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Studies on the Orientation of Brush-Border Membrane Vesicles

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Orientation of rat renal and intestinal brush-border membrane vesicles was studied with two independent methods: electron-microscopic freeze-fracture technique and immunological methods. With the freeze-fracture technique a distinct asymmetric distribution of particles on the two membrane fracture faces was demonstrated; this was used as a criterion for orientation of the isolated membrane vesicles. For the immunological approach the accessibility or inaccessibility of aminopeptidase M localized on the outer surface of the cell membrane to antibodies was used. With both methods we showed that the brush-border membrane vesicles isolated from rat kidney cortex and from rat small intestine for transport studies are predominantly orientated right-side out.

In the last few years methods have been developed to study the role of the plasma membranes from renal proximal-tubular and intestinal-epithelial cells in reabsorptive processes. Thereby the epithelial-cell membrane envelope was resolved into luminal (brush-border) and contraluminal (basolateral) plasma membranes, and transport into osmotically reactive vesicles was measured by a rapid-filtration method. With respect to the physiological relevance of results obtained with these isolated membrane vesicles it is essential to know the orientation of the membrane in the vesicles, i.e. whether the direction of the flux observed *in vitro* is identical with that of the flux occurring *in vivo*.

In the experiments presented below two approaches were used to define the orientation of the membranes in brush-border vesicles isolated from rat proximal tubule and from rat small intestine: electron-microscopic freeze-fracture technique and immunological methods. The former takes advantage of the difference in particle density on the two fracture faces of the plasma membrane and was used by Steck *et al.* (1970) on erythrocyte 'ghosts' and by Altendorf & Staehelin (1974) on *Escherichia coli* plasma-membrane vesicles. The second approach uses the accessibility of aminopeptidase M, an enzyme located at the outer surface of the brush-border membrane (Thomas & Kinne, 1972; Louvard *et al.*, 1975), to inhibitory antibodies as a criterion for membrane orientation. Both methods lead to the conclusion that almost all brush-border vesicles isolated from rat kidney and rat small intestine are oriented right-side out. Thus fluxes observed with these membranes in transport studies *in vitro* are identical in direction with the luminal uptake of substances by the renal and intestinal epithelial cells.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Part of this work was presented at the Frühjahrstagung der Gesellschaft für Biologische Chemie, Regensburg (Haase *et al.*, 1977).

Materials and Methods

Preparation of brush-border membrane vesicles

Brush-border membrane vesicles from rat kidney cortex were isolated either by a calcium-precipitation method by a modification (Evers *et al.*, 1978) of the procedure described by Booth & Kenny (1974), or by free-flow electrophoresis (Heidrich *et al.*, 1972). Brush-border membrane vesicles from rat small intestine were obtained by a calcium-precipitation method by a modification (Sigrist-Nelson *et al.*, 1975; Kessler *et al.*, 1978) of the procedure described by Schmitz *et al.* (1973). Briefly, for the calcium-precipitation method mucosal pieces of kidney-cortex slices were homogenized in a hypo-osmotic medium (Booth & Kenny, 1974; Sigrist-Nelson *et al.*, 1975), and after addition of 10mM-$CaCl_2$ the brush-border membranes were purified by differential centrifugation. For the electrophoretic separation the kidney-cortex slices were homogenized in iso-osmotic sucrose medium, and after differential centrifugation luminal and contraluminal membranes were separated by virtue of their different electrophoretic mobilities in the free-flow-electrophoresis apparatus (FF IV, Desaga, Heidelberg, Germany) (Heidrich *et al.*, 1972). After isolation the brush-border membranes (approx. 6mg of protein) were suspended in 40ml of a buffer containing 100mM-D-mannitol/20mM-Hepes/Tris (pH7.4) by ten strokes with a rapidly rotating (1200 rev./min) tight-fitting Teflon pestle (clearance 0.095–0.119mm). Alkaline phosphatase, a marker enzyme for rat intestinal and proximal-tubular brush-border membranes, was enriched about 17-fold for membranes obtained from

small intestine and about 12-fold for renal membrane vesicles isolated by Ca^{2+} precipitation or free-flow electrophoresis, compared with the original homogenates. Alkaline phosphatase was assayed by the method of Berner & Kinne (1976).

Enzyme assays

Activity of aminopeptidase M was measured by using a LKB reaction-rate analyser which monitored the appearance of 4-nitroaniline at 410nm and 37°C. The incubation medium contained 100mM-mannitol, 20mM-Hepes/Tris, pH7.4, and 0.3mg of L-leucine 4-nitroanilide/ml; final volume was 1ml. For measuring the effect of specific antisera on the membrane-bound or purified aminopeptidase, identical samples of membranes (10μl containing 0.9–1.2mg) were preincubated in 100mM-mannitol/20mM-Hepes/Tris, pH7.4, with increasing concentration of antiserum at room temperature (20°C) (protein content 2–5mg/ml) for 20min before measurement of the remaining aminopeptidase M activity. Identical experiments were performed with purified aminopeptidase M. Cryptic aminopeptidase M activity was detected in the antibody-inhibition experiments in the presence of Triton X-100 (0.05% final concn). Since normal serum and antiserum of rabbit contain aminopeptidase activity, the activity in the sera was determined and subtracted from the activity found in the inhibition assays.

