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Isolation of Brush-Border Membranes from Rat and Rabbit Colonocytes: Is Alkaline Phosphatase a Marker Enzyme?

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Summary. A method for the isolation of brush-border membranes of large intestinal epithelial cells was developed, which is based on the purification of intact brush-border caps by Percoll® density-gradient centrifugation followed by separation of the vesiculated brush-border membranes on sucrose gradients. The procedure has two major advantages in comparison to known methods: 1) its first step does not depend on the determination of marker enzymes and 2) the method is applicable to rats as well as rabbits without major modifications. Due to the lack of an accepted marker for the colonic brush-border membrane the validity of the isolation procedure was tested by its application to the small intestine. Rat small intestinal brush-border membranes were enriched 21-fold when compared to the homogenate. The method was used to evaluate alkaline phosphatase as a marker enzyme for the colonic brush-border membrane. The results suggest that alkaline phosphatase is not exclusively localized in the brush-border membrane since this enzyme was also associated with membranes having different physical properties.

Key Words brush-border membrane · large intestine · small intestine · isolation · alkaline phosphatase

Introduction

The large intestine of the mammals is lined with a moderately tight epithelium [24]. In order to elucidate the cellular and subcellular topology of the different functions of the colon, surface membrane vesicles derived from defined epithelial cell populations, such as colonocytes, would constitute invaluable tools. Surprisingly, attempts to isolate membrane vesicles from the surface membrane of colonocytes have only recently been reported. This might be due to some of the following problems: 1) The colonocytes of the large intestine exhibit much shorter microvilli on their luminal surface as compared to the small intestine which makes isolation of their apical membrane more difficult. 2) No accepted marker has so far been established for the colonic brush-border membrane. In particular, the studies on the value of alkaline phosphatase as such a marker have led to controversial results [12, 30], while numerous marker enzymes are known for the small intestinal brush-border membrane [13]. 3) In the small intestine, the enterocytes are the most abundant cell type of the villus epithelium, while the colonocytes contribute only a small fraction to the total number of cells in the colon [28]. 4) The tight adhesiveness of the colonocytes to the basal membrane prevents direct application of the methods developed for the isolation of small intestinal epithelial cells to the colon. To our knowledge, there are only two publications on the isolation of the colonic brush-border membrane: Gustin and Goodman [9] established a method based on Percoll® density-gradient centrifugation to isolate brush-border membrane vesicles from rabbit distal colon. Brasitus and Keresztes [3] applied a sucrose density-gradient technique to isolate the brush-border membrane from rat proximal colon in a vesicular form. Both groups used alkaline phosphatase as a marker for this membrane relying on a histochemical study of Vengesa and Hopfer [30].

The aim of the present work was to develop a method for the isolation of the colonic brush-border membrane that is less dependent on marker enzymes. The method is based on mild homogenization of isolated colonocytes leaving their brush-border caps intact, a procedure adapted from Eichholz and Crane [6] and Forstner et al. [8]. This enabled us to monitor the purification of the brush-border caps by phase-contrast microscopy after Percoll® density-gradient centrifugation. Subsequently, the brush-border caps were converted into membrane vesicles by Tris disruption [6] and the vesicles were further purified by centrifugation on sucrose gradients. The validity of this approach was assessed by its application to the small intestine from rat and rabbit. This comparison enabled us first to test for a general applicability of the method and second to address the question of marker enzymes for the co-

lonic brush-border membrane. It should be mentioned, that the first procedure for the isolation of small intestinal brush-border membranes which led to the definition of marker enzymes for this membrane was based on the strategy of isolating intact brush-border caps [17].

Materials and Methods

Animals

Female Sprague Dawley rats (230 to 270 g) and female New Zealand white rabbits (4 to 6 kg) were kept in a constant temperature environment and fed a standard chow. The animals were starved overnight and killed the following morning between 8:00 and 8:30 a.m. by a blow on the neck. From both animals, the whole small intestine was used for the experiments. The large intestine of rats was divided into two equal parts which were designated proximal and distal. With rabbits, the separation was made in the area, where the colon became smooth: the upper third was taken as proximal and the lower two-thirds were considered distal.

Isolation of Enterocytes and Colonocytes

Enterocytes from the small intestine were isolated according to [31] with the following modification: After the intestines had been filled with ice-cold Weiser's solution B, they were placed on a glass plate on ice and the enterocytes were released by slightly tapping the intestines with our fingers. The cells were collected by centrifugation and washed once with 5 mM Na_2SO_4, 1 mM Tris/HCl, pH 7.6. All the buffers contained 40 μg/ml PMSF, which was added from a stock solution (40 mg/ml ethanol) immediately before use.

Large intestinal colonocytes were isolated essentially according to [9]. In order to avoid cell damage, the colon was not everted. The colon was rinsed with 0.9% (wt/vol) NaCl, 0.5 mM DTT, 40 μg/ml PMSF and then filled with buffer A (in mM): 30 NaCl, 5 Na_3EDTA, 8 HEPES/Tris, pH 7.6, 0.5 DTT, 40 μg/ml PMSF and ligated. The segments were incubated in buffer A on ice. After 20 min, the intestines were emptied, filled again with buffer A and incubated in fresh buffer A. After 1 hr, the solution in the intestines was collected and the buffers changed. After another hour the content of the sacs was again collected. The solutions were combined and centrifuged in an IEC PR 6 centrifuge for 7 min at 1800 rpm (700 g_{av}). The colonocytes were resuspended in buffer A by gently shaking, and centrifuged for 7 min at 1800 rpm. The pellet was resuspended in buffer B: 5 mM Na_2SO_4, 1 mM Tris/HCl, pH 7.6, 40 μg/ml PMSF and again centrifuged for 7 min at 1800 rpm.

Isolation of Brush-Border Caps

The washed colonocytes of six rats (or of 10 rats for experiments in which proximal and distal colon were separately processed) or of one rabbit were resuspended in 70 ml of buffer B and transferred to a 400-ml beaker of a Sorvall Omnimixer on ice. The mixer was set to position 1 (3000 rpm) for 5 sec to disintegrate the epithelial cell sheets. After a break of 30 sec, the cells were homogenized for 30 sec at instrument setting 6 (10,000 rpm) (except for rabbit small intestine: setting 5 (8900 rpm)). The homogenate was spun for 7 min at 2200 rpm (1060 g_{av}) in an IEC PR 6 centrifuge. The pellet was resuspended in 50 ml of buffer B by shaking the tubes very gently, and recentrifuged for 7 min at 2200 rpm. The pellet was resuspended in 32.0 ml buffer B and 3.56 ml Percoll® were added to a final concentration of 10.0% (vol/vol) in a 50-ml polycarbonate tube. The gradients were formed by spinning the tubes for exactly 20 min at 20,000 rpm in a Sorvall SS 34 rotor (37,000 g_{av}) with a Sorvall RC 2 B centrifuge. For the preparation of the colonocyte brush-border caps, two gradient tubes for each segment were used. For the preparation of the brush-border caps from the small intestine, three gradient tubes were prepared for four rat small intestines and three gradient tubes for one rabbit small intestine. The brush borders were collected by fractionating the gradients from the bottom of the tubes as follows: rat small intestine: discard 1 ml, collect 4.5 ml; rat large intestine: discard 2 ml, collect 4.5 ml; rabbit small intestine: discard 2 ml, collect 4 ml; rabbit large intestine: discard 2 ml, collect 5 ml. The fractions containing the brush-border caps were diluted with buffer B and centrifuged for 75 min at 55,000 rpm in a Beckman Ti 60 rotor (213,000 g_{av}). The supernatant was discarded and the brush-border caps were collected from the surface of the glassy Percoll® pellet by careful resuspension in 1 ml of water.

