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PLASMA MEMBRANES FROM RAT INTESTINAL EPITHELIAL CELLS AT DIFFERENT STAGES OF MATURATION

I. PREPARATION AND CHARACTERIZATION OF PLASMA MEMBRANE SUBFRACTIONS ORIGINATING FROM CRYPT CELLS AND FROM VILLOUS CELLS

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Summary

To determine the mechanism of the maturation of the brush border membrane in intestinal epithelial cells, purification of the plasma membrane from undifferentiated rat crypt cells and of the basal-lateral membrane from villous cells has been performed. The method is based on density perturbation of the mitochondria to selectively disrupt their association with the membrane. With both cell populations, two membrane subfractions displaying the same respective density on sucrose gradient have been obtained with an overall yield of 15—20% and a 10-fold enrichment of the plasma membrane markers 5′-nucleotidase and $(Na^+ + K^+)$-dependent, ouabain-sensitive ATPase chosen to follow their purification. The four fractions were constituted by sheets and apparently closed vesicles of various sizes. Each fraction was characterized by a distinct protein composition and different levels of enzyme activities. The cells, used for the preparation of the membranes, were isolated as a villus to crypt gradient. This separation and that of the membranes led to the conclusion that the $(Na^+ + K^+)$-dependent ATPase is localized principally in the plasma membrane of all cells whatever their state of maturation, while 5′-nucleotidase is predominantly located in the basal-lateral membrane of the villous cells and may serve as a specific marker for the purification of this membrane. Finally it has been shown that aminopeptidase, disaccharidases and alkaline phosphatase do not appear simultaneously in the maturation process of the cells, alkaline phosphatase being absent from the crypt cells and aminopeptidase being the first to be synthesized. This enzyme seems to appear in the crypt cells membrane before being integrated into the mature brush border membrane.

Introduction

The intestinal epithelium composed of two functionally distinct regions, the crypts and the villi, is a tissue which undergoes rapid renewal [1,2]. The undifferentiated crypt cells are mitotically active and do not participate in the digestive process. They then stop dividing and, while moving up the villus toward the lumen, they differentiate (in 36—72 h in the rat [2]) into the absorptive villous cells to form the brush border. This differentiation is accompanied by the acquisition of a cellular polarity such that the brush border membrane at the apical pole of the cell is exposed to the external medium of the lumen, while the lateral and basal faces are in contact with the serosal compartment. This polarity has important physiological consequences expressed by distinct functional characteristics (digestive process and transport). In addition, some modifications in the chemical compositions of the different areas of the cells have been reported [3—7].

To determine the mechanism of this maturation process and to eventually propose a model for the biogenesis of the brush border membrane, it would be relevant to describe as precisely as possible the chemical composition of the plasma membrane from the crypt cells and follow any change occurring in this composition during the evolution of the membrane towards its final state of basal-lateral and brush border membrane. The prerequisites to this study would be (i) a technique to distinguish and prepare the different cell types and (ii) purification methods for the different plasma membranes. Some of these requirements have already been partially satisfied since the cells at different stages of maturation can be separated by several procedures [8,9]. Also, the brush border membrane from the villous cells is easily purified due to the presence of specific hydrolases such as aminopeptidase, alkaline phosphatase and saccharidases [10]. Finally, many procedures for the preparation of the basal-latereal membrane from the villous cells have been proposed [11—19].

By contrast, the plasma membrane from undifferentiated crypt cells has never been isolated. In the present paper an original procedure will be described for the purification of this membrane which could also serve to obtain the plasma membrane from cells at different stages of maturation. This preparation has been used to determine the total lipid composition of these membranes which will be discussed in terms of the maturation process in the following papers.

Materials and Methods

Preparation of suspensions of isolated epithelial cells. Male Wistar rats (from Lagrace, Marseille or Evic Ceba, Bordeaux, France) weighing approximately 200 g were killed by decapitation. Their small intestines were excised after removal of the duodenum and ileum and thoroughly washed with ice-cold 0.9% NaCl. The cells were isolated according to the method of Weiser [9] with slight modifications. Briefly, the method consists in an incubation of the gut with a citrate buffer (solution A from Weiser) composed of 1.5 mM KCl/96 mM NaCl/27 mM sodium citrate/8 mM KH_2PO_4/5.6 mM Na_2HPO_4 (pH 7.3) for 15 min at 37°C. Then the gut is incubated and washed with solution B (10 mM

phosphate-buffered saline (pH 7.3)/1.5 mM EDTA/0.5 mM dithiothreitol) for increasing lengths of time at 37° C to detach sequentially the cells starting from the completely differentiated enterocytes present in the villus tips to the mitotically active cells from the crypts. We performed the series of incubations and washings under controlled shaking speed in a B.&T. Searle Company water bath either with buffer B or with buffer C. Buffer B consisted of the original solution B free of dithiothreitol and supplemented with 1.5 mM EGTA, 1% bovine serum albumin (from Sigma) and 1.5% Dextran T 10 (from Pharmacia, Uppsala) (pH 7.4). This buffer was chosen to obtain morphologically intact and completely dissociated cell suspensions. The efficiency of the cell separation as a gradient from the villus tips to the crypts was controlled either by direct observation under the light microscope or after centrifugation on microscope slides ($5 \cdot 10^3$ cells per slide, 500 rev./min in an Elliott Shandon cytospin), 2.5% glutaraldehyde fixation, staining of the villous cells with the periodic-acid Schiff reagent and of the crypt cells with Methylene Blue. Buffer C consisted of 10 mM Tris · HCl/5 mM $MgCl_2$/0.15 M sucrose/5 mM sodium phosphate/30 mM succinate/1 mM $MnCl_2$ (pH 7.4) and was preferred to buffer B when the cells were prepared for further subcellular fractionation. The rationale for this choice will be discussed under Results. In this case, some cells clumps were occasionally observed.

