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PURIFICATION OF THE HUMAN INTESTINAL BRUSH BORDER MEMBRANE

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SUMMARY

A method is described to obtain brush border, *i.e.* microvillus membranes, from human intestinal epithelial cells. It is based on the sequential use of $CaCl_2$, differential centrifugation and Tris. The final preparation contains no DNA, no succinate dehydrogenase, traces only of (Na^+-K^+)-ATPase, NADPH–cytochrome *c* reductase and β-glucuronidase and is characterized by a high specific activity in brush border membrane markers as well as its appearance on electron micrographs.

The method may be applied to fresh or frozen intestine, to surgical specimens or peroral jejunal biopsies.

INTRODUCTION

The functional elements of the brush border membrane provide for the terminal digestion of carbohydrates and oligopeptides as well as their absorption as monosaccharides and amino acids or dipeptides[1,2]. In man these functions are known to be impaired in a few rare diseases[3,4], and it was of interest for us, in line with previous studies on the structure of the brush border membrane[5], to try to establish whether these defects or abnormalities might affect, for example, the protein composition of the membrane. For this purpose pure microvillus membranes were needed.

An initial attempt of this laboratory to prepare such membranes has been published elsewhere[6]. The attempt was unsuccessful inasmuch as there was sizable contamination by microsomes and undisrupted core material. However, we have taken advantage of this experience and of the fact, recently reported[7,8], that Ca^{2+}-aggregated microsomal vesicles sediment at very low speed to devise a method which yields brush border membranes of acceptable purity. The method may be applied to frozen or fresh intestine, to samples of rather large size as obtained at surgery or to biopsies. It is the subject of this paper.

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MATERIALS AND METHODS

Intestinal samples

Full thickness sections of macroscopically normal human small intestine were obtained from a total of 18 patients. 13 jejunal and 2 ileal specimens are included in this study. In addition 3 peroral jejunal biopsies, weighing between 20 and 30 mg, were obtained with a four-port Rubin tube. All samples were sealed in plastic bags, immediately frozen on solid CO_2 and retained in a deep-freeze at −20 °C, until use. In two cases surgical samples were not frozen but were processed immediately.

Preparation of brush border fragments (Fraction P_2)

Slices were cut from the specimens and 0.5–2 g of the still frozen mucosa were carefully dissected from the underlying tissue, along the muscularis mucosae. A 1% homogenate was made in 50 mM mannitol–2 mM Tris (pH 7.1) at 4 °C, using either a Waring blendor at full speed during 20 s with the Powerstat variable transformer at a setting of 90 or a conical grinding tube (Bellco), depending on the weight of the sample. All following operations were performed at 4 °C. The homogenate was filtered through a piece of nylon mesh of 40 μm pore size (Tobler, Ernst and Traber, Inc. No. 32544). Solid $CaCl_2$ was added with stirring to a final concentration of 10 mM. During 10 min of occasional mixing by inversion an increase in turbidity of the homogenate was observed. $CaCl_2$ did not change the pH of the homogenate. Centrifugation of the Ca^{2+}-treated homogenate at 2000 × *g* during 10 min in a Sorvall centrifuge yielded a heavy whitish pellet (Fraction P_1) which was resuspended for further analysis in 50 mM mannitol–2 mM Tris, and a clear supernatant (Fraction S_1). The supernatant was centrifuged at 20000 × *g* during 15 min in the same centrifuge to yield a small brownish pellet (Fraction P_2) containing the brush border fragments. The final supernatant (Fraction S_2) was kept for analysis. Fraction P_2 was resuspended either in distilled water when Tris disruption (see below) was to follow or in 50 mM mannitol–2 mM Tris when it was to be used for analysis only.

The same procedure as above was applied for the biopsies except that the volume of the homogenate was set arbitrarily at 10 ml and the total quantity of Fraction P_2 was used to prepare Fraction FII (see below), no part of it being kept for assays.

Preparation of the microvillus membrane fraction (Fraction FII)

As previously described[9], Tris was used to disrupt brush border fragments into microvillus membrane and core material. Freshly prepared 1.6 M Tris was added to Fraction P_2 resuspended in distilled water to obtain a final Tris concentration of 0.8 M. The Tris–pellet mixture was frequently stirred during 1 h. The mixture was then layered on top of a 37, 40, 42, 45, 60% glycerol gradient which was 0.05 M in $MgCl_2$ and centrifuged 15 min at 63000 × *g* in an $L_2$65B Spinco Ultracentrifuge using the 39 rotor. 3–4 bands, clearly visible by the Tyndall effect, were recovered in four fractions with a syringe from the top of the gradient, diluted in 2–5 times their volume of distilled water and washed free of glycerol by being centrifuged 60 min at 200000 × *g* using the same ultracentrifuge with the SW 50-1 rotor. The largest band, the second from the top of the gradient, was found to contain the microvillus membrane, and is referred to as Fraction FII. The very small final pellets were resuspended