Purification of aminopeptidase M and preparation of antisera

Aminopeptidase M was isolated from rat kidney cortex by the following modification of the procedure originally described by Pfleiderer (1970). Rat kidney cortical slices (20g) were homogenized in a Potter–Elvehjem glass/Teflon homogenizer with 50ml of 0.1M-Tris/HCl buffer, pH7.3. The suspension was stirred at 4°C for 30min. The homogenate was centrifuged for 10min at 9000*g* and the resulting supernatant was spun for 2h at 100000*g*. The microsomal pellet was resuspended in 50ml of buffer (0.25M-sucrose/10mM-triethanolamine/HCl, pH7.6) containing 24mg of KCN-activated papain (Emmelot *et al.*, 1968). After 15min of incubation at 37°C the suspension was centrifuged at 100000*g* for 3h and the supernatant was concentrated by using an Amicon Diaflo XM 50 apparatus, and passed through a column (2.6cm×30cm) of Sepharose 2B equilibrated with 75mM-Tris/HCl buffer, pH7.4. Fractions containing aminopeptidase M were again concentrated and washed three times with 50ml of 20mM-sodium phosphate buffer, pH7.3, containing 100mM-NaCl in an Amicon Diaflo XM 50 apparatus. Final aminopeptidase purification was obtained by ion-exchange chromatography on a column (2.6cm×30cm) of DEAE-Sephadex A-50. Elution of enzyme from the ion-exchange column was performed with a 100–200mM-NaCl gradient in 20mM-sodium phosphate buffer, pH7.3. Ion-exchange chromatography was repeated once. All operations except proteinase treatment were carried out at 4°C. Concentrated purified enzyme was stored at −20°C. Purified aminopeptidase M (approx. 0.5mg) dissolved in Ringer's solution (140mM-NaCl/4mM-KCl/2.5mM-$CaCl_2$) was mixed with 1ml of complete Freund's adjuvant by vigorous stirring, and multiple injections were given in the neck region and along the spinal cord of the rabbits. Boosting was performed twice after 3 weeks by the injection of 0.25mg of aminopeptidase M dissolved in 0.5ml of the Ringer's solution by intramuscular injection into the hind leg. After another 3 weeks the animals were killed and their blood was recovered from the jugular vein.

Freeze-fracture studies

For freeze-fracture studies, brush-border vesicles and pieces of rat kidney cortex and small intestine were fixed with 2.5% (w/v) glutaraldehyde buffered with 0.1M-sodium cacodylate, pH7.4. Then the specimens were infiltrated stepwise with glycerol up to a final concentration of 30% (v/v) and frozen in solid N_2. Freeze-fracturing was carried out in a freeze-fracture apparatus (EPA 100; Leybold-Heraeus, Cologne, Germany) with the specimen stage at −90°C. Fracturing was immediately followed by carbon/platinum shadowing. Replicas were cleaned by acid digestion and examined in a Philips 300 electron microscope, operating at 60kV.

Materials

Sepharose 2B and DEAE-Sephadex A-50 were purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were obtained from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Boehringer (Mannheim, Germany) and were of the highest purity available.

Results and Discussion

Freeze-fracture studies

Microvilli in situ. Plate 1 shows the freeze-fracture picture of the brush-border region of a proximal-tubular epithelial cell and Plate 2 a small intestinal epithelial cell. The fracture faces belonging to the inner half of the membrane (P-face, P) show a convex curvature and contain in both tissues more particles than the concave outer half of the membranes (E-face, E). The particle density on the P-face is 3361±921 particles/μm^2 for the renal brush-border and 2913±456 particles/μm^2 for the intestinal brush-

EXPLANATION OF PLATE 1

Freeze-fracture picture of a proximal-tubule cell in the brush-border region

Convex half-membranes (P) contacting the cytoplasm show a high particle density, whereas concave half-membranes (E) representing the outer membrane leaflet are characterized by low particle density. The arrow in a white circle indicates the direction of shadowing.

EXPLANATION OF PLATE 2

Freeze-fracture picture of the brush-border region of a small-intestinal cell

The convex fracture faces of the cytoplasmic membrane leaflet (P) are rich in particles, whereas the concave outer-membrane leaflets (E) have a low particle density on their fracture faces. The arrow in a white circle indicates the direction of shadowing.

EXPLANATION OF PLATE 3

Freeze-fracture picture of isolated brush-border membrane vesicles of rat kidney cortex

Convex fracture faces (CV) mostly show a high particle density; concave ones (CC), however, show a smaller particle density, indicating a right-side-out orientation of the vesicles. The arrow in a white circle indicates the direction of shadowing.

EXPLANATION OF PLATE 4

Freeze-fracture picture of isolated brush-border membrane vesicles of rat small intestine

Almost all microvilli membranes have formed spherical vesicles whose convex fracture faces (CV) are particle-rich whereas the concave ones (CC) demonstrate mostly a low particle density. The arrow in a white circle indicates the direction of shadowing.

