Rat Intestinal Microvillus Membranes

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION

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1. A technique is described for the removal of subcellular contaminants from intact rat intestinal brush borders, and for the subsequent separation of a microvillus membrane fraction from a fibrillar residue. 2. Increments in invertase activity, microscopic homogeneity and low nucleic acid content indicate that the microvillus plasma membrane has been extensively purified. Multiple membrane preparations have been shown to be highly reproducible with respect to their invertase specific activity, cholesterol content and phospholipid content. Alkaline phosphatase, leucine aminopeptidase, Mg^{2+} - and Ca^{2+} -dependent adenosine triphosphatase and seven separate disaccharidases were shown to be predominantly confined to the membrane fraction. 3. The fibrillar fraction has been shown to contain approximately 30% of the total protein of purified brush borders, plus most of the residual nucleic acid contaminant. No evidence was found for the localization of any specific enzyme in this fraction.

Present evidence suggests that the intestinal microvillus membrane plays an important role in determining the rate and specificity of intestinal transport processes. Not only do the microvillus projections increase the luminal absorptive surface of the gut almost 25-fold (Palay & Karlin, 1959), but the microvillus surface is rich in enzymes that complete the hydrolysis of selected nutrients to absorbable solutes (Dempsey & Deane, 1946; Nachlas, Monis, Rosenblatt & Seligman, 1960; Miller & Crane, 1961; Holt & Miller, 1962; Hübscher, West & Brindley, 1965; Crane, 1966) and also contains several of the carrier systems for uphill transport (Newey, Parsons & Smyth, 1959; McDougall, Little & Crane, 1960; Kinter & Wilson, 1965). Since the functional characteristics of the membrane must depend to a large extent on its chemical composition, quantitative analysis of the components of the isolated plasma membrane may provide significant clues to the molecular mechanisms underlying intestinal absorption.

Microvillus membranes may be isolated for study *in vitro* as part of a brush-border complex that also includes the fibrils of the terminal web and its microvillus extensions (Miller & Crane, 1961). This preparation has been used with success to localize and study brush-border enzymes (Crane, 1966), but the unknown contribution of the fibrillar com-

ponent greatly limits its usefulness for precise analysis of membrane structure and function. In addition, it is clear that brush borders prepared by available techniques are contaminated to a considerable extent by other subcellular components (Eichholz & Crane, 1963; Porteous & Clark, 1965; Hübscher *et al.* 1965).

Since both the subcellular contaminants and the fibrillar material prevent accurate assessment of the composition of the microvillus membrane, we have directed our efforts to developing a method whereby microvillus membranes can be reproducibly prepared free of these elements. The purpose of this paper is to describe such a method, and to establish by microscopic, enzymic and chemical criteria the relative purity and functional integrity of the resulting microvillus membranes.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass., U.S.A.), each weighing approx. 200–300 g., were used. They were starved overnight.

Preparation of brush borders. Rats were killed by cervical dislocation. The entire small intestine was removed and immediately irrigated with cold 0.9% NaCl. The intestine was then everted, and the mucosa removed with a glass slide. The mucosa was weighed, placed in 75 vol. of 5 mM-EDTA adjusted to pH 7.4 with NaOH ('EDTA buffer') and homogenized in a Waring Blendor for 25 sec. All operations were performed at 4°. After centrifugation at

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450g for 10 min., the sediment was washed three times by suspension and centrifugation in 5 vol. of EDTA buffer. The washed sediment of 'crude' brush-border fraction was then suspended in 2 vol. of 90 mM-NaCl-0.8 mM-EDTA buffer, mixed thoroughly and kept until a well-defined sediment had developed. This step usually required 20-30 min. The supernatant and sediment were then poured in succession through a pad of glass-wool (Pyrex brand Corning no. 3950; approx. dry wt. 300 mg.) to remove aggregated particles. After the glass-wool pad had been washed with a further 20 ml. of 5 mM-EDTA buffer, brush borders in the total washings were sedimented by centrifugation at 450g for 10 min., and washed once with 2.5 vol. of 2.5 mM-EDTA buffer. After centrifugation the final pellet was suspended in a convenient volume of 2.5 mM-EDTA buffer. This fraction is referred to below as 'purified' brush borders.

