An efficient method for the isolation and separation of basolateral-membrane and luminal-membrane vesicles from rabbit kidney cortex

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A procedure for isolation and separation of purified luminal-membrane and basolateral-membrane vesicles from adult and newborn rabbit renal cortex by using $\text{Ca}^{2+}/\text{Mg}^{2+}$ precipitation, differential centrifugation and a self-orienting Percoll-gradient centrifugation is described. The purity of the membrane-vesicle suspensions was examined by electron microscopy and by measuring the activity of several marker enzymes. The activity of $\text{Na}^{+} + \text{K}^{+}$ -stimulated ATPase in the fraction mainly containing adult rabbit basolateral-membrane vesicles was enriched 16-fold, and the activity of alkaline phosphatase in the fraction mainly containing luminal-membrane vesicles was increased 13-fold, compared with the homogenate. Similar results were obtained with kidneys from newborn rabbits. Uptake studies, with a rapid filtration technique and the spectrophotometric method described in an accompanying paper [Kragh-Hansen, Jørgensen & Sheikh (1982) *Biochem. J.* **208**, 359–368], showed that both adult and newborn rabbit luminal-membrane vesicles, in contrast with the basolateral-membrane preparations, possess an Na^{+} -dependent electrogenic transport system for L-proline. Adult rabbit luminal-membrane vesicles take up citrate and L-malate by Na^{+} -dependent electrogenic processes, whereas adult rabbit basolateral-membrane vesicles do not exhibit electrogenic uptake of citrate. By contrast, these vesicles show Na^{+} -dependent electrogenic uptake of L-malate.

In the past the mechanism of renal transport of organic solutes has been studied, by various techniques *in vitro* and *in vivo*, with intact epithelial preparations. It has been demonstrated that extracellular Na^{+} as well as the operation of the $\text{Na}^{+} + \text{K}^{+}$ -stimulated ATPase are crucial prerequisites for the transepithelial transport of a wide variety of organic compounds (Crane *et al.*, 1976; Sacktor, 1977, 1978; Ullrich, 1980; Jørgensen, 1980; Maxild *et al.*, 1981). Although the membrane phenomena involved in these Na^{+} -dependent processes could be indirectly assessed from these studies, the coupling between Na^{+} and organic solute flux at the level of the membrane has not been unequivocally documented (Murer & Kinne, 1980).

A development of great importance in the study of tubular transport processes at the subcellular level is the use of the isolated membrane vesicles. In the rat kidney separation of vesicles derived from the basolateral membranes and luminal membranes has been achieved by the application of free-flow electrophoresis to plasma-membrane fractions prepared from sucrose homogenates of renal cortex by differential centrifugation (Heidrich *et al.*, 1972; Reynolds *et al.*, 1980). The free-flow electrophoresis

method has not given satisfactory results in other species such as rabbit, guinea pig and dog. More recently Scalera *et al.* (1981) have described an alternative method, based on self-orienting Percoll-gradient centrifugation, for isolation of basolateral plasma membrane of rat kidney cortex. However, in preliminary experiments, we were unable to obtain purified preparations of basolateral-membrane and luminal-membrane vesicles from rabbit kidney cortex by using the method of Scalera *et al.* (1981).

In the present paper we describe an efficient method for the isolation of highly purified basolateral-membrane and luminal-membrane vesicles prepared from adult as well as newborn rabbit kidney cortex. The purity of the membrane-vesicle preparations was thoroughly examined by determination of various marker-enzyme activities, electron microscopy and transport studies, with both a filtration technique and spectrophotometric measurements (Kragh-Hansen *et al.*, 1982a).

Experimental

Materials

The carbocyanine dye 3,3'-diethyloxadicarbocyanine iodide was obtained from Eastman Kodak

Co., Rochester, NY, U.S.A. L-Malic acid, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Trizma base and Trizma hydrochloride were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. Citric acid and L-proline were purchased from E. Merck, Darmstadt, West Germany. Radioactive L-[^{14}C]proline (sp. radioactivity 293 mCi/mmol) was bought from New England Nuclear, Boston, MA, U.S.A. Membrane filters (0.45 μm , type Sm 11106) were obtained from Sartorius, Göttingen, West Germany. Luma gel was supplied by Lumac, Scharesberg, The Netherlands.