Isolation of Brush-Border Membranes

The resuspended brush-border caps were diluted with 1 vol 2 M Tris/HCl, pH 8.2, containing 12 mM DTT and left on ice for 15 min. The suspension was then diluted with 4 vol of 6 mM DTT and centrifuged for 30 min at 20,000 rpm in a Sorvall SS 34 rotor. The resulting pellet was resuspended as follows: a) rat small intestine: The membranes were suspended in 900 μl 50 mM $CaCl_2$ and divided into 3 equal portions for 3 gradients: 1 vol of membranes was diluted with 3 vol of 60% (wt/wt) sucrose in 50 mM $CaCl_2$ to give a final sucrose concentration of 45% (wt/wt) and filled into a 4.4-ml tube of a Beckman SW 56 rotor. The tubes were filled with 10% (wt/wt) sucrose in 50 mM $CaCl_2$ and centrifuged for 2 hr at 48,000 rpm (226,000 g_{av}). The brush-border membranes were collected from the 10/45% interface. b) Rat colon: The membranes were resuspended in 400 μl distilled water and layered on the following gradient: 1 ml 35% and 2 ml 10% (wt/wt) sucrose. The tubes were centrifuged as above. c) Rabbit small intestine: The same procedure was used as for the rat small intestine. For one rabbit, usually 2 gradients were run. d) Rabbit colon: The membranes were resuspended in 400 μl of water and loaded on the following gradient: 1 ml 48.5%, 1 ml 35% and 1 ml 10% (wt/wt) sucrose in 50 mM $CaCl_2$ and centrifuged as above. Membranes were collected from the interfaces. For some experiments it was necessary to dilute the membrane fractions with the appropriate buffer and spin the membranes down for 30 min at 20,000 rpm in a Sorvall SS 34 rotor prior to further analysis.

Determination of Marker Enzyme Activities

All measurements were performed at 37°C. Alkaline phosphatase and aminopeptidase N (measured according to [11]) were used as marker enzymes for the brush-border membrane. $(Na^+ + K^+)$-ATPase and ouabain-sensitive K^+-*p*-nitrophenylphosphatase were used as markers for the basolateral membrane. Succinate-cytochrome *c* oxidoreductase (measured according to [7]) and KCN-resistant NADH oxidoreductase (measured according to [27]) were used to monitor mitochondria and endoplasmic reticulum, respectively. Glucosaminidase (measured according to [23]) was used as a lysosomal marker enzyme. Alkaline phosphatase,

aminopeptidase N, $(Na^+ + K^+)$-ATPase and KCN-resistant NADH oxidoreductase were measured using an LKB reaction rate analyzer. Succinate-cytochrome *c* oxidoreductase was measured kinetically in a Kontron Uvikon 820 spectrophotometer. Alkaline phosphatase was measured in 1 M diethanolamine/HCl, pH 9.8, 0.5 mM $MgCl_2$ with 10 mM *p*-nitrophenylphosphate disodium salt as substrate. In cases of low activity, the reaction was carried out in a volume of 1.15 ml in a waterbath for up to 1 hr and terminated by the addition of 100 μl 10 N NaOH. *p*-Nitrophenylphosphatase was measured with 3 mM *p*-nitrophenylphosphate ditris salt as substrate in the presence of 0.015% (wt/vol) saponin using the following buffers: (I) 10 mM KCl, 3 mM $MgCl_2$, 50 mM Tris/HCl, pH 7.4, 5 mM theophyllin; (II) buffer (I) containing 5 mM ouabain; (III) 10 mM NaCl, 3 mM $MgCl_2$, 50 mM Tris/HCl, pH 7.4, 5 mM theophyllin. The incubation was carried out in a total volume of 1.15 ml for up to 1 hr in a waterbath and terminated by the addition of 100 μl 10 N NaOH. The difference between the activities measured with buffers (I) and (II) was taken as $(Na^+ + K^+)$-ATPase activity (dephosphorylation step) and the difference between buffers (II) and (III) as ouabain-insensitive K^+-stimulated *p*-nitrophenylphosphatase according to [9]. $(Na^+ + K^+)$-ATPase was measured according to [1] in the presence of 0.015% (wt/vol) saponin.

Other Methods

SDS polyacrylamide gel electrophoresis was performed on 7.5% polyacrylamide slab gels according to [14]. Protein was determined with the Bio-Rad protein assay kit using protein standard I. Transport experiments were performed as described previously [29] using Millipore filters (type HA, 0.45-μm pore size).

Materials

Saponin was obtained from Merck (Darmstadt, FRG), *p*-nitrophenylphosphate ditris salt and *p*-nitrophenyl-N-acetyl-β-D-glucopyranoside were from Sigma (St. Louis, Mo.), lactate dehydrogenase and pyruvate kinase from Boehringer (Mannheim, FRG). [^{3}H]-D-Glucose was purchased from New England Nuclear (Boston, Mass.). Omeprazol was the generous gift of Dr. Wallmark (AB Hässle, Sweden). All other reagents were of highest purity available and obtained either from Sigma or Merck.

Abbreviations

DCCD: N,N'-dicyclohexylcarbodiimide
DTT: D,L-dithiothreitol
HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Na_3EDTA: ethylenediamine tetraacetic acid trisodium salt
PMSF: phenylmethylsulfonylfluoride
SDS: sodium dodecyl sulfate
Tris: Tris(hydroxymethyl)aminomethane

Results

Isolation of Intact Brush-Border Caps

In preliminary experiments it became clear that colonic brush-border membranes could only be purified successfully if the preparation was started with isolated colonocytes. Gustin and Goodman [9] have developed such a method. However, we found it necessary to modify their procedure in order to increase the yield of cells and to keep cell damage to a minimum. Therefore the intestines were not everted and longer incubation times had to be used. The developed protocol can be applied to both rats and rabbits. For rabbits, it was occasionally necessary to extend the incubation time for up to 4 hr in order to obtain a sufficient amount of colonocytes. This isolation procedure yielded a mixture of single colonocytes and epithelial sheets. A brief outline of the developed protocol for the purification of colonic and small intestinal brush-border membranes from rat and rabbit is presented in Fig. 1. Figure 2 illustrates isolated colonocytes and enterocytes from rabbit colon and small intestine, respectively. It is evident from this figure that part of the problems encountered during the isolation of the colonic brush-border membrane may be due to the much shorter microvilli of the colonocytes as compared to the small intestine. Alternatively, the relevant char-

