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A HIGH YIELD PREPARATION FOR RAT KIDNEY BRUSH BORDER MEMBRANES

DIFFERENT BEHAVIOUR OF LYSOSOMAL MARKERS

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Rat kidney coretx slices were homogenized with a polytron in a isoosmotic medium containing 5 mmol/l EGTA. By two precipitations with $MgCl_2$ (12 mmol/l) and differential centrifugation, brush border membranes were purified. The brush border marker enzymes alkaline phosphatase and aminopeptidase M were found to be enriched 17.0 ± 5.3-fold and 16.7 ± 3.7-fold, respectively. By this method, a high yield of brush border membranes was obtained (48.3 ± 7.9% for alkaline phosphatase; 47.0 ± 9.5% for aminopeptidase M). The acid phosphatase was enriched 5-fold, whereas other lysosomal enzymes (glucosaminidase, glucuronidase, cathepsin D) were enriched only 0.2-fold. Acid phosphatase activity could not be washed out, but could be separated from alkaline phosphatase and leucine aminopeptidase by means of free flow electrophoresis and sucrose density gradient centrifugation. Vesicles prepared by the presently described Mg/EGTA-method show better transport properties, compared to vesicles prepared by the calcium method of Evers et al. (Evers, C., Haase, W., Murer, H. and Kinne, R. (1978) Membrane Biochem. 1, 203–219), whereas by SDS-polyacrylamide gel electrophoresis, no differences in the protein patterns were observed.

Introduction

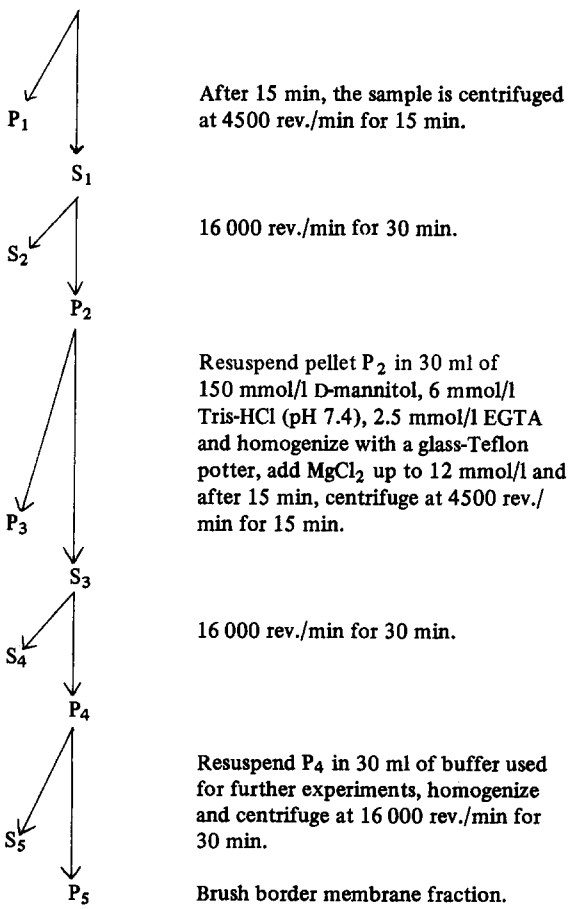
Several methods have been developed in the past to isolate brush border membrane vesicles from kidney cortex [1–5]. Most of these methods are modifications of the method originally introduced by Booth and Kenny [5], which is based on differential centrifugation following addition of 10–20 mmol/l $CaCl_2$ or $MgCl_2$ to the initial homogenate. By these procedures, brush border vesicles can be obtained, which are 10–15-fold enriched with respect to the brush border marker enzymes (e.g., alkaline phosphatase), whereby the yield of these enzymes is only about 5–20% of the amount present in the initial homogenate. Interestingly, often a cross-contamination by lysosomal structures was reported [1,5].

In the present paper, we describe a modified procedure, based on the procedure of Booth and Kenny [5]. Modifications in the buffer composition, in the initial homogenization as well as in the centrifugation conditions lead to a brush border membrane preparation with a yield of about 50% of the marker enzymes and similar enrichment factors as in the preparations mentioned above. A cross-contamination by the lysosomal marker enzyme acid phosphatase was also found in this preparation. However, evidences were obtained that the acid phosphatase is not bound to the brush border membrane nor represents a cross-contamination by lysosomes.

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulphate.