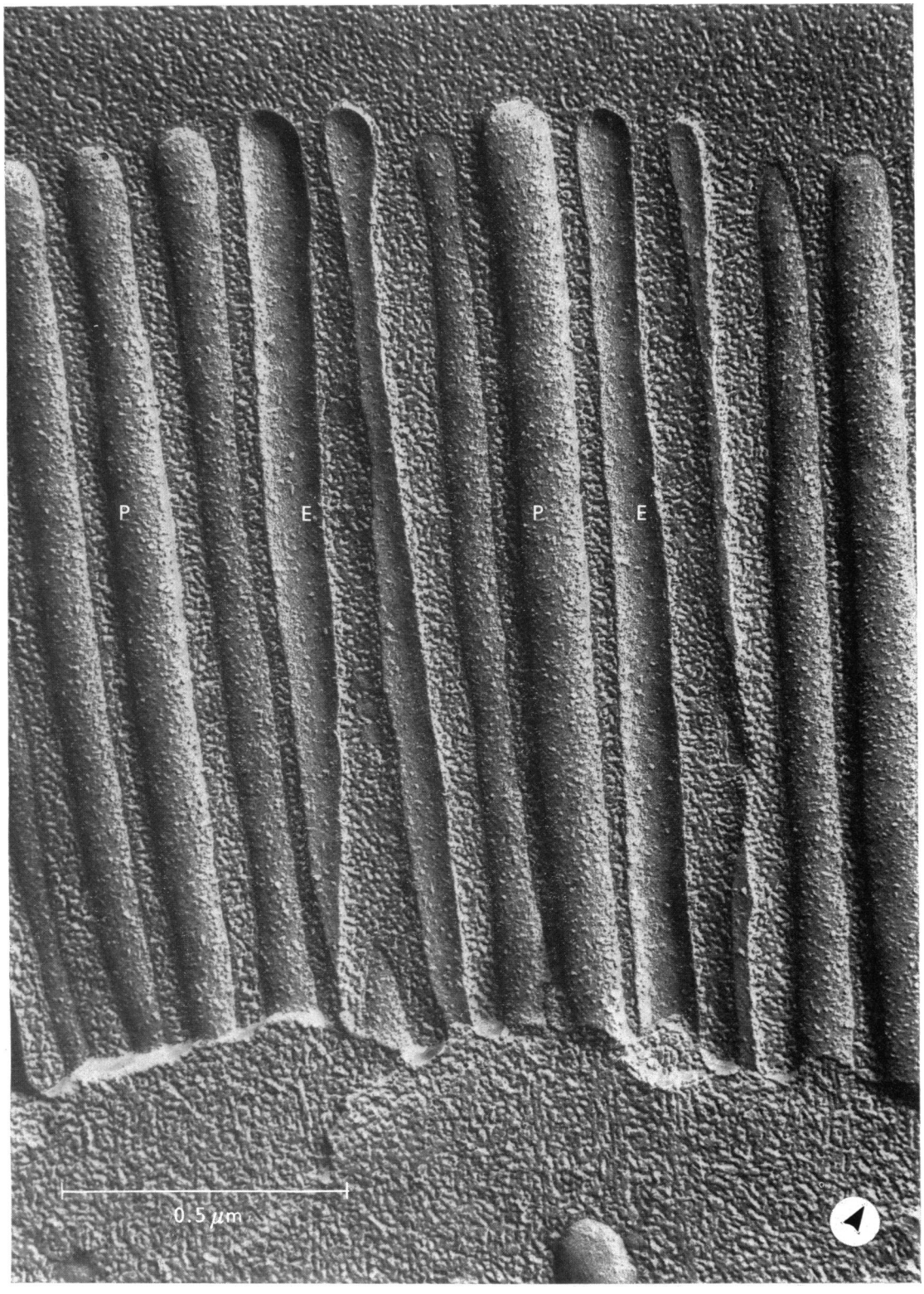
EXPLANATION OF PLATE 5

Comparison between right-side-out orientated and inside-out orientated kidney brush-border vesicles