Preparation of membrane vesicles

The preparation of luminal-membrane and basolateral-membrane vesicles was performed at 4°C. Adult and newborn rabbits (less than 72 h old) were killed after being anaesthetized with sodium pentobarbitone (Nembutal, Abbott). The kidneys were excised and perfused as previously described (Sheikh, 1972). The cortex was dissected, minced and homogenized in an appropriate amount of 'homogenizing medium' containing 0.2 mM-phenylmethanesulphonyl fluoride (to inhibit proteinases) and 300 mM-sucrose in 25 mM-Hepes/Tris buffer, pH 7.0, to make a 20% (w/v) homogenate, in a Potter-Elvehjem homogenizer (30 strokes at maximum speed). Membrane isolation was performed in a Sorvall SS-34 rotor and a Sorvall RC 2B centrifuge (4°C) unless otherwise noted. The homogenate was centrifuged at 3000 rev./min (1080 g) for 10 min, and the supernatant (fraction A) was pipetted out and re-centrifuged at 10000 rev./min (12100 g) for 10 min. The supernatant thus obtained, together with the light fluffy membrane layer (dark-brown pellet was discarded), designated fraction B, was further centrifuged at 20000 rev./min (48400 g) for 30 min. The supernatant was carefully decanted, and the light fluffy membrane layer (fraction C) was separated from the dark-brown pellet and suspended in a homogenizing medium containing 2.5 mM-MgCl₂ and 2.5 mM-CaCl₂. This crude suspension of membrane vesicles was stirred for 2 h at 2°C and then re-centrifuged at 3500 rev./min (1475 g) for 15 min. The supernatant thus formed, containing highly purified luminal-membrane vesicles (fraction D), was collected and concentrated by centrifugation, to obtain a final luminal-membrane protein concentration of approx. 8 mg/ml, and stored at 0°C until use. The pellet, which predominantly consists of basolateral-membrane vesicles (fraction E), was resuspended in a homogenizing medium containing 0.2 mM-EDTA, to remove Mg²⁺ and Ca²⁺ ions from the vesicles. The suspension of basolateral-membrane vesicles was thoroughly mixed by Whirlimixer (Fisons) and centrifuged at 20000 rev./min (48400 g) for 20 min. This procedure was repeated twice, and the pellet thus formed was suspended in

an appropriate amount of homogenizing medium to make a final protein concentration of 5 mg/ml. The suspension of basolateral-membrane vesicles was further purified by adding small samples (2 ml) of the preparation to 38 ml of 12% (w/v) Percoll solution, prepared by diluting stock solution of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) with homogenizing medium. The resultant mixture was centrifuged at 16000 rev./min (30900 g) for 30 min. At least three bands were observed after self-orienting Percoll-gradient centrifugation. A distinct band, having a very high Na⁺ + K⁺-stimulated ATPase activity, located at the bottom of the centrifuge tube was collected and re-centrifuged at 50000 rev./min (226400 g) in a Beckman centrifuge with a Ti-50 rotor for 45 min. After centrifugation, the Percoll formed a very dense glassy pellet, and the highly purified basolateral-membrane vesicles formed a loose fluffy pellet above the Percoll, which could be easily collected. The suspension of purified basolateral-membrane vesicles (fraction F) was further concentrated by centrifugation, to obtain a final protein concentration of approx. 10 mg/ml. They were then stored at 0°C until experimental use. All experiments were performed on freshly prepared membrane vesicles.

Measurement of marker-enzyme activities

Renal homogenates and the various membrane fractions were assayed for different marker-enzyme activities. Na⁺ + K⁺-stimulated ATPase activity was assayed as described by Ottolenghi (1975) and used as a marker for basolateral-membrane vesicles. Alkaline phosphatase and trehalase were measured as reported by Bessey *et al.* (1946) and Sacktor (1968) respectively, and used to detect luminal-membrane vesicles. Degree of contamination of membrane vesicles by mitochondria and lysosomes was examined by measuring succinate dehydrogenase (Sheikh, 1972) and acid phosphatase (Hübscher & West, 1965) activities. The amount of DNA in various fractions was determined by the method of Croft & Lubran (1965). The amount of protein in different fractions was measured by the method of Lowry *et al.* (1951) as modified by Peterson (1977), with human serum albumin (Sigma Chemical Co.) as standard.