Methods

Membrane separation. Brush border membranes from rat kidney cortex slices (male wistar rats, approx. 200 g) were prepared by a two-step Mg^{2+} precipitation method as outlined in Scheme I. Cortex slices from one rat were homogenized with a polytron (PT 10-35, Kinematica GmbH, Kriens, Switzerland), and all centrifugation steps were performed at 4°C in a Sorvall RC-5B centrifuge, using a SS-34 rotor. Kidney cortex slices from one rat are homogenized in 15 ml isolation buffer (300 mmol/l D-mannitol, 5 mmol/l EGTA, 12 mmol/l Tris-HCl, pH 7.4) with the Polytron on setting 5 for 2 min. Add 21 ml of cold bi-distilled water and $MgCl_2$ up to a final concentration of 12 mmol/l.



Scheme I. Flow diagram for the isolation of brush border membranes from rat kidney cortex slices.

Enzyme and protein determination. All enzymes were analysed at the same day. if possible, semi-automatic determination was carried out with a LKB reaction rate analyzer 8600 at 37°C. The activities of alkaline phosphatase (EC 3.1.3.1) and $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) were assayed as described by Berner and Kinne [6] and that of aminopeptidase M (EC 3.4.11.2) as described by Haase et al. [7]. Potassium-stimulated phosphatase was measured as described in Ref. 8. The activities of KCN-resistant NADH-oxidoreductase (EC 1.6.99.2) and succinate-cytochrome *c*-oxidoreductase (EC 1.3.99.1) were determined according to Sottocasa et al. [9] and Fleischer and Fleischer [10], respectively. Acid phosphatase (EC 3.1.3.2) was determined by the amount of *p*-nitrophenyl-phosphate split at pH 4.5 (acetate buffer, 100 mmol/l) in the presence of 0.15% (w/v) Triton X-100 (Merck). Glucosaminidase (EC 3.5.1.33) was assayed in a citrate buffer (200 mmol/l, pH 4.5) containing 20 mg Triton X-100 per 100 ml and 8 mmol/l *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) as the substrate, according to Scalera et al. [11]. The same buffer but containing 4-nitrophenyl-β-D-glucopyranoside uronic acid (Merck) was used for the determination of the glucuronidase (EC 3.2.1.31) activity. Cathepsin D (EC 3.4.4.23) was determined according to the following procedure (Portmann, P., personal communication): 0.75 ml substrate buffer (0.1 mol/l sodium lactate, pH 3.8), containing 2% hemoglobin, were mixed with 0.05 ml of enzyme solution and incubated overnight at 37°C. The reaction was stopped with 1 ml 5% trichloroacetic acid and the samples were centrifuged for 10 min. 1 ml of the supernatant was then mixed with 0.5 ml of 1 N Folin-Ciocalteus reagent and 2 ml 5% NaOH. After 10 min, the optical density was determined at 750 nm. L-Tyrosine was used as a standard. Lactate dehydrogenase (EC 1.1.1.27) was determined according to Scalera et al. [11]. Protein was determined according to the method of Bradford [12] using the Bio-Rad reagent.

Other methods. SDS-polyacrylamide gel electrophoresis was performed on 2-mm thick 8.5% slab gels, prepared according to Laemmli [13]. Brush border membranes were denatured in a Tris-HCl buffer (pH 6.8), containing 2% SDS and 0.7 mol/l mercaptoethanol by rapid boiling for 2 min. Transport studies were performed as described in Refs. 4 and 14,

using a Millipore filtration technique. Membrane vesicles obtained by the Mg/EGTA-method were prepared for electron microscopy by standard procedures (e.g., Ref. 1).