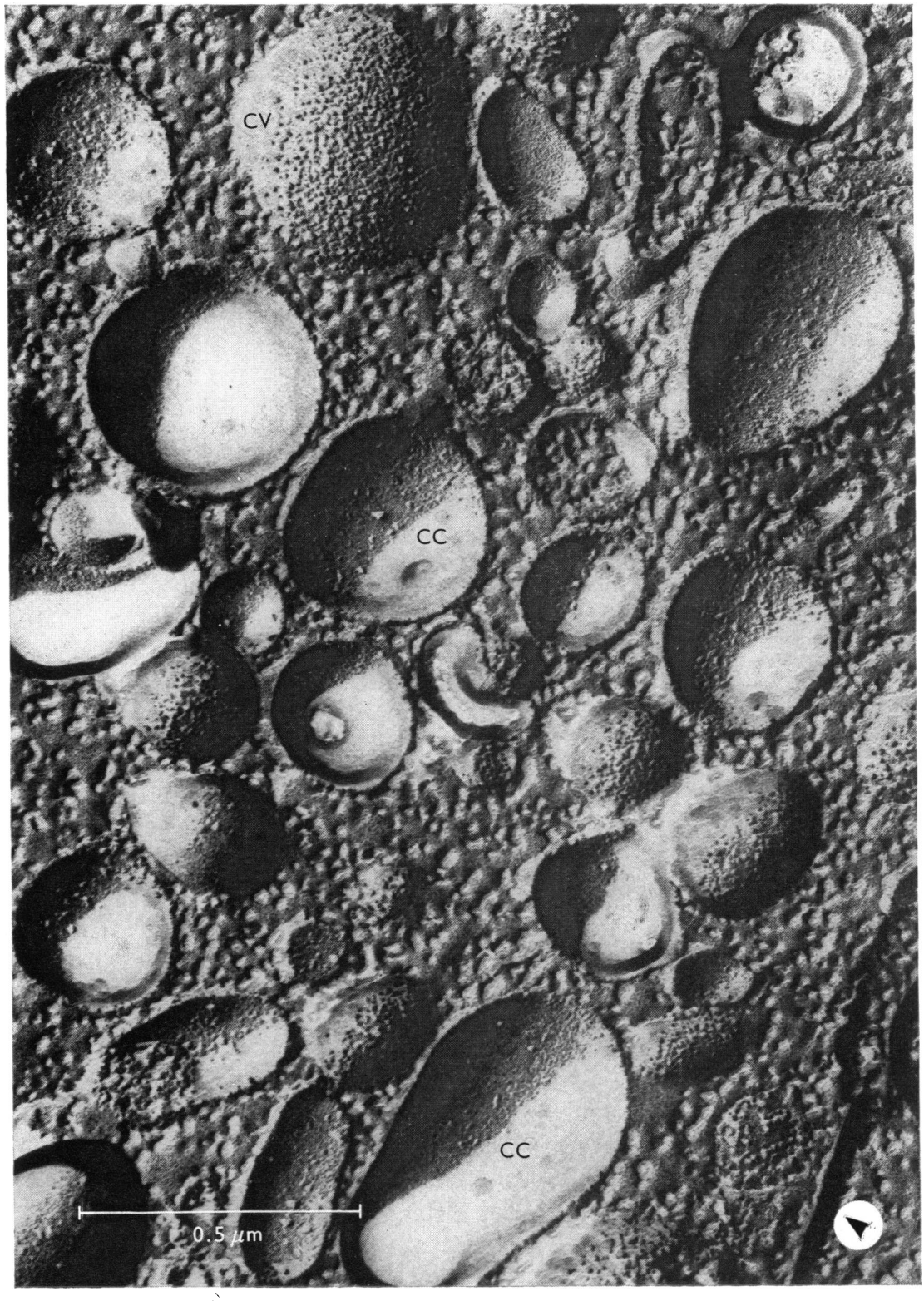
(*a*) Concave fracture faces: the concave fracture face with a high particle density (asterisk) represents an inside-out orientated vesicle. (*b*) Convex fracture faces: the convex fracture face with a low particle density (asterisk) represents an inside-out orientated vesicle.

(*facing p.* 58)


