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THE SIMULTANEOUS PREPARATION OF BASOLATERAL AND BRUSH-BORDER MEMBRANE VESICLES FROM GUINEA-PIG INTESTINAL EPITHELIUM, AND THE DETERMINATION OF THE ORIENTATION OF THE BASOLATERAL VESICLES

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A rapid method is described for the simultaneous preparation of both membranes of guinea-pig enterocytes, using simple differential centrifugation techniques. Basolateral membranes were purified on a Percoll gradient and the final yield of $(Na^+ + K^+)$-ATPase was 12.4% of the original activity with an enrichment factor of 12.6-fold. Purification of the brush-border fraction was achieved by a calcium-precipitation technique. The yield of alkaline phosphatase was 18.9% of the original activity with an enrichment of 17.5-fold. Both fractions could be obtained within 3 h of the original homogenization. The characteristics of the preparations were checked by negative-staining electron microscopy and by the determination of glucose uptake. The orientation of the basolateral vesicles was determined by measuring the Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities and the [^{3}H]ouabain binding before and after treatment of the preparation with a mixture of deoxycholate and EDTA which transforms the vesicles into sheets. There was a 60% rise in $(Na^+ + K^+)$-ATPase activity and ouabain binding, but no change in Mg^{2+}-ATPase activity. It was therefore concluded that 60% of the original preparation consisted of inside-out vesicles and 40% of membrane sheets.

Introduction

The study of the mechanisms involved in the transport of ions and organic solutes across epithelia such as that of the small intestine or the renal tubule received considerable impetus with the development of methods for isolating the different membranes of the cell. In this way, transport mechanisms could be examined directly in the absence of any interference from other cellular events. The majority of the techniques for the isolation of membranes have concentrated on one particular membrane in one particular tissue [1–8], whilst attempts to separate the two types of membrane from the same starting material have generally been long and complicated or have involved highly sophisticated equipment [9–12]. In the present work, we describe a simple and rapid technique to obtain both types of membrane from the same initial material; the method involves simple differential centrifugation and precipitation with divalent cations (Ca^{2+}); it is freely applicable to both intestinal and renal epithelia.

Part of this work was presented to the Physiological Society at a joint meeting with the Société Suisse de Physiologie in Berne [41] and to the 4th meeting of the European Intestinal Transport Group in Berlin.

Materials and methods

Materials. All chemical products were used at the highest available purity. D-(−)-Mannitol, D-

Abbreviation: EGTA, ethyleneglycol-bis(2-aminoethyl)tetraacetic acid.

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(+)-glucose, sucrose, EGTA, EDTA, deoxycholic acid, succinic acid, ouabain (strophanthin-G) and Tris were purchased from Merck (Darmstadt), Na_2ATP and glucose 6-phosphate from Boehringer (Mannheim), β-glycerophosphate from Sigma (St. Louis) and D-[^{14}C]glucose and [^{3}H]ouabain from Amersham International (Amersham).

Preparation of basolateral and luminal membranes. Crude fractions of both membranes were obtained by a modification of the technique of Fitzpatrick et al. [13], as follows.

The small intestine was excised from guinea-pigs weighing 400–500 g and was washed and rapidly placed in cold phosphate buffer. The mucosa was scraped off and weighed before being suspended in a mixture of 250 mM sucrose/2 mM Tris-HCl (pH 7.2) at a level of 1 g tissue per 3 ml solution. The suspension was then homogenized using a motor-driven homogenizer (Braun, Melsungen) with a Teflon pestle, with eight strokes at 1500 rpm. The homogenate was then filtered through gauze to remove foam and inhomogeneous material. The membrane isolation was performed in an MSE High-Speed 25 centrifuge at 0–4°C, according to the flow sheet presented in Scheme I.