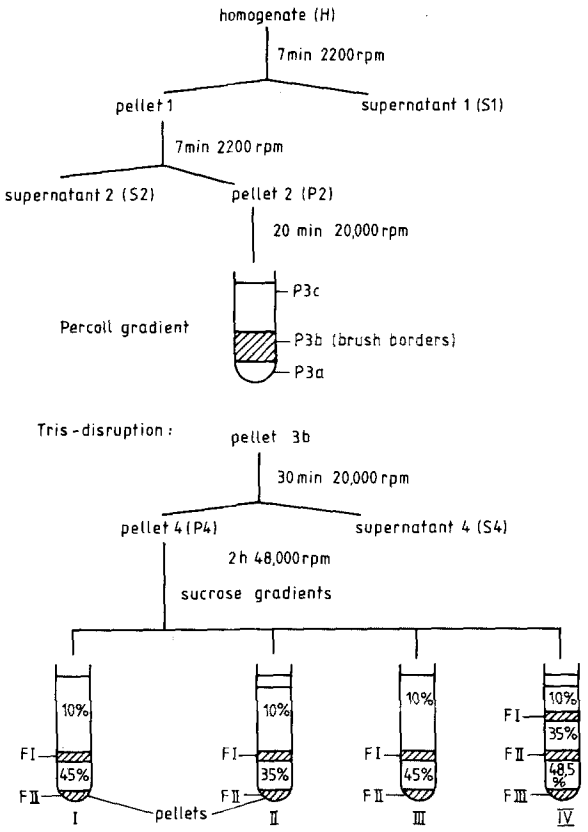


Fig. 1. Purification protocol for the isolation of intestinal brush-border membranes. The Percoll® gradient was run with 10% (vol/vol) Percoll in 5 mM Na_2SO_4, 1 mM Tris/HCl pH 7.6. Except for gradient II, all the sucrose solutions contained 50 mM $CaCl_2$. In gradients II and IV the samples were layered on the gradient, whereas in I and II they were resuspended in 45% sucrose. I: rat small intestine; II: rat colon; III: rabbit small intestine; IV: rabbit colon

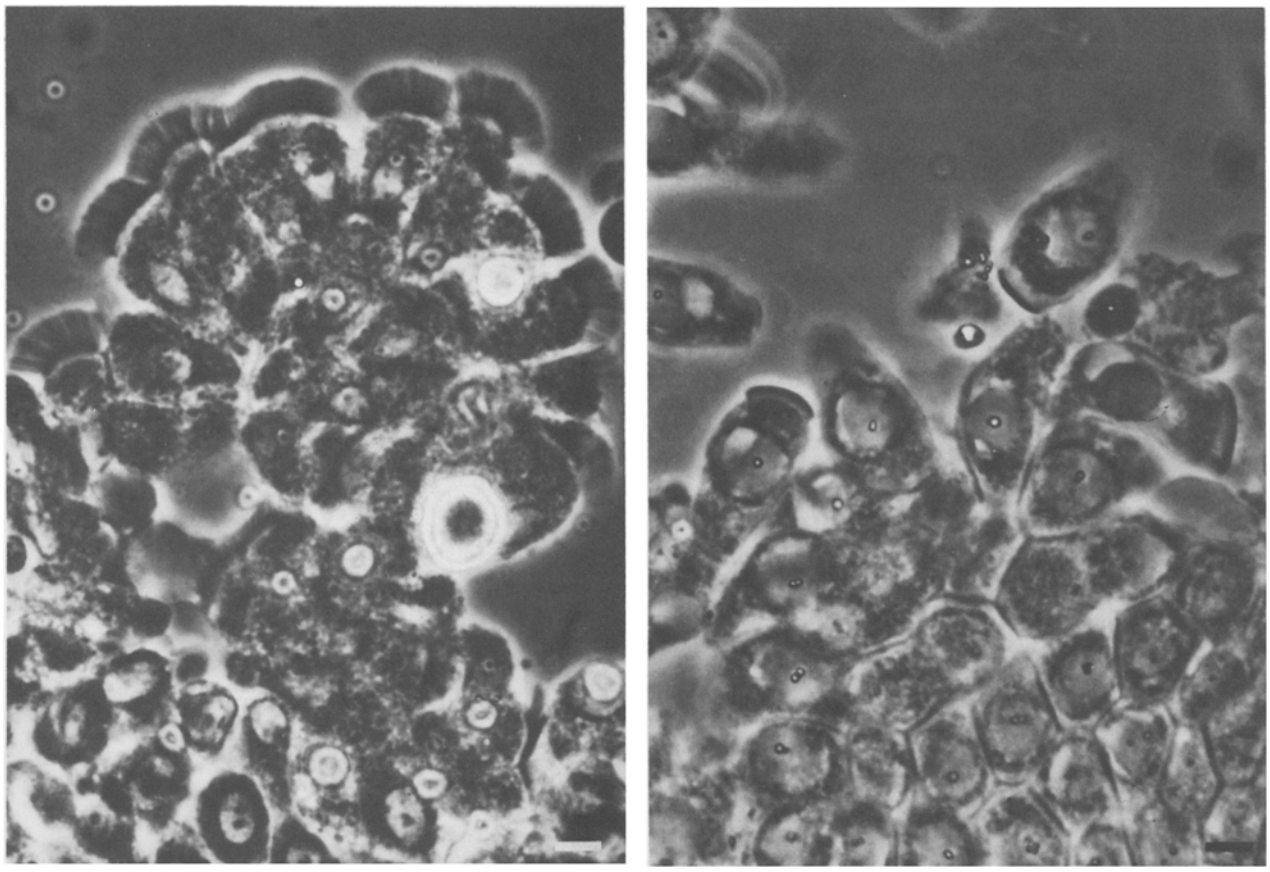


Fig. 2. Isolated cell sheets from the rabbit intestine. Phase-contrast picture of surface epithelial cells from the small intestine (left) and from the distal colon (right); bar = 10 μm

Table 1. Specific activities and enrichment factors of marker enzymes in brush-border caps of rat distal colon

	Specific activity (mU/mg protein)		Enrichment factor
	Homogenate	P3b	
Alkaline phosphatase	3.35 ± 1.79	8.44 ± 2.64	2.9 ± 1.3 (n = 4)
Ouabain-sensitive K^+-*p*-nitrophenylphosphatase	18.8 ± 6.6	61.0 ± 29.1	4.4 ± 4.5 (n = 4)
Ouabain-insensitive K^+-*p*-nitrophenylphosphatase	6.26 ± 1.09	16.9 ± 27.8	3.1 ± 5.2 (n = 4)

acteristics might be the strength of the cytoskeleton and of its interaction with the membrane. Equally well preserved cells and cell sheets were obtained from rat (*not shown*). Figure 3 shows isolated brush-border caps from the rat colon and thus demonstrates that it is indeed possible to isolate this organelle with the brush-border membrane attached without the use of marker enzymes.

Isolation of Brush-Border Membranes

Enzymatic Characterization

As the enzymes of the rat colon have very low activities the recoveries and the enrichment factors for the brush-border caps and the brush-border

Table 2. Specific activities and enrichment factors of marker enzymes in brush-border membranes of rat whole colon

	Specific activity (mU/mg protein)		Enrichment factor	
	Homogenate	FI		
Alkaline phosphatase	1.42 ± 0.09	14.3 ± 8.3	10.3 ± 6.3	(n = 7)
(Na^+ + K^+)-ATPase	183.3 ± 35.1	1087.6 ± 312.8	6.3 ± 2.8	(n = 7)
KCN-resistant NADH-oxidoreductase	274.2 ± 62.1	249.5 ± 166.1	0.92 ± 0.61	(n = 7)
Cytochrome c oxidoreductase	79.5 ± 13.6	55.7 ± 22.6	0.70 ± 0.27	(n = 6)
Glucosaminidase	106.3 ± 16.3	10.6 ± 8.4	0.10 ± 0.08	(n = 7)

Table 3. Recoveries of marker enzymes in brush-border caps of rat whole colon

	S1	S2	P3a	P3b	P3c	Total	
Protein	70.3 ± 2.6	15.2 ± 5.3	0.67 ± 0.32	1.2 ± 0.5	8.5 ± 0.6	95.8 ± 5.4	(n = 7)
Alkaline phosphatase	76.5 ± 3.2	13.7 ± 4.4	2.9 ± 1.4	5.7 ± 2.4	15.0 ± 2.2	113.8 ± 8.3	(n = 7)
(Na^+ + K^+)-ATPase	73.3 ± 11.1	14.6 ± 2.3	0.60 ± 0.26	3.1 ± 0.9	9.0 ± 1.8	100.6 ± 13.8	(n = 7)
KCN-resistant NADH-oxidoreductase	77.7 ± 9.2	25.2 ± 12.5	0.49 ± 0.35	1.6 ± 0.6	9.0 ± 1.5	114.0 ± 21.3	(n = 7)
Cytochrome c oxidoreductase	65.8 ± 4.2	22.6 ± 4.2	0.51 ± 0.22	1.5 ± 1.1	15.4 ± 2.3	105.8 ± 8.0	(n = 6)
Glucosaminidase	73.1 ± 2.8	15.2 ± 4.0	0.28 ± 0.07	0.04 ± 0.03	7.8 ± 0.6	96.4 ± 4.3	(n = 7)

The values are given as the percentage of the amount determined in the homogenate.