P
E
E
0.5 μm



W. HAASE AND OTHERS



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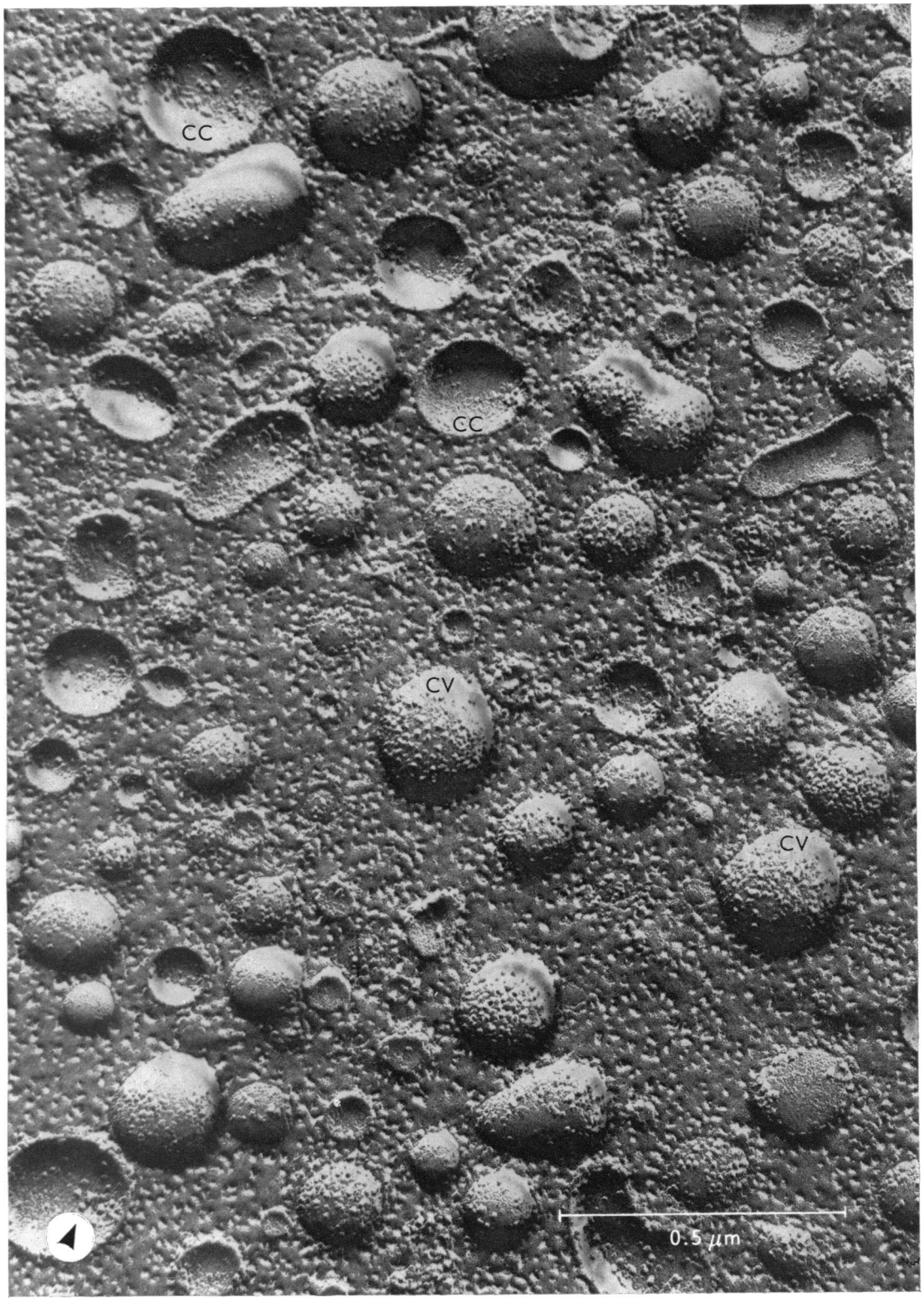
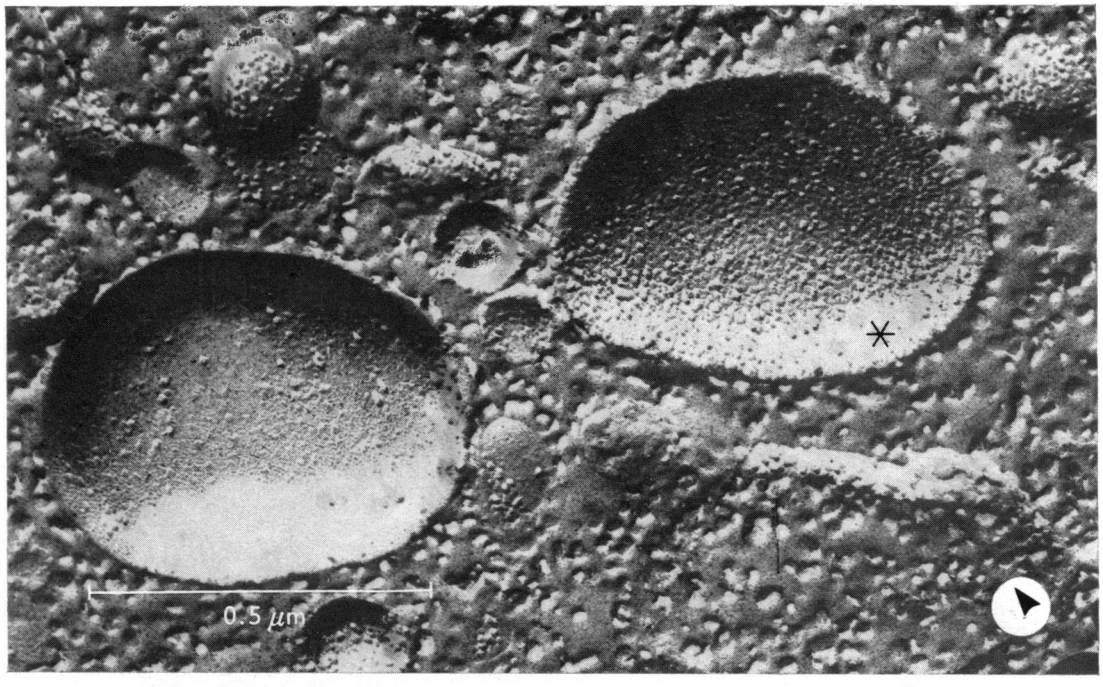
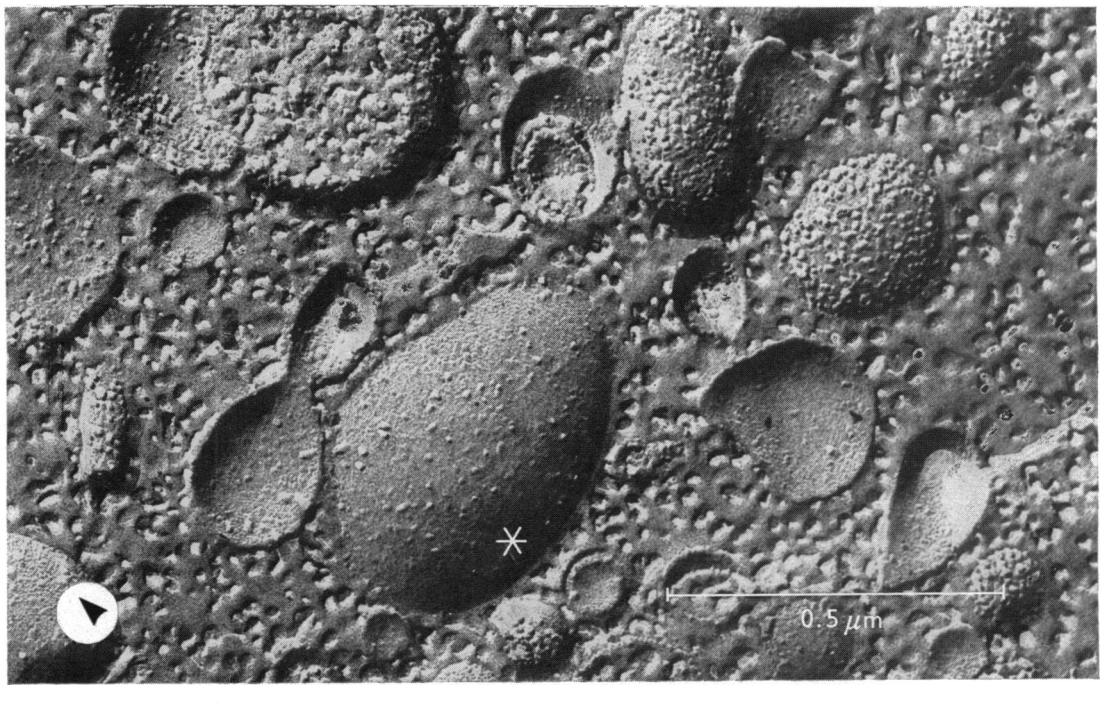

