Biochimica et Biophysica Acta, 552 (1979) 468—477

BBA 78344

ISOLATION OF LUMINAL AND ANTILUMINAL MEMBRANES FROM DOG KIDNEY CORTEX

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(Received October 25th, 1978)

Key words: Plasma membrane isolation; (Kidney cortex membrane)

Summary

Luminal (brush border) and antiluminal (basal-lateral) membranes were isolated from canine renal cortex. The enzyme marker for luminal membrane, alkaline phosphatase was enhanced 19-fold and the antiluminal enzyme marker, $(Na^+ + K^+)$-ATPase, was enhanced 22-fold in their respective membrane preparation, while the amount of cross contamination was minimal. Contamination of these preparations by enzyme markers for lysosomes, endoplasmic reticulum and mitochondria was also low. Routinely, more than 50 mg membrane protein was isolated for each membrane. Electron micrographs showed that the membranes were uniform in size, appearance, and vesicular in nature. An examination of the orientation of these membranes showed that 76.5% of the antiluminal membranes and 86% of the luminal membranes were right-side out.

Introduction

The plasma membrane of the renal proximal tubule cell contains at least two distinct regions, the luminal (or brush border) and antiluminal (or basal-lateral) membranes, which are distinguished morphologically, functionally, and biochemically [1—6]. Studies of the mechanisms of transepithelial membrane transport, hormonal interactions, membrane enzyme activities, and other membrane phenomena, rely on separation and purification of these two types of plasma membrane. The most successful technique for plasma membrane separation is free-flow electrophoresis [3], although there are problems associated with this technique. Liang and Sacktor [5] reported separation of kidney membranes by a series of differential and sucrose gradient centrifugations. Membranes prepared in this manner were not as pure as those obtained after free-flow electrophoresis and the yields, although better, severely limited the extent of studies possible from a single preparation.

More satisfactory technique for separation of kidney plasma membranes would thus be very advantageous. This report describes a procedure for isolating luminal and antiluminal membranes from dog kidney by a method that is reproducible, relatively simple and inexpensive, and that gives high purity and good yields.

Materials and Methods

Membrane separation. Kidneys from a male or female mongrel dog were excised and placed in an ice-cold, isotonic saline (pH 7.0). After the medulla and capsule were removed, the cortex (30—40 g) was minced with scissors in about 400 ml 8% sucrose (pH 7.0). The cortex was homogenized with 10 strokes in a Dounce glass-homogenizer with a tight-fitting pestle.

The steps developed for the isolation of the membranes are outlined in Fig. 1.