Table 4. Specific activities and enrichment factors of marker enzymes in brush-border caps and brush-border membranes from rat small intestine

	Specific activity (U/mg protein) enrichment factor		
	Homogenate	P3b	FI
Alkaline phosphatase	0.4589 ± 0.1265 (n = 8)	5.6886 ± 1.7997 (n = 8) 12.3 ± 2.5	10.430 ± 1.232 (n = 5) 24.6 ± 6.7
Aminopeptidase N	0.0667 ± 0.0115 (n = 10)	0.7871 ± 0.1586 (n = 10) 11.9 ± 2.4	1.426 ± 0.2801 (n = 5) 20.8 ± 1.2
(Na^+ + K^+)-ATPase	0.1795 ± 0.0140 (n = 8)	0.1454 ± 0.0157 (n = 8) 0.82 ± 0.11	0.1175 ± 0.0335 (n = 3) 0.67 ± 0.19
KCN-resistant NADH oxidoreductase	0.1907 ± 0.0582 (n = 8)	0.0247 ± 0.0258 (n = 8) 0.17 ± 0.23	0.0201 ± 0.0089 (n = 3) 0.11 ± 0.06
Cytochrome c oxidoreductase	0.0744 ± 0.0134 (n = 8)	0.0404 ± 0.0146 (n = 8) 0.54 ± 0.14	0.0123 ± 0.0168 (n = 3) 0.15 ± 0.20
Glucosaminidase	0.0368 ± 0.0037 (n = 5)	0.0227 ± 0.0068 (n = 5) 0.62 ± 0.18	0.00251 ± 0.00196 (n = 3) 0.07 ± 0.05

membranes had to be determined in separate experiments. Table 1 summarizes specific activities and enrichment factors of marker enzymes in isolated brush-border caps from rat distal colon. Table 2 shows the same data for isolated brush-border membranes from the entire rat colon and the corresponding recoveries are presented in Table 3.

In order to test the general validity of the established isolation procedure, the same method was applied to the rat small intestine. In Tables 4 and 5