EXPLANATION OF PLATE 1

Electron micrograph of renal membrane vesicles from adult rabbit

(a) Thin section of basolateral-membrane vesicles; magnification $\times 36000$. (b) Negatively stained preparation of luminal-membrane vesicles; magnification $\times 18000$. Inset shows that the vesicles are covered with a coat of small particles; magnification $\times 90000$.

Table 1. *Distribution of marker enzymes during purification of basolateral-membrane and luminal-membrane vesicles*
All results are given as the means $\pm$ S.D. for at least six determinations. Specific activities for $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, alkaline phosphatase and trehalase are μmol of substrate hydrolysed/min per mg of protein, whereas those for succinate dehydrogenase and acid phosphatase have been multiplied by 100, as shown. The amount of DNA in various fractions is given as mg of DNA/g of homogenate protein. The average amount of protein in homogenate from four adult kidneys cortex was 3200 mg. The average yields of basolateral-membrane vesicles (fraction F) and luminal-membrane vesicles (fraction D) were 60 mg and 50 mg respectively. The activity of $\text{Na}^+ + \text{K}^+$ -stimulated ATPase in fraction F was enriched 16-fold as compared with homogenate. The activities of alkaline phosphatase and trehalase in fraction D were enriched 13-fold as compared with homogenate.

Fractions	$\text{Na}^+ + \text{K}^+$ - stimulated ATPase sp. activity	Alkaline phosphatase sp. activity	Trehalase sp. activity	$10^2 \times$ Succinate dehydrogenase sp. activity	$10^2 \times$ Acid phosphatase sp. activity	DNA (mg/g of homogenate protein)
Homogenate	0.203 ± 0.020	0.118 ± 0.013	0.036 ± 0.006	2.94 ± 0.611	1.3 ± 0.2	16.3 ± 1.2
Fraction A	0.243 ± 0.016	0.146 ± 0.018	0.055 ± 0.009	4.52 ± 0.932	1.5 ± 0.2	1.57 ± 0.09
Fraction B	0.318 ± 0.031	0.219 ± 0.021	0.095 ± 0.007	1.87 ± 0.354	1.6 ± 0.1	0.86 ± 0.07
Fraction C	1.105 ± 0.019	0.516 ± 0.033	0.194 ± 0.013	0.65 ± 0.083	1.2 ± 0.2	0.37 ± 0.04
Fraction D	0.115 ± 0.011	1.573 ± 0.045	0.479 ± 0.026	0.053 ± 0.007	0.7 ± 0.1	0.004 ± 0.00
Fraction E	2.15 ± 0.040	0.243 ± 0.012	0.074 ± 0.007	0.072 ± 0.008	0.9 ± 0.1	0.009 ± 0.00
Fraction F	3.18 ± 0.035	0.181 ± 0.015	0.064 ± 0.008	0.027 ± 0.007	0.1 ± 0.1	0 ± 0.00

Electron microscopy

The basolateral vesicles were fixed in a Karnowsky-type mixture (2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M-phosphate buffer, pH 7.3), washed in 0.1 M-sodium cacodylate buffer, pH 7.3, treated with 1% OsO_4 in 50 mM-Veronal buffer, pH 7.3, and washed twice with the same buffer. After dehydration and embedding in Epon they were sectioned and stained with both uranyl acetate and lead acetate.

For negative staining a droplet of the luminal vesicles was applied to a Formvar- and carbon-coated grid and stained with potassium phosphotungstate.

Results and discussion

The distribution of various marker enzymes at each stage of membrane isolation is shown in Table 1. After the early centrifugation steps, most of the contaminating membranes from nuclei, mitochondria and lysosomes were eliminated from fraction C, which is enriched approx. 5-fold in specific activities of luminal-membrane and basolateral-membrane marker enzymes as compared with the initial homogenate. Fraction C was then

