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ISOLATION OF BASOLATERAL MEMBRANES FROM COLUMNAR CELLS OF THE PROXIMAL COLON OF THE GUINEA PIG

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A method for an analytical isolation of plasma membranes from columnar cells (colonocytes) of the proximal colon of the guinea pig is described. Isolation of the colonocytes was performed by a mild EDTA-chelation method. After homogenization, two subsequent sucrose gradient centrifugations (isokinetic and isopycnic) yielded a plasma-membrane fraction which was enriched 12-fold in ($Na^+ + K^+$)-ATPase activity and 8-fold in adenylate cyclase activity. It is suggested that the purified membrane fraction consists mainly of basolateral membranes of the colonocytes. Due to the lack of suitable marker enzymes, no evidence for enrichment of the brush-border membranes was obtained. Histochemical studies demonstrated that alkaline phosphatase is absent from the luminal membrane of the surface epithelial cells of the proximal colon of the guinea pig.

Introduction

The physiology of transepithelial transport processes in the colon has been well characterized [1–6]. As with the small intestine [7], experiments with membrane vesicles isolated from colonocytes might help to delineate membrane transport mechanisms involved in colonic transepithelial transport. In general, attempts to isolate membrane vesicles from an epithelium face two major problems: (a) cell separation in a epithelium with different cell types: and (b) the definition of marker enzymes for the two cell poles.

The colonic epithelium can be roughly divided into a surface and a cryptic region. Besides a small number of goblet cells, the surface epithelium is mainly composed of columnar cells with a morphology similar to that of the small intestinal villous cells [8,22]: At the luminal pole, a typical and well-organized brush border can be observed.

In practically all epithelia, ($Na^+ + K^+$)-ATPase is a reliable marker for the basal-lateral cell membrane. This is also the case for the colonocytes [18]. In contrast, the enzymatic composition of the luminal membrane varies greatly among the different epithelia. In histochemical studies on rat colonocytes, alkaline phosphatase activity has been found in the luminal membrane [18]. Cell fractionation experiments with rabbit colonocytes provided evidence for a K^+-stimulated, ouabain-insensitive phosphatase in the luminal membrane [13]. As shown in the present study, alkaline phosphatase is completely absent from the luminal membrane in columnar cells of the proximal colon of the guinea pig.

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Starting with isolated colonocytes (columnar cells) from the proximal colon of the guinea pig, we developed a method for the isolation of basolateral membranes. Our attempts to isolate the luminal membranes have been unsuccessful so far, due to the lack of specific marker enzymes.

Recently, purification of the basolateral membranes from rat proximal colonic epithelial cells was described [28]. This method yielded in a similar enrichment as described in the present study of the basolateral membranes marker enzymes.