Results

1. Enzymatic characterization

In Table I are compiled the recoveries (Table Ia), specific activities as well as the enrichment factors (Table Ib) of marker enzymes for the brush border membrane (alkaline phosphatase and aminopeptidase M), basal-lateral plasma membranes ($(Na^+ + K^+)$-ATPase)), endoplasmic reticulum (KCN-resistant NADH-oxidoreductase), mitochondria (succinate-cytochrome *c*-oxidoreductase) lysosomes (cathepsin D, glucuronidase, glucosaminidase, acid phosphatase) and the cytosol (lactate dehydrogenase). The reason for the use of several lysosomal marker enzymes is their non-parallel distribution pattern during tissue fractionation, as will be presented and discussed below. The specific activities of brush border marker enzymes, alkaline phosphatase and aminopeptidase M, were enriched in the final brush border membrane fraction (Table Ib; sediment P_5) about 17-fold, as compared to their activities in the starting homogenate. Approx. 50% of total enzyme activity for these two enzymes was recovered with the brush border membrane fraction (Table Ia). Besides the acid phosphatase, only the $(Na^+ + K^+)$-ATPase activity is slightly enriched in the final brush border membrane preparation (Table Ib). This small contamination of the brush border membrane preparation by basal-lateral plasma membranes might, at least in part, be explained by insufficient homogenization, leading to brush border membrane fragments containing lateral tags of lateral-basal membrane fragments. However, since in most of the described brush border membrane preparations a similar cross-contamination by this basal-lateral membrane marker is observed, (e.g., see Refs. 1 and 5), one might speculate that $(Na^+ + K^+)$-ATPase is to a small extent also a constituent of the luminal membrane. Certainly, further experiments will be needed to answer this question definitively. As indicated by the low activities for KCN-resistant NADH-oxidore-

TABLE Ia

RECOVERIES FOR MARKER ENZYMES OBTAINED DURING ISOLATION OF BRUSH BORDER VESICLES FROM RAT KIDNEY CORTEX

The values represent the percentage of the enzyme activities found initially in the homogenate. Means ± S.D. of *n* individual experiments are given.

	Homogenate	P_1	S_2	P_2	$S_4 + S_5$	Brush border fraction P_5	Total recovery
Alkaline phosphatase ($n = 7$)	100	31.7 ± 5.3	10.1 ± 2.1	9.1 ± 2.3	11.7 ± 3.8	48.3 ± 7.9	110.9 ± 10.7
Aminopeptidase M ($n = 7$)	100	20.0 ± 3.3	18.1 ± 8.5	8.1 ± 3.0	11.6 ± 6.8	47.0 ± 9.5	104.8 ± 15.1
Succinate-cytochrome *c* oxidoreductase ($n = 7$)	100	80.1 ± 18.9	3.3 ± 2.2	1.5 ± 0.7	1.7 ± 1.3	0.3 ± 0.1	86.9 ± 19.1
KCN-resistant NADH oxidoreductase ($n = 7$)	100	80.9 ± 15.1	18.7 ± 3.5	1.5 ± 1.0	3.3 ± 0.8	0.1 ± 0.2	104.5 ± 15.5
$(Na^+ + K^+)$-ATPase ($n = 6$)	100	67.5 ± 23.9	2.7 ± 1.8	3.4 ± 0.6	1.0 ± 0.6	3.6 ± 1.3	78.2 ± 24.0
K-phosphatase ($n = 7$)	100	81.3 ± 13.0	7.8 ± 3.1	4.5 ± 0.7	3.6 ± 0.8	6.6 ± 3.8	103.8 ± 13.9
Lactate dehydrogenase ($n = 7$)	100	20.6 ± 5.9	92.1 ± 15.1	0.8 ± 0.7	1.9 ± 0.5	0.2 ± 0.1	115.6 ± 16.2
Acid phosphatase ($n = 7$)	100	19.8 ± 2.5	55.5 ± 7.5	2.1 ± 0.2	6.5 ± 1.2	14.4 ± 4.2	98.3 ± 9.0
Glucosaminidase ($n = 6$)	100	75.4 ± 8.3	21.2 ± 4.0	1.6 ± 0.4	1.2 ± 0.3	0.7 ± 0.2	100.1 ± 9.2
Glucuronidase ($n = 5$)	100	25.3 ± 3.4	75.5 ± 9.5	0.9 ± 0.2	3.7 ± 1.1	0.5 ± 0.1	105.9 ± 10.1
Cathepsin D ($n = 4$)	100	35.6 ± 9.8	70.2 ± 11.4	1.4	2	0.3 ± 0.1	109.5 ± 15.0
Protein ($n = 7$)	100	39.5 ± 4.6	42.8 ± 2.9	1.8 ± 0.4	0.6 ± 0.1	2.9 ± 0.4	87.6 ± 5.5

TABLE Ib

SPECIFIC ACTIVITIES AND ENRICHMENT FACTORS OF THE MARKER ENZYMES FOUND IN THE BRUSH BORDER FRACTION

All enzyme activities are given in $\mu mol \cdot min^{-1} \cdot mg^{-1}$, except for glucuronidase and cathepsin D, which are expressed in $nmol \cdot h^{-1} \cdot mg^{-1}$. Means ± S.D. of the same number of experiments as in Table Ia are given.