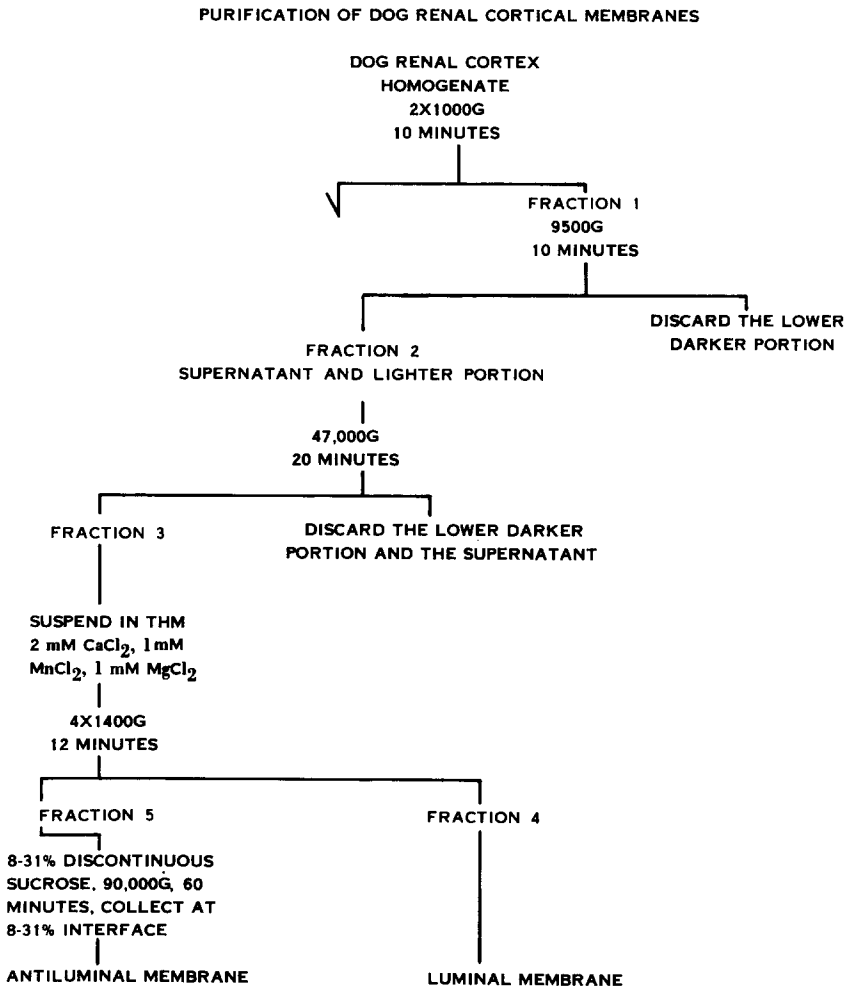

Fig. 1. Flow diagram for the isolation of luminal and antiluminal membranes. THM refers to a 25 mM Hepes buffer, 100 mM mannitol adjusted to pH 7.0 with Tris. For more complete details of the purification procedure see Materials and Methods.

All operations were carried out at 4°C. The cortical homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant saved. The pellet was re-extracted by resuspending it in 8% sucrose (one-fourth the original volume) and re-homogenized with 10 strokes in a Dounce homogenizer. Again this homogenate was spun at 1000 × *g* for 10 min, the supernatant was decanted, combined with the previous supernatant (Fraction 1, Fig. 1).

Fraction 1 was centrifuged at 9500 × *g* for 10 min. The supernatant and the soft lighter portion of the pellet were removed, combined (Fraction 2) and centrifuged at 47 000 × *g* for 20 min. The supernatant was removed and discarded, while the soft, lighter upper portion of the pellet was saved (Fraction 3).

Fraction 3 (Fig. 1) is a plasma membrane-enriched fraction and was purified further by selective precipitation of the antiluminal and contaminating membranes from the luminal membranes. Previously George and Kenney [7] used a 10 mM Tris buffer containing 10 mM $MgCl_2$ to separate plasma membranes. This method was modified by suspending Fraction 3 in medium A (25 mM Hepes (*N*-2[1]-hydroxyethylpiperazine-2-ethanesulfonic acid), 100 mM mannitol, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ adjusted to pH 7.0 with Tris) to a final protein concentration of 6—8 mg/ml, leaving the suspension in an ice bath for 1 h, and centrifuging at 1400 × *g* for 12 min. The supernatant contained luminal membranes, and was saved (Fraction 4). The pellet contained antiluminal membrane, luminal membrane, and membrane systems from other cell organelles. The luminal membrane contamination in this pellet fraction was significantly reduced by repeated extractions with Medium A as described above. The supernatant was removed and combined with Fraction 4 while the pellet was extracted again. Routinely, the pellet was re-extracted three times, resulting in an enhanced yield of purified luminal membranes, while reducing the cross contamination of luminal membranes in the antiluminal preparation. The final pellet from the extraction procedure (Fraction 5) was suspended at 6—8 mg protein/ml in Medium B (25 mM Hepes, 100 mM mannitol, titrated to pH 7.0 with Tris). The membranes contained within the pooled supernatants (Fraction 4) and the resuspended pellet (Fraction 5) were collected by centrifugation at 47 000 × *g* for 20 min, suspended in Medium B at a concentration of 5—7 mg protein/ml, and dialyzed overnight at 4°C against 50 vols. of this buffer. The dialysis step was necessary to remove the divalent cations.

Fraction 4, containing the luminal membranes, was not purified further and was ready for use.