Analytical techniques. Proteins were evaluated by the method of Lowry et al. [20] using bovine serum albumin as reference. Nucleic acids were precipitated by trichloroacetic acid and hydrolyzed as described by Medolesi [21]. DNA was evaluated colorimetrically with diphenyl amine [22] by comparison with a calf thymus DNA sample (from Sigma).

Enzyme activity determinations. Standard methods were used for the assay of aminopeptidase (EC 3.4.11.2) on ala-*p*-nitroanilide [23], alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatases on *p*-nitrophenyl phosphate [24,25], disaccharidases (EC 3.2.1.26) on sucrose, maltose and trehalose with the aid of the glucostat reagent [26], cytochrome *c* oxidase (EC 1.9.3.1) [27] and NADPH cytochrome *c* reductase (rotenone insensitive) on cytochrome *c* [28]. The substrates and reagents were of the best available grade and purchased from Bachem, Sigma, Merck and Worthington. Enzyme units were defined in each case as that amount of enzyme inducing the disappearance of 1 nmol of substrate per min under the assay conditions.

The ($Na^+ + K^+$)-dependent, ouabain-sensitive ATPase (EC 3.6.1.3) activity was measured in a coupled assay with 2.5 mM ATP, 1 mM phosphoenolpyruvate, 50 μg each of pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) (10 mg/ml purchased from Sigma) and the oxidation of 0.5 mM NADH was recorded at 340 nm and 37° C [29]. The buffer consisted of 90 mM Tris · HCl (pH 7.4)/0.1 mM EDTA/2 mM $MgCl_2$/60 mM NaCl/5 mM KCl. After recording the total ATPase activity for 3 min, 0.5 mM ouabain was added and the new activity recorded. The difference in the slopes between the two lines is a measure of the ouabain-inhibited activity. This activity accounts for 20% of the total ATPase activity present in crude homogenates of the rat mucosa. One enzyme unit is expressed in μmol of substrate hydrolyzed per h.

As discussed by Douglas et al. [11], the standard assay conditions for 5′-nucleotidase (EC 3.1.3.5) are not appropriate in the case of the intestinal

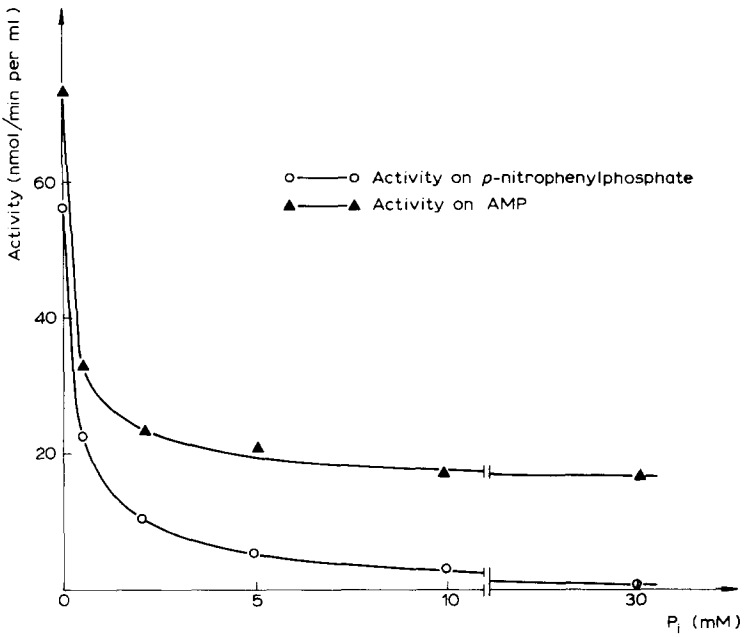


Fig. 1. Effect of P_i concentration for phosphatase and 5′-nucleotidase activities. A supernatant S1 obtained as described in Scheme I was tested spectrophotometrically at 25°C with 0.1 mM *p*-nitrophenyl phosphate and 0.1 mM AMP as substrates at 410 nm and 265 nm, respectively. The assay with AMP as substrate was coupled to an excess (10 μg) adenosine deaminase which was not limiting under these conditions. The appropriate concentration of P_i was added and the ionic strength of the buffer was kept constant in all circumstances by the addition of Tris. The pH of the assay was 7.4 on both substrates. Values were corrected for the initial P_i concentration of the sample tested.

epithelium because of the presence in the brush border of large amounts of alkaline phosphatase which can readily hydrolyze 5′-AMP. However, distinction between the two activities can be achieved by working at pH 7.4 (the optimum pH for 5′-nucleotidase is 7.4 while that for the phosphatase is 8.5) and in presence of 30 mM inorganic phosphate on 0.1 mM AMP as substrate in a coupled assay [30] with an excess adenosine deaminase (EC 3.5.4.4) (10 μg from Boehringer). The validity of the assay was demonstrated on a supernatant S_i prepared as described in Scheme I which contained the majority of the brush border and basal-lateral membranes with the same ratio phosphatase/5′-nucleotidase (=3) as in the original homogenate. Fig. 1 depicts the differential effect of P_i for the hydrolysis of *p*-nitrophenyl phosphate and AMP. *p*-Nitrophenyl phosphate is a substrate only for the phosphatase since 5′-nucleotidase has an absolute specificity for 5′-monophosphate nucleosides, in contrast to AMP which is a substrate for both enzymes. In presence of 30 mM P_i, 99% of the phosphatase activity was competitively inhibited while a substantial activity remained on AMP that could be almost entirely attributed to the 5′-nucleotidase. Controls on pure *Escherichia coli* alkaline phosphatase have shown that the activity and level of inhibition of the enzyme were identical both on AMP and *p*-nitrophenyl phosphate. In these conditions, it is possible to accurately determine a 5′-nucleotidase activity accounting for less than 10% of the total phosphatase activity.

