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DIFFERENTIAL ISOLATION OF MICROVILLOUS AND BASOLATERAL PLASMA MEMBRANES FROM INTESTINAL MUCOSA: MUTUALLY EXCLUSIVE DISTRIBUTION OF DIGESTIVE ENZYMES AND OUABAIN-SENSITIVE ATPase

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SUMMARY

Using density-gradient centrifugation microvillous membranes were isolated from a partially purified brush border fraction, and basolateral plasma membranes from a crude mitochondrial fraction. Alkaline *p*-nitrophenyl phosphatase, sucrase and leucylglycine hydrolase were enriched in the former, and ouabain-sensitive ATPase in the latter. Electron microscopy showed that the microvillous membrane fraction was composed of thick membrane vesicles of regular size, and the basolateral membrane fraction of vesicles more irregular in shape and size. Most of the work was performed with mouse jejunum. Similar results were obtained with rat.

INTRODUCTION

It is well established that ouabain-sensitive ATPase activity is characteristic of the surface membrane of animal cells. It was found, however, that this activity was low in rat intestinal brush borders, whereas it was high in the fractions containing basal and lateral plasma membranes[1].

In the present work we want to present a simple method of isolating what seem to be a microvillous membrane fraction and a basolateral membrane fraction from a single homogenate of intestinal mucosa of mouse and rat.

The enzymic properties of the two membranes were very much in contrast; one being rich in digestive enzymes and the other rich in ouabain-sensitive ATPase, thus confirming the earlier conclusion arrived at by isolating brush borders free of basal and lateral membranes[1].

Apart from their physiological significance, two types of plasma membranes which, as reported in this paper, form a continuous cell boundary but differ in enzymic composition, may be a useful material for the analytical study of membrane organization.

METHODS

Preliminary fractionation

Usually three mice (Swiss strain, about 2 months old, either sex) were sacrificed for one experiment. After discarding the first 5 cm segment adjacent to the pylorus, the upper two-thirds of the remaining small intestine were recovered and handled as previously described for rat[1]. The mucosal scrapings from three mice were taken up in 20 ml of 0.25 M sucrose containing 5 mM histidine–imidazole buffer (pH 7.0) and 0.5 mM neutralised EDTA (sucrose–EDTA, henceforth). It was then homogenized with a Dounce-type homogenizer (glass–teflon) without revolving the pestle. Usually 50 up-and-down strokes using a loosely fitting homogenizer were necessary to obtain the first homogenate. The number of strokes, however, was reduced when the homogenizer was more tightly fitted. The suitable number of strokes to be used with the homogenizer was determined by examining the homogenate under light microscopy, and the appropriate number of strokes was routinely applied with the homogenizer. The criterion of adequacy of the first homogenization was provided by microscopic observation: nuclei were released in the medium and brush borders still retained a considerable amount of granular material, which resulted in faster sedimentation of brush borders than nuclei, facilitating their separation. Too much homogenization would cause increasing loss of brush borders to the supernatant following their fragmentation. The homogenate, 20 ml, was spun at 270 $\times$ g for 5 min (Sorvall rotor SS-34, 1500 rev./min). The pellet was taken up in 10 ml of sucrose–EDTA, homogenized by 30 strokes and centrifuged in the same way. When the number of strokes applied for the first homogenization was other than 50, as necessitated by different clearances of individual homogenizers, half that number of strokes was applied. The pellet was further homogenized by 20 strokes. If the number of strokes applied at the first homogenization had been other than 50, a third of that number of strokes was employed. The pellet was then centrifuged at 360 $\times$ g (1750 rev./min) for 5 min, and suspended in 5 ml of 5 mM histidine–imidazole buffer (pH 7.0) containing 0.5 mM EDTA. This fraction was mainly composed of typical brush borders when examined microscopically. It will be referred to as the brush border fraction (see Fig. 1).

The combined supernatants from the above steps were centrifuged at 1000 $\times$ g for 10 min to sediment nuclei(nuclear fraction). The supernatant was further centrifuged at 20 000 $\times$ g for 10 min (the pellet, mitochondrial fraction). The subsequent centrifugation at 40000 $\times$ g for 30 min yielded heavy microsomes (heavy-microsomal fraction) and a supernatant which was, when necessary, further fractionated into light microsomes and soluble components by centrifuging at 200000 $\times$ g for 30 min.