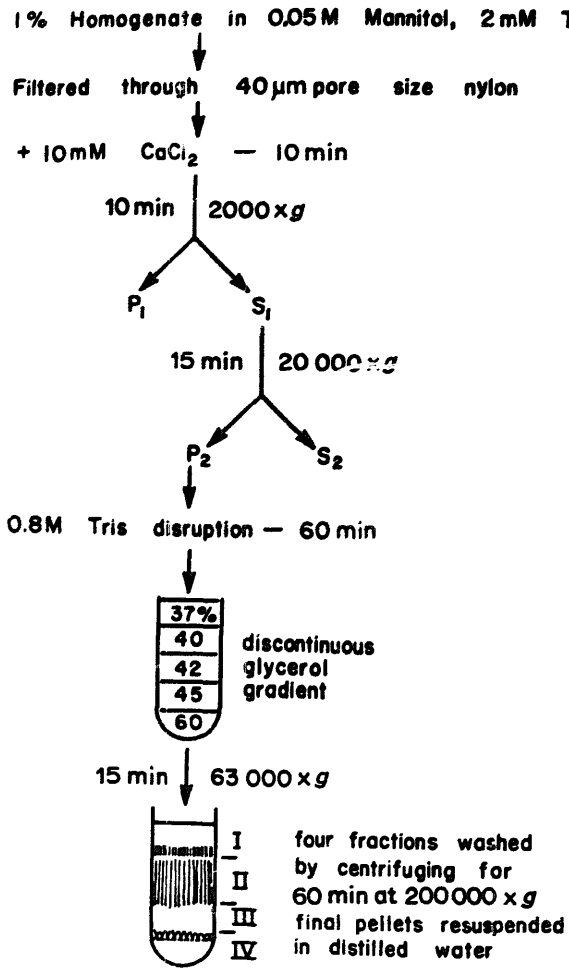


Fig. 1. Schematic representation of the purification procedure of human microvillus membrane as described in Materials and Methods.

in 0.3–0.8 ml of distilled water depending upon the amount of protein expected to be in the fractions. The biopsies were processed identically. The entire procedure is summarized on the flow diagram in Fig. 1.

Assays

Sucrase was used as the microvillus membrane marker. Trehalase, leucylnaphthylamidase and alkaline phosphatase, which are also present in the microvillus membrane, were also determined. The two disaccharidases were assayed according to a modification by Lloyd and Whelan[10] of Dahlqvist's method[11]; leucylnaphthylamidase and alkaline phosphatase according to Goldbarg and Rutenburg[12], and Eichholz[13], respectively.

DNA served as a marker for nuclei and was measured according to Burton's method[14] as modified by Giles and Myers[15].

Succinate dehydrogenase was chosen as a marker for mitochondria and was assayed following Pennington's method[16] as modified by Porteous and Clark[17]. The specific activity is expressed in arbitrary units, by the variation in A at 490 nm, per min and per mg protein.

NADPH–cytochrome *c* reductase was used as a marker for microsomal con-

tamination as proposed by Clark *et al.*[18] and its activity was assayed and expressed according to Masters *et al.*[19]. One unit is defined as an absorbance change of 1.0 per min at 550 nm. The specific activity is the number of units per mg protein.

(Na^+-K^+)-ATPase was chosen as the basolateral plasma membrane marker for reasons that will be discussed later. Total, Mg^{2+}-activated and ouabain-insensitive ATPases were assayed according to Eichholz[13], who used the Fiske and SubbaRow[20] phosphate assay, with the exception of the following points: Tris–ATP (Sigma) was used as substrate; EDTA was added to the assay mixture at the final concentration of 5 mM; the final concentrations of $MgCl_2$ and Tris buffer were 10 and 75 mM, respectively. The addition of EDTA reduced to an acceptably low level an ATP-splitting activity which occurred without any addition of cation and increased the (Na^+-K^+)-ATPase activity which was otherwise very low. Since divalent cations (mainly Ca^{2+}) are known to affect both Mg^{2+}- and (Na^+-K^+)-ATPase activities and their ratio[21], it is inferred that EDTA acts to chelate a contaminating cation. Chelators have a similar effect on the (Na^+-K^+)-ATPase activity of rabbit kidney outer medulla[22]. In previous studies[23–30] added EDTA was not needed presumably because it was already present as specified by Miller and Crane[31] for the preparation of brush borders. Ouabain, when used, was added at the final concentration of 1 mM. Mg^{2+}-activated and ouabain-insensitive activities were very similar and (Na^+-K^+)-activated ATPase was estimated routinely by the difference between total and ouabain-insensitive activities.

β-Glucuronidase was chosen as a marker for lysosomal contamination and was assayed according to Plaice[32].

On the basis of the good recoveries obtained in the assays as well as of a comparison of Mg^{2+} treatment with that of Ca^{2+}, as described later, it can be concluded that none of the assays were importantly affected by the use of 10 mM Ca^{2+} in the method. For all enzyme activities assay conditions were chosen so that the velocity of the reaction was linear with regard to time and protein concentration. When not otherwise stated, specific activities have been expressed in μmoles of substrate hydrolyzed per min (I.U.) and g protein.

Protein determination was by the method of Lowry *et al.*[33] using crystalline bovine serum albumin as standard, with final volumes of 1.4, 2.5 or 6 ml, depending on the expected protein concentration in the sample.