The high sensitivity of this assay, which greatly favours 5′-nucleotidase activity and not that of the phosphatase, allowed the unambiguous detection of 5′-nucleotidase in the rat mucosa with a specific activity of 5—10 units per mg protein.

Polyacrylamide gel electrophoresis. Electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed according to the method of Fairbanks et al. [31]. Membrane proteins were solubilized by SDS treatment [32] and then reduced and alkylated as described by Edelman et al. [33].

Preparative centrifugations and centrifugations in sucrose gradients. A Sorvall superspeed refrigerated centrifuge Model RC2B equipped with a SS 34 angle rotor (8 × 50 ml) was employed for low speed centrifugations. High speed centrifugations and centrifugations in sucrose gradients were carried out in a Spinco Beckman preparative centrifuge Model L5 65 equipped with a swinging bucket rotor SW 27. The *g* values mentioned in the text correspond to the acceleration calculated for the bottom (Sorvall) or the top (Spinco Beckman) of the tubes. Linear 22—46% (w/w) sucrose gradients were prepared with solutions containing 10 mM Tris · HCl and 2.5 mM $CaCl_2$ (pH 7.4). The sucrose concentrations were checked to ±0.5% with the aid of an Abbe refractometer. Samples (1.2 ml) were collected with an Isco density gradient fractionator Model 540. The linearity of the gradient was also checked by refractometry after centrifugation in the eluted fractions.

Electron microscopy. Samples for electron microscopy were prepared by fixation of a membrane pellet first with 2.5% glutaraldehyde in 0.2 M sodium cacodylate (pH 7.4) for 1 h at 4°C and then with 2% OsO_4 in 0.1 M sodium cacodylate for 1 h at room temperature. After dehydration in ethanol and embedding in Epon, all sections obtained with the aid of an LKB Ultratome III were double stained using uranyl acetate and lead citrate and examined in a Siemens Elminskop I electron microscope.

Results

Enzyme activities of rat enterocytes: effects of maturation

The first 3 or 4 fractions of cells isolated as a villus to crypt gradient contained villous cells with their typical columnar shape and well defined brush borders heavily stained by the Schiff reagent. In the following fractions, cells at different intermediate stages of maturation were observed. Finally, fractions VIII and IX were composed only of round crypt cells, contaminated at most by 5% of villous cells. After intraperitoneal injection of ^{3}H-labelled thymidine (100 μCi) half an hour before killing [9] the incorporation reached a significant level only in fractions IX and VIII confirming the presence of mitotically active crypt cells solely in these fractions. The contrasting areas of alkaline phosphatase, aminopeptidase and disaccharidase activities with the maximum values being observed in the villus zone confirmed further the separation (Fig. 2).

However, the brush border enzymes do not appear simultaneously during the maturation process. Indeed, when the first 2/3 of the total cells have been detached, 97% of the total alkaline phosphatase activity is present while there is only 70, 81 and 89% of aminopeptidase, trehalase and maltase, respectively.

The appearance and localization of two general plasma membrane markers

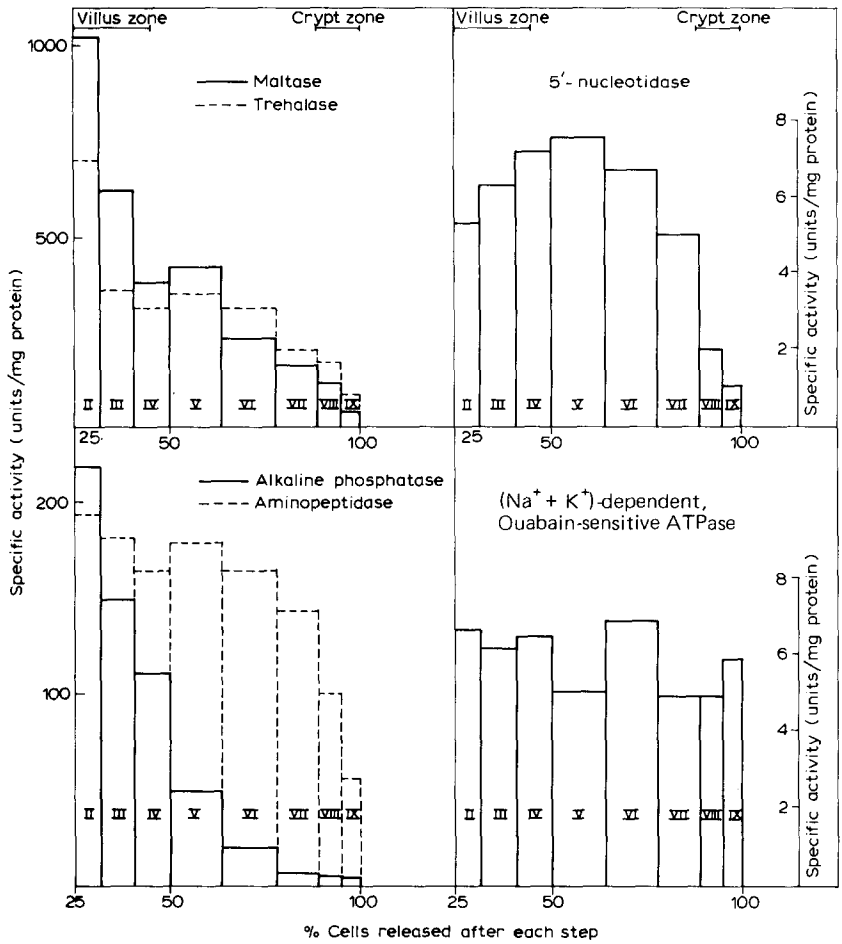


Fig. 2. Enzyme activities as distributed amongst villous and crypt epithelial cells. The cells were isolated as described in Materials and Methods with buffer B. Specific activities on the ordinate are expressed per mg protein in each individual fraction (1 mg protein corresponds to $4 \cdot 10^5$ cells). The percentage of cells released after each step is determined by the proportion of cell protein isolated in each fraction and successively summed up. The first fraction is not represented for simplification (it contains some mucus but has the same specific activity as fraction II). Values are mean values from at least 10 individual gradients except for the disaccharidases which have been tested only once.