The brush border and mitochondrial fractions were kept standing at 0 °C overnight before being applied to density-gradient centrifugation.

Density-gradient centrifugation

The mitochondrial fraction was diluted to 10 ml with the dilute buffer–EDTA described above after homogenization with a Potter–Elvehjem type homogenizer by 10 up-and-down strokes at a medium speed of revolution. The brush border fraction was homogenized by 20 strokes and diluted. Both suspensions were centrifuged at 200000 $\times$ g for 30 min. The pellets were homogenized gently but adequately in 2.0 ml of 50% (w/v) sucrose solution and placed at the bottom of 5-ml centrifuge

tubes, then overlayered with 1.5 ml of 40% (w/v) sucrose, 1.0 ml of 30% and 0.5 ml of 20% sucrose solution. All sucrose solutions contained histidine–imidazole buffer (pH 7.0) and EDTA at 5 and 0.5 mM, respectively.

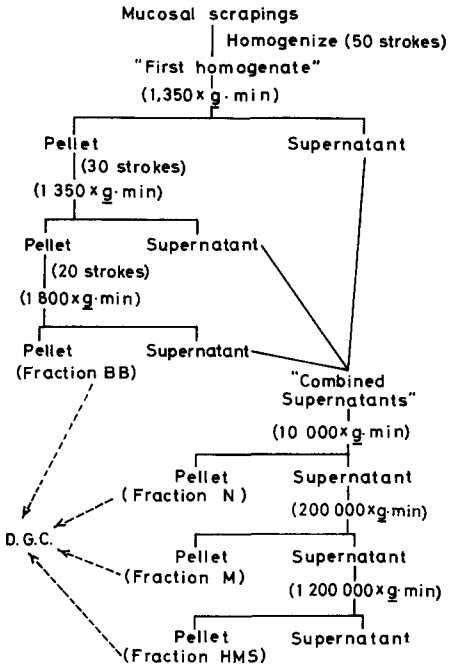

Fig. 1. Fractionation of cellular components. Fraction BB, brush border fraction; Fraction N, nuclear fraction; Fraction M, mitochondrial fraction; Fraction HMS, heavy-microsomal fraction.

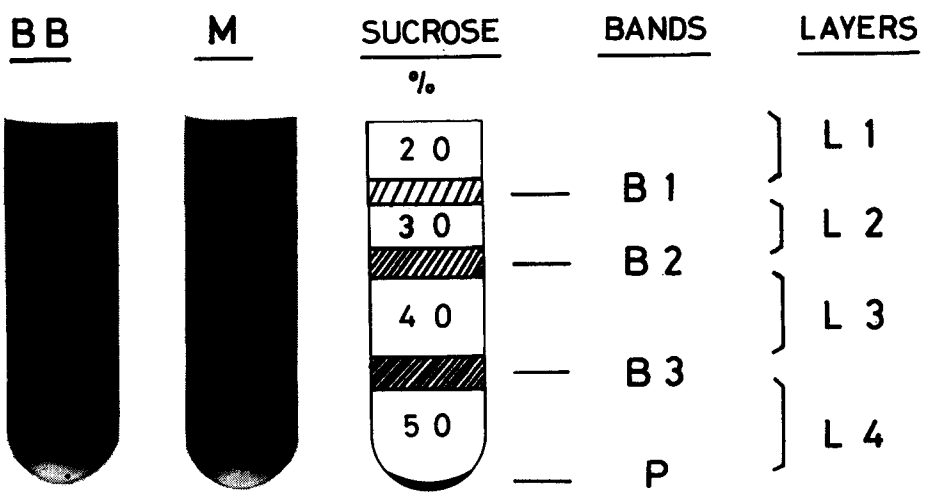

Fig. 2. Band patterns after a density-gradient centrifugation. BB, brush border fraction (see Fig. 1); M, mitochondrial fraction; B1, 2, 3: first, second, and third interfacial band, respectively. L1, 2, 3, 4: first, second, third and fourth layer, respectively. P: pellet.