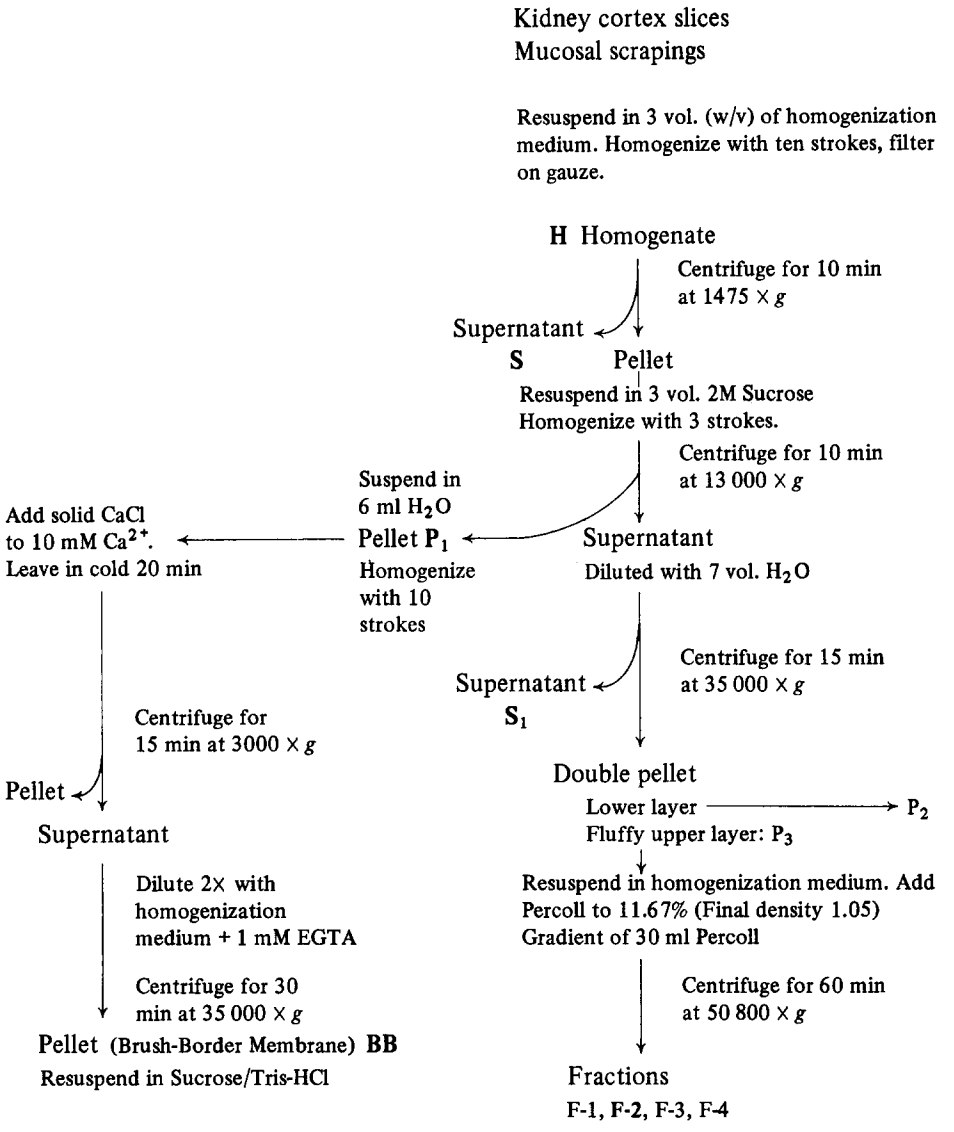
For the isolation of the basolateral membranes, a Percoll gradient, as proposed by Scalera et al. [8], was applied. The fraction P_3 was mixed with a solution of Percoll/sucrose/Tris (pH 7.2) to a final volume of 30 ml (final concentration of Percoll 11.67%, density 1.05). The mixture was centrifuged for 1 h at 50800 × *g* and the spontaneously formed gradient was divided, from top to bottom, into 30 fractions of 1 ml which were removed with the aid of a slow peristaltic pump. After assaying each fraction for $(Na^+ + K^+)$-ATPase and alkaline phosphatase, we combined the first eight as fraction F-1, the next nine as fraction F-2, the next five as fraction F-3, and the last eight as fraction F-4. In order to remove the Percoll from the preparation, the final fraction was diluted in the required resuspension medium and centrifuged twice at 50800 × *g* for one hour. The Percoll strongly adheres to the centrifugation tube, and the membranes form a pellet which is easily separated by addition of resuspension medium.

Luminal membranes were obtained by applying the calcium precipitation method of Schmitz et al. [2], as modified by Kessler et al. [3], to the fraction P_1 (see Scheme I). The excess calcium was removed by washing the brush-border fraction twice with resuspension medium containing 1 mM EGTA.

Enzymatic assays. The determination of enzymatic activities in each fraction obtained during the preparative process was performed on non-frozen samples, within 6 h of the animals' death. All fractions were pretreated with 0.06% deoxycholate/1 mM EDTA before the determinations in order to open any closed vesicles (see below).

ATPase activities. The method of Proverbio and del Castillo [14] was applied. The incubation medium, final volume 1 ml, contained 50–150 mM Tris-HCl (pH 7.0 at 37°C), 5 mM $MgCl_2$ and, where appropriate, 100 mM NaCl, 20 mM KCl and 1 mM ouabain. The concentration of Tris-HCl was varied in order to adjust the osmolality of the medium. The final quantity of protein was between 25 and 50 μg, in which range the ATPase activity revealed a linear relationship with concentration. The enzyme was preincubated for 5 min and the reaction started with the addition of 2 mM Na_2ATP. After 15 min, the reaction was stopped by the addition of 1 ml cold 6% perchloric acid. The samples were mixed and centrifuged, and the orthophosphate in the supernatant was determined [15]. Preliminary experiments showed that under these conditions, there was a linear relationship between the quantity of orthophosphate liberated and the incubation time. All samples were run in triplicate. The ATPase activity is expressed in nmol of phosphate liberated per mg protein and per min, after subtraction of a blank in which the membranes were added to the incubation medium only after the perchloric acid. The activity of $(Na^+ + K^+)$-ATPase was obtained by subtracting from the total activity, measured in the presence of Mg^{2+}, Na^+ and K^+, the activity found in the presence of Mg^{2+}, Na^+, K^+ and ouabain. The Mg^{2+}-ATPase activity is that measured in the presence of Mg^{2+} + ouabain or Mg^{2+}, Na^+ and ouabain. The $(Na^+ + K^+)$-ATPase activity (EC 3.6.1.3) was used as a marker for basolateral membranes [16].

Other phosphatases. Alkaline phosphatase (EC 3.1.3.1), used as a marker for brush-border membranes [17], and acid phosphatase (EC 3.1.3.2), a



Scheme I. Flow diagram for the preparation of renal and small intestinal basolateral and brush-border membrane fractions. Homogenization medium: 250 mM sucrose/20 mM Tris-HCl (pH 7.2). Further details of the procedures are given in the text.

marker for lysosomes [18], were determined according to the method of Hübscher and West [19]. Glucose-6-phosphatase (EC 3.1.3.9), used as a marker for the endoplasmic reticulum [20], was assayed by the method of Heppel and Hilmore [21].

Other enzymes. Succinic dehydrogenase (EC 1.3.99.1) was used as a mitochondrial marker [22] and was assayed according to the method of King [23].