Preparation of membrane for electron microscopy

Fraction FII was resuspended in phosphate buffer, pH 7.0, and was fixed in a mixture made in the same buffer of 1% glutaraldehyde and 1.5% paraformaldehyde (final concentrations) during 30 min at room temperature. The fixed membranes were pelleted by 1 h centrifugation at $30000 \times g$. The very small sediment was washed twice with phosphate buffer and once with veronal acetate buffer, pH 6.2, thoroughly dispersed in a small volume of veronal acetate buffer and then carefully mixed at 40 °C with 1% immunodiffusion agar dissolved in the same buffer. This mixture was layered on a clean glass slide and the thin agar film (2–3 mm thick) was solidified in cold (4 °C) for 5 min. The agar film was then cut into small pieces (1 mm^2) and used for further processing.

The agar blocks containing the aldehyde-fixed specimens were fixed in 1% OsO_4 in veronal acetate buffer[34] and $CaCl_2$ at pH 6.2 for 12–15 h at room temperature

in the dark. The fixed blocks were rinsed in veronal acetate buffer and further fixed for 1 h in 0.5% uranylacetate in veronal acetate buffer. The blocks were then rinsed successively in 30 and 50% alcohol (30 min each) containing 0.1% NaCl. The rinsed blocks were then dehydrated in 70, 95 and 100% alcohol (30 min each step) followed by propylene oxide (30 min, two changes). The dehydrated material was infiltrated at 45 °C for 1 h in a 50:50 mixture of propylene oxide and Dow Epoxy resin[35]. After infiltration the agar blocks were individually encapsulated in fresh Dow Epoxy resin in "Beam" capsules and polymerized by incubation at 37, 45 and 60 °C for 24 h at each temperature. The polymerized blocks were sectioned with a Porter-Blum MT1 ultramicrotome. Sections of 500 Å thickness were collected on 300-mesh uncoated copper grids and stained immediately in Reynold's lead citrate[36]. The freshly stained grids were examined in a JEM 120 electron microscope at 80 kV acceleration voltage and fitted with a high contrast pole piece attachment, 30 μm objective lens apperture and an anticontamination device. Under conditions of experiment the microscope showed 10 Å point to point resolution on biological material.

RESULTS

The effectiveness of Ca^{2+} in the preparation of brush border fragments (Fraction P_2)

It is difficult, if at all possible, to isolate intact brush borders from frozen human tissue. Also the brush border fragments obtained are very small and not readily separable from microsomal fragments (endoplasmic reticulum) either by filtration or by gradient centrifugation. However, the separation can be achieved with Ca^{2+}.

After addition of $CaCl_2$, a first spin at $2000 \times g$ was aimed at pelleting the endoplasmic reticulum and a second one at $20000 \times g$ to collect the brush border fragments. As seen in Fig. 2, Ca^{2+} caused mitochondria and basolateral plasma membranes as well as endoplasmic reticulum to sediment at $2000 \times g$, but did not appreciably affect the brush border fragments. Thus already well purified brush border material was obtained in Fraction P_2.

The effectiveness of Ca^{2+} was further verified by following the distribution of the various possible contaminants during the processing of surgical specimens from 5 different patients with the results presented in Table I. In general recoveries were around 90%. Only for (Na^+-K^+)-ATPase was the recovery from the first centrifugation more variable. Ca^{2+} was probably not responsible for this variability inasmuch as a similar recovery and distribution of (Na^+-K^+)-ATPase was obtained with Mg^{2+}. It is more likely that variable amounts of (Na^+-K^+)-ATPase activity were not assayed in Fraction P_1, since this fraction was always difficult to resuspend uniformly.

Fraction P_1 contained the total recovered DNA and more than 90% of both recovered succinate dehydrogenase and (Na^+-K^+)-ATPase. Fraction P_2 contained no DNA, traces of succinate dehydrogenase and only slightly more than 1% of the original (Na^+-K^+)-ATPase activity. NADPH–cytochrome *c* reductase was fairly evenly distributed between Fraction P_1 (63%) and Fraction S_2 (40%). Only 1.5% was still found in Fraction P_2. β-Glucuronidase went mostly (81%) into Fraction S_2 leaving less than 1% of the original homogenate activity in the second pellet. 41% of the total original sucrase activity was retained in Fraction P_2, where a 16-fold increase in this brush border marker activity was achieved. Identical results were obtained

TABLE I

PURIFICATION OF MICROVILLUS MEMBRANES FROM SURGICAL TISSUE

In the case of succinate dehydrogenase and NADPH–cytochrome *c* reductase the values taken for 3 tissues were the average of determinations from 3 experiments and for 2 tissues from 2 experiments for (Na^+–K^+)-ATPase. Recoveries and increases in spec. act.≈ are means of the recoveries and increases from each experiment, except for (Na^+–K^+)-ATPase where the recovery is the sum of the averages recovered in each fraction. In all Tables, small rounding errors cause totals to be not precisely the sum of the individual percentages.