Preparation of membrane and fibrillar fractions. The sub-fractionation of brush borders was carried out according to the outline shown in Scheme 1. 'Purified' brush borders were added with gentle stirring to 150 vol. of cold water to bring the concentration of EDTA to 17 μ M, and the mixture was allowed to stand at 4° for exactly 12 min. Sufficient EDTA buffer was then added with rapid swirling to bring the concentration to 4.5 mM. The mixture was then centrifuged at 1800g for 20 min., to yield a sediment and a supernatant fraction. The supernatant was centrifuged at 30000g for 30 min., and the pellet obtained (membrane fraction I) was suspended in a convenient volume of water or 2.5 mM-EDTA buffer. A portion (2 ml.) of the 1800g sediment was placed on a gradient consisting of a lower layer of 30% (w/v) sucrose (3 ml.) and an upper layer of 15% (w/v) sucrose (3 ml.) in a 2.5 mM-EDTA buffer. After centrifugation for 20 min. at 1000g, the upper aqueous phase (membrane fraction II) and the lower sucrose phase (fibrillar fraction) were removed with a Pasteur pipette, suspended in 10 vol. of 2.5 mM-EDTA buffer, and centrifuged at 30000g for 30 min. The resulting pellets were suspended in water or in 2.5 mM-EDTA buffer. All supernatants from the 30000g centrifugations were discarded. In experiments requiring large amounts of membrane, fractions I and II were pooled. As shown in Table 3, these fractions appear to be identical.

Chemical analyses. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with twice-recrystallized bovine albumin as standard. DNA and RNA were determined after extraction with cold trichloroacetic acid and lipid solvents at room temperature, and hydrolysis in 5% (w/v) trichloroacetic acid at 90° for 15 min. In some experiments hydrolysis in 0.3 N-KOH for 1 hr. at 37° was employed (Fleck & Munro, 1962). DNA was determined by the method of Croft & Lubren (1965), with highly-polymerized calf thymus DNA (Sigma type 1, organic phosphorus content 6.1%) as standard. The reaction mixture was generally incubated at 30° for 18-24 hr.; no significant difference was noted with incubations at 6-13° for 54 hr. RNA was determined by the orcinol method of Dische (1953) or by measurement of the extinction at 260 μ m (Fleck & Munro, 1962). For the determination of phospholipid and cholesterol, the tissue lipids were extracted according to Folch, Lees & Sloane-Stanley (1956). Phospholipids were determined as P_i after hydrolysis in 70% (v/v) $HClO_4$ by the method of Bartlett (1959) as modified by Marinetti (1962). Cholesterol was separated

from other lipids by thin-layer chromatography on silica gel G (Merck) in a solvent system consisting of benzene-diethyl ether-acetic acid (50:50:1, by vol.) (Heimberg, Weinstein, Dishmon & Fried, 1965). After elution from the silica gel G with three 10 ml. volumes of chloroform, the cholesterol was determined by the method of Zlatkis, Zak & Boyle (1953).

Enzyme determination. Disaccharidases were determined as described by Dahlqvist (1964). Sucrose, lactose, isomaltose, trehalose, turanose and cellobiose were employed as substrates. Glucose 6-phosphatase was determined as described by Hübscher & West (1965). Alkaline phosphatase was determined with *p*-nitrophenyl phosphate (Sigma 104) as substrate. The assay system contained 25 μ moles of glycine buffer, pH 9.2, 2.5 μ moles of $MgCl_2$, 0.5 μ mole of $ZnCl_2$ and 9.0 μ moles of substrate in a final volume of 0.6 ml. Enzyme and substrate were incubated at 37° for 15 min. and the reaction was terminated by the addition of 2.5 ml. of 0.02 N-NaOH. Free *p*-nitrophenol was determined spectrophotometrically at 400 m μ . The concentrations of $MgCl_2$ and $ZnCl_2$ used above were found to be optimum for the assay of alkaline phosphatase in both homogenates and brush borders. Leucine aminopeptidase was determined as described by Porteous & Clark (1965). Adenosine triphosphatase was determined by following the liberation of P_i from ATP. The assay system contained 70 μ moles of tris-HCl buffer, pH 7.4, 5 μ moles of tris-ATP (Sigma) and 5 μ moles of $MgCl_2$ in a final volume of 1.0 ml. Ca^{2+} (5.0 μ moles) was added to the assay system to determine Ca^{2+} -dependent adenosine triphosphatase activity, which was expressed as the increment above the Mg^{2+} -dependent activity. Unless otherwise specified, 1 unit of each enzyme activity is equal to 1 μ mole of substrate hydrolysed/min.