5'-nucleotidase and $(Na^+ + K^+)$-ATPase has also been followed. The $(Na^+ + K^+)$-dependent, ouabain-sensitive ATPase displayed (Fig. 2) an even distribution throughout the gradient. (Charney et al. [34] found it mainly in the villous cells and this discrepancy may be explained by different assay conditions and disruption of their cells with sodium deoxycholate.) By contrast, 5'-nucleotidase was chiefly located in the villous cells. The localization of these two markers was further investigated by following their fate in a purification of brush border membrane vesicles according to Louvard et al. [24]. Neither one copurified with the brush border markers. The homogeneous membrane fraction contained only 3% of the 5'-nucleotidase activity present in the mucosa with a specific activity similar to that in the crude homogenate and no detectable

ATPase activity, which is in agreement with Murer et al. [18]. Consequently, both ($Na^+ + K^+$)-ATPase and 5'-nucleotidase have been used as markers to follow the purification of plasma membranes distinct from the brush border.

Preparation of plasma membranes from isolated cells

(a) Conditions for cells homogenization. The main difficulty encountered when purifying the plasma membrane from enterocytes resides in a strong association between the plasma membrane and the mitochondria resulting in a parallel enrichment of the marker enzymes for the two subcellular fractions. Different techniques have been devised to overcome this situation [12,13,16]; however, they all present some drawbacks, for example they modify the membrane structure.

By working with scrapings of the total mucosa or with isolated cells, all our attempts to disrupt the association led us to the conclusion that once formed it was almost irreversible and had to be avoided immediately when breaking the cells. The procedure described here has been founded on a density perturbation of the mitochondria rather than of the membrane itself and consists in loading the mitochondria with calcium or manganese phosphate in the presence of succinate as substrate [35]. Controls with an equivalent ionic strength in the absence of calcium phosphate and succinate yielded only partial disruption of the interactions. Manganese phosphate, being as effective as calcium phosphate in loading the mitochondria [36], was chosen to minimize an eventual lipolytic degradation of the membranes by phospholipases. Therefore, all homogenizations were carried out in buffer C and the mitochondria loaded for 30 min at 25°C under magnetic stirring, this period ensuring maximum disruption with minimum nuclei damage. The villous and crypt cells prepared from 3 rats fasted overnight, the definition and composition of which is reported in Table I, were thus homogenized without addition of more buffer (this favours a higher yield of plasma membrane) by 75 and 60 strokes, respectively, of a hand driven glass-glass homogenizer (Dounce type B).

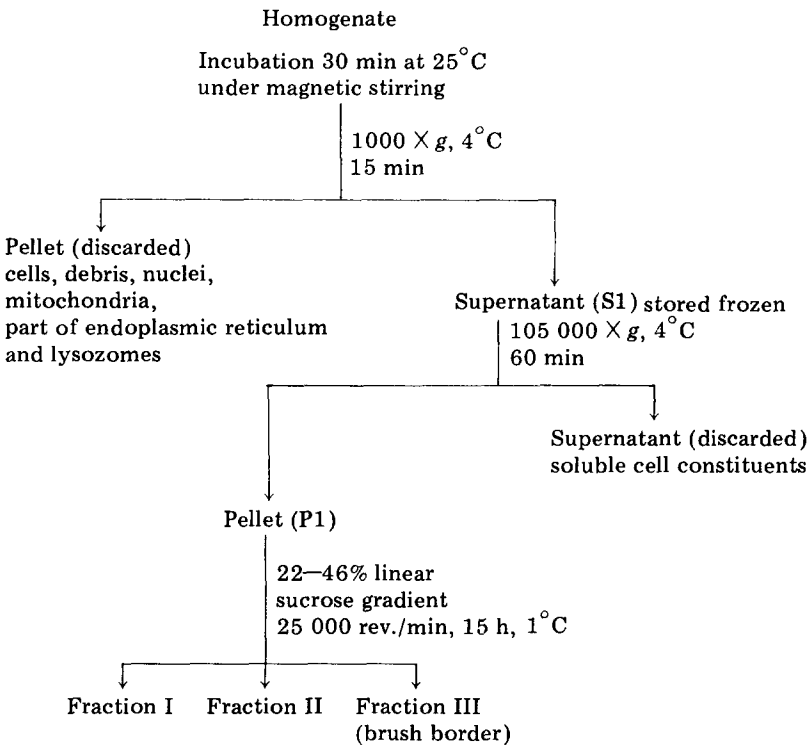
TABLE I

COMPOSITION AND ENZYME CONTENT OF RAT VILLOUS AND CRYPT CELLS

The separation of the cells as a villus to crypt gradient was performed with buffer C. The 'villous cells' were constituted by pooled fractions 1—4 and the 'crypt cells' by pooled fractions 8 and 9. After cooling at 4°C, they were centrifuged at 200 × *g* for 5 min and analyzed. The results are given as the total number of units for each enzyme, total amount of protein and specific activities in units/mg protein are given in parentheses. The percentages are calculated by comparison with the values in the first column. The figures are the average of 31 assays for protein and aminopeptidase, 15 assays for 5'-nucleotidase, 5 assays for alkaline phosphatase and ($Na^+ + K^+$)-dependent, ouabain-sensitive ATPase.