Fraction	*Protein* (%)	*DNA* (%)	*Succinate dehydrogenase* Spec. act.*	%	*NADPH–cytochrome c reductase* Spec. act.	%	*(Na^+–K^+)-ATPase* Spec. act.	%	*β-Glucuronidase* Spec. act.	%	*Sucrase* Spec. act.	%
H	100 (13)***	100 (5)	79±6** (5)	100	0.24±0.03 (5)	100	55±8 (5)	100	2.1±0.1 (5)	100	92±15 (13)	100
P_1	41±2 (13)	93±3 (5)	132±39 (5)	77±9	0.33±0.07 (5)	63±12	91±18 (5)	65±10	0.7±0.03 (5)	14±2	64±11 (13)	30±3
P_2	2.5±0.2 (13)	0	8.3±3.1 (5)	0.2±0.08	0.10±0.03 (5)	1.4±0.7	23±9 (5)	1.2±0.4	0.8±0.2 (5)	0.8±0.1	1457±214 (13) ×16±0.6≈	41±3
S_2	50±2 (13)	0	11±1 (5)	6.3±0.7	0.17±0.01 (5)	40±10	5.1±1.3 (4)	5.0±1.5	3.4±0.2 (5)	81±7	29±6 (13)	17±2
Recovery	93±2	93±3		83±9		104±8		71±9		96±5		88±2
F_{II}	0.9±0.1 (10)	—	traces (3)		traces (3)		6.03 (3)	0.07	0.3±0.1 (5)	0.2±0.1	2410±326 (10) ×26.1±1.9≈	23±2
Recovery from the gradient	66±4 (10)								30±2.4 (4)		78±4	

* Spec. act. = specific activity in I.U./g protein except for succinate dehydrogenase and NADPH–cytochrome *c* reductase where it is expressed in ΔA/min per mg protein.

** Mean ± S.E.

*** Number of different tissues studied.

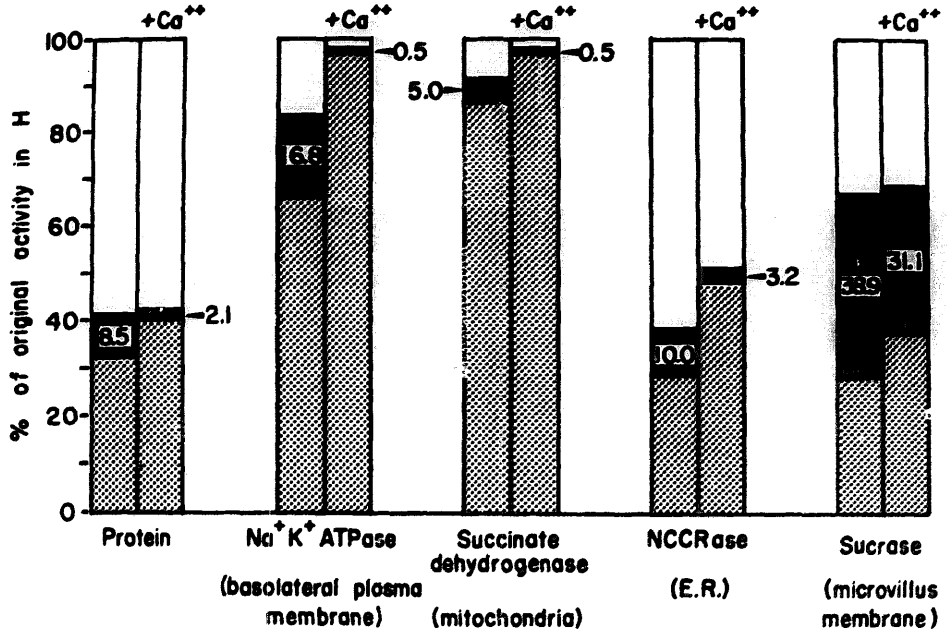


Fig. 2. Distribution of basolateral plasma membrane, mitochondria, endoplasmic reticulum (E.R.) and brush border membrane in Fraction P_1 (dotted area), Fraction P_2 (dark area) and Fraction S_2 (empty area) when Fraction P_2 was prepared from a plain (left columns) or 10 mM Ca^{2+}-treated (right columns) homogenate. Figures in or beside the bars represent percentages of starting homogenate activities found in P_2. NCCRase, NADPH–cytochrome *c* reductase.

when fresh tissues were processed. The results were pooled with those obtained with frozen tissue and are included in Table I.

Studies on the nature of the Ca^{2+}-effect

The specificity of the effect of Ca^{2+} on the centrifugation pattern of mitochondrial, microsomal and brush border membranes was checked by varying its concentration and by replacing Ca^{2+} by other cations. It can be seen in Fig. 3 that 8 mM Ca^{2+} was fully effective, that Mg^{2+} mimicked Ca^{2+}, and that K^+, tested at two concentrations, was ineffective.

In other experiments, millipore filters of graded porosity were used to determine that Ca^{2+} acted to aggregate the sedimentable endoplasmic reticulum and the mitochondria but not the brush border membrane fragments, into large particles which sediment at lower gravitational forces.