	Specific activities in		Enrichment factors
	Homogenate (H)	Brush border fraction (P_5)	
Alkaline phosphatase	0.69 ± 0.18	11.70 ± 1.98	17.0 ± 5.3
Aminopeptidase M	0.072 ± 0.011	1.199 ± 0.197	16.7 ± 3.7
Succinate-cytochrome *c* oxidoreductase	0.0217 ± 0.0015	0.00275 ± 0.0065	0.1 ± 0.3
KCN-resistant NADH oxidoreductase	0.4824 ± 0.027	0.0065 ± 0.0014	0.01 ± 0.003
$(Na^+ + K^+)$-ATPase	0.150 ± 0.043	0.253 ± 0.125	1.7 ± 1.0
K-phosphatase	0.016 ± 0.0034	0.0374 ± 0.010	2.3 ± 0.8
Lactate dehydrogenase	0.777 ± 0.236	0.048 ± 0.011	0.06 ± 0.02
Acid phosphatase	0.057 ± 0.008	0.307 ± 0.140	5.4 ± 2.6
Glucosaminidase	0.0915 ± 0.0103	0.025 ± 0.006	0.3 ± 0.1
Glucuronidase	43.5 ± 13.2	10.44 ± 8.4	0.2 ± 0.2
Cathepsin D	0.47 ± 0.04	0.05 ± 0.02	0.1 ± 0.04

ductase and succinate-cytochrome *c*-oxidoreductase, there is only very little cross-contamination of the brush border membrane fraction P_5 with endoplasmic reticulum and mitochondria, respectively. As judged by the recovered activities and enrichment factors (far below 1) for cathepsin D, glucuronidase and glucosaminidase, the described brush border membrane preparation is only contaminated very little by lysosomes. Interestingly, acid phosphatase, an enzyme routinely used as a lysosomal marker, does not follow the distribution of the other lysosomal marker enzymes, but the specific activity of this enzyme is enriched in the final membrane preparation by a factor of about 5, as compared to its activity in the initial homogenate. The phosphatase activity measured at low pH values cannot be attributed to a rest activity of alkaline phosphatase because the presence of 5 mmol/l theophylline – 50% inhibition is observed at 300 μmol/l for alkaline phosphatase – did not reduce the activity at low pH values (data not shown). The acid phosphatase activity associated to the brush border membrane fraction could be inhibited by 5 mmol/l L-tartrate to an extent of 82% and by 2 mmol/l NaF to an extent of 64% (data not shown).

2. Different behaviour of lysosomal marker enzymes

For the lysosomal enzymes tested in this study, a different sedimentation behaviour was found (Table Ia). Thereby, the enzyme activities recovered in the first low-speed pellet (P_1) were compared with the activities found in the first high-speed supernatant S_2. As indicated by the lactate dehydrogenase activity, soluble enzymes are predominantly found in the supernatant S_2 (92 ± 15%). Also most of the lysosomal enzymes are found in the supernatant S_2 (acid phosphatase: 55 ± 7%; glucuronidase: 75 ± 10%; cathepsin D: 70 ± 11%). Among the lysosomal markers tested, only the glucosaminidase activity shows a different sedimentation behaviour. Its activity is predominantly recovered in the first pellet P_1 (75 ± 8%). Thus, acid phosphatase, cathepsin D and glucuronidase behave like soluble proteins or are bound to particles which cannot be sedimented under these experimental conditions, whereas glucosaminidase can be centrifugated. Since homogenization by the Polytron is powerful, we can assume that lysosomes are largely destroyed by this procedure. This finding would therefore indicate, that glucosaminidase but not the other lysosomal enzymes, is localized in lysosomal substructures (membranes) or is