Preparation of brush borders and brush-border fractions for electron microscopy. Brush-border and membrane pellets were fixed without resuspension in sodium cacodylate-buffered glutaraldehyde, pH 7.2, for 1 hr. at 4°. The pellets were washed in four changes of buffer, stored overnight at 4° in a fifth rinse and postfixed in 1% OsO_4 for 30 min. at 4°. The pellets were then dehydrated at 4° in a graded series of ethanol concentrations, allowed to come to room temperature in 100% ethanol and passed through three rinses of propylene oxide. The pellets were then spun at 400g for 10 min. and infiltrated for 1 hr. in a mixture of equal volumes of propylene oxide and Epon. The pellets were next infiltrated overnight at 4° in pure Epon, brought to room temperature and embedded. Section showing silver-interference colours were cut on a Porter MT-1 ultramicrotome, with diamond knives, and then stained first with saturated uranyl acetate in 50% ethanol and then with lead citrate (Venable & Coggeshall, 1965). The preparations were examined with a Philips 200 electron microscope operated at 60 kv.

RESULTS

Purification of brush borders. Initial experience indicated that crude rat intestinal brush borders prepared according to Miller & Crane (1961) were associated with a large quantity of viscous contaminant. Filtration through no. 11 bolting silk, as suggested by Miller & Crane (1961), failed to remove this material satisfactorily. However, the

Table 1. Purification of rat intestinal brush borders : nucleic acid content and activity of invertase and alkaline phosphatase

Brush borders were prepared as described in the text. 'Crude' brush borders were washed three times in cold EDTA buffer before analysis. 'Purified' brush borders were analysed after saline-sedimentation of contaminant and filtration through glass wool. Analyses were performed as described in the text.

Preparation	Protein		DNA		RNA		Invertase		Alkaline phosphatase	
	(mg.)	(% recovery)	(μ g./mg. of protein)	(% of initial DNA)	(μ g./mg. of protein)	(% of initial RNA)	(units/mg. of protein)	(% of initial invertase)	(units/mg. of protein)	(% of initial alkaline phosphatase)
Homogenate	1290	100	32.0	100	35.0	100	0.05	100	0.23	100
'Crude' brush borders	49	3.8	41.0	4.8	22.4	2.5	0.50	38	2.50	40
'Purified' brush borders	15.5	1.2	7.8	0.3	13.9	0.45	0.99	24	4.90	25

addition of brush borders to 90–150mm-sodium chloride resulted in the aggregation of non-brush-border material. Initially this aggregated material was removed by low-speed centrifugation; later, greater yields of brush borders could be obtained by removing the contaminant on glass wool, as suggested by Harrison & Webster (1964).

In Table 1 brush borders 'purified' by removal of aggregated contaminant are compared with the 'crude' brush borders obtained essentially by the method of Miller & Crane (1961). Brush-border invertase and alkaline phosphatase specific activities in the 'purified' brush borders were twice those of the crude fraction. When compared with the activities present in the original mucosal homogenate this represented a 20-fold purification. More than 80% of the nucleic acid of the crude fraction was removed by the saline and glass-wool procedures, and the purified preparation contained less than 1% of the total mucosal DNA and RNA.

Although the data in Table 1 represent the analysis of a typical experiment, in a total of 20 experiments the mean ($\pm$ s.d.) invertase specific activity of the purified preparation was 0.96 ± 0.18 , with an overall purification factor (compared with the mucosal homogenate) of 17.5 ± 4.3 . In four sets of experiments, the DNA content of the purified brush borders ranged from 7.8 to 22 μ g./mg. of protein (mean 14.9); the RNA content ranged from 13.9 to 24 μ g./mg. of protein (mean 18.8).

Several attempts were made to remove additional contaminants by density-gradient centrifugation of brush borders. In the experiment shown in Fig. 1, 'purified' brush borders were placed on a sucrose zone gradient and centrifuged at low speed for

Fig. 1. Density-gradient centrifugation of 'purified' brush borders. Brush borders were layered on the gradient and centrifuged for 30 min. at 200g. The gradient consisted of five zones each containing 3.0 ml. of sucrose in the concentrations shown in the Figure, in 2.5mm-EDTA, pH 7.4. After centrifugation, individual zones were removed with a Pasteur pipette and analysed for invertase (●—●) and nucleic acid (DNA + RNA) (○---○) as described in the text. 'Recovery (%)' values refer to % recovered of the total amount fractionated.