The state of purity of the microvillus membranes (Fraction FII)

The state of purity of the microvillus membrane fraction is given in the bottom part of Table I. Since contamination by non-brush border material was very low in the whole gradient, marker activities were often barely assayable. Because of this, only the composition of Fraction FII out of the 4 fractions is detailed. DNA was not assayed in the gradient since it was already absent from Fraction P_2. Only traces of succinate dehydrogenase and NADPH–cytochrome *c* reductase could be measured in the gradient, making it impossible to estimate specific activities or recoveries. Less than 0.1% of the original (Na^+–K^+)-ATPase could be found in Fraction FII and at a specific activity reduced to only 1/10 of the original in the homogenate. Very small amounts of β-glucuronidase were detectable in all fractions (0.15% in Fraction FII) with about the same specific activities. In contrast, 23% of sucrase was found in

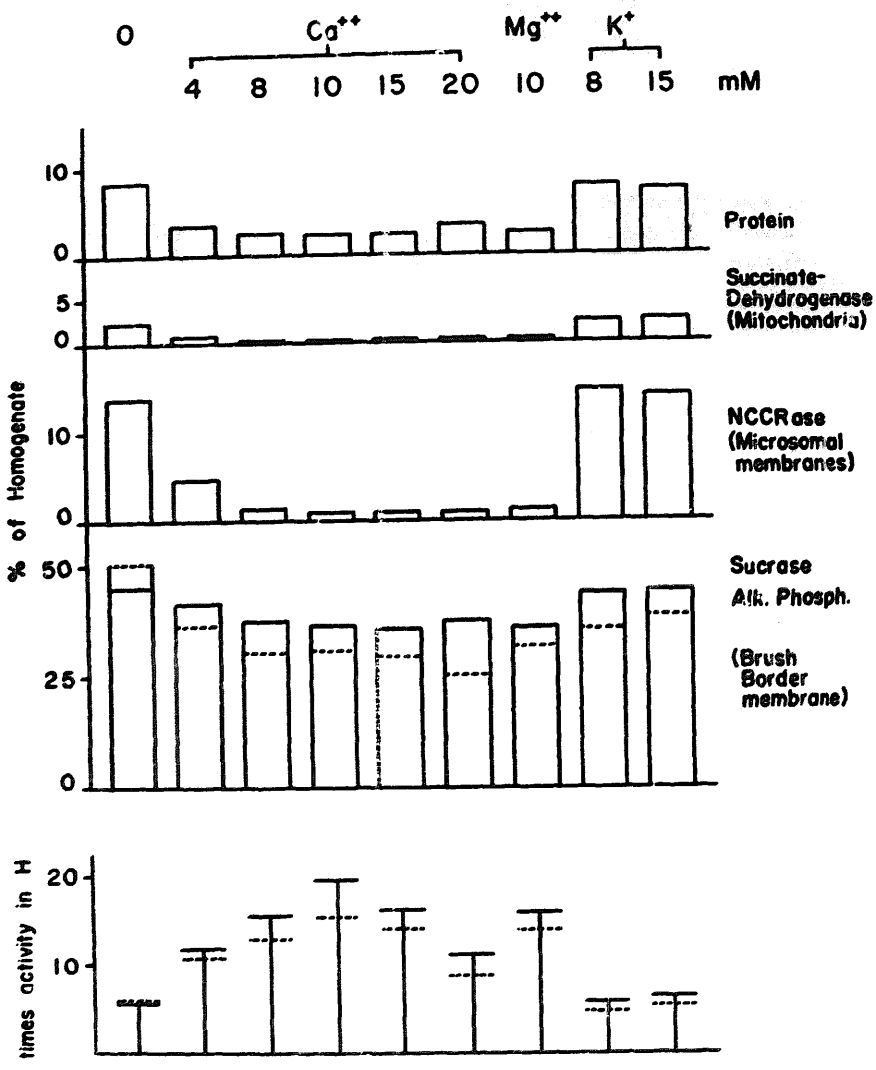
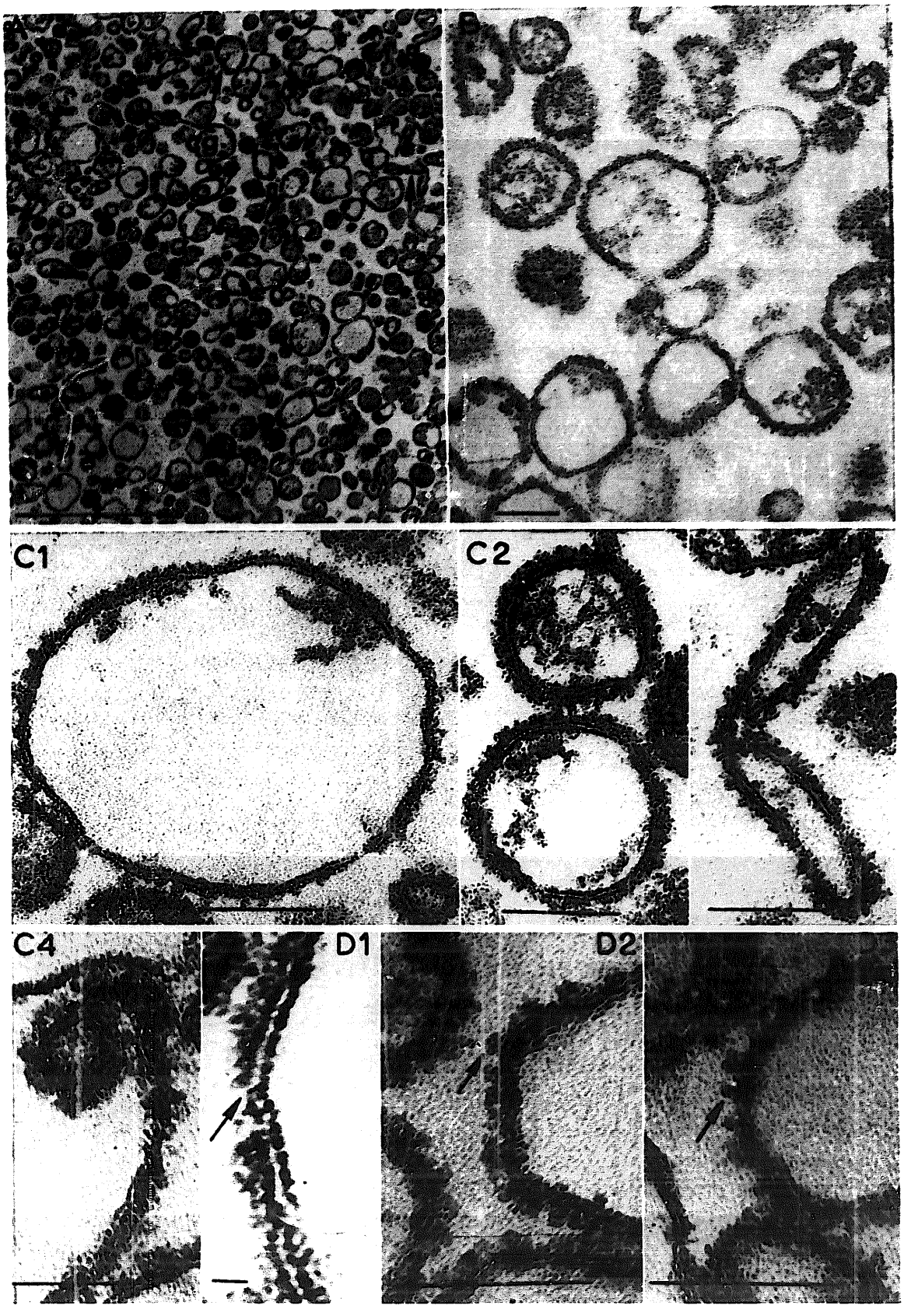


