Characterization of brush borders purified in iso-osmotic medium and microvillar membranes subfractionated from mouse small intestine

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Brush borders free of nuclei were isolated by repeated homogenization and centrifugation in iso-osmotic medium. They showed typical morphology under electron microscopy. The mean recovery and enrichment of alkaline phosphatase activity in the brush-border fraction were 50% and 17.5-fold respectively. γ -Glutamyl transpeptidase showed a close parallelism with alkaline phosphatase and sucrase in subcellular distribution. Microvillar membranes were purified from isolated brush borders; they showed a further enrichment for alkaline phosphatase and were composed of homogeneous vesicles. Both brush-border and microvillar-membrane preparations were analysed for contamination by basolateral and endoplasmic-reticular membranes. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the microvillar-membrane preparation in six different systems revealed approx. 40 components in the mol.wt. range 15 000–232 000. They were grouped into seven major classes on the basis of molecular weight and electrophoretic patterns.

Intestinal brush borders are an important subject of study not only for their chemical and enzymic compositions but for their biogenesis and turnover.

Several different preparation techniques have been reported. These are based on three types of homogenization media: hypo-osmotic EDTA (Miller & Crane, 1961), iso-osmotic sucrose/EDTA (Porteous & Clark, 1965) and hypo-osmotic CaCl_2 (Schmitz *et al.*, 1973). All three methods have their disadvantages: hypo-osmotic EDTA induced nuclear aggregation which interfered with purification of brush borders (Harrison & Webster, 1964). The calcium precipitation method yielded fragmented brush borders, and their purity was unsatisfactory in our experience. In iso-osmotic medium nuclei co-sedimented with brush borders (Porteous & Clark, 1965).

In the present work nuclei-free brush borders were obtained by repeated centrifugation in iso-osmotic medium. This was made feasible by mild homogenization and effective separation of crude brush borders and free nuclei at initial steps of centrifugation. Moreover, as the medium was iso-osmotic, the post-brush-border supernatant could be fractionated into other relevant organelles which are important elements in the study of biogenesis and turnover of the brush border and its membrane.

Abbreviation used: SDS, sodium dodecyl sulphate.

Microvillar membranes were prepared from purified brush borders by density-gradient centrifugation (Fujita *et al.*, 1972). They were characterized for degree of contamination by basolateral and endoplasmic-reticular membranes. The protein composition was analysed by SDS/polyacrylamide-gel electrophoresis.

Experimental

Animals

Adult mice of either sex were used.

Homogenizers

Loose-fitting Dounce-type homogenizers were used for preparation of brush borders. The fit was such that one could apply a stroke to the Teflon pestle (diameter 18.4 mm) in an empty cylinder (vol. 25 ml) with minimum resistance.

Buffers and media

Medium A contained 0.32 M-sucrose, 5 mM-imidazole/HCl buffer, pH 7.1, and 0.5 mM-EDTA. Medium B was medium A free of sucrose.

Tissue preparation

Two to ten animals were killed for each experiment. The small intestine (jejunum) was flushed with ice-cold iso-osmotic 0.9% NaCl immediately after

removal. After blotting, the mucosal surface was gently scraped with a glass microscope slide.

Cell fractionation

The combined scrapings were transferred to a homogenizer containing medium A and the total volume was adjusted to 3 ml/animal. Five gentle strokes were applied manually without rotation. The homogenate was diluted with the same volume of medium A and centrifuged at $270g_{\max}$ for 5 min. Resuspension of the pellet and repetition of homogenization are summarized in Table 1.

The final pellet was recovered in medium B. This fraction was referred to as 'purified intact brush borders'. The recovered and combined supernatant could be used for further purification of cell organelles other than brush borders.

Subfractionation

The purified intact brush borders were, after thorough homogenization, suspended in 50% (w/v) sucrose containing 5 mM-imidazole/HCl buffer, pH 7.1, and 0.5 mM-EDTA. It was overlaid with 40, 30 and 20% sucrose in the same buffer with EDTA as previously described (Fujita *et al.*, 1972).

Enzyme assay procedures

Alkaline phosphatase and sucrase. The assay methods were described previously (Fujita *et al.*, 1972).

γ -Glutamyl transpeptidase. The activity was assayed with L- γ -glutamyl *p*-nitroanilide and glycylglycine. The reaction mixture was similar to that of Szasz (1974).