Table 2. *Glucose 6-phosphatase in brush-border preparations and the effect of glucose on its activity*

Homogenate and brush-border preparations were obtained as described in Table 1. One unit of glucose 6-phosphatase activity is equivalent to 1 μ mole of P_i liberated/min.

Preparation	Invertase (units/mg. of protein)	Glucose 6-phosphatase (units/mg. of protein)		Inhibition of glucose 6-phosphatase by glucose (%)
		No glucose	0.4 M-Glucose	
Whole homogenate	0.04	6.9	3.5	49.5
'Crude' brush border	0.526	10.5	9.8	7.0
'Purified' brush border	0.88	15.3	14.9	2.0

15 min. Most of the brush borders (measured by invertase activity) were subsequently found in the 20 and 30% zones, and some nucleic acid contaminant had migrated into the higher-density zones. However, most of the nucleic acid could not be removed from the brush borders by this technique, and therefore density-gradient centrifugation was not generally employed.

Markers of contamination other than nucleic acid were not generally used. In previous studies, we did not find cytochrome oxidase in purified brush borders (Forstner, Riley, Daniels & Isselbacher, 1965). Hübscher *et al.* (1965) suggested the use of glucose 6-phosphatase as a marker for microsomal contamination. However, we noted that there was a parallel increase of both glucose 6-phosphatase and invertase during purification (Table 2). This observation suggested that the glucose 6-phosphatase content of the purified brush-border fraction might indeed not be a reflection of microsomal contamination. To investigate this possibility further, enzyme activity was measured in the presence of glucose, which has been shown to inhibit hepatic and intestinal glucose 6-phosphatase (Field, Epstein & Egan, 1965). In the whole homogenate, 0.4 M-glucose resulted in a 50% inhibition, but in the brush-border fractions no significant inhibition occurred (Table 2). Thus it appears likely that most of the 'glucose 6-phosphatase' activity in the brush-border preparations was due to the action of non-specific phosphatases.

Fractionation of 'purified' brush borders into membrane and fibrillar components. In preliminary experiments a variety of conditions, including the use of tris, Mg^{2+} , Ca^{2+} , thiol reagents and water, were employed in an attempt to separate microvillus membranes from fibrillar material. The addition of hyperosmotic tris (Eichholz & Crane, 1965), as well as water, produced generalized swelling of the brush border, followed by a gradual loss of membrane material and finally complete disruption of the complex. This sequence could not be interrupted when tris was used; however, with water it was found that restoring the EDTA

concentration to 4 mM immediately arrested the swelling and disruption. The latter observation was used to establish optimum osmotic and temporal conditions for the release of membrane and the preservation of an intact fibrillar substructure. Further, the fibrillar substructure of the brush border maintained the shape of the isolated brush borders throughout the several centrifugations in spite of the loss of membrane. The fact that these structures could readily be identified by phase-contrast microscopy greatly improved the ease and accuracy of their separation from the microvillus membrane.

The optimum conditions for the subfractionation of brush borders are shown in Scheme 1. With this technique two membrane fractions (I and II) and a fibrillar fraction were obtained. In most experiments these three fractions contained approximately 80% of the original protein and invertase activity. The protein and enzyme yield could not be increased by prolonged centrifugation in the final stages, and the supernatant fractions did not contain trichloroacetic acid-precipitable protein. Thus the failure to recover 100% of the brush-border material was probably due to unavoidable losses during the various transfer steps.

As shown in Scheme 1, most of the invertase activity (i.e. 70%) was recovered in the two membrane fractions. Further (Table 3), the mean specific activity of invertase in the membrane fractions (I and II) was 1.6 times that of the 'purified' brush border and 4–6 times that of the fibrillar fraction. Thus, assuming invertase to be a valid marker of the microvillus membrane (Hübscher *et al.* 1965), the data in Table 3 indicate that an effective separation of membrane and fibrillar material was consistently achieved.

Further evidence in support of an effective separation of membranes from fibrillar material was obtained from the relative distribution of cholesterol and phospholipid. These lipids are major components of cell membranes (van Deenen, 1965). Table 3 shows that the specific content of both of these constituents paralleled the invertase

Scheme 1. Flow-sheet of the method and procedure for the preparation of membrane and fibrillar fractions from purified brush borders. Percentages of protein and invertase refer to the percentages of the initial total protein and invertase that are found in each fraction.