precipitated by magnesium. The second puzzling finding in the study on the behaviour of lysosomal markers was that, among the lysosomal enzymes found in supernatant S_2, only acid phosphatase 'co-purifies' somehow during further differential centrifugation with brush border membrane markers. Certainly, these findings indicate that the acid phosphatase activity found in the final brush border membrane preparation is not a measure for contamination of the membrane preparation by intact lysosomes. What are the possible explanations for these findings? (i) Acid phosphatase might be localized in lysosomal (sub)structures which have a similar sedimentation behaviour as the brush border membranes. (ii) Solubilized acid phosphatase in the presence of divalent cations forms aggregates which show sedimentation behaviour similar to that of brush border membranes. (iii) Acid phosphatase is a native constituent of the brush border membrane or is trapped into the vesicles. (iv) Solubilized acid phosphatase specifically interacts with the brush border membrane surface similarly to the known interaction between lysosomal enzymes and plasma membranes in other tissues [15–17]. Various agents were therefore tested for their capacity to wash out the acid phosphatase from the brush border vesicles. Treatments with high salt concentrations, EDTA or dithioerythritol were without effect. It was also not possible to wash out the acid phosphatase by various sugars and sugar derivatives, known to be competitive for the interactions of lysosomal proteins with plasma membranes [15–17]. Especially, D-mannose-6 phosphate, which has been shown to compete for glucuronidase-plasma membrane interaction [15] was without effect. Also no effect of this sugar was observed in the presence of theophylline to prevent hydrolysis of mannose-6 phosphate by alkaline phosphatase (data not shown). Therefore, a binding of acid phosphatase to brush border membranes is unlikely. Since the addition of the detergent Triton X-100 has no effect on the acid phosphatase activity found in the brush border membrane fraction, a trapping of the acid phosphatase in the vesicle interior is also unlikely (data not shown).

However, it was possible to separate partially the activities of the acid phosphatase from the ones of alkaline phosphatase and leucine-aminopeptidase M. By free-flow electrophoresis (data not shown) and

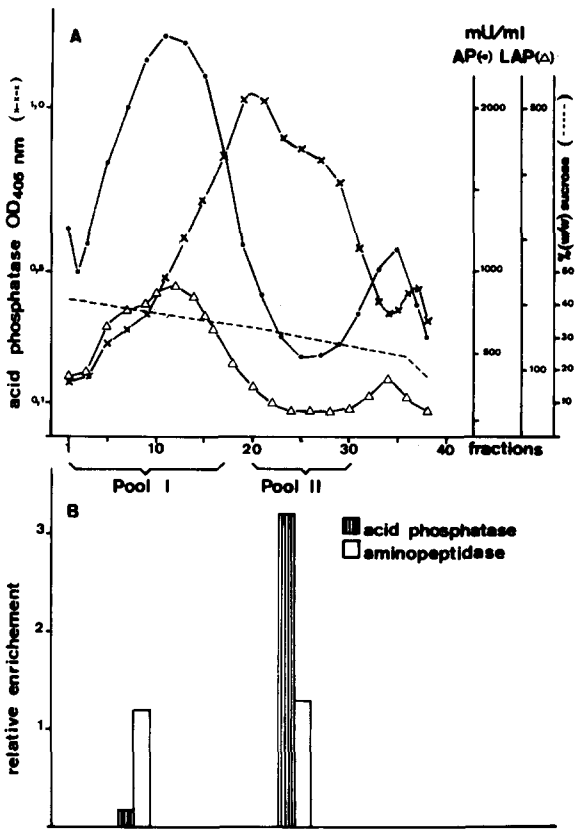


Fig. 1. (A) Distribution pattern of alkaline phosphatase (AP, •), leucine aminopeptidase M (LAP, △) and acid phosphatase (X) after sucrose density centrifugation of the purified brush border vesicles (3 X 20 swing out, MSE; 90 min at 30 000 rev./min). (B) Relative enrichment factors of acid phosphatase and leucine aminopeptidase M. Membranes were collected in pooled fractions by centrifugation at 100 000 X g for 60 min. The specific activities found in pool I and pool II were compared with the specific activities found in membrane fraction (P_5) put on top of the gradient. The means of two experiments are given.

sucrose density gradient centrifugation (Fig. 1A) a different distribution pattern of these enzymes was found, indicating different physical properties (surface charge, density) of the acid phosphatase containing particles and the brush border membrane vesicles. Accordingly, there is a negative correlation between the specific activities found for alkaline phosphatase and acid phosphatase (Fig. 1B). We conclude therefore that the acid phosphatase is connected with particles which are different from the brush border membrane vesicles.