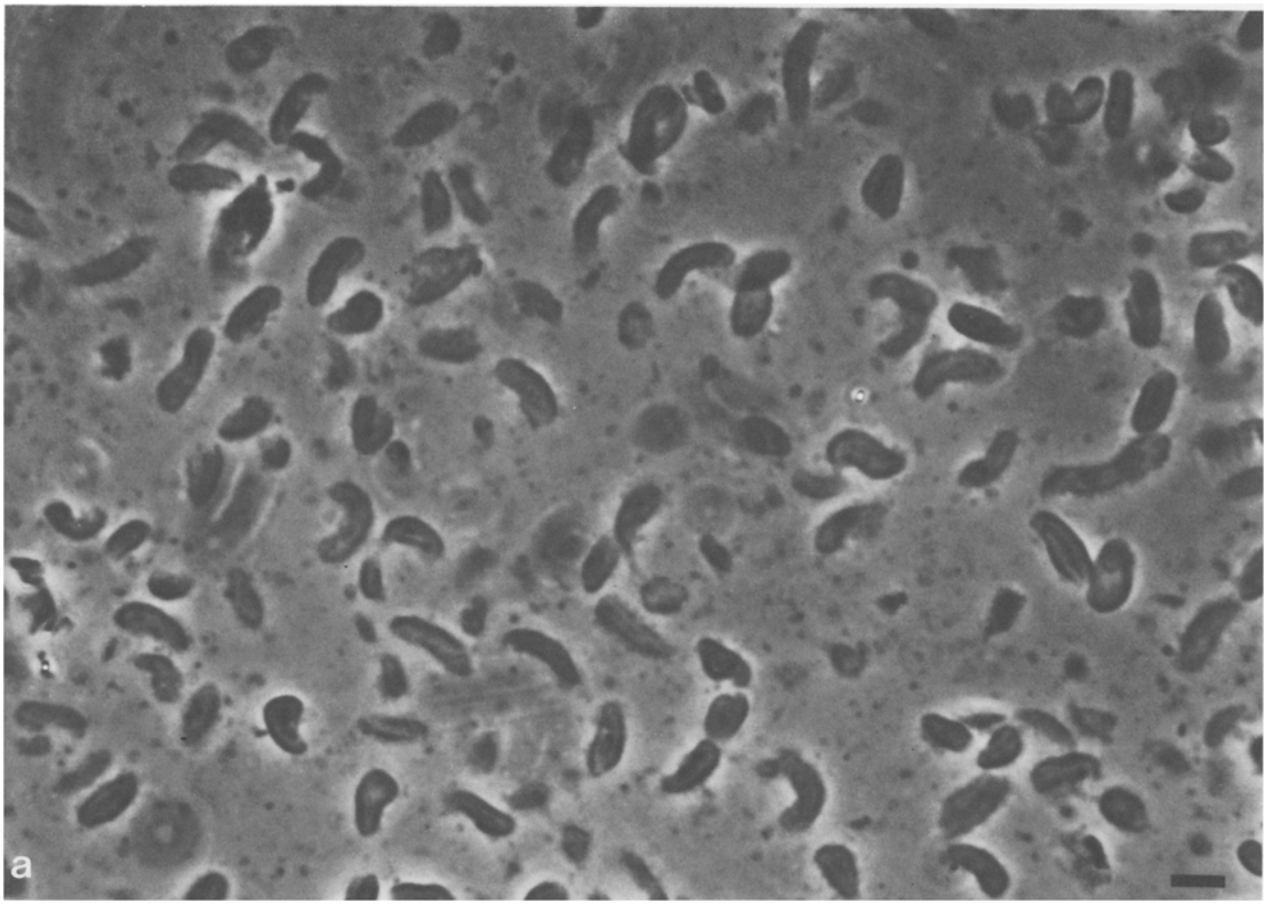


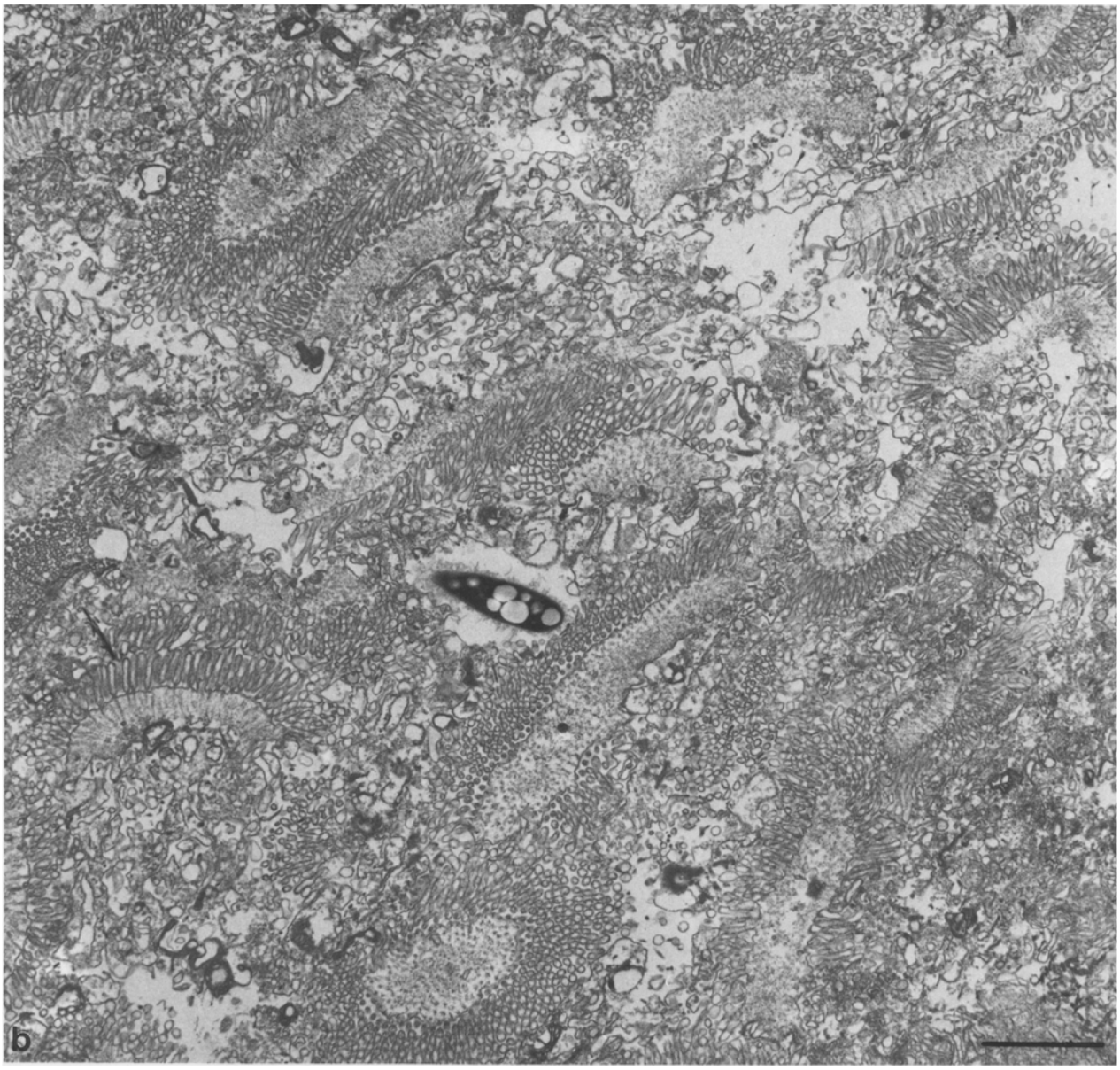
Fig. 3. Isolated brush-border caps (fraction P3b) from rat colon. (*a*) Phase-contrast micrograph (bar: 10 μm); (*b facing page*) electron micrograph (bar: 1 μm, magnification 9000-fold). The isolated brush-border caps were fixed in suspension with 1% glutaraldehyde in phosphate-buffered saline at 4°C overnight followed by 1% osmium tetroxide in phosphate-buffered saline for 1 hr at 4°C and then processed for transmission electron microscopy.

specific activities, enrichment factors and recoveries of marker enzymes of rat small intestinal brush-border membranes are given. The majority of alkaline phosphatase and aminopeptidase N were found in the fractions containing the purified brush-border caps (P3b). Furthermore, the disruption of the brush-border caps by Tris followed by sucrose density gradient centrifugation led to an additional approximately twofold purification of the brush-order membranes (FI). The final purity of the small intestinal brush-border membrane was comparable to that of other widely used procedures (for reviews *see* [18, 25]). The same experiments were performed with rabbit proximal and distal colon (Tables 6 and 7) and with small intestine. They led to results that were comparable to those obtained with rats. We therefore conclude, that the developed method is indeed suitable to isolate brush-border membranes from rat and rabbit colon as suggested by its application to the small intestine.

Properties of the Membranes

To study the protein composition of the isolated colonic brush-border membranes, we subjected them to SDS-polyacrylamide gel electrophoresis and compared the pattern with that of the small intestinal brush-border membranes. The three most prominent bands above 100 kDa of rat small intestine were found to be absent from the rat colonic brush-border membrane (Fig. 4). As these bands are mainly due to sucrase-isomaltese and aminopeptidase N [13], their lack in the colonic brush-border membrane of the rat can be explained by the known absence of these hydrolases in the colon. A very similar gel pattern was obtained for rabbit colonic brush-border membranes (*not shown*).

An important question was, whether the vesicles isolated by this procedure retained their functional properties. One valid criterion which can be applied is the measurement of solute transport. As



this function is not so well characterized in the colon, we measured a small intestinal transport function, the uptake of D-glucose. This uptake was stimulated by sodium and it was faster in the presence of chloride than in the presence of sulfate (Table 8). Thus, these membranes exhibit the well-known characteristics of the D-glucose transport system of the small intestinal brush-border membranes [18] and can therefore also be used for transport studies.

Evaluation of Alkaline Phosphatase as a Marker for the Brush-Border Membrane of Rat and Rabbit Colonocytes

As previous histochemical studies on the localization of alkaline phosphatase in the rat colon have led to controversial results [12, 30] special attention was paid to this potential marker enzyme. The yield of alkaline phosphatase in the isolated brush-border caps (P3b) of rat colon was only 5% (Table 3), whereas in the small intestine 70% were recovered (Table 5). This is in our view not enough to consider alkaline phosphatase as a suitable marker for the rat colonic brush-border membrane despite the 10-fold enrichment. Such a low yield of alkaline phosphatase may even entirely reflect cross-contamination as illustrated by the fact that $(Na^+ + K^+)$-ATPase, a well-known marker for the basolateral membrane, was recovered to a similar extent in the brush-border cap fraction (large intestine: 3%, small intestine: 4%). Studies on the distribution of alkaline phosphatase on the Percoll® gradient showed a bimodal

Table 5. Recoveries of marker enzymes in brush-border caps of rat small intestine (n = 3)

	S1	S2	P3a	P3b	P3c	Total
Protein	64.0 ± 9.0	15.0 ± 4.9	0.62 ± 0.06	5.0 ± 0.8	11.7 ± 2.1	96.4 ± 2.0
Alkaline phosphatase	37.8 ± 3.8	9.9 ± 0.3	0.90 ± 0.58	70.2 ± 12.4	6.4 ± 1.8	125.3 ± 8.5
Aminopeptidase N	38.2 ± 7.1	8.4 ± 1.2	1.6 ± 0.4	59.1 ± 3.8	12.7 ± 6.1	120.0 ± 5.7
(Na^+ + K^+)-ATPase	63.2 ± 5.3	14.2 ± 1.9	0.13 ± 0.06	4.1 ± 0.4	10.4 ± 1.8	92.0 ± 4.8
KCN-resistant NADH-oxidoreductase	59.4 ± 33.6	22.8 ± 0.5	0.31 ± 0.04	0.68 ± 0.72	15.9 ± 3.0	99.1 ± 36.2
Cytochrome c oxidoreductase	51.8 ± 10.6	23.7 ± 3.9	0.33 ± 0.03	3.1 ± 0.8	12.5 ± 3.0	91.4 ± 3.7
Glucosaminidase	62.4 ± 8.1	18.3 ± 4.1	0.85 ± 0.35	3.4 ± 1.5	9.4 ± 2.2	94.4 ± 3.6

Recoveries after Tris disruption:

	FI	FII	Supernatants	Total
Protein	16.7 ± 5.5	31.3 ± 5.0	26.8 ± 2.3	74.6 ± 5.1
Alkaline phosphatase	23.7 ± 3.3	20.0 ± 6.7	15.6 ± 0.9	59.3 ± 9.8
Aminopeptidase N	29.9 ± 10.8	27.3 ± 7.5	16.3 ± 3.0	73.5 ± 9.7

The values are given as the percentage of amount determined in the homogenate. The recoveries after Tris disruption are presented as percentage of the amounts measured in the brush-border caps before Tris disruption.