The antiluminal membranes were separated from the others contained within Fraction 5 by the following procedure: 5-ml portions of Fraction 5 were layered over a discontinuous sucrose gradient comprised of 10 ml 31% (w/w) sucrose solution and 12 ml 8% (w/w) sucrose solution and spun at 90 000 × *g* for 60 min in a Beckman SW 25.1 rotor. The membranes at 8—31% sucrose interface were collected and dialyzed overnight against 50 vols. Medium B. This final product contained the antiluminal membranes.

Assays. $(Na^+ + K^+)$-ATPase (EC 3.6.1.4) was measured by the linked enzyme assay method of Schwartz et al. [8] at 37°C with 2.5 mM ATP and 2.5 mM Mg^{2+}. Enzyme activity in all fractions were measured in the presence of deoxycholate. The amount of total ATPase inhibited by 0.1 mM ouabain was taken

as the ($Na^+ + K^+$)-ATPase activity. Alkaline phosphatase (EC 3.1.3.1) activity was measured at ambient temperature as described [9]; and succinate dehydrogenase (EC 1.3.99.1) activity determined according to Earl and Korner [10] at 37°C. Trehalase (EC 3.2.1.28), maltase (EC 3.2.1.20), and sucrase (EC 3.2.1.26) were determined at 37°C by the method of Berger and Sacktor [11], γ-glutamyltransferase (EC 2.3.2.2) was determined at 37°C by the method of Glossmann and Neville [12]. Both glucose-6-phosphatase (EC 3.1.3.9) and acid phosphatase (EC 3.1.3.2) were determined by the method of Hübscher and West [13]. Phosphate determination were made according to Chen et al. [14] and protein estimated according to Lowry et al. [15] using bovine serum albumin as standard.

Membrane orientation. Antiluminal membrane orientation was determined by a method similar to Walter [16]. The orientation of the antiluminal membrane can be inferred from the asymmetry of the ($Na^+ + K^+$)-ATPase, where ouabain acts on the intact sodium pump from the exterior while ATP is effective only when added to the interior. By measuring total ATPase activity and ouabain-sensitive ATPase activity in intact antiluminal membranes and in disrupted membranes (0.1% deoxycholate and freezing and thawing), an estimation of the percentage of normal, everted, and sheets could be made.

Since glycoproteins are intrinsic to membranes, the luminal membrane orientation was determined by labelling exposed sugar moieties. The method used was similar to Steck and Dawson [17] and assumes that sugar moieties are found only on the exterior of the plasma membrane. 0.5 mg of both intact and disrupted (0.1% deoxycholate, freeze-thaw) luminal membrane were incubated in tritium-labeled $NaBH_4$ in a final concentration of 0.5 mequiv. $NaBH_4$/mg protein in the presence or absence of glucose and galactose oxidase (0.3 I.U.). The membranes were incubated at 37°C for 1 h and the reaction was stopped with 25% trichloroacetic acid. The proteins were collected on 0.3 μm Millipore filters (PHWP) and counted in a Beckman S-351 scintillation counter after adding 20 ml Instrabray scintillation cocktail (Yorktown Research). The ratio of counts between intact and disrupted membranes was determined after correcting for non-specific borohydride labelling by assaying the membranes without glucose and galactose oxidase. This ratio is the proportion of right-side out luminal membranes.

RNA and DNA analysis. In order to determine if the membrane preparations were contaminated with RNA or DNA, kidney cortex homogenates, luminal membranes, and antiluminal membranes from 2 dogs were extracted by the method of Schmidt and Thannhauser [18] as modified by Fleck and Munro [19] and Blobel and Potter [20]. RNA and DNA concentrations were determined by the method described by Mueller and Argyris [21].

Electron microscopy. Samples for electron microscopy were fixed with 2% glutaraldehyde in isotonic cacodylate buffer (pH 7.3) and were postfixed in 1% OsO_4 in the same cacodylate buffer, dehydrated through graded alcohols and propylene oxide, and embedded in Araldite 502. Thin sections were cut with diamond knives on LKB, stained with lead citrate and uranyl acetate and examined under a Philips 300 electron microscope operating at 60 kV.