Protein determination. Proteins were routinely determined by the folin method [24], using crystalline bovine albumin as standard. Since high concentrations of Percoll can interfere with this method, blanks were prepared from gradients run without membranes. In addition, a modified Coomassie blue method [25,26] with which Percoll does not interfere [27] was also used to check the results obtained for fractions emerging from Percoll gradients.

Treatment with deoxycholate/EDTA. In order to open closed vesicles, the technique of Jørgensen and Skou [29] was applied. Membrane fractions suspended in the sucrose/Tris-HCl medium at pH 7.2 and containing 2–3 mg protein per ml were treated with a mixture of deoxycholate and EDTA (final concentrations 0.06% and 1 mM, respectively) for 30 min at room temperature. Immediately after this treatment, the samples were tested for enzyme activities and [^{3}H]ouabain binding.

[^{3}H]Ouabain binding. The method used is a modification of that of Lane et al. [30]. The reaction was performed at 37°C in the presence of 50 mM Tris-HCl (pH 7.2), 1.25 mM Na_2ATP, 1.25 mM $MgCl_2$ and 100 mM NaCl (all final concentrations) in a total volume of 200 μl. The quantity of protein added was 0.2 mg. The reaction was initiated by the addition of [^{3}H]ouabain (final concentration, 0.01 mM), and 2 min later the reaction was stopped by removal of 50 μl of the suspension which were immediately diluted in 1 ml of washing solution (identical to the incubation medium, but containing in addition 1 mM unlabelled ouabain). This mixture was then immediately collected on a Millipore filter (HA 025, 0.45 μm). The filters were washed with 3 ml of the same solution to remove adherent incubation medium. They were then allowed to dry and were dissolved in 'Scintillator 299' emulsifier (Packard) for counting in a liquid scintillation spectrometer. The counts were corrected for the background and for quenching by means of the channel ratio technique. These assays yield the so-called total binding. In order to determine the nonspecific component of binding, samples of membranes were exposed for 15 min to 5 mM ouabain before being incubated with the labelled ouabain. The difference between the total binding and the nonspecific binding provides a measure of the specific binding.

Glucose transport. Glucose uptake into brush-border and basolateral membranes was determined by the Millipore filtration technique [31]. Membrane samples were centrifuged and resuspended in a mixture of 100 mM mannitol/2 mM Tris-HCl (pH 7.2). They were incubated [10,32] in a medium which contained 100 mM mannitol, 2 mM Tris-HCl (pH 7.2), 100 mM NaCl or 100 mM KCl (when appropriate) to which D-[^{14}C]glucose was added at a concentration of 1 or 2 mM. The incubations were performed at 25°C over different time periods and were terminated by removal of 20 μl of the medium which were diluted in 1 ml of cold buffer and immediately collected on a Millipore filter. The filter was washed with 3 ml of cold buffer which comprised 150 mM NaCl/10 mM Tris-HCl (pH 7.2)/0.2 mM phloridzin. The filters were then dissolved and counted using the technique outlined above.

Negative staining for electron microscopy. Samples of the fractions B-B and F-2 were washed by means of three successive centrifugations at 50800 × *g* for 15 min in a solution of 250 mM Tris-HCl (pH 7.2), in order to remove all traces of sucrose from the medium, since this can interfere with the staining. Thereafter, the samples were suspended in the same medium and subjected to negative staining [28] as follows. A drop of the material was placed on a 400-mesh copper grill and covered with a film of 0.25% Formvar reinforced with carbon. The excess material was removed by touching the grill with filter paper. Next, a drop of 2% phosphotungstic acid (pH 7.2) was placed on the grill, and the excess was again removed 30 s later with filter paper. The specimen was then allowed to dry. The samples thus prepared were examined with a Zeiss EM 9 electron microscope, operating at 60 kV with an amplification of 12000–20000 ×. The photographs were taken at an 18000 magnification (as shown in Fig. 4: ×13150).

Results

Yield of basolateral membranes

Table I illustrates the recovery of proteins, the specific activity and relative specific activity and the percentage recovery of each of the marker enzymes at every stage of the preparation. These results concern the guinea-pig small intestinal epithelium; corresponding data for renal cortex can be found in ref. 14. The $(Na^+ + K^+)$-ATPase, marker for basolateral membranes, is principally enriched in the fraction P_3 (6.0-times), where there is a recovery of 18.1% of the total activity of the original homogenate, although the protein recovery was only 3.0%. Nevertheless, this fraction is