Materials and Methods

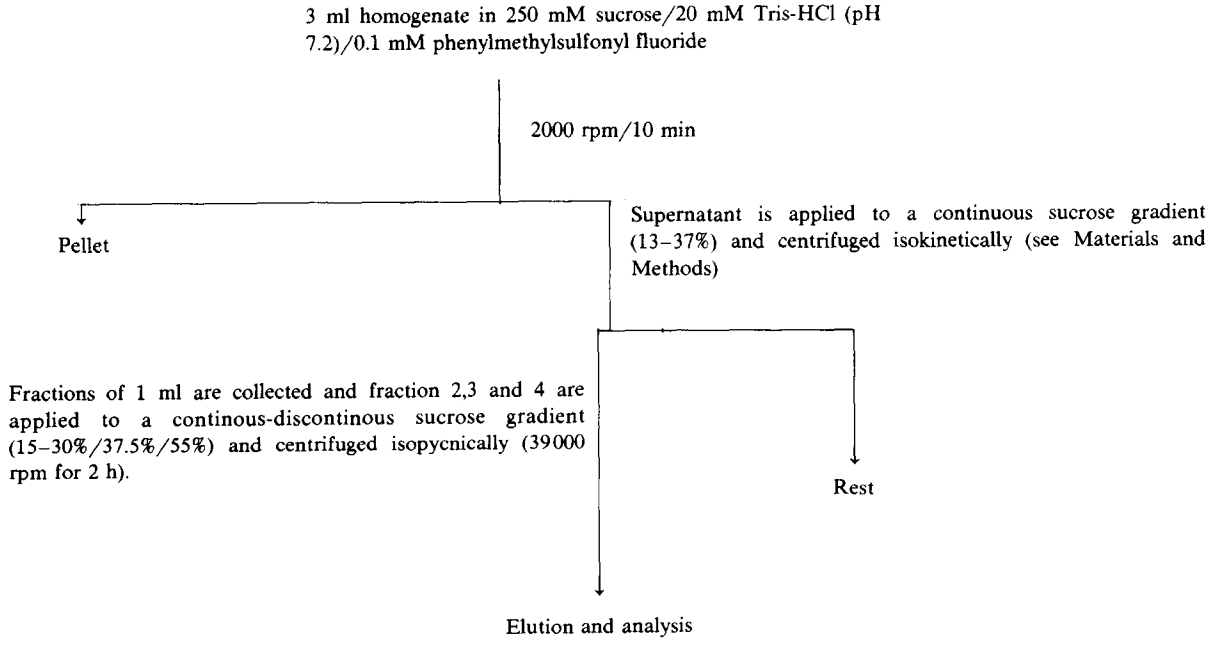
Isolation of colonocytes

Routinely, four guinea pig (male, approx. 500 g) were used for one preparation. The proximal colons (15 cm in length) were removed by colectomy after anesthesia of the animals with 40 mg ketalar and 40 mg nembutal given intramuscularly.

Colonocytes were isolated as originally described by Roediger and Truelove [9]. The colons were washed with buffer A (118.4 mM NaCl/24.9 mM $NaHCO_3$/4.7 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$ (pH 7.4)). The everted colons were filled with buffer A containing 2.5% bovine serum albumin (Sigman No. A-4503) ligated at both ends and incubated in 300 ml of buffer A containing 0.25% bovine serum albumin and 5 mM EDTA at 37°C for 30 min in a shaking water-bath (70 oscillations/min) and continuously gassed with 95% O_2/5% CO_2. The colons were then rinsed for a short time with buffer A and suspended in buffer A containing 0.25% bovine serum albumin. Colonocytes were released by gently shaking the inverted colons. Released cells were collected by a brief centrifugation (1 min at 2000 rpm, Sorvall RC-5B, GSA-Rotor) and resuspended as described below.

Membrane purification protocol

Fractionation of membranes was performed according to the scheme shown in Scheme I. All steps were carried out at 4°C. The collected colonocytes were resuspended in 3 ml 250 mM sucrose/20 mM Tris-Hepes (pH 7.2)/0.1 mM phenylmethylsulfonyl fluoride/1 mg/ml of DNAase I (Sigma No. D-4763).



Scheme I. Purification of colonocyte membranes.

Homogenization was performed using a Potter-Elvehjem homogenizer and checked by phase-contrast microscopy. About 20–40 strokes were necessary for complete disruption of the cells. The homogenate was centrifuged at 2000 rpm for 10 min (Sorvall, RC-5B, SS-34 Rotor) to dispose of the nuclei and large cellular fragments. The resulting supernatant was applied directly onto a continuous sucrose gradient (13 to 37%, w/w) and spun until the average *g*-force reached $100\,000 \times g$ (28 000 rpm in a Rotor TST-41, Kontron, Switzerland, using a Sorvall Ultracentifuge OTD 75). Fractions of 1 ml were collected and fractions 2–4 (see Fig. 4) were pooled and homogenized again (Potter-Elvehjem, ten strokes) after dithiothreitol had been added up to 0.2 mM. This membranous suspension was layered onto a 10 ml continuous-discontinuous sucrose gradient (15 → 30%/37.5%/55%) and centrifuged for 2 h in a TST 41 swing-out Rotor at 39 000 rpm ($200\,000 \times g_{av}$). The gradient was fractionated into 0.8 ml portions and analyzed.

Enzyme and protien determinations

The activities of alkaline phosphatase (EC 3.1.3.1), leucine-amino-peptidase (EC 3.4.11.2), KCN-resistant NADH oxidoreductase (EC 1.6.99.2), succinate-cytochrome *c* oxidoreductase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), lactate dehydrogenase (EC 1.1.1.27) and ($Na^+ + K^+$-ATPase (EC 3.6.1.3) were determined as described in Ref. 10.