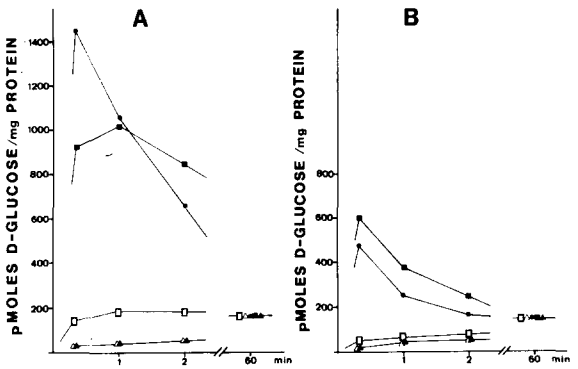


Fig. 2. D-Glucose uptake by renal brush border vesicles. Vesicles were prepared by the Mg/EGTA method (Fig. 3A) and by a Ca^{2+} method [1] (Fig. 3B). Transport was measured, using 0.1 mmol/l D-[^{3}H]glucose under the following conditions: (i) inwardly directed gradient of 100 mmol/l NaSCN (●); 100 mmol/l NaCl (■) and 100 mmol/l KCl (▲). (ii) Vesicles were preequilibrated at room temperature for 60 min, either with 100 mmol/l NaCl (□) or 100 mmol/l KCl (△).

3. Transport experiments

The uptake of 0.1 mmol/l D-[^{3}H]glucose was measured under two different conditions and compared with vesicles prepared according the procedure published by Evers et al. [1] (Fig. 2). When an inwardly directed electrochemical gradient of sodium ($[Na^+]_{out} > [Na^+]_{in}$) was applied to the vesicles isolated by the Mg/EGTA-method, D-glucose is accumulated about 10-fold, using NaSCN and 6.5-fold using NaCl. D-Glucose uptake into vesicles which have been pre-equilibrated either with 100 mmol/l NaCl or 100 mmol/l KCl for 60 min at room temperature, showed a 10-fold stimulation of D-glucose transport (measured at 20 s) by sodium. The overshoot, the effect of anion replacement *, as well as the sodium effect under equilibrated conditions is more pronounced in Mg/EGTA-vesicles than in membrane vesicles isolated by the method described in Ref. 1 (Fig. 2B). Thus, membrane vesicles isolated by the Mg/EGTA precipitation method seem to possess the better specific transport characteristics than membranes isolated by Evers et al. [1]. In addition, the Mg/EGTA vesicles also exhibit higher rates for phosphate transport (data not shown).

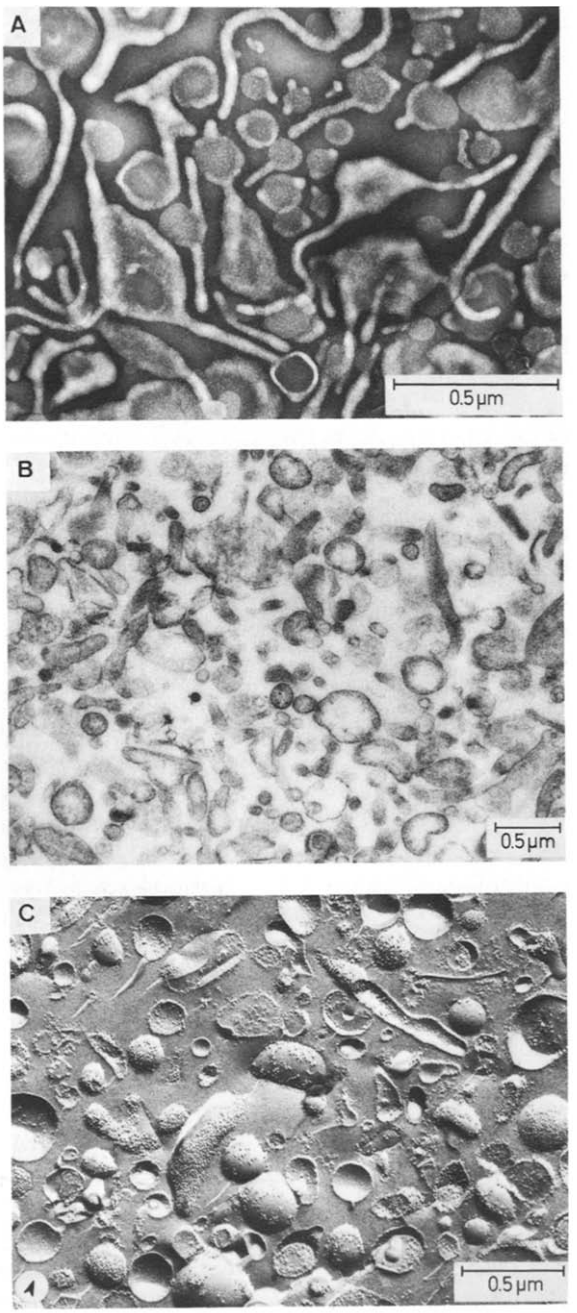


Fig. 3. Electron micrographs of rat renal brush border membranes (fraction P_5). (A) Negative-stained preparation; (B) Thin-sectioned preparation; (C) Freeze-fractured preparation.