TABLE I

ENZYME ACTIVITIES AND RECOVERIES IN SMALL INTESTINE HOMOGENATE (H) AND OTHER FRACTIONS

	Fractions:	H	S	P_1	S_1	P_2	P_3	Total recovery (%)
Proteins	*R*%	100	52.9±1.49	27.4±1.00	10.7±0.55	4.6±0.37	3.0±0.29	98.6±1.59
Mg^{2+}-ATPase	S.A.	123 ± 3.8	95 ±6.8	129 ±11.5	33 ± 4.6	312 ±15.4	224 ±17.0	
	R.S.A.	1	0.77	1.05	0.27	2.54	1.82	
	R%	100	40.9±2.98	28.7± 2.16	2.9± 0.42	11.6± 0.92	5.5± 0.41	89.6±2.87
Acid phosphatase	S.A.	5.9± 0.79	5.1±0.67	4.8± 0.56	6.8± 1.13	11.2± 1.63	8.4± 1.19	
	R.S.A.	1	0.86	0.80	1.14	1.88	1.42	
	R%	100	45.4±2.53	22.0± 1.68	12.2± 0.98	8.6± 0.70	4.3± 0.31	92.5±2.75
Glucose-6-phosphatase	S.A.	31 ± 2.0	31 ±2.3	29 ± 2.1	19 ± 3.5	44 ± 4.2	50 ± 5.5	
	R.S.A.	1	0.98	0.93	0.61	1.40	1.61	
	R%	100	52.0±2.84	25.5± 1.61	6.6± 1.11	6.4± 0.71	4.8± 0.41	95.3±1.77
Succinate dehydrogenase	S.A.	2.8± 2.3	19 ±1.4	43 ± 8.5	17 ± 0.4	84 ± 4.8	20 ± 1.3	
	R.S.A.	1	0.67	1.55	0.61	3.01	0.72	
	R%	100	35.5±4.52	42.5± 6.00	6.5± 0.36	13.7± 0.62	2.2± 0.16	100.4±1.57
Alkaline phosphatase	S.A.	204 ±35.1	55 ±8.4	440 ±70.6	59 ±11.0	317 ±63.9	450 ±70.6	
	R.S.A.	1	0.27	2.15	0.29	1.55	2.20	
	R%	100	14.2±1.30	59.1± 1.32	3.1± 0.61	7.1± 0.63	6.6± 0.94	90.1±1.36
$(Na^+ + K^+)$-ATPase	S.A.	43 ± 3.0	26 ±4.0	61 ± 6.1	15 ± 2.4	47 ± 8.8	256 ±30.3	
	R.S.A.	1	0.62	1.43	0.36	1.11	6.02	
	R%	100	32.5±1.56	39.1± 2.40	3.8± 0.71	5.0± 0.55	18.1± 1.85	98.5±2.44

The different fractions are named as indicated in Materials and Methods. Abbreviations: S.A., specific activity, defined as nmol liberated P_i/mg protein per min for the phosphatases and μmol succinate oxidized/mg protein per min for the succinate dehydrogenase. R.S.A., relative specific activity, calculated as the ratio of specific activity of any particular fraction to that of the homogenate. *R*%, percentage of total activity of the original homogenate recovered in the fraction. In this and in the following tables, the values are expressed as the mean±S.E. In the present table, $n = 7$.

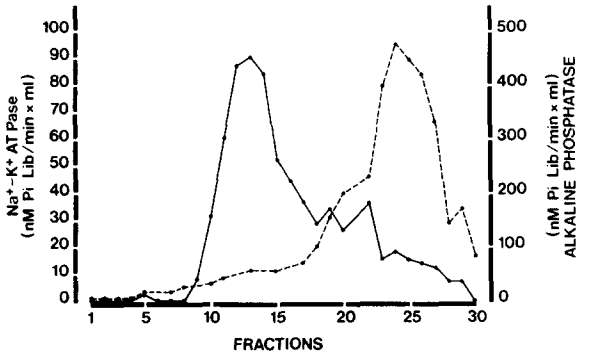


Fig. 1. Distribution of $(Na^+ + K^+)$-ATPase (————) (basolateral membranes) and alkaline phosphatase (- - - - -) (brush-border membranes) on Percoll gradient using Percoll (11.67%) in 250 mM sucrose/20 mM Tris-HCl (final density, 1.05). The gradient was separated from the top into 30 fractions of 1 ml.

contaminated with luminal membranes, as witnessed by the specific activity of alkaline phosphatase, and contains a slight contamination with endoplasmic reticulum and lysosomes. In order to remove these contaminants, this fraction was mixed with Percoll (final concentration: 11.67%) and centrifuged to form a spontaneous gradient. Fig. 1 shows the distribution of $(Na^+ + K^+)$-ATPase and alkaline phosphatase on the Percoll gradient. There is a clear separation of the two enzyme activities, so that the basolateral membrane fraction can be practically freed of luminal contamination. As described in the Materials and methods section, the fractions from the Percoll gradient were amalgamated into four principal fractions, the enzyme activities and protein contents of which are given in Table II. The fraction that contains the highest activity of $(Na^+ + K^+)$-ATPase is F-2 which reveals an enrichment of 12.6-times and a recovery of 12.4% of the total activity of the original homogenate. In contrast, neither alkaline phosphatase nor the other contaminants is enriched in this fraction with respect to the original homogenate.

Fig. 2 illustrates the enrichment and recovery of the different enzymes in the P_3 fraction and the F-2 fraction obtained from the Percoll gradient. The reduction in the contaminants and the enrich-