The tubes were spun at 200 000 × *g* for 90 min (Hitachi rotor RPS 40T, 39 000 rev./min). Band patterns are shown in Fig. 2. The band formed at the 30–40% interface (Fraction B2, Fig. 2) of a mitochondrial fraction was carefully collected with a micropipette cf 200–300 μl volume (Fraction M-B2). From the brush border fraction all layers lighter than the 50% sucrose layer (including the 40–50% interface) were removed with a micropipette connected to vacuum, and the remaining 50% layer (Layer L4, Fig. 2) was recovered by decantation, leaving a tight pellet at the bottom. When indicated, these fractions were diluted with redistilled water and centrifuged at 200 000 × *g* for 30 min, and the pellets suspended in redistilled water.

Enzymic assays

Ouabain-sensitive ATPase or (Na^+,K^+)-ATPase activity was assayed as described previously[1]. Ouabain concentration was 0.1 mM for mouse enzyme.

Alkaline phosphatase activity was assayed using *p*-nitrophenyl phosphate as substrate at pH 10.0. The reaction mixture contained 100 mM $NaHCO_3$ buffer (pH 10.0), 5 mM $MgCl_2$, 0.5 mM $ZnSO_4$ and 3 mM substrate.

Glucose-6-phosphatase activity was assayed at pH 6.2 using 20 mM substrate in the presence of EDTA[2]. NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase activities were assayed from the increase of absorbance at 550 nm (refs 3, 4). NADH oxidase activity was assayed from the absorption decrease at 340 nm in the absence of respiratory inhibitors.

Sucrase activity was assayed in the presence of 50 mM NaCl at pH 6.8 (ref. 1). A glucose oxidase–peroxidase system was used for glucose determination[5].

L-Leucylglycine hydrolase activity was assayed by determining the liberated L-leucine with L-amino acid oxidase coupled to peroxidase and orthodianisidine[16]. All assays were carried out at 37 °C.

Electron microscopy

The pelleted fractions, mitochondrial fraction M-B2 and brush border fraction BB-L4, were fixed with glutaraldehyde at pH 7.4 for 2 h, then washed three times with water and postfixed with 2% MnO_4^-. After dehydrating and embedding in Epon they were cut out into "gray" sections using a Sorvall microtome and stained with uranyl acetate followed by lead citrate. Electron micrographs were taken with a Hitachi electron microscope Model HU-12 at 50 and 75 KV. OsO_4 fixation was tried but gave poor results possibly due to technical failure.

RESULTS

Distribution of alkaline phosphatase and ouabain-sensitive ATPase activities at preliminary fractionation

A preliminary fractionation was performed as described in Methods.

About 60% of the ouabain-sensitive ATPase activity was recovered in the combined mitochondrial and nuclear fractions and 30% in the heavy-microsomal fraction. Very little activity was found in the brush border fraction and the light-microsomal fraction. No activity was detected in the final supernatant (Table I).

About 40% of the alkaline phosphatase activity was associated with the brush border fraction while the rest was distributed in the mitochondrial and microsomal

TABLE I

DISTRIBUTION OF OUABAIN-SENSITIVE ATPase ACTIVITY AND ALKALINE PHOSPHATASE ACTIVITY AT PRELIMINARY FRACTIONATION

Figures are in μmoles of P_i (ATPase) and *p*-nitrophenol (alkaline phosphatase) liberated per fraction per h.

Fraction	*Ouabain-sensitive ATPase*	*Alkaline phosphatase*
Homogenate	220	72
Brush border	3	30
Nuclear and mitochondrial	140	21
Heavy-microsomal	60	13
Light-microsomal	1	1
Supernatant	0	0

TABLE II

DISTRIBUTION OF OTHER MARKER ENZYMES AT PRELIMINARY FRACTIONATION

Figures are in μmoles of cytochrome *c* reduced per min (per mg protein).