Materials. ATP, phospho*enol*pyruvate, and pyruvate kinase/lactate dehydrogenase were purchased from Boehringer. Glucose oxidase, galactose oxidase,

NADPH, NADP, γ-glutamyl-*p*-nitroanilide, glucose-6-phosphate, *p*-nitrophenyl phosphate, ouabain (octahydrate), sodium succinate, 2,6-dichlorophenolindophenol, sodium deoxycholate, and $NaBH_4$ were all obtained from Sigma. NaB^3H_4 (661 Ci/mol) was purchased from Amersham/Searle.

Results

Isolation of antiluminal and luminal membranes

Table I shows the recovery of activity of $(Na^+ + K^+)$-ATPase (the antiluminal membrane marker) and alkaline phosphatase (the luminal membrane marker) during a typical purification. After the early centrifugation steps (Fig. 1) at $9500 \times g$ and $47\,000 \times g$, much of the contaminating membranes from lysosomes and endoplasmic reticulum were removed from Fraction 3, which retained 52% of the $(Na^+ + K^+)$-ATPase activity and 30% of the alkaline phosphatase activity. The addition of the buffer containing Mg^{2+}, Mn^{2+}, and Ca^{2+} to Fraction 3 caused a selective aggregation of antiluminal and mitochondrial membranes into Fraction 5. This latter fraction retained nearly all of the $(Na^+ + K^+)$-ATPase and about one-third of the alkaline phosphatase activities present in Fraction 3 (Table I). In the final step, the luminal membrane retains 1% of the total $(Na^+ + K^+)$-ATPase and 16% of the alkaline phosphatase activities.

It was obvious that Fraction 5 could be further purified by lowering the mitochondrial and the luminal membrane contamination. A single discontinuous sucrose gradient from 8 to 31% sucrose was found to yield the purest antiluminal membranes. Although a great deal of $(Na^+ + K^+)$-ATPase activity was lost in this step (46—12% recovery), more of the contaminating membranes were lost.

Table I also shows the protein yields of this method. 0.7% of the total crude homogenized protein was isolated in the antiluminal fraction, and 0.9% was isolated in the luminal membrane fraction. The various preparations of antiluminal membranes ranged in eight experiments from 35 to 75 mg of membrane pro-

TABLE I

TYPICAL RECOVERY OF ACTIVITY OF $(Na^+ + K^+)$-ATPase AND ALKALINE PHOSPHATASE IN THE ISOLATION PROCEDURE

The total activity is expressed in μmol inorganic phosphate produced/min.

Sample	Total mg protein	$(Na^+ + K^+)$-ATPase		Alkaline phosphatase	
		Total activity	Yield (%)	Total activity	Yield (%)
Homogenate	7649	1060	100	924	100
Fraction					
1	5500	935	88	825	89
2	2370	830	78	592	64
3	610	549	52	280	30
5	390	483	46	98	11
Antilumen	55	132	12	18	2
Lumen	70	7	1	147	16

tein or about 1.4 mg protein/g cortex. Luminal membrane protein recovery varied in eight experiments from 50 to 110 mg protein or nearly 2 mg protein/g cortex.

The activities of the membrane marker enzymes are shown in Table II. From the specific activities, one can compare the degree of enhancement between the homogenate with the final preparation. The table shows that the homogenate contained measurable amounts of all the markers for luminal (alkaline phosphatase, maltase, sucrase, trehalase, γ-glutamyltransferase), antiluminal ($Na^+ + K^+$)-ATPase, endoplasmic reticular (glucose-6-phosphatase), mitochondrial (succinate dehydrogenase), and lysosomal (acid phosphatase) membranes. The antiluminal membrane showed about a 22-fold purification in specific activity (0.11—2.40 μmol P_i/min per mg protein) of its membrane marker compared to homogenate, while the major contaminating membrane in this fraction was luminal membrane which doubled its marker enzymes. There was also some mitochondrial contamination and small amounts of endoplasmic reticulum and lysosomal markers. Table II shows that there is some difference in enhancement between the various luminal enzyme markers. This difference may be due to a loss of enzyme activity by some of the enzymes during the isolation procedure and has also been seen in other laboratories [12,17,22—24]. Luminal membranes show an average of 15-fold enhancement in specific activity of all its membrane markers. Contamination by other membrane markers is minimal with barely detectable amount of endoplastic reticulum, mitochondria, and lysosomes. The specific activity of ($Na^+ + K^+$)-ATPase is about the same as that found in the homogenate.