Glucose 6-phosphatase. The reaction mixture contained 25 mM-imidazole/HCl, pH 7.1, 0.25 mM-

EDTA, 25 mM-glucose 6-phosphate and the enzyme source. Incubation was for 30 min at 37°C and the liberated P_i was determined colorimetrically.

ATPases. Mg^{2+} -stimulated ATPase (Mg-ATPase) activity was determined with 5 mM-ATP in 50 mM-imidazole/HCl, pH 7.1, containing 5 mM- $MgCl_2$ and 0.5 mM-EDTA. For the total ATPase assay 100 mM-NaCl and 10 mM-KCl were present in addition. Other procedures were as described previously (Fujita *et al.*, 1967). Na^+ - and K^+ -stimulated ATPase (Na,K-ATPase) activity was calculated as the difference between the total and the Mg-ATPase activities.

Galactosyltransferase. The activity was assayed with 25 μ M-UDP- $[^{14}C]$ galactose (6.5 μ Ci/ μ mol) and ovalbumin (15 mg/ml) as donor and acceptor of galactose residue. The incubation was carried out at 37°C for up to 40 min in the presence of 30 mM-2-mercaptoethanol and 0.2% Triton X-100.

Protein. Protein was determined with the Lowry method with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis

The buffer system of Laemmli (1970) was used throughout the experiment. The compositions of separation gels were (final %, w/v, of acrylamide: *NN'*-methylenebisacrylamide): (I) 12.5:0.34, (II) 10.0:0.36, (III) 7.5:0.22, (IV) 6.7:0.31, (V) 5.0:0.26 and (VI) 4.0:0.22. That for the stacking gel was 3.3:0.22. Cylindrical gels were used. Molecular-weight standards (and their values) were: myosin heavy chain (200 000), β -galactosidase (116 000), phosphorylase *b* (92 500), bovine serum albumin (68 000), ovalbumin (43 000), trypsin (23 000), haemoglobin (15 500) and cytochrome *c* (11 700).

Table 1. Cell fractionation procedures to obtain 'purified intact brush borders'
For further details see the text.

Step	Final volume of suspension (ml/animal)	Medium	No. of strokes	Centrifugation		Duration (min)
				Speed (rev./min)*	$g_{\max}$	
1	6	A	5	1500	270	5
2	6	A	5	1500	270	5
3	6	A	10	1750	350	5
4	3	A	15	2000	470	5
5	3	A	20	2250	600	5
6	3	A	20	2500	720	5
7	1.5	B	10			
			(10 min on ice)			
			10†	3000	1100	5
8	1.5	B	20	3000	1100	5

* SS-34 rotor (Sorvall) or equivalent rotor ($r_{\max} = 10.8$ cm).

† Homogenized again after 10 min.

EXPLANATION OF PLATE 1

Morphological examination of steps to obtain purified intact brush borders

(A) Isolated villi (mucosal scrapings). Not stained. Bar represents 100 μm . (B) Initial homogenate. Not stained. Bar represents 10 μm . (C) Purified intact brush borders stained with Acridine Orange. Bar represents 10 μm . (D) The same fraction under the electron microscope. Bar represents 1 μm . The fraction was fixed with 2% osmic acid and embedded in Epon. Sections were stained with uranyl acetate.

EXPLANATION OF PLATE 2

Electron micrographs of (a) individual brush borders in the purified fraction and (b) purified microvillus membrane fraction

(a) (A) A typical brush border (note the intact terminal web and a few smooth-surfaced vesicles). (B) Remaining 'lateral membrane tags' (Quigley & Gotterer, 1969) of two adjoining cells. (C) A lateral membrane vesicle entrapped within the brush border. (b) Bar represents 1 μ m.

Results and discussion

Mucosal scrapings

The jejunal scrapings were found by microscopy to be composed of individual villi, mostly devoid of lamina propria (Plate 1A).

Cell disintegration

The initial homogenate largely consisted of fragmented sheets of epithelial cells which had lost their basal part (Plate 1B). The brush borders in these sheets were seen as strongly refractive plates.

Cell fractionation

The *g*-min value applied at the initial centrifugation was one of the lowest ever reported for brush-border separation. It was effective for sedimenting crude brush borders before free nuclei, despite the statement by Porteous & Clark (1965) that 'the nuclei and microvillus sheets sedimented together and it has been impossible to separate these subcellular components by centrifugal methods'.