Fraction	*NADH–cytochrome c reductase*	*Succinate–cytochrome c reductase*
Brush border*	0.6	Not tested
Nuclear*	1.7	Not tested
Mitochondrial	3.8 (0.28)	2.6 (0.20)
Heavy-microsomal	2.2 (0.38)	0.15 (0.025)
Light-microsomal	1.2 (0.30)	0.05 (0.011)

* Protein of the fraction was not determined.

fractions which will be shown to be separable from ouabain-sensitive ATPase by density-gradient centrifugation.

Distribution of other marker enzymes

As shown in Table II, NADH–cytochrome *c* reductase activity was distributed from the nuclear to light-microsomal fractions with the highest specific activity in the heavy-microsomal fraction. In contrast, succinate–cytochrome *c* reductase activity was high in the mitochondrial fraction but poor in the microsomal fractions.

Separation of ouabain-sensitive ATPase activity and alkaline phosphatase activity by density-gradient centrifugation

In order to estimate the distribution of ouabain-sensitive ATPase activity in the density gradient, a combined mitochondrial and heavy-microsomal fraction on the one hand and a brush border fraction on the other were fractionated after density-gradient centrifugation as shown in Fig. 3. For the former the highest ouabain-sensitive ATPase activity was found at the interface between the 30 and 40% sucrose layers, whereas the alkaline phosphatase activity was concentrated at the 40–50% interface and in the 50% layer. Thus the two activities were separated after density-gradient centrifugation.

The brush border fraction showed a similar pattern except for the absence of ouabain-sensitive ATPase activity at the 30 and 40% interface and the higher peak of alkaline phosphatase activity in the 50% layer. Those findings suggest strongly that the latter activity in both fractions arises from a species of membrane different from that which contains ouabain-sensitive ATPase.

A 30–40% interface was isolated from each of the nuclear, mitochondrial and heavy-microsomal fractions in order to know which yields the most active subfraction (Table III). The M-B2 subfraction derived from the mitochondrial fraction was found to be the most active.

About 17% of the total ouabain-sensitive ATPase activity of the homogenate was recovered in the mitochondrial fraction M-B2. The specific activity was enriched 11-fold over that of the homogenate (Table IV). Although the relative specific activity was fairly constant, the absolute specific activity varied from preparation to preparation, the lowest value being about 35 and the highest about 100 in terms of μmoles of P_i liberated per h per mg protein.

In contrast to the ouabain-sensitive ATPase activity the alkaline phosphatase activity was lowest in this fraction. It was highest in brush border fraction BB-L4 (50% sucrose layer derived from the brush border fraction). The specific activity relative to that of the homogenate was 18. The activity recovery in this fraction was

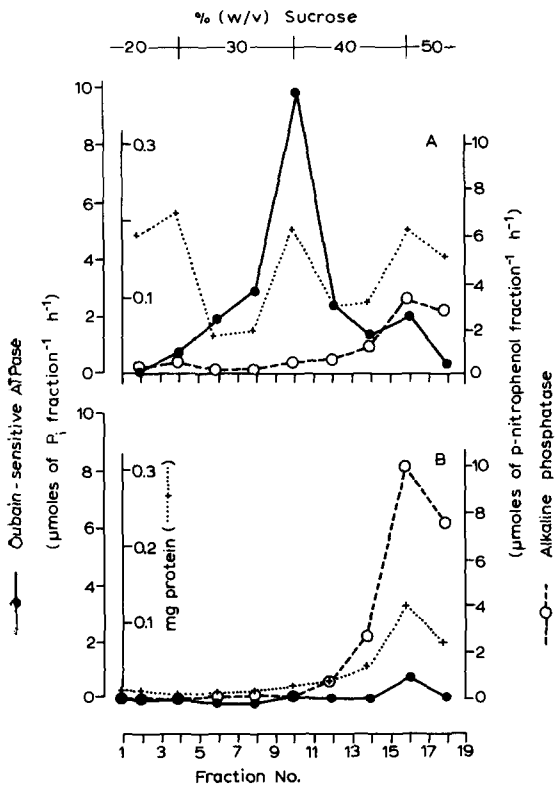

Fig. 3. Distribution of alkaline phosphatase and ouabain-sensitive ATPase activities in the density gradient. A: combined mitochondrial and heavy-microsomal fraction. B: brush border fraction.