Table 6. Specific activities and enrichment factors of marker enzymes in brush-border caps and brush-border membranes from rabbit colon

	Specific activity (U/mg protein) enrichment factor				
	Homogenate	P3b	FI	FII	
Proximal Colon:					
Alkaline phosphatase	59.1 ± 37.8	341.8 ± 168.9	753.6 ± 185.7	567.4 ± 427.4	(n = 4)
		7.2 ± 3.1	19.7 ± 16.9	15.5 ± 13.0	
Ouabain-sensitive	22.7 ± 9.0	26.5 ± 8.4	48.7 ± 38.8	33.1 ± 27.1	(n = 4)
p-nitrophenylphosphatase		1.3 ± 0.6	2.4 ± 2.2	1.8 ± 1.4	
KCN-resistant	273.6	310.4			(n = 2)
NADH oxidoreductase		1.1			
Cytochrome c	44.4	67.9			(n = 2)
oxidoreductase		1.5			
Glucosaminidase	43.1	17.5			(n = 2)
		0.42			
Distal Colon:					
Alkaline phosphatase	10.1 ± 13.1	21.9 ± 21.8	54.9 ± 44.2	54.4 ± 56.3	(n = 4)
		3.4 ± 1.6	19.9 ± 21.4	11.3 ± 11.0	
Ouabain-sensitive	12.0 ± 0.9	12.5 ± 7.0	42.4 ± 11.6	16.8 ± 8.2	(n = 4)
p-nitrophenylphosphatase		1.1 ± 0.7	3.6 ± 1.9	1.4 ± 0.7	
Ouabain-insensitive	3.1 ± 2.1	11.1 ± 7.4	39.3 ± 16.1	15.2 ± 8.4	(n = 4)
p-nitrophenylphosphatase		3.9 ± 1.9	16.2 ± 11.3	7.5 ± 6.7	
KCN-resistant	246.9	174.2			(n = 2)
NADH oxidoreductase		0.58			
Cytochrome c	37.1	51.2			(n = 2)
oxidoreductase		1.4			
Glucosaminidase	81.7	37.9			(n = 2)
		0.29			

Table 7. Recoveries of marker enzymes in brush-border caps of rabbit colon

	S1	S2	P3a	P3b	P3c	Total	
Proximal Colon:							
Protein	37.1 ± 9.2	24.2 ± 7.5	3.3 ± 2.8	5.1 ± 1.4	37.1 ± 11.9	106.5 ± 21.8	(n = 3)
Alkaline phosphatase	20.1 ± 7.6	8.8 ± 2.8	2.8 ± 0.9	33.4 ± 13.6	29.7 ± 10.6	94.9 ± 11.3	(n = 3)
Ouabain-sensitive p-nitrophenylphosphatase	32.8 ± 7.0	23.8 ± 8.6	0.27 ± 0.05	6.0 ± 3.9	31.3 ± 6.0	94.1 ± 21.6	(n = 3)
KCN-resistant NADH oxidoreductase	30.6	24.4	0.90	5.3	29.0	90.0	(n = 2)
Cytochrome c oxidoreductase	38.0	30.8	1.3	6.9	31.7	98.7	(n = 2)
Glucosaminidase	62.5	20.7	1.9	1.9	11.7	98.7	(n = 2)
Distal Colon:							
Protein	51.5 ± 10.0	15.4 ± 5.7	3.6 ± 3.8	2.6 ± 1.7	27.8 ± 14.9	100.7 ± 6.7	(n = 3)
Alkaline phosphatase	60.5 ± 9.3	20.4 ± 4.1	0.91 ± 0.57	8.1 ± 1.2	39.4 ± 4.1	129.3 ± 2.9	(n = 3)
Ouabain-sensitive p-nitrophenylphosphatase	68.9 ± 27.1	20.3 ± 7.2	0.54 ± 0.80	4.0 ± 4.3	22.7 ± 5.8	116.5 ± 33.4	(n = 3)
Ouabain-insensitive p-nitrophenylphosphatase	33.4 ± 14.6	6.9 ± 2.4	0.39 ± 0.30	10.0 ± 0.6	21.4 ± 24.7	69.9 ± 13.5	(n = 3)
KCN-resistant NADH oxidoreductase	54.8	22.2	1.3	1.7	24.9	104.0	(n = 2)
Cytochrome c oxidoreductase	49.7	30.6	2.5	4.6	38.6	136.0	(n = 2)
Glucosaminidase	69.0	13.9	1.1	1.0	11.9	96.7	(n = 2)

The values are given as the percentage of the amount determined in the homogenate.

distribution, but only one of the enzyme peaks was associated with the brush-border caps on the Percoll gradient (*not shown*).

Working with rabbit intestines proved to be easier since the enzyme activities and the amount of membranes obtained were usually higher than with rats. It was therefore possible to study the distribution of alkaline phosphatase activity during subcellular fractionation in more detail. The distribution of marker enzymes after centrifugation of the P2 fraction of rabbit small intestine on a Percoll® gradient is given in Fig. 5. Aminopeptidase N was exclusively found in the fractions that contained the intact brush-border caps. Thus, using the small intestine as a standard, it is evident that an enzyme which is exclusively localized in the brush-border membrane should form only a single peak on the gradient. The distribution of marker enzymes on the same gradient run with the P2 fraction from rabbit proximal and distal colon is shown in Figs. 6*a* and 6*b*. In the proximal colon, alkaline phosphatase is found strictly localized with intact brush-border caps. In contrast, in distal colon this enzyme shows a bimodal distribution, which indicates that it is also associated with membranes different from the brush border. To further explore the distribution of alkaline phosphatase in rabbit colon, we analyzed the brush-border membrane vesicles after Tris disrup-

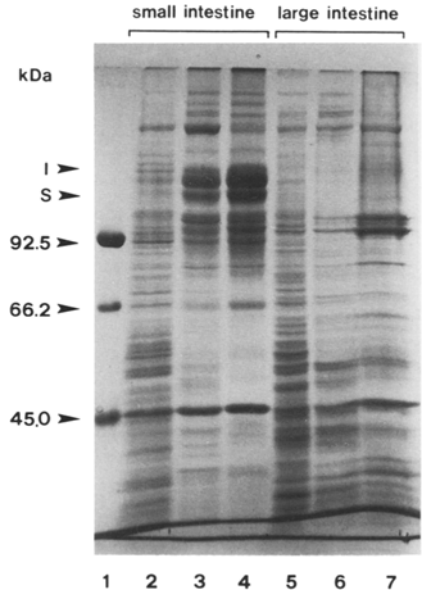


Fig. 4. SDS-polyacrylamide gel of purified brush-border membranes from rat intestine. The reduced samples were separated on a 7.5% slab gel. Lane 1: molecular weight markers (phosphorylase B = 92.5 kDa, bovine serum albumin = 66.2 kDa, ovalbumin = 45.0 kDa); lane 2, homogenate; lane 3, brush-border caps; lane 4, brush-border membranes (FI) from small intestine; lane 5, homogenate; lane 6, brush-border caps; lane 7, brush-border membrane (FI) from large intestine. I, isomaltase; S, sucrase