The assay medium for the ($Na^+ + K^+$)-ATPase contained in addition 0.05% (w/v) saponin. In the absence of saponin less ($Na^+ + K^+$)-ATPase activity was determined and the values were highly variable. Adenylate cyclase activity (EC 4.6.1.1) was determined according to Rosselin and Freychet [11]. Cyclic AMP was determined using the Amersham kit No. TRK 432. Dipeptidylpeptidase IV (EC 3.4.14.5) was measured similarly to the method described by Gossrau [12]. 0.020 ml of the sample was incubated in 0.250 ml of 100 mmol/l cacodylate-HCl (pH 7.5) containing 1 mg/ml (2.5 mmol/l) L-Lys-L-Pro-4-methoxy-2-naphthylamide (Bachem, Switzerland). The substrate was solubilized first in dimethylformamide at 20 mg/ml. The incubation was performed at 37°C for 30 min and the reaction was stopped by boiling the samples for 2 min. After centrifugation of the samples in an Eppendorf table centrifuge, fluorescence in the supernatant was determined at λ_{ex} 357 nm and λ_{em} 424 nm. 4-Methoxy-2-naphthylamide (Bachem) was used as standard. Potassium-stimulated, ouabain-insensitive phosphatase activity was determined as described in Ref. 13.

Protein was determined according to Bradford [14], using the dye reagent and the standard protein mixture from Bio-Rad.

For electron microscopy, membranes were pelleted by high-speed centrifugation and resuspended in a small volume of 0.1 mol/l sodium cacodylate (pH 7.2)/2.5% glutaradehyde. Thin sectioning was performed as described by Evers et al. [15].

SDS-gel electrophoresis was performed on 8.4% polyacrylamide gels according to Laemmli [23]. The samples were denaturated in a Tris buffer (0.116 M, pH 6.8) containing 2% SDS and 0.7 M β-mercaptoethanol by heating for 2 min at 95°C. Gels were fixed and stained in 50% methanol/5% acetic acid/0.02% Coomassie blue (Bio-Rad).

Histological and histochemical methods

Specimens of the everted colon were fixed in Bouin's solution for 24 h. The samples were embedded in paraffin (Autotechnicon) and thin sections (2 μm) were cut. The sections were stained with haematoxylin-eosin (Fig. 1).

Isolated cells were studied with phase-contrast microscopy with a Zeiss photomicroscope III equipped with phase-contrast and optovar. Photographs were taken on Agfapan 25 professional or on Ilford HP 5 black-and-white films.

Alkaline phosphatase activity was demonstrated by the Gomori-calcium-cobalt method [24]. Tissue was fixed for 30 min in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Small pieces were cut with a tissue sectioner (Sorvall TC 2) and incubated for 20 min in Gomori's medium, post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 30 min, dehydrated in alcohol and embedded in Epon-araldite. 2-μm sections were cut and then photographs were taken with a Zeiss photomicroscope III.