The isolated brush borders were easily identified with light- (Plate 1C) and electron- [Plates 1(D) and 2a(A)] microscopy. Even a very mild homogenization as adopted in the present work fragmented some brush borders (21% of the alkaline phosphatase activity was recovered in combined heavy- and light-microsomal fractions). It is not surprising then that some earlier workers recovered most of the brush-border enzymes in the microsomal fraction (Borgström & Dahlqvist, 1958; Carnie & Porteous, 1962; Robinson, 1963; Hübscher *et al.*, 1965).

Subfractionation

There was very little turbidity in the fractions

lighter than the 50%-sucrose layer after density-gradient centrifugation. Only a slight turbidity was seen at the interface between the 40%- and 50%-sucrose layers. The colourless pellet was comparatively bulky. Under the electron microscope the 40%-/50%-sucrose interface fraction largely consisted of microvillar-membrane vesicles, with minor contamination by basolateral membranes. The 50%-sucrose layer contained homogeneous vesicles (Plate 2b). The pellet revealed membranous vesicles and filamentous materials.

Localization and enrichment of enzymes

Alkaline phosphatase, sucrase and γ -glutamyl transpeptidase. The mean enrichment of the alkaline phosphatase in the purified intact brush-border fraction was 17.5-fold, with 50% recovery (Table 2). After density-gradient centrifugation the 50%-sucrose layer showed the highest enrichment (21.7-fold averaged over eight preparations). The 40%-/50%-sucrose interface and the pellet showed 14.5- and 5.9-fold enrichment respectively.

Sucrase activity showed a close correlation with alkaline phosphatase activity in subcellular fractionation. In one experiment the alkaline phosphatase/sucrase ratio was 2.0–3.3 for homogenate, microvillar membrane fraction, 40%-/50%-sucrose interface and pellet.

γ -Glutamyl transpeptidase activity was also concentrated in the microvillar-membrane fraction and showed a close association with alkaline phosphatase and sucrase in subcellular distribution (results not shown). The finding supports its asserted localization in the brush border (Kokot *et al.*, 1965; Greenberg *et al.*, 1967).

Table 2. Activities of some marker enzymes in the homogenate, brush-border and microvillar-membrane fractions of mouse jejunal-mucosal epithelial cells

The activities are given in μ mol of substrate consumed/h per mg of protein, as means $\pm$ s.d. for the numbers of preparations in parentheses. 'r' represents relative contamination for brush border and microvillar membrane fractions, which is estimated as the enrichment of another marker enzyme relative to alkaline phosphatase. Recovery values (%) are shown in square brackets.

Marker enzymes	Homogenate	Brush-border fraction			Microvillar-membrane fraction		
		Enrichment	r		Enrichment	r	
Alkaline phosphatase	3.6 $\pm$ 1.5 (8) [100]	63 $\pm$ 19 (8) [50 $\pm$ 25]	17.5		78 $\pm$ 43 (8) [26 $\pm$ 9]	21.7	
Na,K-ATPase	4.9 $\pm$ 2.9 (27) [100]	10.0 $\pm$ 8.9 (9) 2.5 $\pm$ 1.9 (4)* [2.8 $\pm$ 2.8]	2.0 0.51*	0.12 0.06*	6.2 $\pm$ 5.1 (20) 1.7 $\pm$ 1.9 (7)* [0.20 $\pm$ 0.28]	1.27 0.35*	0.072 0.008*
Glucose 6-phosphatase	1.30 $\pm$ 0.80 (14) 1.5† [100]	0.9†	0.6†	0.034†	1.42 $\pm$ 0.82 (9) [1.3 $\pm$ 1.0]	1.1	0.051
Cytochrome c oxidase	0.36	Not determined			0.037	0.10	0.005
Galactosyltransferase	1.34 $\pm$ 0.31 (4)	Not determined			0.18	0.14	0.006

* Exhaustively purified at the expense of yield.

† Single experiment.

ATPases. (1) Mg-ATPase. The activity was generally more dominant than Na,K-ATPase activity, except for those fractions in which basolateral membranes were enriched (results not shown).

(2) Na,K-ATPase. The specific activity of this enzyme doubled in the purified intact brush borders (Table 2). This apparent enrichment of the activity could be accounted for by the 'lateral membrane tags' (Quigley & Gotterer, 1969) attached to the free end of the brush borders [Plates 2a(B) and 2a(C)]. Nevertheless, it was possible to obtain a brush-border fraction with less Na,K-ATPase activity if the number of homogenization and centrifugation steps was increased at the expense of the final yield (Table 2). In such preparations the specific activity was half that of the homogenate and further decreased in the microvillar membrane fraction. The recovery of the activity was 5.6% of that for alkaline phosphatase in the purified intact brush border and only 0.8% in the microvillar-membrane fraction (Table 2).