Fig. 3. Specificity of the effect of Ca^{2+}. Fraction P_2 was prepared from a plain (left bars, under 0), Ca^{2+} (at various concentrations), Mg^{2+} or K^+-treated homogenate and percentages in protein, succinate dehydrogenase, NADPH–cytochrome *c* reductase (NCCRase), sucrase in solid line and alkaline phosphatase in dotted line, of the original activities in the homogenate found in Fraction P_2 were calculated. On the bottom row is represented the degree of purification of sucrase and alkaline phosphatase in Fraction P_2, in the same conditions.

Fraction FII with a satisfactory recovery from the fractions. Sucrase specific activity was increased on the average 26 times in Fraction FII over the starting specific activity in the homogenate.

Electron microscopy

At low magnification (Fig. 4A) it can be seen that Fraction FII contained only one kind of mostly vesiculated membranes, although the vesicles were often still filled with electron dense material which presumably originated from the core of the microvilli. Contaminating membranes from organelles other than brush borders could not be detected. Fig. 4B,C show the outer granulated coat of the membrane which gives to the vesicles their appearance of cogwheels in certain instances (Fig. 4 C_2). At high magnification (Fig. 4D) the fine structure of the membrane, unaltered by the freezing of the tissue, is clearly seen. The 3-layered structure was of uniform


C1
C2
C4
D1
D2

thickness (70–75 Å) and the two dense layers were of equal density. Knob-like structures were seen on the outer face of the membrane.

In the course of preliminary electron microscopic examinations, Fraction FIV was found to contain mostly very electron-dense material, convoluted in dark hairy strings which have been assumed to represent fibrillous core material[37].

Other enzymes of the microvillus membrane fraction

In addition to sucrase, which is the commonly used marker, we investigated particularly the purification of trehalase, leucylnaphthylamidase and alkaline phosphatase in jejunal surgical samples from 9 patients. The results concerning these activities are summarized in Table II. The latter three activities were purified to a similar extent (about 14 times in Fraction P_2 and 23 times in Fraction FII over the homogenate), but somewhat less than sucrase (16 and 26 times, respectively). Since the recoveries for the 4 enzymes were identical, it is worth noting that their partition among the 3 fractions (Fractions P_1, P_2 and S_2) is not identical. Compared to sucrase, the lesser amounts of alkaline phosphatase and leucylnaphthylamidase found in Fraction P_2 are due to a bigger loss in Fraction P_1 for the first, and in Fraction S_2 for the second. The partition of trehalase is intermediate between those of sucrase and alkaline phosphatase. In any case, the four activities are similarly distributed along the gradient. so that the differences recorded in Fraction P_2, between sucrase and the three other enzymes, are reflected in Fraction FII.