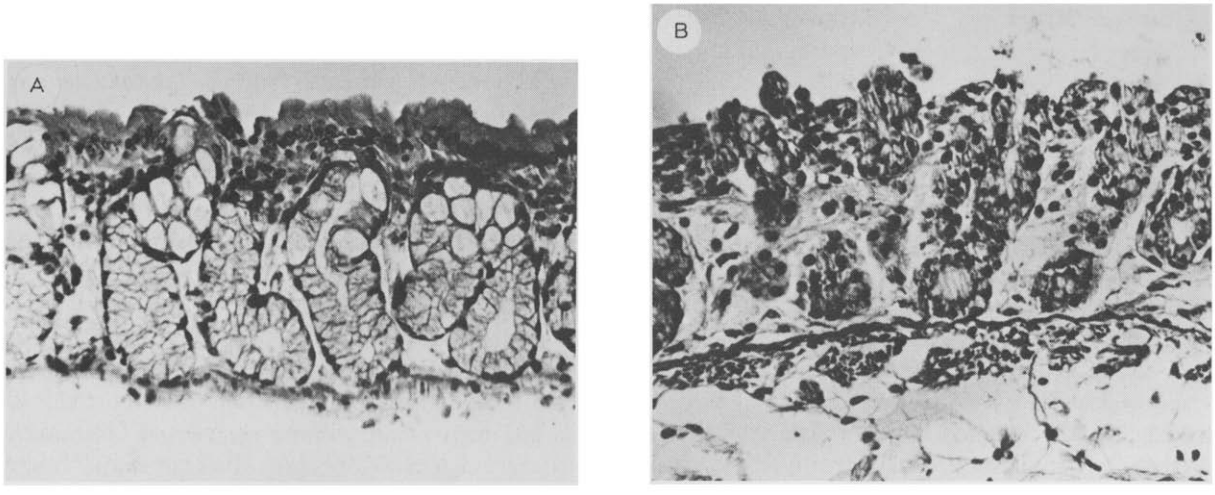


Fig. 1. Proximal colonic epithelium of the guinea pig before (A) and after (B) isolation of surface columnar epithelial cells (colonocytes). Specimens were fixed in Bouin's solution and embedded in paraffin, 2-μm sections were cut and stained with hematoxylin-eosin. In (B) surface cells are efficiently removed, while crypt cells are retained on the lamina propria. Magnification 234×.

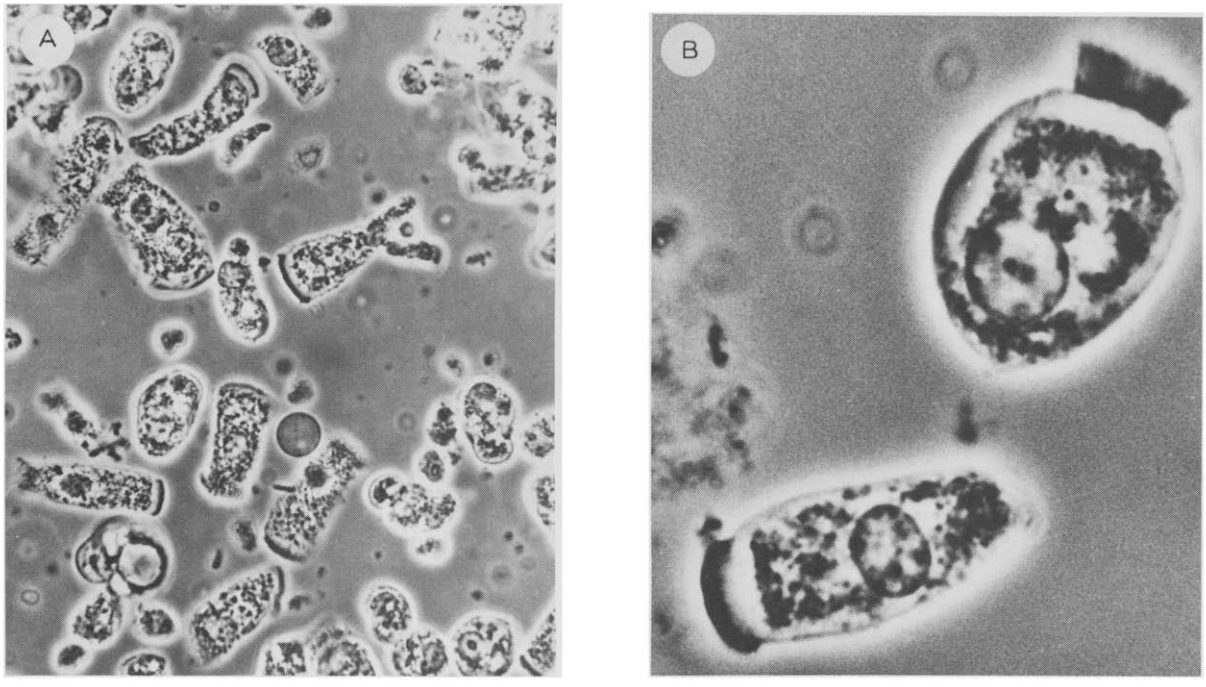


Fig. 2. Phase-contrast micrographs of isolated cells from the proximal colon of the guinea pig. In (A) an overview is given and it can be seen that mainly columnar cells with brush border are present in the preparation, either isolated cells or cells that stick together (3–4 cells maximally). In (B) isolated cells are shown in higher magnification and the brush border can be clearly seen ; cells are columnar cells with basally situated nuclei. Some cells have rounded up in the preparation but they still retain the brush border at one cell pole (B). Magnification (A) 440×; (B) 1520×.