Table 3. *Membrane components and nucleic acids in rat intestinal brush borders and membrane and fibrillar fractions*

Results are presented as means $\pm$ s.d. The numbers of preparations analysed are indicated in parentheses. Assay procedures are described in the text.

Fraction	Membrane components			Nucleic acids	
	Invertase (units/mg. of protein)	Phospholipid (μ g. of P/mg. of protein)	Cholesterol (μ g./mg. of protein)	DNA (μ g./mg. of protein)	RNA (μ g./mg. of protein)
'Purified' brush borders	0.952 ± 0.15 (8)	103 ± 18 (6)	53 ± 5.9 (5)	17.6 (2)	18.7 (2)
Membrane					
Fraction I	1.53 ± 0.28 (8)	126 ± 8 (5)	85 ± 5.0 (3)	4.6 (3)*	10.3 (2)*
Fraction II	1.53 ± 0.26 (8)	127 ± 15 (5)	86 ± 5.1 (4)		
Fibrillar fraction	0.33 ± 0.22 (8)	75 (3)	33 (2)	71 (3)	41.4 (3)

* Results from fractions I and II pooled.

specific activity in the various fractions. By contrast, DNA and RNA were found predominantly in the fibrillar fraction, and the membrane fractions had considerably less nucleic acid/mg. of protein than the original 'purified' brush borders. Also the cholesterol, phospholipid and invertase contents were virtually the same in membrane fractions I and II, which suggests that they are identical.

Electron-microscopic observations. The 'purified' brush-border preparations (Plate 1a) were morphologically homogeneous, consisting of isolated brush borders that were free of nuclear and cytoplasmic contamination except for variable amounts of vesicular membranous material that remained attached to the non-microvillus surface of individual brush borders. These vesicular membranes

Table 4. *Enzymic composition of brush-border membrane and fibrillar fractions*

Membrane and fibrillar fractions were prepared from 'purified' brush borders as described in the text. Protein and enzyme recoveries in the subfractions are expressed as percentages of the total protein or enzyme activity in the brush borders before disruption. S.A., specific activity (units of enzyme activity/mg. of protein); Mg²⁺-ATPase and Ca²⁺-ATPase, Mg²⁺- and Ca²⁺-stimulated adenosine triphosphatase activities.

Preparation	Protein (mg./ fraction)	Invertase		Alkaline phosphatase		Leucine amino-peptidase		Mg ²⁺ -ATPase		Ca ²⁺ -ATPase	
		(% of initial protein)	(% of initial invertase)	(% of initial alkaline phos- phatase)	(% of initial leucine amino- peptidase)	(% of initial leucine amino- peptidase)	(% of initial leucine amino- peptidase)	(% of initial Mg ²⁺ - ATPase)	(% of initial Mg ²⁺ - ATPase)	(% of initial Ca ²⁺ - ATPase)	(% of initial Ca ²⁺ - ATPase)
'Purified' brush borders	25.3	100	100	100	100	100	100	100	100	100	100
Membrane (fractions I and II)	12.2	48	74	54	61	61	61	82	82	102	102
Fibrillar fraction	8.9	35	7	7	19	19	19	24	24	13	13
Recovery (%)		83	81	61	80	80	80	106	106	115	115

were devoid of ribosomes and presumably were remnants of the smooth endoplasmic reticulum. The ultrastructural characteristics of the microvilli, microvillus membranes and terminal web were normal.

To demonstrate the effect of hypo-osmotic exposure on these brush borders, several preparations were examined after short exposure to water. Varying degrees of microvillus swelling and vesiculation were seen (Plate 1b), indicating that the whole or part of each microvillus behaved as a semi-independent sac-like compartment that eventually ruptured at a critical point of stretch. The vesiculated membranes were devoid of the microfibrils that comprise the internal structure of the normal microvillus. This observation suggested that during the process of hypo-osmotic swelling the fibrils are retracted from the microvilli, resulting in the formation of hollow sacs and vesicles at the time of disruption. Of interest was the linear sheet of membrane overlying the terminal web that apparently resulted from fusion of the membranes at the base of microvilli as their peripheral portions break off.