Enzyme	Total cell population from one rat small intestine	Villous cells		Crypt cells	
		Units	%	Units	%
Aminopeptidase	25 450 (110)	16 220 (140)	64	1620 (45)	6
Alkaline phosphatase	35 250 (151)	29 220 (225)	84	307 (8)	1.1
5'-nucleotidase	1 390 (6)	920 (6)	66.3	66 (1.8)	4.8
ATPase	1 300 (5.6)	785 (7.0)	60	151 (4.2)	11.6
Protein (mg)	233	117	50.2	36.3	15.6

(b) Fractionation of the homogenates. Further fractionation of the resulting homogenates is given in Scheme I.



Scheme I

Flow sheet for the purification of plasma membranes originating from rat villous and crypt cells.

As shown in Tables II and III, the first step not only removed from the supernatants S_1 a high percentage of the cytochrome oxidase activity but also left in them the largest part (70%) of the two plasma membrane markers. All enzymes tested behaved similarly with either cell population, thus yielding supernatants S_1 of comparable composition which were stored frozen until enough material was collected. No significant loss of marker enzyme activities was observed upon storage for at least 3 weeks, with a possible exception of $(Na^+ + K^+)$-ATPase, which will be discussed later.

(c) Sucrose gradient centrifugation. The pool of thawed supernatants was centrifuged at high speed and the pellets P1 were homogenized and layered on a 22—46% continuous sucrose gradient. Two typical distribution diagrams are depicted in Fig. 3. The main difference between them resides in the case of crypt cells in a great decrease of the amount of brush border membrane characterized by a peak of aminopeptidase and alkaline phosphatase activity corresponding to 43% sucrose.

TABLE II

COMPOSITION AND ENZYME CONTENT OF VARIOUS FRACTIONS FROM RAT VILLOUS CELLS

The separation of the fractions is described in Scheme I. For each enzyme the first figure indicates the number of units found in the fraction per 100 units in the homogenate. The figure in parenthesis gives the specific activity in units/mg protein. The figures are the average of assays carried out on 67 animals and 7 complete purifications. The two membrane fractions I and II are taken together as a sum or as an average when specific activities are given.

Fraction	5′-Nucleotidase	$(Na^+ + K^+)$-ATPase	Amino-peptidase	Alkaline phosphatase	Protein	DNA	Cytochrome oxidase	Cytochrome reductase	Acid phosphatase
Homogenate	100 (5.5)	100 (2.6)	100 (143)	100 (150)	100	100	100 (40)	100 (16)	100 (33)
Supernatant S_1	73 (11)	69 (4.5)	50 (178)	45 (170)	40	8	10.5 (12)	11 (4.5)	34 (27)
Pellet P1	47 (20)	39 (7.5)	40 (326)	35 (269)	13	2	7 (15)	4.3 (4.8)	15 (30)
Fractions I + II	21 (54)	15 (24)	4 (460)	3 (310)	1.5	0	0.14 (6)	<0.1	0.7 (25)

TABLE III

COMPOSITION AND ENZYME CONTENT OF VARIOUS FRACTIONS FROM RAT CRYPT CELLS

For details, see legend to Table II. The figures are the average of assays carried out on 135 animals and 3 complete purifications.

Fraction	5′-Nucleotidase	$(Na^+ + K^+)$-ATPase	Amino-peptidase	Alkaline phosphatase	Protein	DNA	Cytochrome oxidase	Cytochrome reductase	Acid phosphatase
Homogenate	100 (1.8)	100 (2.2)	100 (45)	100 (8)	100	100	100 (41)	100 (6)	100 (30)
Supernatant S_1	67 (3.7)	65 (4.4)	48.5 (52)	52.5 (25)	32.5	9	20 (26)	16 (4.6)	24.5 (25)
Pellet P1	40 (6.0)	30 (6)	35 (100)	35 (30)	12	2	11.5 (23)	5 (4)	8 (22)
Fractions I + II	19 (22)	14 (26)	10 (440)	8 (59)	0.9	0	0.1	<0.1	0.7 (22)