Table 8. Uptake of D-glucose into brush-border membrane vesicles from rat small intestine

Incubation time	15 sec	1 min	2 min	90 min
NaCl	34.6%	45.0%	61.0%	100.0%
KCl	—	14.3%	30.5%	100.0%
Na_2SO_4	2.3%	10.2%	29.4%	100.0%
K_2SO_4	1.1%	10.6%	23.4%	100.0%
Equilibria: NaCl:	84.4 pmol D-glucose/mg protein			
KCl:	97.3 pmol D-glucose/mg protein			
Na_2SO_4:	91.9 pmol D-glucose/mg protein			
K_2SO_4:	79.1 pmol D-glucose/mg protein			

Membrane vesicles were resuspended in 400 mM D-mannitol, 20 mM HEPES/Tris, pH 7.4, and incubated in the following buffer: 200 mM D-mannitol, 20 mM HEPES/Tris, pH 7.4, 0.1 mM D-glucose, 100 mM NaCl, KCl, or 50 mM Na_2SO_4, K_2SO_4.

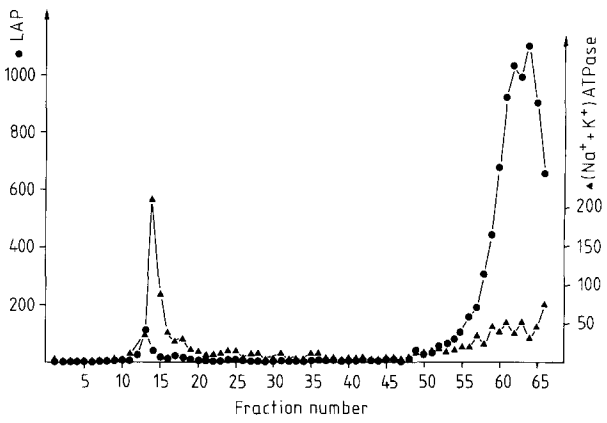


Fig. 5. Distribution of marker enzymes on a Percoll® gradient for the isolation of brush-border caps from rabbit small intestine. The activities of (Na^+ + K^+)-ATPase and aminopeptidase N (LAP) are expressed in mU/ml. Brush-border caps were found concentrated in fractions 59 to 66

tion on linear sucrose density gradients (Fig. 7). Again, there is a difference between the two colon segments: in the proximal colon, alkaline phosphatase shows a bimodal distribution, whereas in the distal colon only one peak can be found. The analysis of the same gradient with membranes from the small intestine revealed only one peak for aminopeptidase N (*not shown*). These results further support the notion that alkaline phosphatase is not a suitable marker for the colonic brush-border membrane.

Gustin and Goodman [9] found a novel K^+-stimulated, ouabain-insensitive ATPase in the brush-border membrane of rabbit distal colon. In our experiments this activity was found to comigrate with the brush-border caps of rabbit distal colon (Fig. 6*b*) which is in line with [9]. In isolated brush-border caps from rabbit distal colon, this enzyme was already enriched four times whereas ouabain-sensitive K^+-*p*-nitrophenylphosphatase was not enriched. However, we found no such enzyme activity in proximal colon or small intestine. In brush-border caps of rat distal colon, both enzymes are enriched to about the same extent (Table 1).

Since on the other hand the K^+-stimulated *p*-nitrophenylphosphatase is completely inhibited by ouabain in rabbit small intestine and proximal colon, but only to about 50% in the rat small intestine (*data not shown*), we conclude that the ouabain-insensitive K^+-*p*-nitrophenylphosphatase is absent in the apical membrane of rat distal colon. A similar conclusion was drawn by Brasitus and Keresztes [3]. The residual ouabain-insensitive activity may nevertheless be due to (Na^+ + K^+)-ATPase, as the rat enzyme is known for its low sensitivity to ouabain [20]. The distribution of ouabain-insensitive K^+-*p*-nitrophenylphosphatase on the gradients from rabbit distal colon (Figs. 6*b* and 7*b*) indeed suggests, that this enzyme is a marker for the brush-border membrane of the rabbit distal colon.

Gustin and Goodman [10] suggested, that the ouabain-insensitive K^+-*p*-nitrophenylphosphatase from rabbit distal colon and the (H^+ + K^+)-ATPase in the stomach might be related enzymes. This enzyme might play an important role in the cellular mechanism of potassium transport of the colon [26]. To test this possibility, we studied the effect of omeprazol and DCCD on this enzyme. Both compounds are known to inhibit the gastric (H^+ + K^+)-ATPase [16, 21]. The results in Table 9 show that both substances have a considerable inhibitory effect on the activity of ouabain-insensitive K^+-*p*-nitrophenylphosphatase. This indeed strongly indicates a similarity in the structure and/or reaction mechanism of these two enzymes.

Discussion

The isolation of brush-border membranes described in the present publication offers two main advantages: 1) It can be applied to animals of at least two different species without major changes and it is not restricted to the colon. 2) Its first step does not require the use of marker enzymes, as the purification of the brush-border caps can be visually followed by phase-contrast microscopy. To achieve this goal, the homogenization of the isolated cells and cell sheets had to be optimized in such a way, that most of the cells were broken, but that the brush-border caps remained intact. After a wash step (careful resuspending of the brush-order caps is essential) by low-speed centrifugation, the brush-

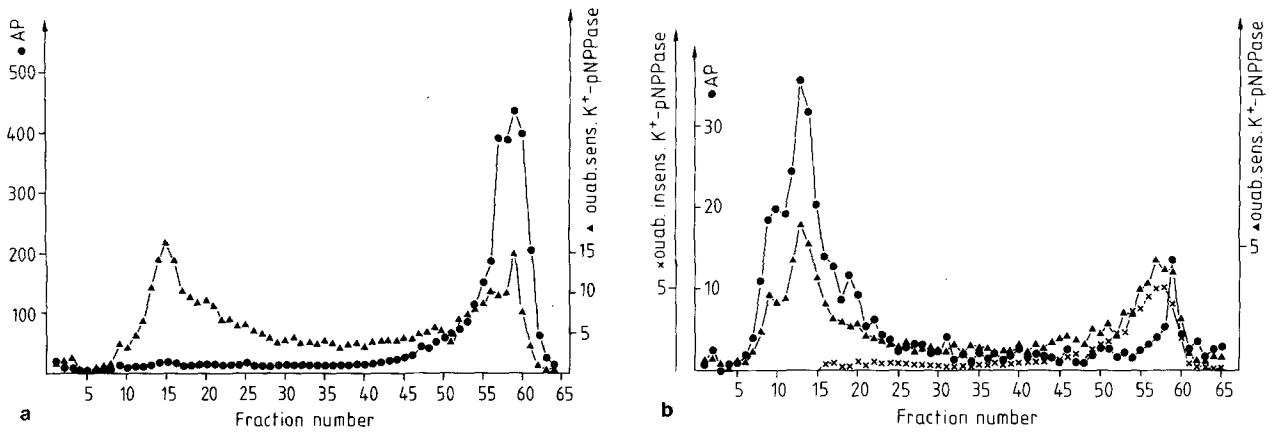


Fig. 6. Distribution of marker enzymes on a Percoll® gradient of rabbit proximal (*a*) and distal (*b*) colon for the isolation of brush-border caps. The activities of ouabain-sensitive K^+-*p*-nitrophenylphosphatase (ouab. sens. K^+-*p*NPPase), alkaline phosphatase (AP) and ouabain-insensitive K^+-*p*-nitrophenylphosphatase (ouab. insens. K^+-*p*NPPase) are expressed in mU/ml. In both gradients the brush-border caps were found concentrated in fractions 53 to 61. Ouabain-insensitive K^+-*p*NPPase was not found in fractions 1 to 15