Membrane preparations (Plate 2a) consisted of an extremely homogeneous array of membrane sheets and hollow vesicles. Membranes were well-preserved bilaminar structures (Plate 2b) in which the unit-membrane structure was intact and of the characteristic thickness of undisrupted microvillus membranes.

In the fibrillar fraction (Plate 2c) many of the fragments could be readily identified as terminal webs since they had maintained the semilunar shape of whole brush borders. Admixed with the fibrillar material were some membranous sheets that remained attached to the terminal web during disruption of the microvilli. In some pellets a darker-staining granular material of amorphous shape was observed. This appeared to represent a contaminant, possibly of nuclear origin. Admixed with the terminal web material were fibrillar strands (Plate 2d) presumably representing the

EXPLANATION OF PLATE I

(a) Sectioned pellet of a 'purified' brush-border preparation. The microvilli (MV) and terminal web (TW) appear normal. Some smooth-walled membranes, presumably remnants of the endoplasmic reticulum (ER), remain attached to the non-microvillus surface. Magnification $\times 15345$. (b) Vesiculation of microvilli during hypo-osmotic exposure. Varying degrees of microvillus swelling and vesiculation (V) are apparent. The vesicles are devoid of microfibrils. A linear sheet of membrane (M) that overlies the terminal web (TW) may have formed by fusion of the membranes at the base of the microvilli as the peripheral portions broke off. Magnification $\times 23750$.

structural internal fibrils of the microvilli. In addition a filamentous meshwork extended from the undersurface of the web. Similar filaments are seen in the intact brush borders (Plate 1a).

Enzyme composition of brush-border fractions. Since disruption of 'purified' brush borders might damage the substructures, enzymes known to be present in brush borders were studied to obtain evidence of the functional state of the isolated fractions. In the experiments summarized in Tables 4 and 5, the activities of various enzymes were determined in the 'purified' brush borders and in the membrane and fibrillar fractions. All enzymes assayed were much more active in the membrane than in the fibrillar fraction, and in each case there was some enhancement of activity over that associated with the brush borders. Protein and enzyme recoveries were similar (i.e. about 80%) for the disaccharidases and for leucine aminopeptidase, indicating that they were not affected by the preparative techniques. Both Mg^{2+} - and Ca^{2+} -dependent adenosine triphosphatase recoveries were considerably greater, suggesting that they may have been activated during disruption. By contrast, only 60% of the alkaline phosphatase activity was recovered.

Contribution of membrane and fibrillar material to total protein of the brush border. Although it is assumed that most of the brush-border protein is present in the plasma membrane, the literature provides insufficient data to permit a quantitative evaluation of this point. A rough approximation can be derived from the experimental results shown in Table 6, which represent the mean results of four experiments, giving the yields of invertase and protein in membrane and fibrillar fractions. The mean recovery $\pm$ s.d. of protein and enzyme in these experiments was $82.7 \pm 3.2\%$ and $83.8 \pm 8.9\%$ respectively. Unrecovered material was therefore

EXPLANATION OF PLATE 2

(a) Low-power electron micrograph of sectioned membrane preparation. The pellet consists of a homogeneous array of membranous sheets and vesicles. Magnification $\times 6075$. (b) Membrane preparation at high magnification. The vesicles are surrounded by well-preserved bilaminar membranes that are structurally similar to intact microvillar membranes. Magnification $\times 75000$. (c) Sectioned pellet of fibrillar fraction, among which are some fragments identified as terminal web because of their semilunar shape. Membranous sheets are adherent to some of these fragments. Magnification $\times 9750$. (d) Terminal-web (TW) material of the fibrillar fraction, structurally similar to that in the intact brush-border preparations (compare with Plate 1a). A filamentous meshwork extends from the cytoplasmic surface of the sedimented fragments of the terminal web. Magnification $\times 59500$.

Table 5. Disaccharidase composition of brush-border, membrane and fibrillar fractions

Experimental details are identical with those described in Table 4. S.A., specific activity (units/mg. of protein).