When comparisons are made between the purified membranes it can be seen that ($Na^+ + K^+$)-ATPase in the antiluminal membrane is enriched 22-fold compared to the luminal membrane. All of the luminal membrane markers are enriched about 7-fold in the luminal membrane compared to the antiluminal membrane.

TABLE II

SPECIFIC ACTIVITY OF MARKER ENZYMES IN STARTING MATERIAL AND IN PURIFIED MEMBRANES

Each datum represents the mean ± S.E. for 6—8 preparations.

Enzyme	Homogenate	Antilumen	Lumen
($Na^+ + K^+$)-ATPase [a]	0.11 ± 0.01	2.40 ± 0.18	0.10 ± 0.03
Maltase [b]	23.02 ± 0.63	39.53 ± 0.75	258.72 ± 1.67
Sucrase [b]	2.30 ± 0.31	5.09 ± 0.97	38.68 ± 5.73
Trehalase [b]	10.75 ± 0.49	21.32 ± 1.11	175.63 ± 1.42
γ-Glutamyltransferase [c]	0.15 ± 0.01	0.32 ± 0.04	1.99 ± 0.10
Alkaline phosphatase [a]	0.11 ± 0.03	0.32 ± 0.05	2.10 ± 0.25
Glucose-6-phosphatase [d]	0.22 ± 0.03	<0.01	<0.01
Acid phosphatase [d]	0.31 ± 0.07	<0.01	<0.01
Succinate dehyrogenase [e]	1.72 ± 0.21	0.19 ± 0.08	0.04 ± 0.01

[a] μmol inorganic phosphate produced/min per mg protein.
[b] nmol glucose/min per mg protein.
[c] μmol glucose/min per mg protein.
[d] μmol inorganic phosphate produced/h per mg protein.
[e] μmol succinate oxidized/h per mg protein.

RNA and DNA contamination

The values for RNA and DNA (see Table III) were 2.45 and 3.54 μg per mg of cortex, respectively, and compare quite closely to the values of 4.5 μg of DNA or RNA per mg of kidney reported by Malt and Lemaitre [25] in mice. Contamination of the membranes by RNA and DNA was less than 0.5% of that contained in the original homogenate.

Electron micrographs

Electron micrographs of each membrane are shown in Figs. 2A and 2B. Each purified membrane appears homogeneous and mostly vesicular, very few open vesicles or sheets are present especially in the luminal membrane (Fig. 2A). Luminal membranes seem to be further characterized by small infolding in the membrane. All of the vesicles in both preparations are less than 0.5 μm in diameter, with luminal membranes being more homogeneous in size and usually larger than antiluminal membranes.

Membrane orientation

The membrane orientation was determined in eight separate preparations. In the antiluminal the total ATPase activity was 0.61 ± 0.10 μmol inorganic phosphate/min per mg protein before disruption and the $(Na^+ + K^+)$-ATPase accounted for 0.25 ± 0.07 of this activity. After disruption the total ATPase activity increased to 2.60 ± 0.15 and the $(Na^+ + K^+)$-ATPase activity to 2.40 ± 0.18 μmol inorganic phosphate/min per mg protein.