Results and Discussion

Cell isolation

Superficial colonic epithelial cells (called colonocytes) were harvested by a mild divalent chelation method. As shown in Fig. 1, the superficial epithelial cell layer was efficiently removed by this method whereas the cryptic epithelial cell layer remained mainly intact. Examination of the isolated cells by light microscopy revealed a rather homogeneous population of columnar cells, with a distinct and well-developed brush border (Fig. 2). Based on this morphological criteria, it was estimated that the isolated cell population was approx. 85% pure with respect to the columnar epithelial cells.

Membrane isolation

For one membrane preparation, proximal colons from four guinea pigs were harvested giving 41 ± 11 ($n = 15$) mg of protein as starting material. The colonocytes were homogenized in an isotonic sucrose buffer by a glass-Teflon homogenizer. Although most of the cells were disrupted by this method, rather large cellular fragments (containing intact brush borders) could still be observed. By the inclusion of 1 mg/ml of DNAase, to destabilize actin filaments [16] presumably present in the microvillous core, an improvement of the glass-Teflon homogenization was achieved. In contrast to a homogenization in the absence of DNAase I, no structure could be identified as intact brush border after homogenization in the presence of

TABLE IA

RECOVERIES FOR MARKER ENZYMES OBTAINED DURING THE ISOLATION OF PLASMA MEMBRANES FROM COLONCYTES

The values represent the percentage of the enzyme activities found initially in the homogenate. Means ± S.D. of n individual experiments are given. For all enzymes tested, total recoveries were between 80% and 110%.

	Homogenate	Supernatant	Pellet	Peak I
(Na^+-K^+)ATPase ($n = 6$)	100	68 ±11	29 ± 8	12.5 ±2.5
Alkaline phosphatase ($n = 6$)	100	55 ± 5	35 ±11	2.7 ±0.2
Dipeptidylpeptidase IV ($n = 4$)	100	57 ± 8	44 ± 9	2.2 (2)
Succinate-cytochrome c oxidoreductase ($n = 3$)	100	65.7± 8.8	44.9± 3.5	1.3 (2)
KCN-resistant NADH oxidoreductase ($n = 4$)	100	62.4± 2.9	32.4± 3.4	1.1 ±0.2
Lactate dehydrogenase ($n = 2$)	100	62	15	0
Acid phosphatase	100	n.d.	n.d.	2 (2)
Protein ($n = 5$)	100	64 ± 8	30 ± 9	1.50±0.25

TABLE IB

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN THE HOMOGENATE AND IN PEAK I AND PEAK II AFTER ISOPYCNIC SUCROSE GRADIENT CENTRIFUGATION

All enzyme activities are given in $\mu mol \cdot min^{-1} \cdot mg^{-1}$, except for adenylate cyclase which is expressed in $pmol \cdot min^{-1} \cdot mg^{-1}$. Means ± S.D. of n individual experiments.

	Specific activities in:			Enrichement factors in Peak I
	Homogenate	Peak I	Peak II	
($Na^+ + K^+$)-ATPase ($n = 10$)	0.101 ±0.024	1.198 ±0.136	0.124 ±0.014	11.9
Adenylate cyclase ($n = 4$)	10.4 ±1.7	84 ±11	14.3 ±2.0	8.1
Alkaline phosphatase ($n = 8$)	0.038 ±0.010	0.055 ± 0.020	0.027 ±0.011	1.5
Dipeptidyl peptidase IV ($n = 4$)	0.0013±0.0002	0.0023 (2)	0.0016 (2)	1.8
Succinate-cytochrome c oxidoreductase ($n = 4$)	0.029 ±0.005	0.0082± 0.0164	0.072 ±0.040	0.3
KCN-resistant NADH oxidoreductase ($n = 8$)	0.323 ±0.047	0.340 ±0.087	0.213 ±0.059	1
Lactate dehydrogenase ($n = 2$)	0.436	0.400	0.203	0.9
Acid phosphatase ($n = 2$)	0.034	0.066	0.028	2

DNAase I (not shown). As shown in Table I, homogenization in the presence of DNAase I leads to a recovery of more than 50% of the enzymes in the suprenatant after the first low-speed centrifugation. The corresponding values obtained after homogenization in the absence of DNAase I were consistently 10–20% lower. Because DNAase I affected neither the specific activities nor the distribution patterns of the enzymes, DNAase I was routinely included in the homogenization buffer. Alternatively, the cells were homogenized by a polytron homogenizer. Thereby a more complete disruption of the cells was achieved; however, for unknown reasons, no successful purification of the plasma membranes was possible afterwards.