TABLE III

COMPARISON OF OUABAIN-SENSITIVE ATPase ACTIVITIES OF CORRESPONDING BANDS FROM DIFFERENT CELL FRACTIONS

Figures are in μmoles of P_i liberated per fraction per h for the total activity of the fraction and per mg protein per h for the specific activity. N-B2, M-B2 and HMS-B2 are the interfacial fractions between 30 and 40 % (w/v) sucrose after density-gradient centrifugation of nuclear, mitochondrial and heavy-microsomal fractions, respectively (see Methods for details).

Fraction	*Total activity*	*Specific activity*
Homogenate	161	3.6
N-B2	3.0	9.4
M-B2	32	100
HMS-B2	25	69

TABLE IV

DISTRIBUTION OF OUABAIN-SENSITIVE ATPase ACTIVITY AND ALKALINE PHOSPHATASE ACTIVITY IN PLASMA MEMBRANE FRACTIONS OF MOUSE INTESTINAL MUCOSA

M-B2, BB-L4, see Fig. 2.

Fraction	*Ouabain-sensitive ATPase*	*Alkaline phosphatase*
Homogenate	160* (5.1)**	116* (3.7)**
M-B2	27 (68)	0.5 (1)
BB-L4	1 (3)	19 (57)
Relative specific activities:		
Homogenate	1	1
M-B2	11 $\pm$ 0.6 (5)***	0.5 $\pm$ 0.1 (7)***
BB-L4	0.1 $\pm$ 0.1 (5)	18 $\pm$ 1.4 (7)

* Total activity of the fraction in μmoles of P_i (ATPase) and *p*-nitrophenol (alkaline phosphatase) liberated per h.

** Specific activity in μmoles per h per mg protein.

*** Number of separate preparations.

TABLE V

DISTRIBUTION OF BRUSH BORDER ENZYMES AND OUABAIN-SENSITIVE ATPase

Enzyme activities were assayed as described in Methods. Figures are in μmoles of substrates hydrolyzed per fraction per h; those in parentheses per mg protein. M-B2, BB-L4, see Fig. 2.

Fraction	*Alkaline phosphatase*	*Sucrase*	*L-Leucylglycine hydrolase*	*Ouabain-sensitive ATPase*
Homogenate*	320 (11)	150 (5.2)	170 (5.7)	340 (12)
M-B2	1 (2)	0.3 (1)	1.2 (1.8)	58 (78)
BB-L4	54 (110)	24 (47)	31 (82)	0.6 (4)
% *recovery and* (*relative specific activity*):				
Homogenate*	100 (1)	100 (1)	100 (1)	100 (1)
M-B2	0.2 (0.2)	0.2 (0.2)	0.7 (0.3)	17 (6.5)
BB-L4	17 (10)	16 (9)	18 (12)	0.6 (0.3)

* Total sediment from a homogenate after centrifugation at 200000 $\times$ *g* for 20 min.

15–20%. The band formed at the 40–50% interface was also rich in alkaline phosphatase activity but slightly contaminated with ouabain-sensitive ATPase activity.

Sucrase and L-*leucylglycine hydrolase*

The distribution patterns of sucrase and L-leucylglycine hydrolyzing activity were examined along with alkaline phosphatase and ouabain-sensitive ATPase activities (Table V). There was a marked parallelism in the distribution patterns of the first three activities, whereas that of the ATPase was quite different from these.