The proportion of $(Na^+ + K^+)$-ATPase before disruption compared to after treatment reflects the presence of sheets of membranes. Therefore about 10% (0.25/2.40 × 100%) occurred as sheets. The total ATPase before membrane disruption is equivalent to ouabain-sensitive and -insensitive ATPase in the sheets and in everted membrane vesicles. Since $(Na^+ + K^+)$-ATPase is 92.3% (2.4/2.6 × 100%) of the total ATPase in these preparations after disruption, the total of ouabain-sensitive and -insensitive ATPase in the sheets is 10.4% (0.27/2.6 × 100%). Therefore, the total ATPase in everted vesicles is 0.61 μmol P_i/min per mg protein minus 0.27 μmol P_i/min per mg protein. This difference (0.34 μmol P_i/min per mg protein) divided by the total ATPase activity after disruption of the membranes gives the amount of inside out vesicles, which is 13.1% (0.34/2.6 × 100%) of the membranes. Thus the remaining ATPase activity (76.5%) arises from right-side out vesicles.

Experiments were also conducted to determine the orientation of the luminal

TABLE III

RNA AND DNA CONCENTRATION IN KIDNEY CORTEX AND MEMBRANES

Each datum is the mean of two experiments.

	Cortex (μg/mg wet weight)	Homogenate (μg/mg protein)	Antilumen (μg/mg protein)	Lumen (μg/mg protein)
RNA	2.45	58	0.05	0.19
DNA	3.45	84	0.03	0.14

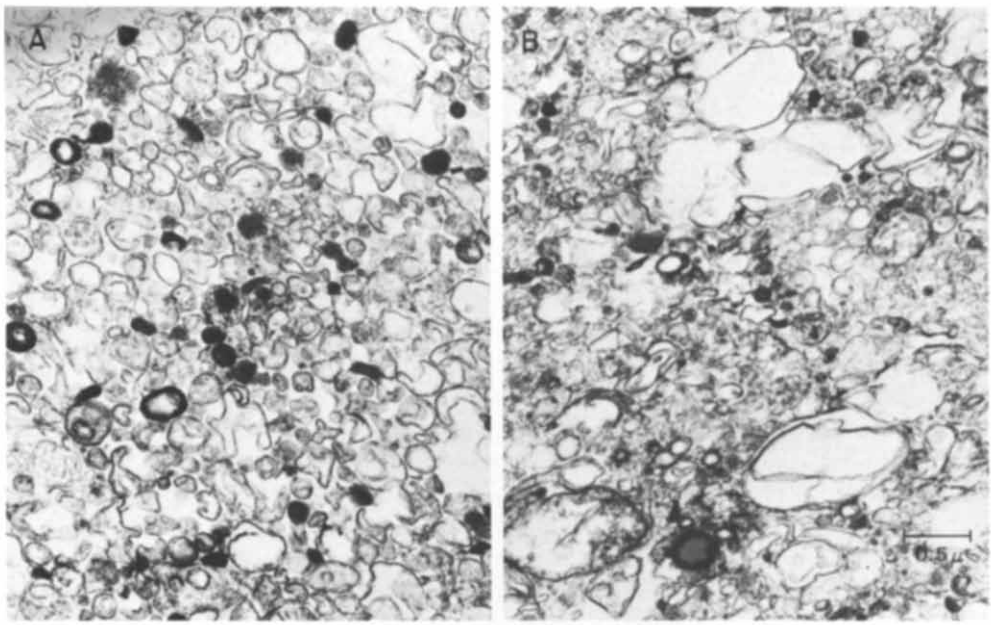

Fig. 2. Electron micrographs of renal cortex luminal (A) and antiluminal (B) membrane preparations viewed after a thin sectioning.

membranes. The carbohydrate moieties in plasma membrane glycoprotein are oriented to the outside [17]. The percent of right-side out vesicles can be determined by first oxidizing the carbohydrate substrates on the glycoproteins with glucose and galactose oxidase and then reducing the carbohydrate with tritium-labeled $NaBH_4$ before and after disruption (0.1% deoxycholate and freezing and thawing). Controls without glucose and galactose oxidase were run concurrently to determine the non-specific labelling. The results obtained did not allow us to discriminate between right-side out vesicles and sheets, but since no sheets could be seen in the electron micrographs, the amount isolated as sheets was not considered. Accumulated data show that 86 ± 6% (n = 8) of the luminal membrane were right-side out.