CC
CC
CV
CV
0.5 μm

(*a*)



(*b*)



border. At the E-face the particle density is 951±284 for the kidney and 1281±345 for the intestine (see Table 1 for details). Thus the particle density on the P-face of the microvillous membrane is 3.5 times that on the E-face in the proximal tubule, and 2.2 times as much in the small intestine; the difference is in both tissues sufficient to allow an unequivocal distinction between the two membrane faces.

Isolated brush-border membrane vesicles. In Plates 3 and 4 freeze-fracture pictures of isolated renal and intestinal brush-border vesicles are shown. Almost all membranes show spherical structures which most probably represent closed membrane vesicles. The diameter of the vesicles ranges from 0.2 to 0.7 μm in the membranes isolated from the kidney and from 0.2 to 0.4 μm in the intestinal preparation; in the latter preparation the size of the vesicles seems to be more uniform. Further, the convex fracture faces of the vesicles show in general more particles than the concave faces. Exceptions (convex, particle-poor and concave, particle-rich) are shown in Plates 5(*a*) and 5(*b*). As shown in Table 1, the particle densities of the particle-rich convex faces and of the particle-poor concave faces correspond very well to the particle densities observed *in situ* on the P-face and E-face respectively. It therefore can be concluded that most of the vesicles of the brush-border membrane are oriented in the same way as *in situ*, i.e. that the vesicles are oriented right-side out. The quantitative evaluation given in Table 2 reveals that in the renal brush-border-vesicle preparation approx. 85%, and in the intestinal preparation approx. 90%, of the identifiable vesicles have a right-side-out orientation. Such an evaluation is, however, only valid if the distribution of particles between and within the two planes of the membranes is not altered during the isolation of the membrane. This seems to hold for the isolated brush border as indicated by the close agreement of the particle density observed *in situ* and the particle density found in the isolated membranes. The value of a quantitative analysis may also be limited by the inhomogeneity of the preparation. In our experiments the orientation of approx. 40% of the vesicles could not be determined owing to unfavourable shadowing, particle aggregation, or because the size of the vesicles was too small. Since most of the unidentifiable vesicles are very small they contribute only to a small extent to the vesicular transport volume. Therefore the quantitative evaluation and its relation to transport properties seem to be justified.

Immunological studies: accessibility of aminopeptidase M to inhibitory antibodies

From immunohistochemical and enzymological investigations it can be concluded that aminopeptidase M is located at the outer surface of the renal and intestinal brush-border membrane (Thomas & Kinne, 1972; H. Pockrandt-Hemstedt & R. Kinne, unpublished work). Since on the other hand it can be assumed that, because of their size, antibodies do not penetrate plasma membranes readily, the degree of accessibility of aminopeptidase in a given membrane fraction to antibodies might provide information on the orientation of the membranes in the

Table 1. *Particle density on the fracture faces of microvilli in situ and brush-border membrane vesicles in vitro*
Particle density per μm^2 was obtained by counting intramembranous particles in small membrane areas (0.01–0.1 μm^2). The results represent mean values ± S.D. for *n* membrane areas counted in three different freeze-fracture preparations.