Preparation of microvillus membranes from peroral biopsies

Study of three biopsies gave the results in Table III. Recoveries were somewhat lower than for surgical samples whereas purification was slightly higher. Differences

Fig. 4. Electron microscopic study of the microvillus membranes (Fraction FII). (A) Low magnification view of the membrane preparation. This micrograph is the average representative of 30 such micrographs which were obtained from 10 grids containing sections prepared from 5 different blocks. The specimen contained vesicles which varied in sizes, shapes, and in the amount of material contained within the cavity. There are some membrane fragments which did not form any closed cavity (arrows). Irrespective of these variations the surface of the membrane appeared rough in general. The vesicles with the rough membrane surface have typically a cogwheel appearance (cg). × 30000. (marker, 1 μm). (B) A collection of intact vesicles: some of these have smooth membrane surface suggesting the removal of material which gives the outer membrane a rough appearance. × 115000. (marker, 0.1 μm) (C1–C4). Varieties of membranous bodies usually found in the preparations; (C1)A smooth surface vesicle. (C2)A rough surface vesicle having a cogwheel appearance. (C3)Rough surfaced membrane reformed into an oblong membranous body which were very commonly seen in the preparation. (C4)Another common membranous formation is a fragment of membrane whose one end is formed into vesicle; such rolling of membrane shows how the coated surface of the membranes forms the outer face of the vesicles. × 230000. (marker, 0.1 μm) (D1–D3) Fine structure of the membrane material of the preparation. (D1), High magnification micrograph of the membrane to show the two electron dense layers of the membrane of uniform thickness (dense layers – 25 Å each and lucid layer, 20 Å; total thickness is 70–75 Å); it is seen that the inner membrane surface is free of any material whereas the outer surface has variable amounts of material associated to it; note in some regions this material is removed (arrow), × 744000 (marker 0.01 μm). (D2 D3), Portions of membrane showing intact "knob"-like structures (arrow) on the outer surface of the vesicle membrane (giving the cogwheel appearance); 40 Å in diameter and 75 Å in height, these knob-like structures remain embedded in structureless material, which generally obscures their visualization (compare C2, D2 and D3); × 390000 (marker 0.1 μm).

TABLE II

ENZYMATIC COMPOSITION OF MICROVILLUS MEMBRANES FROM SURGICAL SPECIMENS

Fraction	*Sucrase*		*Trehalase*		*Leucyl-naphtylamidase*		*Alkaline phosphatase*	
	*Spec. act.**	%	*Spec. act.*	%	*Spec. act.*	%	*Spec. act.*	%
H	92±15** (13)***	100	32±8 (8)	100	54±6 (9)	100	255±34 (9)	100
P_1	64±11 (13)	30±3	27±5 (8)	35±2	38±5 (9)	27±2	213±41 (9)	37±3
P_2	1457±214 (13)	41±3	455±120 (8)	36±4	745±82 (9)	36±3	3453±458 (9)	34±3
$P_{2/H}$††	×16±0.6		×14±0.6		×14±0.4		×14±0.6	
S_2	29±6 (13)	17±2	10±2 (8)	17±2	26±5 (9)	23±3	70±8 (9)	16±2
Recovery		88±2†		88±4		86±3		87±3
F_I	1188±272 (10)	5±1§	307±73 (8)	4.4±0.9	801±101 (9)	6.5±1	3002±455 (9)	4.7±0.9
F_{II}	2410±326 (10)	68±3 (23±3)§§	721±180 (8)	70±3 (21±3)	1185±105 (9)	65±3 (20±2)	5438±566 (9)	65±2 (20±2)
$F_{II/H}$††	×26±2		×23±2		×23±2		23±2	
F_{III}	1525±359 (6)	13±2	478±87 (4)	12±3	1043±138 (5)	11±2	4825±634 (5)	14±3
F_{IV}	1045±244 (6)	20±4	353±84 (4)	18±2	767±209 (5)	22±5	3843±915 (5)	20±1
Recovery from the gradient	83±9† (10)		83±7 (8)		86±5 (9)		88±4 (9)	

* Spec. act. = specific activity in I.U./g protein.

** Mean ± S.E.

*** Number of different tissues studied.

†, †† Recoveries (†) and increases in specific activity (††) are means of the recoveries and increases from each experiment.

§Percentages in the fractions refer to the total amount of activity (100%) recovered in the gradient.

§§ Percentage of activity in Fraction F_{II} compared to the original amount in the homogenate.

among the 4 measured enzymes in their partition during the preparation are not taken to be significant because of the small number of experiments.

DISCUSSION

Our criteria for accepting an intestinal sample were only that it be macroscopically normal and not be taken from an apparently or probably diseased region. Thus we received samples from different parts of the intestine, from people of different ages, on different diets. Furthermore, of the surgical patients operated for obesity most were later determined to be diabetic or paradiabetic. For example, after completion of the electron microscopy study we learned that the patient from whom

TABLE III

PURIFICATION OF MICROVILLUS MEMBRANES IN 3 BIOPSIES

Fraction	*Protein*	*Sucrase*		*Trehalase*		*Leucyl-naphtylamidase*		*Alkaline phosphatase*	
	%	*Spec. act.**	%	*Spec. act.*	%	*Spec. act.*	%	*Spec. act.*	%
H	100	90** (70–105)***	100	23 (13–43)	100	43 (39–50)	100	224 (185–277)	100
P_1	35	67	26	20	31	33	28	185	30
S_2	56	47	29	9	19	19	24	96	22
F_I	0.30	337	1.2	57	1.3	157	1.1	849	3.8
F_{II}	0.53	2735 (2560–2980)***	17	567 (314–912)	15	1158 (1067–1221)	15	6964 (5167–8301)	17
F_{II-H}†		×31 (25–36)***		×27 (21–36)		×27 (25–31)		×32 (27–40)	
F_{III}	0.14	1087	0.9	237	0.9	526	1.0	3234	1.4
F_{IV}	0.90	427	2.7	75	2.5	269	2.9	1320	3.6
Recovery	93		76		70		71		78

* Specific activities in I.U./g protein.