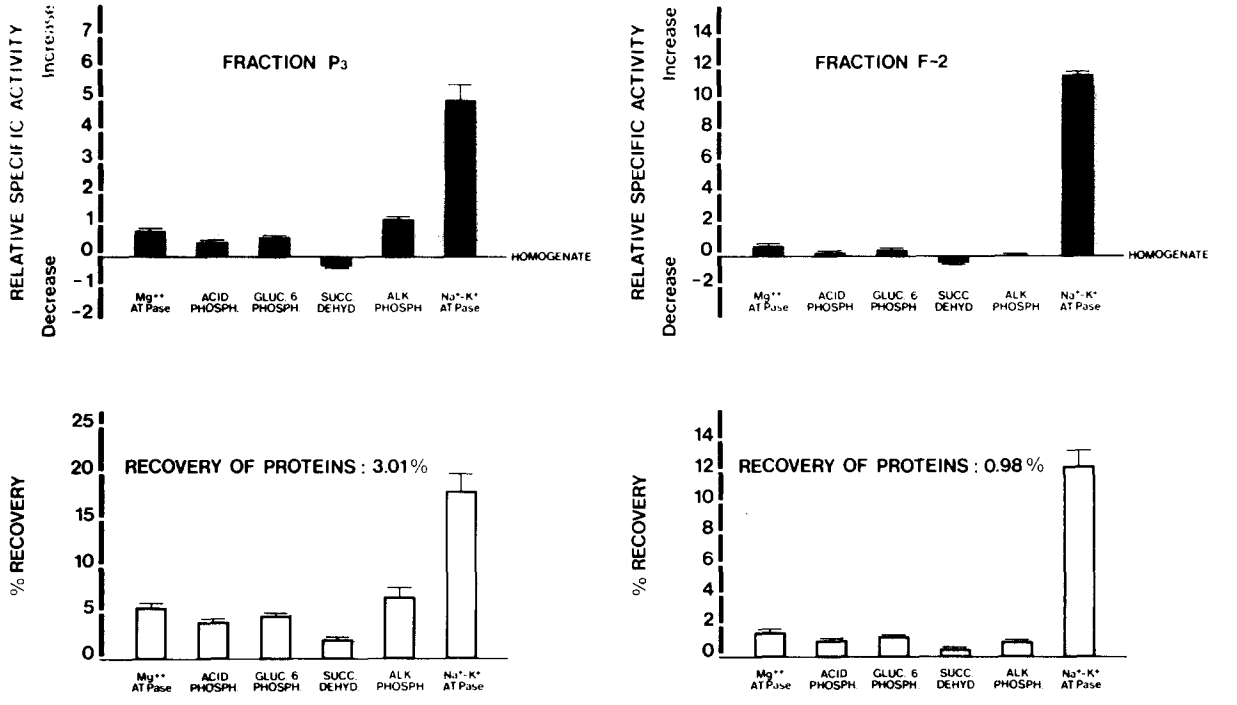


Fig. 2. Relative specific activity (upper panels) and percentage of recovery with respect to the original homogenate (lower panels) of the different marker enzymes in basolateral membrane fractions before (P_3) and after (F-2) passage through the Percoll gradient. In this and the following graph, the zero line refers to the activity of the homogenate. In order to determine the relative specific activity, 1 is subtracted from each value, so that the enrichment or impoverishment of a given marker enzyme can be expressed as an increment or a reduction respectively. Data taken from Tables I and II ($n = 7$).

TABLE II

ENZYME ACTIVITIES AND RECOVERIES OF DIFFERENT FRACTIONS FROM THE PERCOLL GRADIENT

	Fractions:	P_3	F-1	F-2	F-3	F-4	Total recovery (%)
Proteins	*R*%	100	6.2±0.79	32.6±2.71	15.2±1.28	41.3±2.69	95.3±1.65
Mg^{2+}-ATPase	S.A.	224 ±17.0	42 ± 6.0	204 ±16.0	274 ±23.0	294 ±16.2	
	R.S.A.	1	0.19	0.91	1.23	1.31	
	R%	100	1.2± 0.26	29.6± 0.20	18.6± 2.40	54.1± 2.67	103.5±4.97
Acid phosphatase	S.A.	8.4± 1.19	16.1± 2.45	6.9± 0.75	11.7± 1.83	7.6± 0.51	
	R.S.A.	1	1.92	0.82	1.40	0.90	
	R%	100	11.8± 0.93	26.7± 2.02	21.2± 3.22	37.3± 2.26	97.0±5.02
Glucose-6-phosphatase	S.A.	50 ± 5.5	22 ± 2.1	43 ± 2.9	76 ± 8.6	50 ± 4.8	
	R.S.A.	1	0.43	0.85	1.52	0.99	
	R%	100	2.7± 0.45	27.6± 0.35	23.0± 1.52	40.7± 3.39	94.0±4.61
Succinate dehydrogenase	S.A.	20 ± 1.3	n.d.	14 ± 0.6	39 ± 0.8	n.d.	
	R.S.A.	1	0	0.68	1.92	0	
	R%	100	0	22.2± 1.32	29.1± 2.95	0	51.3±1.95
Alkaline phosphatase	S.A.	450 ±70.6	138 ±23.8	206 ± 7.5	376 ±36.0	768 ±32.6	
	R.S.A.	1	0.31	0.46	0.84	1.71	
	R%	100	1.9± 0.13	14.9± 0.71	12.7± 1.47	70.5± 2.08	100.0±2.19
$(Na^+ + K^+)$-ATPase	S.A.	256 ±30.3	n.d.	538 ± 6.8	247 ±34.3	85 ± 8.5	
	R.S.A.	1	0	2.10	0.96	0.33	
	R%	100	0	68.5± 6.33	14.6± 1.26	13.7± 1.66	96.8±5.59

S.A., specific activity; R.S.A., relative specific activity; *R*%, percentage of total activity. R.S.A. and *R*% refer to the P_3 fraction. For details, see the legend of Table I. (n=7). n.d., not detectable.

ment of the basolateral membranes is evident. A P_3 fraction obtained from guinea-pig kidney cortex behaves similarly when applied to a Percoll gradient (results not shown).

Yield of brush-border membranes

Brush-border membranes were obtained by applying the calcium precipitation technique [3] to an intermediate pellet, P_1. The choice of this pellet lay in the recovery of 59.1% of the total alkaline phosphatase activity with an enrichment of 2.15-fold (Table I). However, the fraction was highly contaminated. Nevertheless, following the Ca^{2+} precipitation, the final brush-border fraction (B-B) contained 18.9% of the total alkaline phosphatase activity, with an enrichment factor of 17.5 (Table III). Fig. 3 illustrates the enrichment and recoveries of the different marker enzymes before and after the calcium precipitation and shows that the contaminants are reduced to low levels.