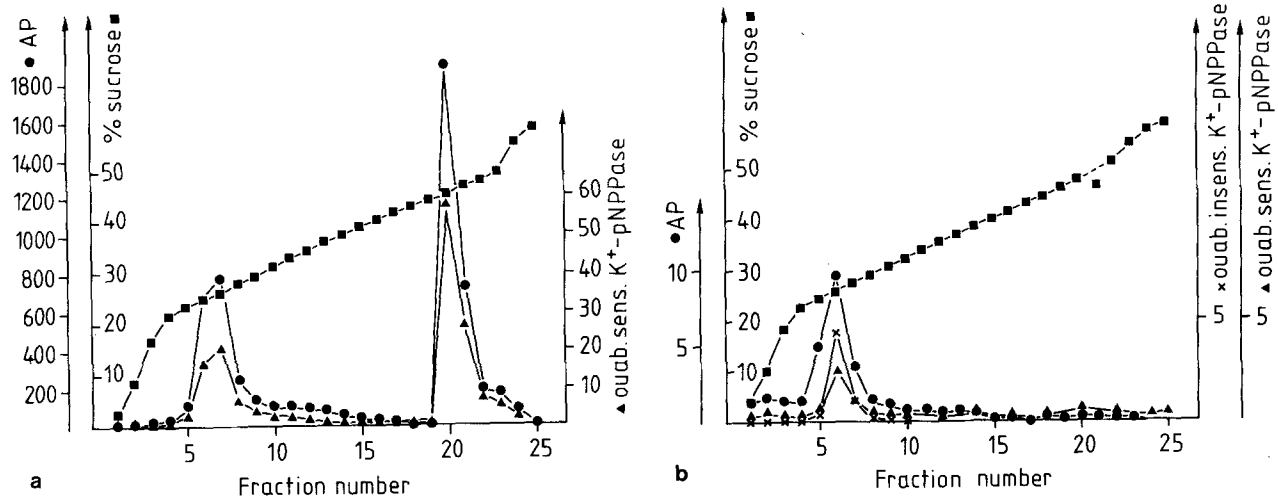


Fig. 7. Distribution of marker enzymes on sucrose density gradients with brush-border membranes from rabbit proximal (*a*) and distal (*b*) colon. The concentration of sucrose is shown as % (wt/wt). The activities of ouabain sensitive K^+-*p*-nitrophenylphosphatase (ouab. sens. K^+-*p*NPPase), ouabain insensitive K^+-*p*-nitrophenylphosphatase (ouab. insens. K^+-*p*NPPase) and alkaline phosphatase (AP) are expressed in mU/ml

border caps were further purified by Percoll® density-gradient centrifugation. This step was introduced to avoid the cumbersome filtration through glass wool which had been applied for the purification of small intestinal brush-border caps by Forstner et al. [8]. The distribution of brush-border caps on the Percoll® gradient can easily be assessed by phase-contrast microscopy and therefore this isolation is independent on marker enzymes. The introduction of this step is the most important modification with respect to the method developed by Gustin and Goodman [9]. These authors used a buffer of high pH for the Percoll gradient. However, under this condition the brush-border caps were converted into vesicles and therefore reliable marker enzymes are mandatory to recover the brush-border membrane vesicles from the gradient. In the present procedure the purified brush-border caps were converted into membrane vesicles by incubation in 1 M Tris at pH 8.2, a step previously introduced by Eichholz and Crane [6] for the small intestine. The subsequent isolation of the brush-border membranes by sucrose density-gradient centrifugation led to an interesting finding: $CaCl_2$ had to be omitted if the gradients were run with membranes from rat colon. In this case the addition of $CaCl_2$ resulted in a complete aggregation of the brush-border membranes at the bottom of the gradi-

Table 9. Inhibition of ouabain-insensitive K^+-*p*-nitrophenylphosphatase of rabbit distal colon by omeprazol and DCCD

	Omeprazol	DCCD
Homogenate	74.1 ± 22.8 (n = 3)	39.6 (n = 2)
P3b	56.9 ± 8.5 (n = 3)	98.6 (n = 2)
FI	63.6 ± 31.9 (n = 3)	96.0 (n = 2)
FII	59.7 ± 40.4 (n = 3)	100.0 (n = 2)

The activity of the enzyme was determined as the difference between buffers (II) and (III) as described in Materials and Methods at pH 7.0. The inhibitors were added from a stock solution in methanol to give a final concentration (vol/vol) of 0.9% for methanol, 0.5 mM for omeprazol and 1.0 mM for DCCD. The values indicate the percentage of inhibition with respect to the control containing only methanol.

ent. This result confirmed findings from our preliminary experiments (*not shown*), that colonic brush-border membranes from rats could not be isolated by the usual cation precipitation procedure as reviewed in refs. 18 and 25. The sucrose concentrations of the gradients used for recovering the small intestinal brush-border membrane vesicles were determined according to the distribution of marker enzymes. For the colonic membranes, the concentrations were chosen according to the protein distribution as shown in Fig. 7 for the rabbit large intestine.

This leads to the question of whether marker enzymes for the colonic brush-border membrane can be defined. According to a postulate of DeDuve [5] a marker enzyme should be associated with a single organelle and uniformly distributed within the membrane in question. Vengesa and Hopfer [30] using histochemical methods found a cysteine-sensitive alkaline phosphatase in the brush-border membrane of rat and rabbit colon. Since we found no inhibition by cysteine of alkaline phosphatase in the homogenate of rat colonocytes (*data not shown*) we made no further measurements of this enzyme activity. The findings of Vengesa and Hopfer are at variance with those of Helander [12] and Ono [19], who found a remarkable decrease or even a disappearance of the enzyme during the postnatal development of rats. Moreover, both the presence [15] or absence [4] of alkaline phosphatase in the apical layers of human colon and its absence in the brush-border membrane of the guinea pig [2] have been reported. These reports together with our own results make it difficult to accept alkaline phosphatase as a marker of the colonic brush-border membrane. Our data rather demonstrate that this enzyme is not exclusively associated with the brush-border membrane, since it co-purifies with membranes of different physical properties after repeated density-gradient centrifugation (Figs. 6 and 7). In conclusion the results strongly suggest that alkaline phosphatase does not meet the above-mentioned conditions for a marker enzyme.

The data of Table 9 suggest a similarity in the reaction mechanism of gastric ($H^+ + K^+$)-ATPase and the K^+-stimulated ouabain-insensitive *p*-nitrophenylphosphatase of rabbit distal colon. This is in line with the observations of Sachs et al. [22] who reported preliminary findings on the cross-reactivity of antibodies against the stomach enzyme with that of the rabbit distal colon. The gastric ($H^+ + K^+$)-ATPase and the colonic ouabain-insensitive K^+-ATPase thus may share structural and/or functional features.

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