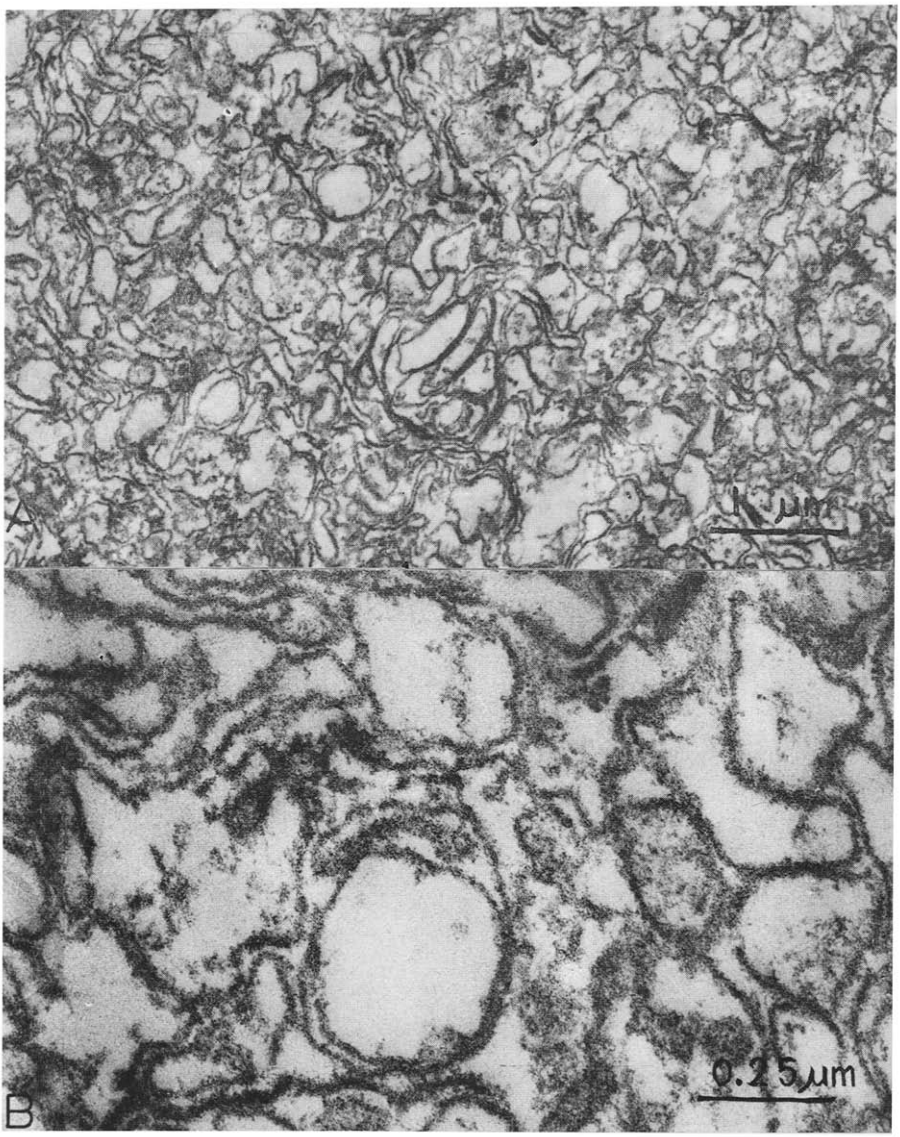

Fig. 4. Electron micrograph of the mitochondrial fraction M-B2. Original magnification: A, × 5000; B, × 20000.

Electron microscopy

Mitochondrial fraction M-B2 and brush border fraction BB-L4 were examined in thin sections under the electron microscope (Figs 4 and 5). Both fractions were composed of vesicles. However, there was a marked difference in their appearances. Those of the mitochondrial fraction M-B2 were irregular in shape and size, while those of the brush border fraction BB-L4 showed less diversity. The membrane of the former was thinner than that of the latter. Some structures in the mitochondrial fraction M-B2 resemble desmosomes and adjacent lateral membranes. The vesicles of