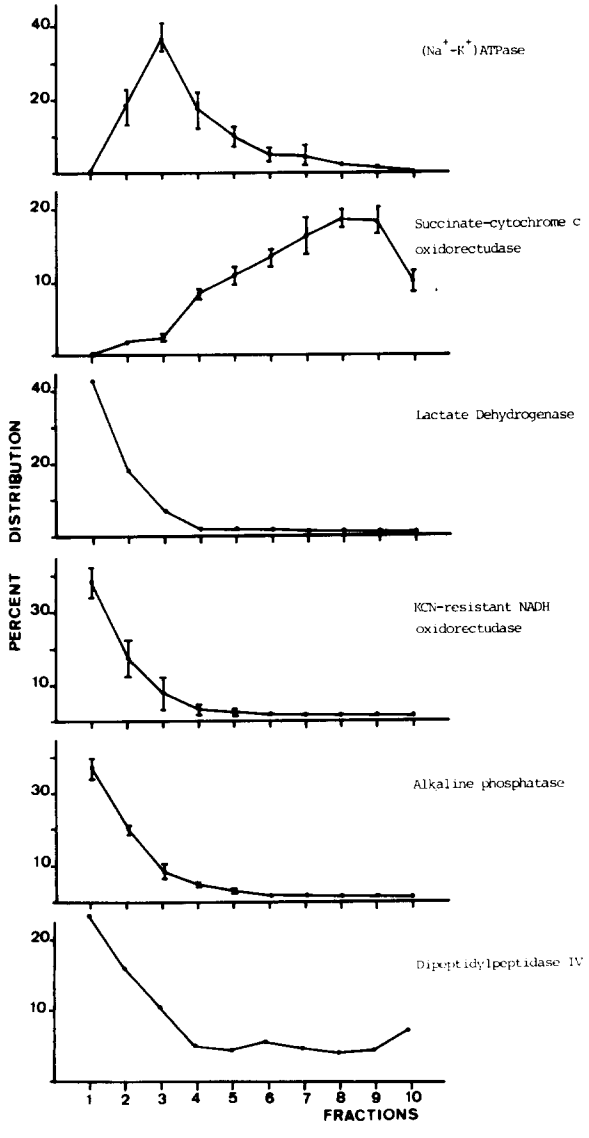


Fig. 3. Distribution of the marker enzymes following isokinetic surcose density gradient centrifugation. The values are given as means ± S.D. of at least three experiments or as means of two experiments. The fraction volume was 1 ml.

The supernatant obtained by the first low-speed centrifugation was directly applied to a continous sucrose gradient (13–37%), which was centrifuged isokinetically. The distribution pattern of the enzymes after isokinetic density-gradient centrifugation is illustrated in Fig. 3. In agreement with Rodriguez and Edelman [17], mitochondria could be clearly separated from the plasma membranes. Most of the cytochrome-*c*-oxidoreductase activity was recovered from the lower third of the gradient, whereas most of the $(Na^+ + K^+)$-ATPase activity was recovered from the upper third of the gradient. The activities of KCN-resistant NADH-oxidoreductase (endoplasmic reticulum), lactate dehydrogenase (cytosol) alkaline phosphatase and dipeptidyl peptidase IV (potential markers for the brush-border membrane) hardly penetrated into the gradient.

For further purification, fractions 2–4 of the isokinetic gradient were pooled and applied to a continuous-discontinous sucrose gradient (15 → 30%/37.5%/55%) which was centrifuged isopycnically. Enzymatic analysis of such a gradient is shown in Fig. 4. Membraneous material was collected from the interphases 30/37.5% (peak I) and 37.5/55% (peak II). Peak I is characterized by a high $(Na^+ + K^+)$ATPase and adenylate cyclase activity. These enzymes were enriched 11.9- and 8-fold (Table Ib). $(Na^+ + K^+)$-ATPase activity could be recovered from peak I with a yield of 12.5%, whereas other enzymes (mitochondria, endoplasmic reticulum, lysosomes) were recovered to much smaller extends (Table Ia). The suggestion that in peak I mainly basolateral membranes were enriched seems reasonable because $(Na^+ + K^+)$-ATPase has been shown to be present specifically in the contraluminal membrane of rat colonocytes [18] and in analogy to small intestinal and renal proximal tubular epithelial cells [19,20]. For the same reason, adenylate cyclase can be regarded as a reliable marker for the contraluminal membrane. In contrast to peak I, no enrichment for any of the