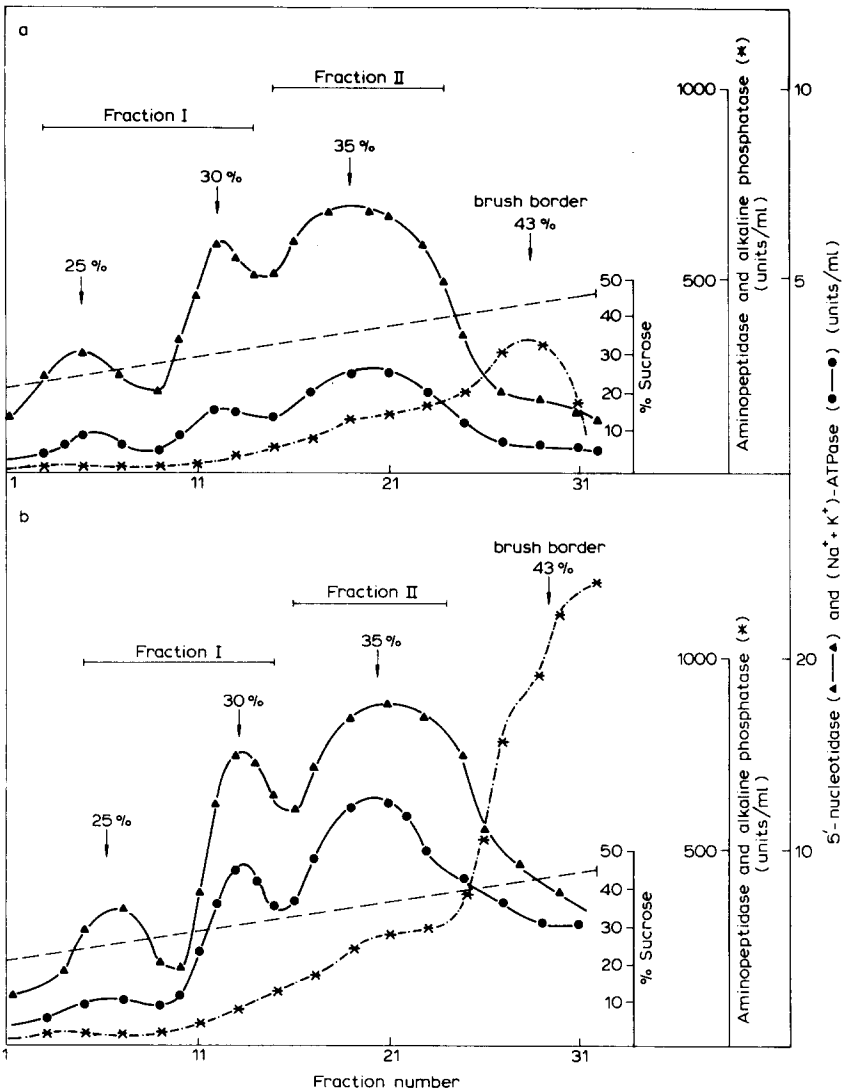


Fig. 3. Distribution of plasma membranes prepared from villous and crypt cells after centrifugation on a continuous sucrose gradient. 2.5 ml of the pellets P1 (Scheme I) from the villous and crypt cells containing approximately 7500 aminopeptidase units, 500 5′-nucleotidase units and 35 mg protein were layered on top of a 36 ml 22—46% continuous sucrose gradient and centrifuged for 15 h at 25 000 rev./min and 1°C. 32 fractions were collected from the top of the tube and directly analyzed for 5′-nucleotidase (▲——▲), ($Na^+ + K^+$)-dependent, ouabain-sensitive ATPase (●——●), aminopeptidase and alkaline phosphatase (*— · — ·*) activities. Cytochrome oxidase, cytochrome reductase and acid phosphatase activities (not shown for simplification) were found in the region of 40—45% sucrose concentration. (a). Distribution of the membranes from the crypt cells; (b). Distribution of the membranes from the villous cells.

By contrast, in both cases the bulk of the material was partitioned into two major fractions, 'Fraction I' and 'Fraction II', corresponding to the same sucrose concentration, 30 and 35% respectively. Occasionally, the first fraction was dissociated in a lighter one (25% sucrose) which was not considered independently due to its low percentage. Tables II and III give the composition of

the membrane fractions. The two markers, 5′-nucleotidase and $(Na^+ + K^+)$-ATPase were purified approximately 10-fold when compared to the crude homogenate with an overall yield of 15—20% and the membranes were essentially free of other organelles. In the case of crypt cells, the ATPase activity was especially unstable, resulting in general in lower yields. However, it was demonstrated on a qualitative scale, by using freshly prepared supernatants S_1 and performing the whole procedure and enzymatic assays within a week, that this marker copurified with the same yield and enrichment factor as that for 5′-nucleotidase. Therefore the values in Tables II and III have been corrected assuming for the ATPase the same recovery as that for 5′-nucleotidase.

The purification procedure described above could be applied to scrapings of the mucosa with only minor changes. The scrapings were suspended in 8 times their weight of buffer C, homogenized by 3 strokes of a motor-driven Teflon glass homogenizer operated at 1200 rev./min, filtered once through gauze to remove the mucus and further homogenized by two additional up and down strokes of the pestle operated at 600 rev./min. There was no need for storage of the supernatant S_1 since it is possible to collect as much mucosa as desired all at once. The distribution diagram of the membrane fractions on sucrose gradient, the overall yields, enrichment factors and homogeneity of the fractions were identical to those presented in Fig. 3 and Tables II and III.

Comparative properties of membrane fractions I and II from villous and crypt cells

The relative proportions of the two fractions obtained from both cell types were constant from one preparation to the other. The amount of protein in fractions I represented 60 and 40%, respectively, of that in the corresponding fractions II (Table IV). With villous cells as starting material, the 5′-nucleotidase to $(Na^+ + K^+)$-ATPase activity ratio was higher in fraction I than in fraction II (2.7/1.8) and the specific activity of 5′-nucleotidase was greater in fraction I. This ratio was inferior to one for the crypt cell fractions, reflecting the situation with intact cells (Table I), and the 5′-nucleotidase specific activity was lower than in the villous cell fractions. By contrast, the $(Na^+ + K^+)$-ATPase displayed approximately the same specific activity in the four fractions whatever their origin.

All the purified fractions still contained some of the specific brush border membrane enzyme activities with the exception of alkaline phosphatase, the specific activity of which was very low in the fractions originating from the crypt cells.

To answer some of the questions raised by all these results, each individual fraction was further submitted to the same centrifugation as in Fig. 3 and the results depicted in Fig. 4 were obtained. The four fractions behave as an apparent single species with cosedimentation of all markers tested and equilibrating at the same sucrose density as in the first centrifugation with no evidence of any further transformation. Also, no peak corresponding to the position of the brush border membrane appeared (Fig. 4, c and d) eliminating the hypothesis of a contamination by brush border membrane vesicles. Increase of the length of the centrifugation from 15 h to 38 h did not alter the apparent density of the fractions.