The results obtained with marker enzymes indi-

TABLE III

ENZYME ACTIVITIES AND RECOVERIES OF B-B FRACTION

S.A., specific activity; R.S.A., relative specific activity; *R*%, percentage of total activity. R.S.A. and *R*% refer to (P_1) fraction. For details see the legend of Table I ($n=5$).

Marker enzymes	S.A.	R.S.A.	*R*%
Proteins	–	–	3.9±0.44
Mg^{2+}-ATPase	201± 24.0	1.56	6.5±0.91
Acid phosphatase	9.7± 0.86	2.03	6.5±0.66
Glucose-6-phosphatase	11± 0.8	0.39	1.4±0.18
Succinate dehydrogenase	19± 0.5	0.59	2.7±0.07
Alkaline phosphatase	3573±304.4	8.12	68.9±1.50
($Na^+ + K^+$)-ATPase	39± 5.1	0.64	3.6±0.66

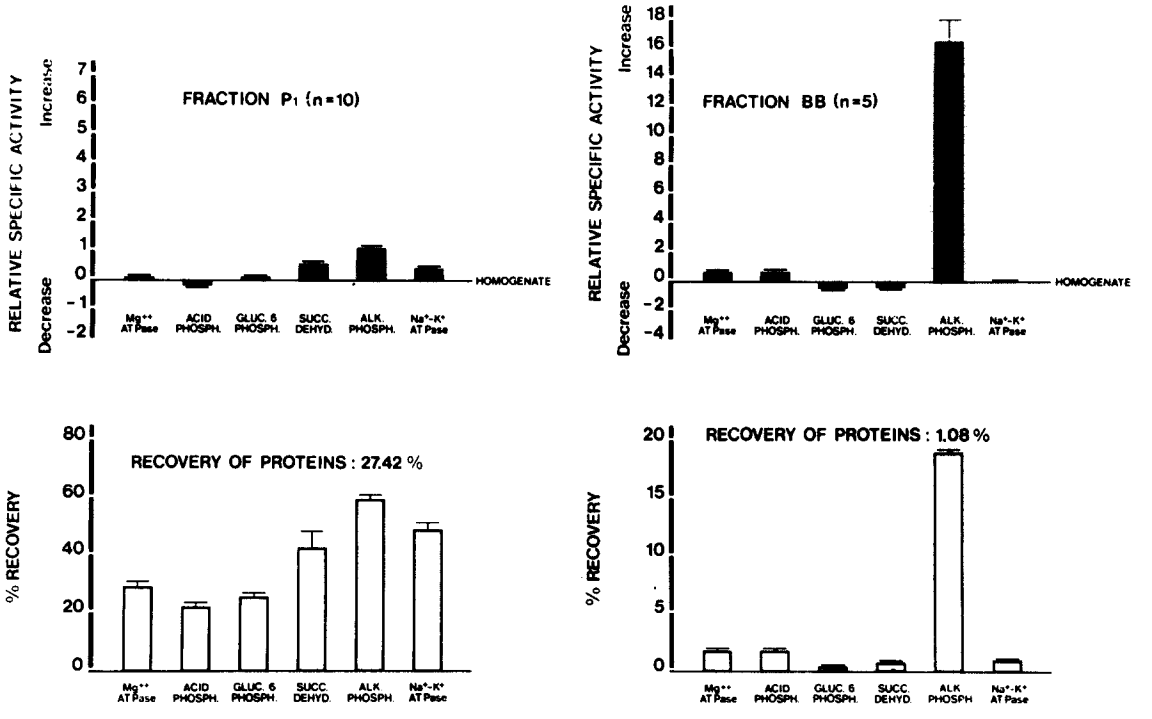


Fig. 3. Relative specific activities (upper panels) and percentage of recovery with respect to the original homogenate (lower panels) of different marker enzymes in brush-border fractions before (P_1) and after (B-B) treatment with calcium. Representation as in Fig. 2. Data taken from Tables I and III ($n=7$).

cate that fractions F-2 and B-B are composed of basolateral and brush-border membranes, respectively. These conclusions are corroborated by the investigation of suspensions using negative staining electron microscopy (Fig. 4). The basolateral fraction contains structures that could be ribosomes, but control experiments indicate that they consist of Percoll particles remaining in the preparation.

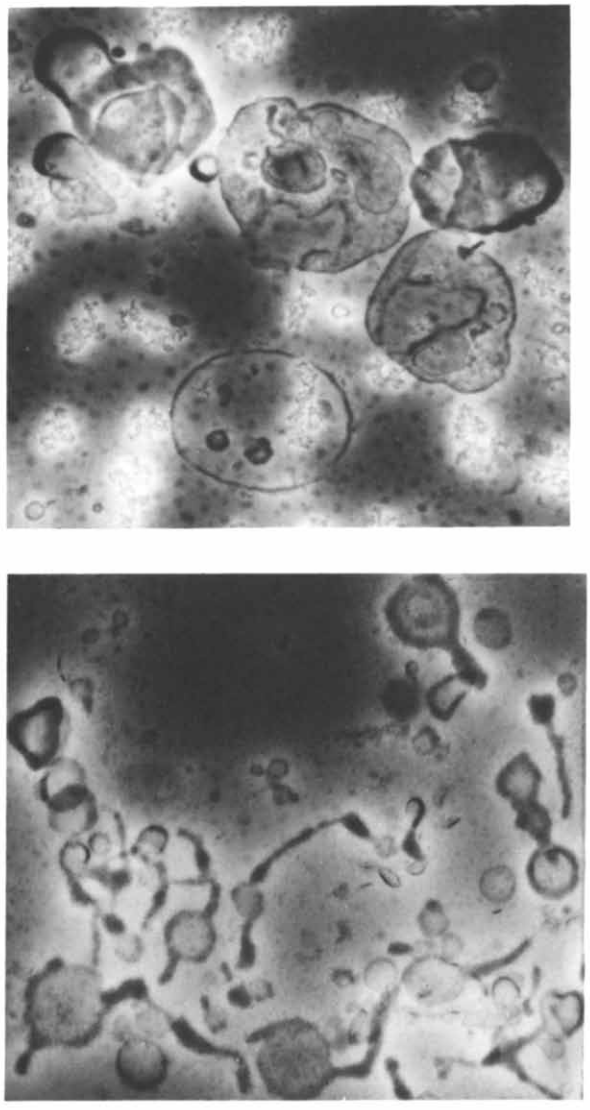
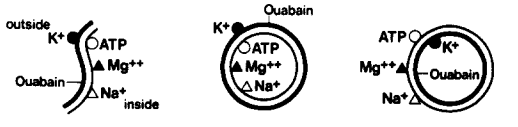