Discussion

In comparing the results of different procedures for isolating membranes it is necessary to use several criteria rather than just rely on enzyme enhancements. Absolute specific activities as well as relative specific activities, together with recovery values of marker enzymes both for the membranes and for likely impurities, provide information useful in judging the purity of the product and the efficiency of the separation process. Thus we discuss a number of criteria below.

Specific activity of marker enzymes

Although we have measured several luminal enzyme markers, we will use

alkaline phosphatase as the basis for comparison purpose, since it is the luminal marker commonly measured by other laboratories. The specific activity of alkaline phosphatase (2.10 μmol P_i/min per mg protein) in the luminal membrane is in the upper range of the specific activities (0.52–2.75 μmol P_i/min), reported by others [7,11,26–28] while the activity of cross contaminating ($Na^+ + K^+$)-ATPase is lower. Likewise, the specific activity of the ($Na^+ + K^+$)-ATPase in the antiluminal membrane (2.40 μmol P_i/min per mg protein) is greater than that reported in other communications [1,3–5,29].

Enhancement of activity of marker enzymes

Enhancement of the specific activity of alkaline phosphatase is of the same magnitude, that is about 19-fold, as other isolation techniques [1,3–4,11,27,28]. These techniques have resulted in enhancements ranging from 4.5 to 19. Enhancement of ($Na^+ + K^+$)-ATPase is about 22-fold which is at the upper range (10.5–22) of enhancement reported by others [7,22,31].

Possibly a better way to evaluate the purity of a preparation, especially when there is concern for contamination of luminal membranes with antiluminal membranes, or vice versa, is to consider the amount of cross contaminating marker enzymes in the purified preparations. Purified luminal membrane contains about seven times the specific activity of alkaline phosphatase compared to the antiluminal membrane. This separation of alkaline phosphatase in the two membranes meets or exceeds other preparations in quality [5,7,11,27,28, 30]. It is not known whether this dual location reflects a limitation of separation techniques or whether alkaline phosphatase is located in membranes other than luminal membranes.

The specific activity of the ($Na^+ + K^+$)-ATPase is 22 times as great in the antiluminal membrane compared to the luminal membrane. This very large separation of ($Na^+ + K^+$)-ATPase between the two membranes is much greater than the 2.4-fold found by Liang and Sacktor [5] and the 10-fold found with free-flow electrophoresis [1,3,4,30]. Kyte [32] using antibodies specific for ($Na^+ + K^+$)-ATPase found small amounts of antigen on the luminal membrane of renal convoluted tubules. The ($Na^+ + K^+$)-ATPase in the isolated luminal membrane may be related to this.

Amounts of ($Na^+ + K^+$)-ATPase related to total ATPase

Another way to assess the purity of the antiluminal membrane is to relate the amount of total ATPase to that portion which is ouabain inhibitable. In other studies of purified membranes the ratio of ($Na^+ + K^+$)-ATPase to other ATPases in the antiluminal membrane is 2.3 for membranes separated by free-flow electrophoresis [3], and 4.1 for membranes separated by a series of sucrose gradients [29]. Antiluminal membranes isolated by our technique have a ratio of 12. Thus, over 90% of the ATPase activity in the antiluminal membrane is inhibited by ouabain.

This techniques also has further advantages over other techniques which isolate both membranes, in that one gets a higher yield of purified membranes. An average of 1400 and 2000 μg of membrane protein per g of cortex is isolated as antiluminal and luminal membranes, respectively. The method employed by Liang and Sacktor [5], isolated less than 200 μg of protein per g of cortex.

While it is not clear what the yield of membrane protein is for free-flow electrophoresis techniques [3], it is probably lower than our yields.

This method for the isolation of luminal and antiluminal membranes provides those interested in cortical membrane transporters, hormone receptors, and other plasma membrane phenomena, with membranes from kidney of high purity, low contamination, in high yields.

Acknowledgements

The authors would like to thank Dr. D.B. Jones for the electron micrographs and Dr. T.S. Argyris for the RNA and DNA determinations. This study was supported by Public Health Service Grant HL-02835.

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