	Microvilli *in situ*		Brush-border vesicles	
	Convex P-face	Concave E-face	Convex particle-rich face	Concave particle-poor face
Kidney cortex	3361±921 ($n=7$)	951±284 ($n=13$)	3025±382 ($n=10$)	795±326 ($n=10$)
Small intestine	2913±456 ($n=24$)	1281±345 ($n=23$)	3005±642 ($n=30$)	915±393 ($n=43$)

Table 2. *Orientation of isolated brush-border membrane vesicles*
In three different membrane preparations the particle density found on the convex fracture planes was used as indicator for the orientation of the vesicles. Right-side-out oriented vesicles were identified by a high particle density, inside-out-oriented vesicles by a low particle density.

	Kidney cortex		Small intestine	
	Number	%	Number	%
Identified vesicles	459	100	772	100
Right-side-out oriented vesicles	390	85	724	94
Inside-out oriented vesicles	69	15	48	6

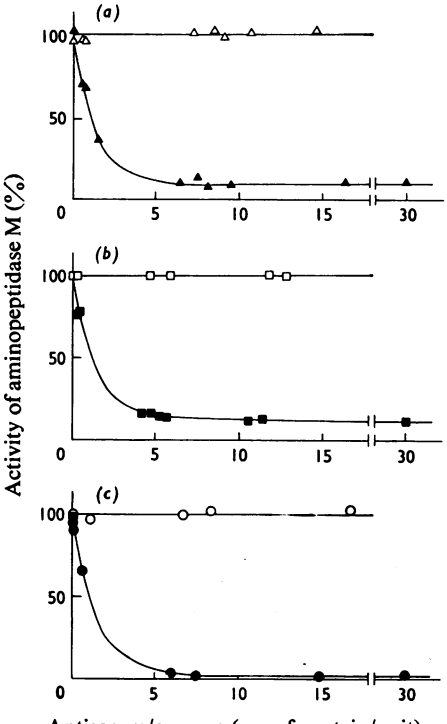


Fig. 1. *Inhibition of aminopeptidase M by antiserum* (*a*) Inhibition of renal-membrane-bound aminopeptidase M (▲, antiserum; △, normal serum). (*b*) Inhibition of intestinal-membrane-bound aminopeptidase M (■, antiserum; □, normal serum). (*c*) Inhibition of purified renal aminopeptidase M (●, antiserum; ○, normal serum). A 100% activity of aminopeptidase M is the blank value where serum is replaced by buffer. Ratio of antiserum/enzyme is given in mg of antiserum protein per enzyme unit (1 μmol of substrate converted/min) of aminopeptidase M.

vesicles. Figs. 1(*a*) and 1(*b*) show the inhibition of aminopeptidase activity in renal and intestinal brush-border vesicles by increasing amounts of antiserum raised against purified renal aminopeptidase. For both the renal and intestinal vesicles the inhibition approaches a maximum value of approx. 90%. This indicates that almost all of the aminopeptidase of the membranes can be reached by and interact with the antibodies.

Before these data can be interpreted with respect to the orientation of the membrane, the degree of vesiculation of the membrane preparation has to be considered, since both the aminopeptidase molecules located at the surface of right-side-out oriented vesicles as well as the aminopeptidase present on open membrane sheets can interact with the antibodies. According to the electron-microscopic pictures (Plates 3 and 4) the membrane preparation investigated in this study shows a very high degree of vesiculation. Therefore it seems reasonable to assume that the great extent of inhibition of the aminopeptidase by antibodies reflects the almost complete right-side-out orientation of the brush-border vesicles.

Noteworthily, as shown in Fig. 1(*c*), the water-soluble isolated aminopeptidase is inhibited by about 96–98% by the antibodies. The small but consistently observed difference between the inhibition of the membrane-associated aminopeptidase in the vesicle preparation and of the soluble enzyme might be interpreted, if steric hindrance of the antigen–antibody reaction at the membrane can be excluded, to be caused by inside-out vesicles, whose aminopeptidase molecules cannot be reached by the antibodies. This hypothesis was tested by subjecting the membrane preparations, before incubation with antibodies, to procedures known to open up membrane vesicles. The first procedure consisted of the addition of small amounts of Triton X-100 to the vesicles. Indeed the maximum inhibition increased from 89.6 to 94.7% in the renal brush-border vesicles and from 87.1 to 93.8% in the intestinal brush-border vesicles (Table 3). The same increase was observed if intestinal membranes were subjected to an osmotic-shock procedure. For these experiments brush-border membrane vesicles were isolated in 500mM-mannitol (instead of 100mM-mannitol) and membrane–antibody reaction was carried out in hypo-osmotic media (10mM-mannitol/2mM-Hepes/Tris, pH 7.4). A similar approach failed for the renal brush-border vesicles, since the preloading of the membranes with hyperosmotic mannitol solution already increased the maximum inhibition to 94% and the osmotic shock was ineffective. These data indicate that procedures that open up vesicles (inside-out as well as right-side-out oriented) lead apparently to an increased accessibility of the aminopeptidase to the antibodies. Therefore the increase in accessibility might be a tool to determine the amount of inside-out-oriented vesicles in a given membrane population. To use this tool properly, however, it seems to be necessary to fulfil the following prerequisites. First, it has to be established that the substrate for the enzyme readily permeates the membrane, otherwise the enzyme activity would be cryptic in the control experiments and no restricted accessibility could be observed. In the renal and intestinal brush-border membranes this seems to be the case for leucine *p*-anilide, which is used as substrate for determination of the aminopeptidase activity. In control experiments, removal of the diffusion barrier by treatment of the membrane preparation with Triton X-100 did not increase the aminopeptidase activity. Secondly, the procedures used to open the vesicles should not alter the associa-