Fig. 4. Electron micrographs of fractions F-2 (upper panel) and B-B (lower panel).

Orientation of basolateral membrane vesicles

The orientation of basolateral membrane vesicles can be explored by making use of the asymmetric orientation of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase in the membranes. The rationale for the techniques applied is illustrated in Fig. 5, which shows the position of the binding sites of the enzymes in each type of orientation. If the membranes are present as sheets, the sites for Mg^{2+}, Na^+ and ATP at the internal face and the sites for K^+ and ouabain at the external face would all be accessible to their ligands, with the result that both Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities would be maximal, as would the binding of ouabain. In the case of right-side-out vesicles, only the K^+ and ouabain sites would be accessible; in particular the ATP site would be inaccessible. Thus in this case, the activities of both ATPases would be non-existent but ouabain binding would be maximal. In the case of inside-out vesicles, the sites for Mg^{2+}, Na^+ and ATP would be accessible, but those for K^+ and ouabain would be absent. Thus the activity of Mg^{2+}-ATPase would be maximal, that of $(Na^+ + K^+)$-ATPase would be low and ouabain binding would be non-existent. Finally, vesicles can be transformed into sheets by treatment with a mixture of deoxycholate and EDTA [34], or by repeated freezing and thawing.

Thus the activities of the two ATPases and of the binding of tritiated ouabain were studied before and after treatment with deoxycholate/EDTA. The results are demonstrated in Tables IV



	SHEETS	RIGHTSIDE-OUT VESICLES	INSIDE-OUT VESICLES
Na^+-K^+-ATPase activity	Maximal	Absent	Low
Mg^{++}-ATPase activity	Maximal	Absent	Maximal
Ouabain binding and inhibition	Complete	Complete	Absent

Fig. 5. Theoretical explanation of the changes in activity of $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase and ouabain binding in accordance with the accessibility of the possible ligands to their binding sites, as a function of the possible orientations of basolateral membranes in a preparation.

TABLE IV

EFFECT OF TREATMENT WITH DEOXYCHOLATE/EDTA ON Mg^{2+}-ATPase AND $(Na^+ + K^+)$-ATPase IN BASOLATERAL MEMBRANES OF SMALL INTESTINE

Incubation medium	ATPase activity (nmol P_i/mg protein per min)	
	−DOC/EDTA	+DOC/EDTA
$Mg^{2+} + Na^+ + K^+$ + ouabain	294 ± 10.7	278 ± 15.8
$Mg^{2+} + Na^+ + K^+$	573 ± 12.3	912 ± 26.8
$(Na^+ + K^+)$-ATPase	+279 (44%)	+634 (100%)

The deoxycholate (DOC)/EDTA treatment of F-2 fraction was carried out as indicated under Materials and Methods. The assays were performed at pH 7. Mg^{2+} concentration, 5 mM; Na^+, 100 mM; K^+, 20 mM; ouabain, 1 mM. ($n = 6$).

and V. The activity of Mg^{2+}-ATPase is not modified by the treatment, but the $(Na^+ + K^+)$-ATPase activity is increased by 56%. Ouabain binding is increased by 61% after treatment with deoxycholate/EDTA. Thus it can be concluded that the preparation is composed approximately of 60% inside-out vesicles and 40% sheets. Unfortunately, no analogous method is available for the determination of the orientation of brush-border membrane vesicles.

All other enzymes studied exhibited the same specific activities before and after pretreatment of the requisite fraction with deoxycholate/EDTA.

TABLE V

EFFECT OF TREATMENT WITH DEOXYCHOLATE/EDTA ON [^{3}H]OUABAIN BINDING IN BASOLATERAL MEMBRANES OF SMALL INTESTINE

The deoxycholate (DOC)/EDTA treatment of the F-2 fraction was carried out as indicated under Materials and Methods. Mg^{2+} concentration, 1.25 mM; Na^+, 100 mM; ATP, 1.25 mM; ouabain, 0.01 mM. ($n = 4$).