TABLE IV

COMPARATIVE COMPOSITION OF MEMBRANE FRACTIONS I AND II FROM VILLOUS AND CRYPT CELLS AND OF BRUSH BORDER MEMBRANE VESICLES

The membrane fractions are the same as in Table II and III and the figures are those obtained for each fraction analyzed separately. Protein content is expressed as the percentage of that in the crude homogenate, and activities are given in units/mg protein. The brush border membrane vesicles were purified according to Louvard et al. [24] as modified by Rietsch (personal communication) with scrapings of the mucosa as starting material.

Membrane fractions	Protein	5'-Nucleotidase/-$(Na^+ + K^+)$-ATPase	5'-Nucleotidase	$(Na^+ + K^+)$-ATPase	Aminopeptidase	Alkaline phosphatase	Maltase	Trehalase
Fraction I villous cells	0.54	2.7	61	23	290	310	260	150
Fraction II villous cells	0.95	1.8	46	26	640	335	560	200
Fraction I crypt cells	0.27	0.9	25	28	290	59	530	360
Fraction II crypt cells	0.63	0.8	20	25	590	59	not measured	not measured
Brush border membrane	1.2	—	10	undetectable	2200	2500	4000	300

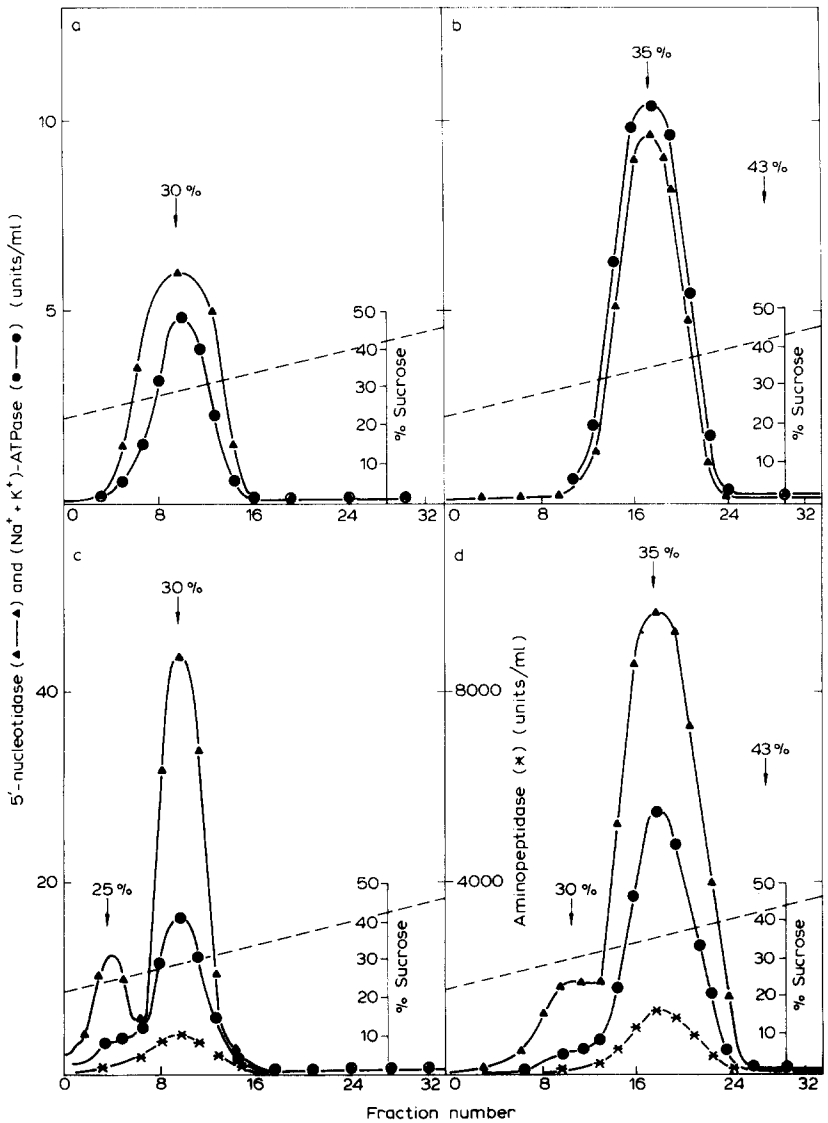


Fig. 4. Distribution after centrifugation on sucrose gradient of each individual membrane fraction originating from villous and crypt cells. Fractions I and II collected as indicated in Fig. 3 from villous and crypt cells were concentrated by high speed centrifugation after dilution with 0.9% NaCl and submitted individually to a centrifugation on a continuous sucrose gradient in the same conditions as in Fig. 3. For details and symbols, see legend to Fig. 3. a, membranes prepared from crypt cells Fraction I and (b) Fraction II. c, membranes prepared from villous cells Fraction I and (d) Fraction II. The small percentage of Fraction I in Fraction II results from a contamination (see pool in Fig. 3).

To test a possible influence of the homogenization conditions, the membranes were submitted to a treatment mimicking that used in the first step (incubation with buffer C for 30 min at 37°C, standing for 1 h at 4°C, homogenization and stirring for 30 min at 25°C). This did not modify their distribution pattern on sucrose gradient. Finally, incubation of the purified membranes in conditions known to solubilize extrinsic components (10 mM EDTA or 1 M

NaCl for 3 h at 37°C and 12 additional hours at 4°C) did not change their densities nor release any of the activities originally present. The general impression of homogeneity given by the chemical and enzymatic determinations was confirmed by electron microscopy. The membranes from both origins were composed of a mixture of apparently closed vesicles and short membrane sheets. Fraction I from villous cells and fraction II from crypt cells possessed a relatively greater proportion of vesicles, the size of which is not homogeneous. The trilamellar membrane structure was easily visible but when the fractions prepared from crypt cells were examined its periphery appeared somewhat less defined as if some material was still attached.