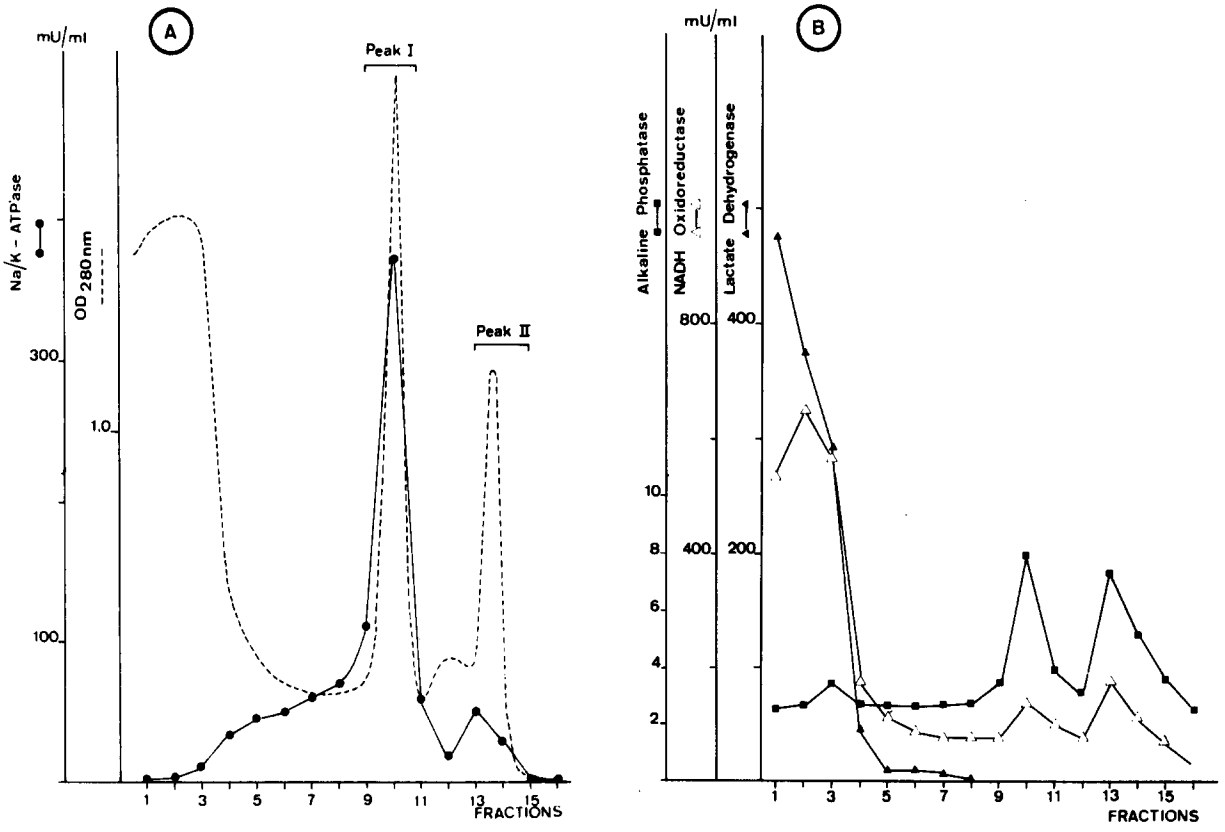


Fig. 4. Distribution of the marker enzymes following isopycnic sucrose gradient centrifugation. Fractions of 0.8 ml were analyzed for: (A) Protein at 280 nm (- - - - -); $(Na^+ + K^+)$-ATPase (●); (B) alkaline phospahtase (■); KCN-resistant NADH oxidoreductase (△); lactate dehydrogenase (▲).

enzyme activities tested was found in peak II (Table Ib).

Electron microscopy

The 30/37.5% sucrose fraction showed a fairly uniform population of membrane vesicles with a diameter of 0.08 to 0.4 μm (Fig. 5). Most of the membrane vesicles contained a more-or-less dense matrix of undefined material. No significant contamination of structure suggestive of other subcellular fractions was observed.