The asymmetry in distribution (Fujita *et al.*, 1971, 1972; Douglas *et al.*, 1972) was also reflected in the ratio of the two enzymes in various subcellular fractions. The Na,K-ATPase/alkaline phosphatase activity ratio ranged from 0.08 in the microvillar-membrane fraction to 56.7 in the basolateral-membrane fraction. The correlation value for alkaline phosphatase and Na,K-ATPase activities in 26 different subcellular fractions was calculated; it was -0.26 , testifying to independent or rather mutually exclusive distribution of the two enzymes.

Glucose 6-phosphatase. The mean specific activity for homogenate, brush-border, nuclear, mitochondrial, heavy-microsomal and light-microsomal fractions were, in one experiment, 1.5, 0.9, 0.9, 1.7, 5.2 and $5.3 \mu\text{mol}$ of product released/h per mg of protein respectively. The enrichment in the brush-border fraction was 0.6-fold; thus the relative contamination (see the legend to Table 2) was $0.6/17.5 = 0.034$ (3.4%). This low degree of contamination was in accord with the electron-microscopic images of the purified brush borders (Plate 2a) in which a very small number of smooth-membrane vesicles was found. The relative contamination calculated on the basis of NADH-cytochrome *c* reductase was even smaller. The mean glucose 6-phosphatase activity in the microvillar-membrane fraction was 1.42, with a 1.1-fold enrichment (Table 2). The relative contamination of the fraction by endoplasmic-reticular membranes was therefore $1.1/21.7 = 0.051$ (5.1%).

SDS/polyacrylamide-gel electrophoresis

We reported previously that microvillar and basolateral membranes had entirely different protein compositions (Fujita *et al.*, 1973). However, the

method was later found insufficient for high resolution of the proteins of the two membranes. In the present work we used the improved gel systems to obtain better separation of the microvillar membrane proteins. Some of the results are shown in Fig. 1.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified microvillus-membrane fraction

Of six different gel systems adopted in the present work, patterns in 5%- (a), 7.5%- (b) and 12.5%-acrylamide (c) gel systems are shown here. Symbols under the tracings represent protein classes (see the text).

The proteins detectable by the present method were grouped into seven major classes on the basis of the electrophoretic pattern and apparent molecular weight. The class-AI polypeptides had mol.wts. greater than 190 000 and showed at least six members, two of which were prominent (Fig. 1a). No remarkable peaks were present in the molecular-weight range larger than 232 000 (4%-acrylamide gel system; results not shown). Seven to eight components were detected in class AII (mol.wt. range 190 000–116 000). The members of this class were among the most dominant of the microvillar-membrane fraction. The two components of class B (mol.wt. range 116 000–86 000) were characteristically prominent in the microvillar membrane preparations not only of mouse but of other species (M. Fujita, H. Ohta & T. Uezato, unpublished work).

The members of class C (mol.wt. range 86 000–61 000) were not very conspicuous, and those of class D (mol.wt. range 61 000–44 000) were even less prominent (Fig. 1a). On the other hand, the protein class with molecular-weight range similar to that of the class D was the most dominant one in the endoplasmic-reticular membrane fraction from the same homogenate (M. Fujita, H. Ohta & T. Uezato, unpublished work). In contrast, class E (mol.wt. range 44 000–35 000) showed two conspicuous peaks, one of which had mol.wt. 42 000 (Fig. 1b). The members of class F (mol.wt. range 35 000–27 000) included one relatively prominent peak (Fig. 1c). The components with molecular weight lower than 25 000 were grouped as class S, which was subdivided into Sa (mol.wt. range 25 000–20 000) and Sb (mol.wt. <20 000); altogether, the class showed at least seven components (Fig. 1c).

In the light of the one-dimensional analysis as adopted here, the minimum number of peaks detected in the microvillar-membrane preparation was about 40, which seems too large to be accounted for by the known number of brush-border enzymes. Although there have been a few attempts to correlate the protein bands in such gels with known microvillar enzymes (Maestracci *et al.*, 1975; Hauri *et al.*, 1977), it is evident that more work should be done with the improved systems in order to identify the individual protein peaks.

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