The protein content of the fractions was examined by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate. The four fractions were different from each other and from the brush border membrane. Only two proteins of apparent molecular weights of approx. 105 000 and 50 000 seemed to be common to all four fractions and the molecular weights were distributed rather in the 140 000—35 000 range, in contrast with the brush border membrane (200 000—60 000). Finally it must be emphasized that the fractions from the crypt cells did not resemble their counterpart from the villous cells.

Discussion

The isolation and characterization of plasma membrane subfractions originating from rat intestinal crypt cells have been reported in this article. This in itself justifies a new purification procedure, since those already available mainly dealt with the preparation of the basal-lateral membrane from the mature enterocytes essentially for transport mechanisms studies. The method proposed provides an important step forward for the study of the enterocytes differentiation process by allowing the preparation of plasma membranes from cells at different stages of maturation. Furthermore, since the starting cell population is reasonably well defined, this technique reduces the controversy about the origin of the purified membranes.

The overall yield and homogeneity of the membrane subfractions obtained herein compare well with that of other authors. A point of interest resides in the density perturbation of the mitochondria.

In this work, two general plasma membranes markers: 5′-nucleotidase and $(Na^+ + K^+)$-ATPase have been used to purify the enterocyte plasma membranes which have no distinguishing morphological features. In most of the previous publications (except in that by Murer et al. [18]), only one enzyme was considered, generally the ATPase because of the assumption that sodium pump sites are localized on the basal-lateral membrane [17,28]. As pointed out by De Pierre [37] for example, this approach might favour the isolation of a specialized domain of the cell surface which may be representative neither of the total cell area nor of the total cell population. Therefore, the effects of the enterocytes' maturation for the two general markers have been investigated. The $(Na^+ + K^+)$-dependent, ouabain-sensitive ATPase is localized principally in the plasma membrane from all cells whatever their state of maturation, suggesting a need for Na^+ pump sites even in mitotically active cells, as supported by (i)

the absence of this enzyme from the brush border membrane (this work and refs. 12—14,18) and (ii) its constant specific activity throughout the villus to crypt gradient of cells (Fig. 2) which is in turn reflected by a constant specific activity in the purified membrane fractions (Table IV). By contrast, and by using the same type of argument, a predominant location of 5′-nucleotidase can be assessed in the basal-lateral membrane from the differentiated cells, which suggests its utility as a specific marker for this membrane. If 5′-nucleotidase is an intrinsic component of the highly specialized brush border which could have preserved to some extent the attributes of a normal plasma membrane [11,19], it certainly represents a very small percentage of its activity initially present in the mucosa.

Furthermore, the shape of 5′-nucleotidase distribution in the gradient of cells (Fig. 2) suggests that its appearance represents an event in the maturation process that may be related to the cessation of the crypt cells' proliferation and induction of the differentiation. This assumption is based on observations that 5′-nucleotidase activity varies with the cell cycle [38] and that it could play a role in the mechanism that slows down and eventually stops the growth by depleting the cell of key intermediates in nucleic acid synthesis [39].

The collection of two distinct membrane subfractions, from the mature cells as well as from the crypt cells, deserves some comments. These membrane fractions could come from different parts of the cell surface, as suggested in the case of the villous cells, by significant differences in their enzyme activities and protein content. Indeed, it is conceivable that the lateral and basal regions do not play the same role and probably contain specific constituents. However, the same reasoning does not hold with the crypt cells since it is more difficult to envisage in this case the same kind of specialization on the cell surface. A more likely hypothesis invokes the homogenization conditions which could favour an artefactual protein segregation into multiple subfractions [40] although no further fractionation of the purified membranes could be demonstrated by mimicking the initial treatment. In any event, the final answer must await the identification of more specific markers.

Finally, the last point of discussion concerns the effects of the cells' maturation for the enzyme activities known to be specific for the brush border membrane. Alkaline phosphatase is poorly represented, if at all, in the crypt cells while disaccharidases occupy an intermediary position and aminopeptidase is certainly one of the first to be synthesized. The distribution pattern for aminopeptidase does not reflect the successive synthesis of several enzymes with different specificities (two aminopeptidases have been described in the rat [41]). A constant ratio of activity on other substrates such as Ala-, Leu- or Asp-β-naphthylamides or dipeptides (Arg-Leu, Phe-Leu, Asp-Leu or Lys-Ile) versus that on ala-*p*-nitroanilide was obtained throughout the gradient of cells.

A screening of the appearance of enzyme activities in different types of plasma membranes may provide a partial answer to the question of knowing when brush border enzymes are integrated into a brush border membrane. The centrifugation pattern on sucrose gradient indicates that in the villous cells the greatest part of the aminopeptidase and alkaline phosphatase activities are present in a typical brush border membrane (density, 1.19). A small amount of this latter type of membrane in the crypt cells is consistent with the light

microscope observation of the cell preparation (less than 5% of villous cells and approx. 10% showing nascent microvillosities). The presence of a non-negligible proportion of aminopeptidase in the other plasma membranes, especially in the case of the crypt cells, suggests that the enzyme is localized on the total cell surface before being integrated in a brush border membrane at the apical pole. The same reasoning probably holds for the other enzymes appearing later in the maturation process. This hypothesis implies, of course, a high degree of homogeneity of the prepared membranes and should be confirmed by immunological and electron microscopy techniques. Redistribution, once the tight junction between the cells was destroyed, of the enzymes [42] and /or purification of membrane fragments composed of brush border and lateral membranes still attached to one another cannot be completely dismissed at the moment. However, that would still not explain a high aminopeptidase activity in the membranes from the crypt cells.

In conclusion, the preparation of membranes from cells at two extreme stages of maturation has allowed a detailed investigation of their lipid composition which will be described in following papers and discussed with respect to the maturation process.

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