** Mean.

*** Range.

§ Increase in spec. act. in Fraction II over the homogenate; final recovery is the sum of what is recovered in Fractions P_1 and S_2 and the gradient.

the sample was taken was a diabetic under insulin. The wide range of specific activities for a given brush border enzyme in the homogenate (37–209 I.U./g protein for sucrase), in Fraction P_2 and in Fraction FII (1089–4558 I.U./g protein for sucrase) could therefore be expected[38].

Despite the constraint of using frozen tissue, we achieved a purification of human microvillus membranes which is satisfactory for the kind of structural studies we intended to do (see the following paper[39]). The mean increase in sucrase specific activity of 26-fold over the homogenate and the yield of 20% of the starting sucrase in Fraction FII, compare well to the 29-fold purification at a similar yield obtained with rat tissue by Forstner *et al.*[40]. Sucrase specific activity was taken as a measure of the extent of microvillus membrane purification. Trehalase, leucylnaphthylamidase and alkaline phosphatase also located in the microvillus membrane[13,40,41] were assayed but not used as markers. Of these, alkaline phosphatase is a marker not only for the brush border membrane but also for plasma membrane in general[42–44]. Leucylnaphthylamidase is found in many plasma membranes[42–45] and some of the activity may also be cytoplasmic, as seems to be characteristic for many peptidases[2]. Trehalase is not known to be other than a brush border enzyme but it differs from sucrase in being present in the membrane of the proximal renal tubule from which sucrase is absent[46]. The variability in recovery and gradient location of these enzymes compared to sucrase may be related to these differences.

Since microvillus membranes were isolated directly from whole cell homogenates and were found to have the thickness usually reported for basolateral plasma membranes[47], contamination of Fraction FII by the latter was important to consider.

In this regard it was decided to assay the (Na^+-K^+)-ATPase in Fraction FII. This activity has been shown to be associated with the plasma membrane of intestinal epithelial cells[25], as it is in other types of cells[48]. However, its partition between basolateral and brush border membrane is still in discussion[26–28]. Since at least 85% of the total epithelial cell activity is located in the basolateral part of the plasma membrane, it has already been used as a distinguishing marker for the basolateral plasma membrane[30] because the brush border membrane possesses all the other markers usually used to identify plasma membrane in other types of cell. We found the (Na^+-K^+)-ATPase activity reduced in Fraction FII to slightly more than traces. It is then clear that, whatever the presence or absence of a small percentage of the total (Na^+-K^+)-ATPase in the brush border membrane, there is no sizable contamination of our preparation by basolateral plasma membrane. This result agrees with recent high resolution autoradiographic studies which found no ouabain-binding sites on the brush border[49], if one assumes that the (Na^+-K^+)-ATPase is the *in vitro* expression of the Na^+-pumping function of the plasma membrane[50]. Also the membrane shown as mostly vesiculated on the electron micrographs of Fraction FII is well preserved despite the freezing of the tissue, and the characteristic microvillus membrane feature, the electron dense glycocalyx, coats its luminal face[47].

Ca^{2+}-induced microsomal aggregation as used here is a well-known phenomenon[51]. It has been used by Kamath *et al.*[7] as the basis for a simple method to prepare microsomes under conditions that we have adapted to our own purpose. Although we found, as have others[51], that microsomal aggregation can be obtained as well with Mg^{2+}, we did not find the effect of K^+ indicated by Dallner and Nilsson[51]. Also, in our case, Ca^{2+} aggregation affected only 15% of the total microsomes, whether from fresh or frozen tissue, instead of the 100% effect reported by Kamath *et al.*[7] and Dallner and Niisson[51]. However, what is most striking, and useful, is the fact that mitochondria, and very probably basolateral plasma membranes as well, are also aggregated by Ca^{2+}, while microvillus membranes are nearly unaffected. Since Ca^{2+} is not specific its effect is likely to be related to electrostatic interactions between the cation and membrane surface charges. Its failure to aggregate brush border would indicate that, despite the negatively-charged glycocalyx[52], the brush border fragment surface, at or near neutrality, is more positively charged than microsomal, mitochondrial and basolateral plasma membrane surfaces. This difference in charge has been recently reported for, and applied to the purification by free flow electrophoresis[53] of the similar kidney tubule microvillus membrane. In our preparation core contamination is lesser than in Welsh's[6] but it has not been completely removed. We tried sonication of Fraction P_2 as well as polymerization with Mg^{2+} of the core material as proposed by Hopfer *et al.*[54] and several modification of the Tris disruption process without significant improvement of our results suggesting that in man as in the pig[55], the core is more tightly linked to the membrane than in either the hamster or the rat. However, the extent of disruption was always the same on the same tissue when it was processed several different times although it was variable from tissue to tissue and the range of sucrase specific activity in Fraction FII extended from 20 to 35 ($m = 26 \pm 6.0$ S.D.).

In our view, the means are now in hand to study human intestinal microvillus membrane from a variety of subjects. Wide application of the method should increase our knowledge of the normal membrane and its enzymic activities in man. As applied

to disease states the method holds the promise of helping to identify the molecular basis of some brush border membrane diseases.

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