Protein	Invertase		Lactase		Isomaltase		Maltase		Trehalase		Turannase		Cellobiase	
	(% of initial protein)	(S.A.)	(% of initial invertase)	(S.A.)	(% of initial lactase)	(S.A.)	(% of initial isomaltase)	(S.A.)	(% of initial maltase)	(S.A.)	(% of initial trehalase)	(S.A.)	(% of initial cellobiase)	(S.A.)
'Purified' brush border	19.3	100	1.14	100	0.14	100	1.96	100	6.5	100	1.14	100	0.05	100
Membrane (fractions I and II)	9.8	51	1.96	87	0.21	75	3.1	80	9.8	76	1.62	72	0.07	75
Fibrillar fraction	6.9	36	0.27	9	0.05	13	0.4	7	1.7	10	0.38	12	0.01	7
Recovery (%)		87		96		88		87		86		84		82

Table 6. *Contribution of plasma membrane and fibrillar material to the total protein of the brush border*

The experimental results are mean yields and activities obtained from four subfractionation experiments. Estimations of total membrane and fibrillar protein include corrections for membrane present in the fibrillar fraction, as described in the text. Specific activity (S.A.) of invertase is expressed as units/mg. of protein.

Fraction	Experimental			Estimated	
	Protein (mg.)	Invertase		Membrane protein (mg.)	Fibrillar protein (mg.)
		(units)	(S.A.)		
Membrane	7.71	12.6	1.63	7.71	0.00
Fibrillar	5.3	1.6		0.98	4.32
Total	13.01	14.2		8.69	4.32
% of total protein				66	34

virtually identical with that actually assayed with respect to invertase and protein, and it can be assumed that the results in Table 6 are representative of the total brush-border material that was fractionated. The mean membrane protein and invertase yields were 7.71mg. and 12.6units, respectively, giving a membrane invertase specific activity of 1.63units/mg. of protein. If this invertase specific activity is considered to be characteristic of pure membrane, it can be calculated that the 1.6units of invertase activity in the fibrillar fraction corresponds to the presence of 0.98mg. of membrane protein as a contaminant. As shown in Table 6, total estimated membrane and fibrillar protein is 8.69mg. and 4.32mg. respectively. Thus only two-thirds of the brush-border protein is in the plasma membrane; one-third is in the fibrillar residue.

DISCUSSION

A technique for the subfractionation of whole brush borders into their membranous and fibrillar constituents has been described. In view of the absence of specific criteria for establishing the identity and degree of purity of insoluble macromolecular complexes, the fractions have been assessed with regard to a number of parameters. The choice of these parameters was based on prior biochemical observations relating to the brush border. Thus previous work has shown (a) that the complex contains many hydrolytic enzymes, at least two of which have been shown to be located in the microvillus membrane (Eichholz & Crane, 1965; Johnson, 1967), (b) that it contains cholesterol and phospholipid (Millington & Finean, 1963), (c) that the microvillus membrane is microscopically distinguishable from the lateral plasma membrane (Millington, 1964), and (d) that brush borders prepared by previous techniques are contaminated with DNA and RNA (Eichholz & Crane, 1963; Hübscher *et al.* 1965; Porteous & Clark, 1965).

The electron-microscopic appearance of the final products of the separation technique is perhaps the most satisfactory evidence of their identity. In Plate 2(a) it is evident that the pooled membrane fractions consist almost entirely of membranous structures. These structures (Plate 2b) have the cross-sectional width of microvillus membranes (90–110Å) (Palay & Karlin, 1959; Millington & Finean, 1962; Millington, 1964), and appear to have retained the bilaminar structure characteristic of the intact membrane (Palay & Karlin, 1959). In contrast, the fibrillar fraction consists almost entirely of material fibrillar in nature, much of which can be identified as being derived from the brush border by the characteristic semilunar appearance and the occasional attachment of membrane tags.

In developing the separation methods the fractions were identified and monitored by their invertase activity. The use of invertase as a membrane marker was amply justified by the demonstration that almost 90% of the brush-border activity of this enzyme was recovered in the microscopically-identified membrane fractions (Table 5). It is unlikely that errors were introduced by the inadvertent purification of a membrane sub-unit containing invertase, since parallel distributions were found for other disaccharidases, as well as alkaline phosphatase, leucine aminopeptidase, adenosine triphosphatase, cholesterol and phospholipid.

A mean invertase purification of 17.5-fold (compared with the whole homogenate) was obtained in the 'purified' brush borders and these served as the starting material for the membrane isolations. Although no comparable data exist for the rat, these results compare favourably with the 11-fold purification obtained in the guinea pig by Hübscher *et al.* (1965) and the 13.6-fold purification reported in the hamster by Miller & Crane (1961). Since isolation of the plasma membrane resulted in a

further mean increment in invertase specific activity of 60%, the final purification of the membrane was about 29-fold. This value suggests that the purification obtained was at least twice that of any previously reported.