treated with homogenizing medium containing 2.5 mM- MgCl_2 and 2.5 mM- CaCl_2 . These bivalent cations have been shown to interact selectively with basolateral-membrane, mitochondrial and microsomal fractions, resulting in aggregation of these membranes (Booth & Kenny, 1974), which can be easily sedimented by low-speed centrifugation [3500 rev./min (1475 g)]. The resultant supernatant (fraction D) contained purified luminal-membrane vesicles, as shown by the specific activities of alkaline phosphatase and trehalase, which are enhanced 13-fold. This procedure is a modification of the method originally described by Booth & Kenny (1974). The sediment (fraction E), which predominantly consists of basolateral membrane, is further purified by the use of self-orienting Percoll-gradient centrifugation (Scalera *et al.*, 1981). Basolateral-membrane vesicles thus obtained are essentially free of nuclei, mitochondria and lysosomes and largely free of luminal membranes. Table 1 shows that the specific activity of the marker enzyme for basolateral membranes, $\text{Na}^+ + \text{K}^+$ -stimulated ATPase, is enhanced 16-fold by this procedure. The results summarized in Table 1 were obtained with membrane vesicles derived from adult rabbit kidney cortex. However, we have also prepared basolateral-membrane and luminal-membrane vesicles from newborn rabbits (less than 72 h old) by the same method. The distribution of different marker enzymes in various fractions of newborn rabbit kidney-cortex homogenate (results not shown) was similar to that shown in Table 1.

The purity of basolateral-membrane and luminal-membrane vesicles was further examined by electron microscopy. Plates 1(a) and 1(b) show, in accordance with the enzymic measurements, that fraction F

EXPLANATION OF PLATE 2

Electron micrograph of renal membrane vesicles from newborn rabbit

(a) Thin section of basolateral-membrane vesicles; magnification $\times 20000$. (b) Negatively stained preparation of luminal-membrane vesicles; magnification $\times 70000$.

Fig. 1. Uptake of L-proline by renal membrane vesicles

(a) Uptake of 100 μ M-L-proline (including tracer amounts of 14 C-labelled L-proline) by adult (● and ○) or newborn (■ and □) rabbit luminal-membrane vesicles, as studied by a filtration technique with Sartorius membrane filters. The pH was 7.0, and the temperature 20°C. The intravesicular medium was 320 mM-mannitol/1 mM-Hepes/Tris buffer, whereas the external medium was 150 mM-NaCl/25 mM-Hepes/Tris buffer (● and ■) or 150 mM-KCl/25 mM-Hepes/Tris buffer (○ and □). Protein concentrations were approx. 2 mg/ml. (b) Uptake of 5 mM-L-proline by adult rabbit membrane vesicles, as studied by spectrophotometry. The pH was 7.0, and the temperature 20°C. Intravesicular medium was 320 mM-mannitol/1 mM-Hepes/Tris buffer. External media: curves 1 and 3, 150 mM-NaCl/25 mM-Hepes/Tris buffer, curves 2 and 4, 150 mM-KCl/25 mM-Hepes/Tris buffer. Curves 1 and 2 were obtained with luminal-membrane vesicles (0.25 mg of protein/ml), whereas curves 3 and 4 represent results with basolateral-membrane vesicles (0.20 mg of protein/ml). (c) Uptake of 5 mM-L-proline by newborn rabbit membrane vesicles. Curves 1, 2 and 3 were obtained with the same experimental conditions as described for curves 1, 4 and 3 in part (b). Protein concentrations were approx. 0.25 mg/ml.

Fig. 2. Uptake of citrate or L-malate by renal membrane vesicles

(a) Uptake of 5 mM-citrate (curves 2 and 3) or 5 mM-L-malate (curves 1 and 4) by adult rabbit luminal-membrane vesicles. Intravesicular medium was 320 mM-mannitol/1 mM-Hepes/Tris buffer. External media: curves 1 and 2, 150 mM-NaCl/25 mM-Hepes/Tris buffer; curves 3 and 4, 150 mM-KCl/25 mM-Hepes/Tris buffer. Protein concentration was approx. 0.25 mg/ml. (b) Uptake of 5 mM-L-malate (curve 1) or 5 mM-citrate (curve 2) by adult rabbit basolateral-membrane vesicles. Intravesicular medium was 320 mM-mannitol/1 mM-Hepes/Tris buffer. External medium was 150 mM-NaCl/25 mM-Hepes/Tris buffer. Protein concentration was approx. 0.3 mg/ml.

and fraction D predominantly contain basolateral-membrane and luminal-membrane vesicles isolated from adult rabbit renal cortex respectively. It appears from the inset of Plate 1(b) that the luminal-membrane vesicles are covered with a coat of small particles on their outer surface, indicating right-side-out and intact vesicles (Rostgaard & Møller, 1971). Plates 2(a) and 2(b) show that the present isolation procedure also can be used for preparing highly purified preparations of newborn rabbit basolateral-membrane and luminal-membrane vesicles respectively.

