucrose of basal-lateral membranes of the rabbit is in the same range as the density of basal-lateral membranes of the rat [8].

The preparation of basal-lateral membranes from the rabbit reported here is a notable improvement over the only other preparation of basal-lateral membranes from this species. Using sucrose gradients, Liang and Sacktor prepared a membrane fraction enriched only 6-fold for $(Na^+ + K^+)$-ATPase [32]. Similar to us, they found that the specific activities of acid phosphatase and glucose-6-phosphatase were not appreciably decreased by placing a crude membrane fraction on a sucrose gradient, but they gave no quantitative data.

Renal basal-lateral membranes have been prepared from the rat and dog on gradients of both sucrose [8,9] and modified colloidal silica [10–12], and by free flow electrophoresis [7]. Ebel achieved a 22-fold purification of $(Na^+ + K^+)$-ATPase from the rat, but this preparation recovered only 2.6% of the $(Na^+ + K^+)$-ATPase activity found in the homogenate [8]. When rat basal-lateral membranes were prepared by free flow electrophoresis, an 8–12 fold purification of $(Na^+ + K^+)$-ATPase was obtained [7,15,29]. When fractions enriched for $(Na^+ + K^+)$-ATPase were re-electrophoresed, this purification increased to about 15-fold [7].

One of us (RDM) has reported that modified colloidal silica (Percoll) was capable of separating membranes containing $(Na^+ + K^+)$-ATPase from membranes containing alkaline phosphatase derived from a crude membrane fraction of rat renal cortex [15]. Recently, these findings have been extended [10–12]. In these reports $(Na^+ + K^+)$-ATPase was purified about 11–22-fold. These basal-lateral fractions contained about 1% of the total protein of the homogenate, comparable to 0.9% from our preparation. As in our preparation, there was a slight enrichment of acid phosphatase [10,12] in the basal-lateral fraction. One of the preparations from rat also contained a slight purification of glucose-6-phosphatase [12]. Although Percoll is facile to use, it will form gradients only in fixed angle rotors, and not in zonal or swinging bucket rotors [33]. Because of the limiting size of fixed angle rotors, it would be difficult to scale up a preparation to a size comparable to that which can be obtained in a zonal rotor. Thus our preparation achieves a purification of $(Na^+ + K^+)$-ATPase comparable to other preparations of basal-lateral membranes. The appearance of the membranes of pellet P_α on electron microscopy was also similar to electron micrographs of basal-lateral membranes prepared by others [7–9].

Our experiments document the feasibility of using the zonal rotor to process a crude membrane fraction for the purpose of purifying a subpopulation of enzymes. The zonal rotor, because of its large volume, enables one to load a large amount of protein onto a gradient at one time. In fact, we could probably double or triple the amount of protein processed by using a larger part of the rotor for the gradient, since the gradient described in this paper extended over only one third of the rotor's total volume. We estimate that by fully utilizing the rotor, the kidneys of at least nine rabbits could be processed in one preparation. One major goal of preparing transporting membranes is the purification of proteins involved in transport. It is likely that these proteins comprise a very small fraction of the total protein associated with the membrane. The gel electrophoresis indi-

cates the magnitude of this problem, since many proteins are found even in a fraction relatively enriched for one type of membrane. Thus a severely limiting factor in attempts to purify membrane bound transport systems is the amount of starting material and it would be advantageous to have a simple method which can handle large volumes of membranes. The use of a zonal rotor will help remove this obstacle.

Acknowledgements

The authors would like to thank Dr. Klaus Bensch for performing the electron microscopy; Dr. David Warnock for helpful discussion; and Ms. Christine O'Keeffe for excellent secretarial work. This work was supported by grants from the National Institutes of Health, AM 27212 and RR 05353, and a Research Starter Grant from the Pharmaceutical Manufacturers Association Foundation. R.D.M. is a recipient of a Faculty Development Award from the Pharmaceutical Manufacturers Association Foundation. S.S.T. is supported by a training grant from the National Institutes of Health, GM 07065.

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