Tables 1 and 3 indicate that most of the contaminating DNA and RNA had been removed from the membrane preparation. In their brush border + nuclei preparations Hübscher *et al.* (1965) found 82% of the invertase and 102% of the DNA present in the original mucosal homogenate; Porteous & Clark (1965) found 71% of the invertase, 99% of the DNA and 21% of the RNA. Thus in these preparations the invertase/DNA ratio was 0.8 and the invertase/RNA ratio was 3.5. Our results with purified brush borders (Table 1) show an invertase/DNA ratio of 80, and an invertase/RNA ratio of 53. These ratios are probably larger in the microvillus membrane subfraction, since its specific content of DNA and RNA is less than, and its invertase specific activity is greater than, that of the whole brush-border fraction (Table 3). Hübscher *et al.* (1965) obtained an invertase/DNA ratio of about 12 in their microvillus membrane fraction. Thus the present techniques permit a greater degree of purification of both intact brush borders and the microvillus membrane than has previously been reported.

It is conceivable that the residual nucleic acid in the purified microvillus membrane is not a contaminant but actually a structural component of the plasma membrane. A DNA-containing receptor for haemagglutinating enteroviruses has been isolated from human erythrocyte plasma membranes (Philipson & Zetterqvist, 1964; Philipson, Bengtsson, Brishammar, Svennerholm & Zetterqvist, 1964). Further, density-gradient centrifugation of the purified membrane preparation has shown that the nucleic acid component is evenly distributed with the membrane particles (G. G. Forstner & K. J. Isselbacher, unpublished work). Such a distribution might be expected if the membranes contained a specific nucleic acid component; however, similar results could occur if nucleic acid was adsorbed to the membranes during their isolation.

The characterization of microvillus membrane preparations with respect to enzymic activity, lipid content and nucleic acid content appears to possess considerable merit for the comparison of data obtained from different animal species or by other preparative techniques. In our experience the pronounced localization of invertase in the microvillus membrane (Tables 5 and 3), its stability and its great reproducibility in different preparations (Table 3) make this enzyme a most useful membrane marker. The results presented in Table 5 suggest, however, that other disaccharidases may

be equally useful. Alkaline phosphatase and adenosine triphosphatase appear to be less suitable as markers. In Table 4 it is evident that an appreciable amount of alkaline phosphatase activity was lost during the subfractionation of the brush borders, and that activation of adenosine triphosphatase appears to have taken place during disruption.

Both microscopic and chemical evidence indicates that the fibrillar fraction contains considerable non-brush-border material (Plate 2c and Table 3). Unfortunately the assessment of the actual amount of fibrillar material present in this fraction is prevented by the lack of a suitable marker for the fibrils. None of the brush-border enzymes examined in this study were located in the fibrillar fraction, and it did not appear to contain either cholesterol or phospholipid. Electron microscopy suggests that most of this fraction is derived from the fibrillar substructure of the brush border. Since calculations based on the data shown in Table 6 indicate that the fraction as a whole accounts for approx. 34% of the total protein of the brush borders, the fibrils may be composed largely of protein. This conclusion is supported histologically by the affinity of the fibrils for protein stains (Puchtler & Leblond, 1958). The apparent lack of Mg^{2+} - and Ca^{2+} -dependent adenosine triphosphatase activity suggests that the fibrils do not contain a contractile system analogous to actomyosin (Wins & Schoffeniels, 1966); this does not exclude the possibility that the fibrils may have a contractile activity as suggested by Millington & Finean (1962).

It is evident microscopically (Plate 1a) that purified brush borders contain tags of endoplasmic reticulum on their undersurface. Much of this material remains attached to the terminal web in the fibrillar fraction, but some may be present in the membrane fractions. Unfortunately the very low activity of specific glucose 6-phosphatase (Table 2) precluded the use of this marker for following the fate of the endoplasmic reticulum during fractionation.

While the details of our subfractionation method were being worked out, Eichholz & Crane (1965) published a method for the fractionation of hamster brush borders. Their fraction C appears to correspond closely to our microvillus membrane fraction (Eichholz, 1967). A comparison of membranes prepared by both techniques would be of interest, since each method may have specific advantages for the study of membrane function.

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