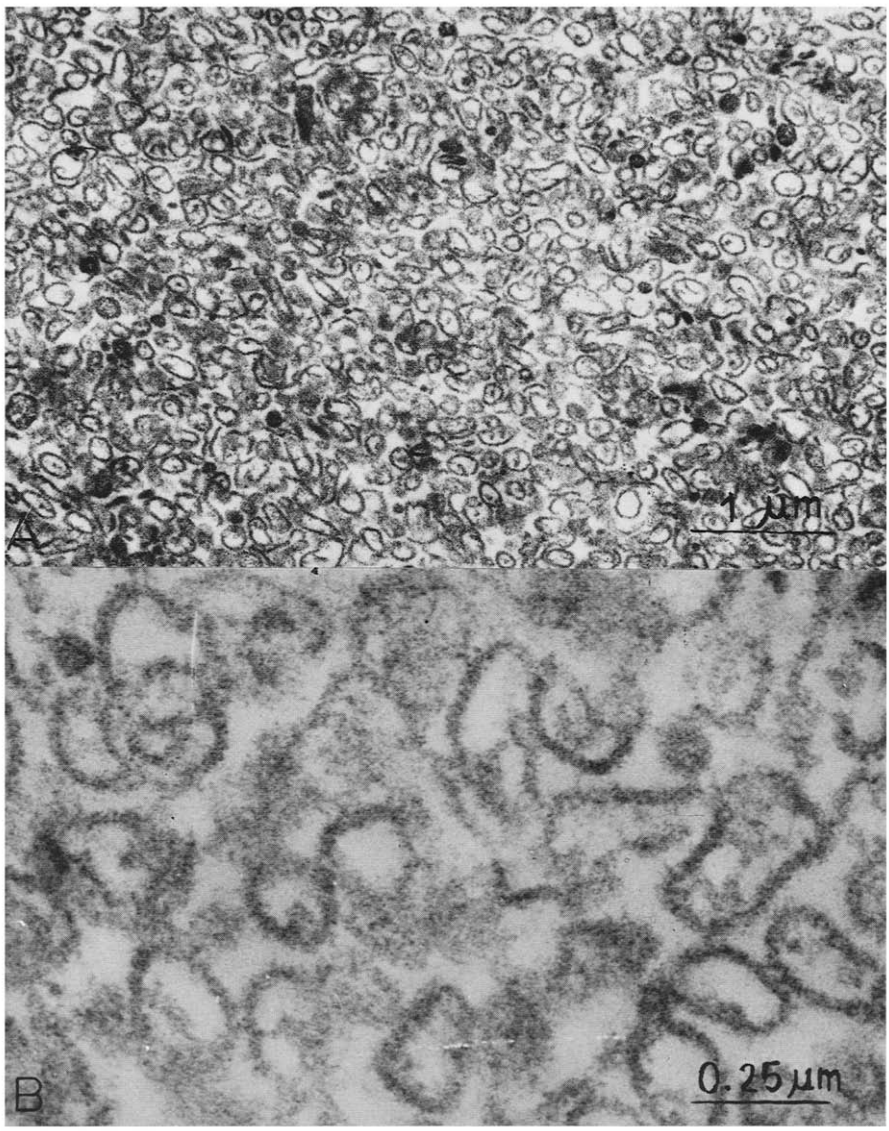

Fig. 5. Electron micrograph of the brush border fraction BB-L4. Original magnification: A, × 5000; B, × 20000.

the brush border fraction BB-L4 are mostly devoid of fibrils such as seen in intact microvilli. However, the general appearances of them bear a close resemblance to those of surface membranes of intact microvilli. A good electron micrograph of an intestinal epithelial cell[6] is valuable in convincing one of a resemblance between microvillous membranes and the present brush border fraction BB-L4 on the one hand and between lateral plasma membranes and the present mitochondrial fraction M-B2 on the other. Although the basal plasma membranes have no characteristic structures to distinguish them, it seems quite natural that they are present in the mitochondrial M-B2 fraction.

Other enzymes

While the recovery of glucose-6-phosphatase activity was low, the increased relative specific activity was constantly encountered in mitochondrial fraction M-B2 and brush border fraction BB-L4 (Table VI). The behavior of NADH–cytochrome *c* reductase, however, did not agree with that of glucose-6-phosphatase. In brush border fraction BB-L4 the relative specific activity of glucose-6-phosphatase was 18 times higher than that of NADH–cytochrome *c* reductase, while it was only three times higher in the mitochondrial fraction M-B2.

There was very little or no succinate–cytochrome *c* reductase activity in either

TABLE VI

DISTRIBUTION OF OTHER ENZYMES

Enzyme assays were performed as described in Methods. Figures in the upper-half of the table are in μmoles substrates used per fraction per h (glucose-6-phosphatase) or per min (other activities): those in parentheses per mg protein. M-B2, BB-L4, see Fig. 2.