* Due to the potential sensitive character of the sodium-glucose co-transport mechanism, gradients of more permeant anions show better stimulation of sodium-dependent glucose transport (e.g., Ref. 23). The higher effect of anion replacement in Mg/EGTA vesicles could therefore be a hint for a lower unspecific membrane permeability.

4. Membrane protein composition

The polypeptide patterns of membrane prepared by the Mg/EGTA-method and that of membranes prepared by Evers et al. [1] were compared by means of SDS-polyacrylamide gel electrophoresis (data not shown). No significant difference of the two polypeptide patterns could be observed, indicating no substantial loss of proteins from the membrane induced by the addition of EGTA to the isolation medium.

5. Morphological characterization

Fig. 3 shows the morphological appearance of renal brush border membranes isolated by the Mg/EGTA precipitation method. As described also for the brush border membrane vesicles isolated according to Ref. 1, spherical vesicles of different sizes as well as small elongated structures can be observed. One might speculate that in the finger-like elongated structures representing probably microvilli, the intramicrovillus core is intact, whereas in the more spherical structures, the actin containing core material is at least partially disintegrated. In agreement with this notion is (i) the appearance of transition structures observed especially in negative contrast pictures where the microvilli are partially blown up and (ii) the preferential appearance of more electron-dense content in small diameter structures and/or elongated structures, as seen in thin section pictures.

Discussion

By inclusion of EGTA into the isolation medium and homogenization with a Polytron, brush border membranes could be purified 17-fold, as was described before [1,5], however, the yield of brush border membranes is higher. This high yield of membrane vesicles can be explained by the inclusion of EGTA, which helps to destroy intercellular junctions [18] and the powerful homogenization used. This high-yield preparation is of interest if only small amount of tissue (or cells) is available.

Recently Hauser et al. [19] showed that in rabbit intestinal brush border vesicles prepared by a calcium precipitation method [20], the content of lysophospholipids is abnormally high. By inclusion of EGTA and, therefore, presumably inactivating membrane bound Ca^{2+}-dependent phospholipases, the content of lysophospholipids decreased to a lower level [19]. The lipid composition of biological membranes is one of the determinants of their transport properties. Thus, artificially induced alterations of the lipid composition might also alter transport characteristics. One might therefore speculate that the reduced transport activity, found in vesicles prepared according to Evers et al. [1] reflects altered lipid composition – increased lyso compounds – compared to that in the Mg/EGTA method. Further experiments are required to substantiate this speculation on differences in the lysophospholipid content.

No contamination by the routinely checked marker enzymes of mitochondria and endoplasmic reticulum was observed. The only enzyme activity, besides the typical brush border enzymes found to be enriched in the final membrane preparation, was that of the acid phosphatase, a marker usually used to detect lysosomal contamination. However, this enrichment of the acid phosphatase does not indicate a contamination of the brush border membrane preparation by intact lysosomes, as discussed above (see Results). Thus, although partial co-purification is found, acid phosphatase is not necessarily a native component of a membrane fraction. This is further confirmed by the fact that in rat renal brush border vesicles isolated from the same group of animals according to Heidrich et al. [22] by free-flow electrophoresis, acid phosphatase is enriched only 0.5 ± 0.2-fold (n = 4). During this preparation, the tissue is homogenized very mildly, leaving most of the lysosomes intact, as indicated by the distribution of the lysosomal enzymes. All of the lysosomal enzymes tested in this study are predominantly found in the low-speed pellets (about 70%), whereas after homogenization with a Polytron, most of the activities are found in the high-speed supernatant (Table Ia).

The non-parallel distribution of the different lysosomal markers, during all fractionation steps might also represent an alternate explanation for the finding on rat enterocyte acid phosphatase activity [21]. In our opinion, it could be possible that acid phosphatase 'solubilized' during the homogenization procedure is bound to basal-lateral membranes or that sub-lysosomal particles (membranes?) co-purify with basal-lateral membranes.

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