Binding (pmol/mg protein)	−DOC/EDTA	+DOC/EDTA
Total	778 ± 91.9	1818 ± 140.6
Nonspecific	116 ± 34.1	129 ± 25.4
Specific	662 (39%)	1689 (100%)

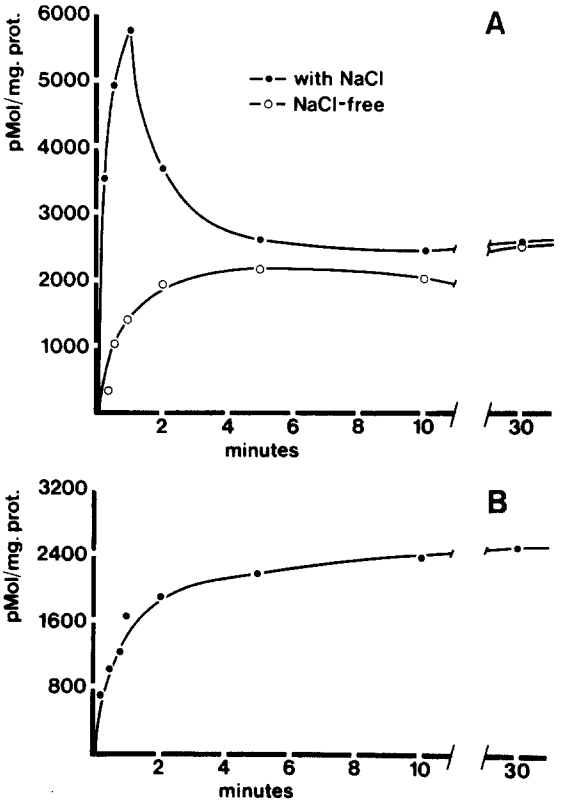


Fig. 6. Uptake of D-[^{14}C]glucose in brush-border (1 mM D-glucose) (A) and basolateral (2 mM D-glucose) (B) membranes as a function of time. Assays performed at 25°C in 100 mM mannitol with 100 mM NaCl or 100 mM KCl. D-Glucose: 1 mM.

TABLE VI

EFFECT OF ABSENCE OF SODIUM AND OF PHLORETIN ON GLUCOSE UPTAKE IN BASOLATERAL MEMBRANES

Results are means ± S.E. of four parallel measurements within a typical experiment with basolateral membranes incubated for different time periods at 25°C in 100 mM mannitol with 100 mM NaCl, or 100 mM KCl, or 100 mM NaCl + 0.2 mM phloretin.

Condition	Uptake of glucose (pmol/mg protein)		
	1 min	2 min	30 min
Presence of sodium gradient	1500 ± 180	2975 ± 299	3031 ± 144
Absence of sodium gradient	1573 ± 218	2844 ± 313	3125 ± 281
Presence of phloretin	979 ± 97	1333 ± 135	1107 ± 131

Functional characteristics of vesicles

The functional properties were examined by determining glucose uptake in the presence of a sodium gradient (exterior > interior). The results are illustrated in Fig. 6. The luminal membranes reveal an overshoot in the uptake of glucose (upper panel), whereas the basolateral membranes reveal an uptake of the sugar that is not affected by a sodium gradient, but is inhibited by phloretin (Table VI). These results correspond with those described by numerous authors [10,32].

Discussion

In the present article, we have described a simple and rapid method for the preparation of brush-border and basolateral membranes from the epithelial cells of the intestine. The fractions can be obtained approximately 3 h after the original homogenization. The method can be applied without modifications to renal tissue. In both final fractions, enrichment and recovery are satisfactory and contamination is minimal. The F-2 fraction contains 12.4% of the total $(Na^+ + K^+)$-ATPase activity with an enrichment factor of 12.6-fold, whereas the fraction B-B contains 18.9% of the alkaline phosphatase with an enrichment factor of 17.5-fold. Negatively stained electron micrographs of each fraction show characteristic images of the two types of cell membrane (Fig. 4).

Using a simple experimental device [35], we have determined the orientation of the basolateral membranes of our preparation. This is possible because of the asymmetric distribution of the $(Na^+ + K^+)$-ATPase complex in the membrane [36–39]. The Mg^{2+}-ATPase has binding sites for both ligands at the internal face of the membrane. When the activities of the two ATPases are determined before and after treatment with deoxycholate/EDTA which transforms the vesicles into sheets, a 56% increase in $(Na^+ + K^+)$-ATPase is observed in the absence of any change in Mg^{2+}-ATPase activity. This result indicates that the sites for Mg^{2+} and ATP are all accessible before the vesicles are transformed into sheets; therefore the preparation can only contain inside-out vesicles and/or sheets. These observations are corroborated by the results on ouabain binding, in which there is an increase of 61% when the preparation is treated with deoxycholate/EDTA. The slight difference between the two results may be due to the fact that a small quantity of potassium ions may diffuse into the inside-out vesicles, which would cause a small increase in the $(Na^+ + K^+)$-ATPase activity before treatment with deoxycholate/EDTA. For this reason, we have indicated on the theoretical figure (Fig. 5) that inside-out vesicles may reveal a small $(Na^+ + K^+)$-ATPase activity. We can conclude from our experiments that our fraction contains about 60% inside-out vesicles and 40% sheets. Interestingly, only one of the many groups that have prepared basolateral membranes has attempted to determine the orientation of their preparation, using the ATPase technique that we have described. Kinsella et al. [11] found, in contrast to ourselves, that the majority of the vesicles obtained from dog renal cortex were right-side-out, since they observed a huge increase in both $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities when the vesicles were opened.

Unfortunately, an analogous technique for determining the orientation of brush-border vesicles is not available, though we can conclude that since deoxycholate/EDTA treatment of the brush-border fraction had no effect on alkaline phosphatase activity in our hands, all our vesicles were right-side-out. Kinsella et al. [11] applied a technique to label exposed sugar moieties of the glycoproteins of the membrane. Otherwise, the determination of the orientation of brush-border vesicles has been performed by freeze etching [40] which makes use of the characteristic structure of the two faces of this vesicle. Such an approach, on the other hand, has not been applied to basolateral vesicles.

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