Table 3. *Effect of Triton X-100 on the inhibition of renal and intestinal aminopeptidase M by antiserum*
The brush-border vesicles or the isolated enzyme were incubated for 20min at 20°C in 100mM-mannitol/20mM-Hepes/Tris buffer with antiserum (15–30mg of protein/unit of aminopeptidase activity) in the presence or absence of 0.05% Triton X-100. The values represent the difference between the enzyme activity found in the samples preincubated with antiserum and the activity found in samples preincubated with normal serum and are expressed as percentage of the latter. The results from two experiments with different membrane preparations are given; they were derived from enzyme determinations performed in duplicate. In all experiments a correction was made for the enzyme activity found in the sera. Membranes were prepared by the methods of: *Heidrich *et al.* (1972), †Evers *et al.* (1978), ‡Sigrist-Nelson *et al.* (1975). Abbreviation: n.d., not determined.

		Enzyme activity (%)		
		Preincubation with antiserum	Preincubation with antiserum and Triton X-100	Preincubation with antiserum and addition of Triton X-100 shortly before measurements of enzyme activity
(A) Membrane-bound enzyme, brush-border membrane vesicles	Kidney*	89.5 89.7	94.8 94.7	90.3 90.0
	Kidney†	90.1 91.3	93.8 94.9	n.d. n.d.
(B) Membrane-bound enzyme, brush-border membrane vesicles	Small intestine‡	86.7 87.6	93.8 93.8	88.6 87.0
(C) Isolated enzyme		95.5 98.0	96.6 96.0	96.6 98.2

tion between the enzyme and the membranes, since soluble enzymes might be more sensitive to inhibition by antibodies than the membrane-associated form. This prerequisite is fulfilled only partly in the experiments with Triton X-100; approx. 50% of the aminopeptidase can be recovered in the high-speed supernatant after treatment with detergent. During the osmotic-shock procedure, however, the release of enzymes from the membranes is minimal.

In this context it should be mentioned that for the elucidation of membrane orientation, the investigation of enzymes that span the membrane is even more suitable. With Na^++K^+-activated adenosine triphosphatase, for example, the catalytic ATP site is located on the cytosolic face and the ouabain-binding site is located at the exterior face of a plasma membrane which is impermeable to ATP and ouabain. When the membrane vesicles are opened up by addition of small amounts of detergent, a stimulation of the adenosine triphosphatase activity is to be expected if the membrane preparation contained vesicles that were oriented right-side out. Inside-out oriented vesicles (provided that K^+ is present at the inside) show an adenosine triphosphatase activity that, in the absence of detergent, is ouabain-insensitive, but which becomes ouabain-sensitive when the vesicles are opened. A similar approach has been used by Walter (1975) to assess the orientation of renal basolateral plasma membrane vesicles.

Conclusions

The two independent approaches used in this study to determine the orientation of brush-border membrane vesicles gave qualitatively the same result, namely that the brush-border vesicles in the membrane fractions used in this laboratory for transport studies are predominantly oriented right-side out. This is an acceptable orientation for studies on ion-gradient-driven transport systems, since these systems are likely to be symmetrical and the gradients involved in the luminal uptake of substances *in vivo* thus can easily be mimicked *in vitro*. However, for ATP-dependent systems, whose catalytic site is at the cytoplasmic face of the membrane and therefore not accessible in this kind of preparation, a higher population of inside-out-oriented vesicles would be preferable. Attempts to invert the brush-border membranes by methods reported to change the orientation of other plasma-membrane vesicles, such as sonication and nitrogen cavitation in media of different ionic composition, treatment with polyethylene glycol and variation of the bivalent-cation concentration, have failed so far in our laboratory. The reason for this unusual stability of the right-side-out orientation of the brush-border membrane might be 3-fold. First, electrostatic repulsion as well as steric hindrance between the hydrophilic heads of the amphipathic hydrolases located at the outer

surface of the membrane (Louvard *et al.*, 1976) might oppose an inversion. Secondly, asymmetric distribution of the phospholipids (Zwaal *et al.*, 1973) could favour the strong curvature of the microvillous membrane (Thompson, 1976) and therefore could also stabilize the right-side-out orientation of membrane vesicles isolated from this region. An involvement of the lipids in maintaining the orientation is further supported by physicochemical measurements of the lipid fluidity, which was shown to be lower in brush-border membranes than in other plasma membranes (Schachter & Shinitzky, 1978). Therefore, during inversion and reorientation of the membrane, a very high frictional resistance has to be overcome. Thirdly, a network of actin filaments has been described in the microvilli (Booth & Kenny, 1976), which also might stabilize the configuration of the microvillous membranes *in vivo*.

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