Fraction	*Glucose-6-phosphatase*	*NADH–cytochrome c reductase*	*NADH oxidase*	*Succinate–cytochrome c reductase*
Homogenate	36 (1.2)	7.6 (0.26)	0.30 (0.010)	0.43 (0.015)
M-B2	1.3 (2.6)	0.084 (0.17)	0.00	0.00
BB-L4	1.1 (2.2)	0.010 (0.02)	0.00	0.00
% *recovery and* (*relative specific activity*):				
Homogenate	100 (1)	100 (1)	100 (1)	100 (1)
M-B2	3.6 (2.1)	1.1 (0.7)	0.0	0.0
BB-L4	3.1 (1.8)	0.1 (0.1)	0.0	0.0

TABLE VII

RESULTS WITH RAT INTESTINAL MUCOSA

Activities are in μmoles of P_i (ATPase) or *p*-nitrophenol (alkaline phosphatase) liberated per h (per mg protein). M-B2, BB-L4, see Fig. 2.

Fraction	*Ouabain-sensitive ATPase*	*Alkaline phosphatase*
Homogenate	140 (2.1)	710 (11)
M-B2	14 (26)	5 (10)
BB-L4	0.4 (0.5)	206 (240)

fraction. Most of the activity was found in the pellet after density-gradient centrifugation. NADH oxidase activity was also negligible.

Results with rat intestinal mucosa

Similar results were obtained with rat intestinal mucosa (Table VII). A partially purified brush border fraction was prepared as described previously[1] except that the procedures after the fourth step were omitted in the present work. Corresponding fractions, mitochondrial fractions M-B2 and brush border fraction BB-L4, were obtained as described in Methods.

DISCUSSION

It is known that sucrase activity is concentrated in the brush border region of the intestinal mucosal cell[7]. It was also reported that alkaline phosphatase activity and sucrase activity behaved in association in fractional centrifugation[8] and density-gradient centrifugation[9]. These findings were confirmed in the present work. Although alkaline phosphatase is generally regarded as one of the marker enzymes of plasma membranes, it could be suggested that, at least in intestinal epithelial cells, the enzyme is concentrated in the apical region of the plasma membrane together with sucrase. A similar pattern seems to be applicable to L-leucylglycine hydrolase.

Contrary to the distribution patterns of the above enzymes, ouabain-sensitive ATPase activity was concentrated in a fraction presumably composed of basal and lateral plasma membranes. There was very little or no activity in the microvillous membrane fraction.

A (Na^+,K^+)-ATPase preparation reported by Quigley and Gotterer[10] seems to correspond to the present mitochondrial M-B2 fraction. Another intestinal (Na^+,K^+)-ATPase preparation was obtained by Nakao *et al.*[11].

The present findings could be summarized as follows: digestive enzymes such as sucrase and L-leucylglycine hydrolase are located at the digestive surface of the mucosal cell, whereas the enzyme system concerned in active transport of solutes is located at the opposite border.

Contamination of plasma membrane fractions with mitochondria seems to be negligible. The presence of glucose-6-phosphatase activity, however, could not be excluded. The specific activity increased rather than decreased, whereas that of NADH–cytochrome *c* reductase was lower than that of the homogenate; more-over, the ratio of the two activities differed six-fold between mitochondrial fraction M-B2 and brush border fraction BB-L4. These findings suggest the possibility that the plasma membrane of mouse small intestinal mucosal cells contains glucose-6-phosphatase activity.

The presence of low NADH–cytochrome *c* reductase activity in the mitochrondrial fraction M-B2 (basolateral plasma membranes) may be explained by either contamination with endoplasmic reticulum or its natural presence in plasma membrane. Although the latter explanation is in line with similar arguments for plasma membranes of liver cells[12] and erythrocytes[13], further purification of the basolateral plasma membrane fraction must be achieved along with that of endoplasmic reticulum to reach a final conclusion.

The present electron microscopic finding that the microvillous membrane was

thicker than the lateral plasma membrane agrees with Sjöstrand's observation on intact cells[14]. Interestingly enough the (Na^+, K^+)-ATPase-rich membrane of the electroplax of *Electrophorus electricus* was thinner than its acetylcholine-rich membrane[15].

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