To test whether the membrane vesicles prepared by the above-mentioned method retained transport properties, the renal uptake of various organic solutes was studied by using a filtration technique and the spectrophotometric method described in an accompanying paper (Kragh-Hansen *et al.*, 1982a).

Fig. 1(a) shows that luminal-membrane vesicles from both adult and newborn rabbits (less than 72 h old) take up L-[14 C]proline by an Na^+ -dependent process. Furthermore, it is seen that both proline curves exhibit 'overshoot' phenomena. Spectropho-

metric measurements, with the dye 3,3'-diethyloxadibenzocyanine iodide, indicate that the Na^+ dependent uptake of L-proline is electrogenic in luminal-membrane vesicles isolated from both the adult (Fig. 1b, curve 1) as well as the newborn animal (Fig. 1c, curve 1). By contrast, basolateral-membrane vesicles prepared from adult or newborn rabbits do not take up L-proline electrogenically (curves 3 in Figs. 1b and 1c). The very small optical responses in the last-mentioned examples also show that the preparations of basolateral-membrane vesicles are not contaminated to any significant extent by luminal-membrane vesicles.

Marked prolinuria is normally observed in newborn mammals, including man (Baerlocher *et al.*, 1971a,b; Brodehl & Gellissen, 1968). The results in Fig. 1 may at least partly explain these findings, since the maximal uptake of L-proline by the newborn rabbit membranes is considerably less than that of the adult rabbit membranes. Goldmann *et al.* (1979) have studied the uptake of L-proline by newborn rat kidney brush-border-membrane vesicles by the Millipore-filtration technique. The most

notable observation in their study was that the uptake of L-proline by these vesicles, in contrast with the uptake of the amino acid by membrane vesicles isolated from adult rats, did not show an overshoot. The apparent discrepancy between our experimental results and those obtained by Goldmann *et al.* (1979) on the mechanism of uptake of proline by luminal-membrane vesicles derived from newborn animals may be due to species difference, since it has been reported that the tubular localization of Phenol Red in rat and rabbit kidney is basically different (Sheikh & Møller, 1980). However, it seems reasonable to avoid further speculation on this before a more detailed study on the renal uptake of proline in newborn animals is provided.

Fig. 2 shows spectrophotometric results obtained with citrate and L-malate. It is seen that the tricarboxylic acid is taken up by adult rabbit luminal-membrane vesicles by an Na⁺-dependent electrogenic process (Fig. 2a, curve 2). These characteristics of citrate transport are in accordance with the fluorescence studies reported by Wright *et al.* (1981). In contrast with the luminal-membrane vesicles, basolateral-membrane vesicles do not exhibit electrogenic citrate uptake (Fig. 2b, curve 2).

In accordance with previous studies (Kragh-Hansen *et al.*, 1982b), L-malate is taken up electrogenically, in the presence of Na⁺, by adult rabbit luminal-membrane vesicles (Fig. 2a, curve 1). Furthermore, it is also seen that basolateral-membrane vesicles are transiently depolarized by L-malate (Fig. 2b, curve 1). However, the optical response with the basolateral-membrane vesicles is only about one-quarter of the response observed with the luminal-membrane vesicles. The possibility that the absorbance change of the dye is the result of contamination by luminal-membrane vesicles can be ruled out, since addition of citrate, L-proline, D-glucose or L-phenylalanine to luminal-membrane vesicles produces large optical responses, whereas addition of the same solutes to basolateral-membrane vesicles gives only very small absorbance changes of the dye (cf. Figs. 4 and 5 in Kragh-Hansen *et al.*, 1982a). The latter results would not have been observed if the basolateral-membrane-vesicle preparations were significantly contaminated by luminal-membrane vesicles. The result shown by curve 1 in Fig. 2(b) was obtained by membrane vesicles prepared in the same way as those used in the above-mentioned experiments. Therefore the curve represents the first published example of electrogenic solute uptake by renal basolateral-membrane vesicles.

We conclude that the present procedure is suitable for the isolation of highly purified and functioning luminal-membrane and basolateral-membrane

vesicles from rabbit kidneys. The method is applicable to both the adult and the newborn animal.

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