SDS-polyacrylamide gel electrophoresis

Analysis of the protein pattern of the 30/37.5% fraction and the homogenate (Fig. 6) indicates the loss of several proteins during the purification steps. The polypeptide pattern of the isolated membranes is characterized by a few prominent proteins of apparent molecular weight 30000, 43000 (actin?) and approx. 95000.

What about the brush-border membrane?

Generally, the enzymatic composition of the brush-border membrane of the colonocyte is not well characterized. In order to follow the brush-border membrane along the purification steps, the following enzymes were tested as possible candidates for markers of the brush-border membranes.

(i) Alkaline phosphatase. Histochemical and cytochemical techniques have been used in the past to characterize the alkaline phosphatase activity in the large intestine. The information about the localization of this enzyme in the colon epithelium has so far been contradictory. Ono [25] was unable to demonstrate alkaline phosphatase activity in the colon of adult rats whereas Vengesa and Hopfer

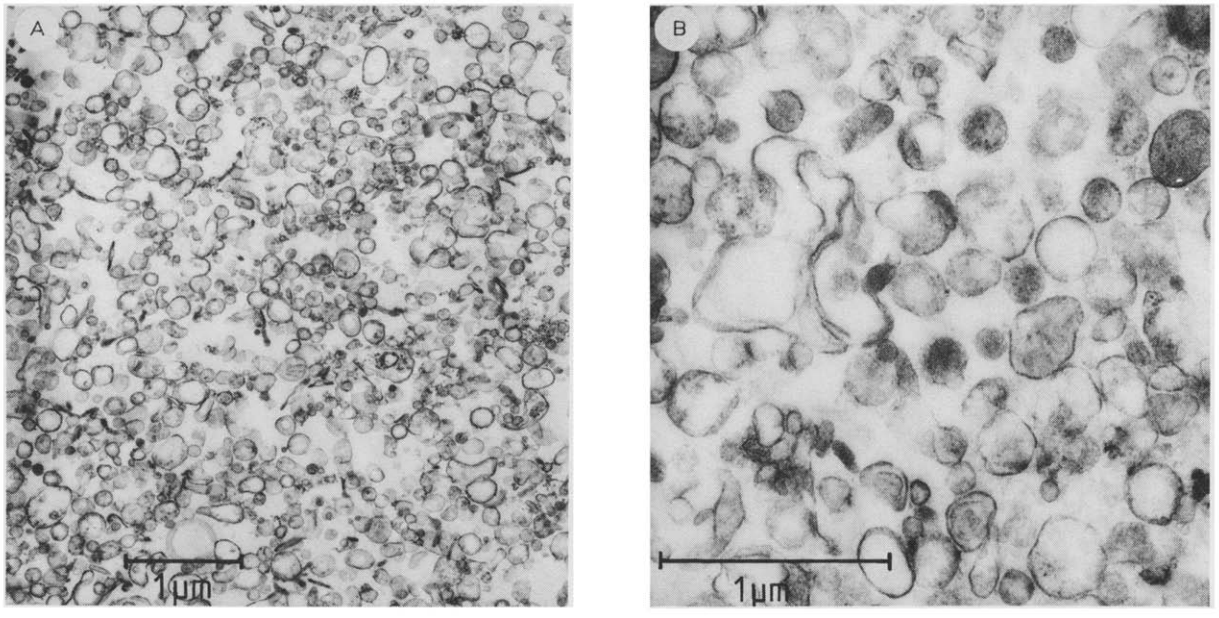


Fig. 5. Electron microscope appearance of the membrane fraction recovered from the 30/37.5% sucrose interphase. Magnification: (A) 15400×; (B) 30150×.

[18] were able to show slight alkaline phosphatase activity located at the apical cell membrane in the adult rat distal colon. However, the activity of alkaline phosphatase in the colon was rather low as opposed to the high activity in kidney or small intestine. Recently, Benjawatanapon et al. [26] and Lev and Griffiths [27] demonstrated alkaline phosphatase activity in the adult human large intestine. Activity appeared to be localized on the surface of the absorptive cells in the upper crypts, on the luminal surface in some lamina propria cells and on the luminal surface of some blood vessels [26]. Biochemical studies showed a ratio of 7.7 : 1 of alkaline phosphatase activity of the small intestine and colon, respectively [27].

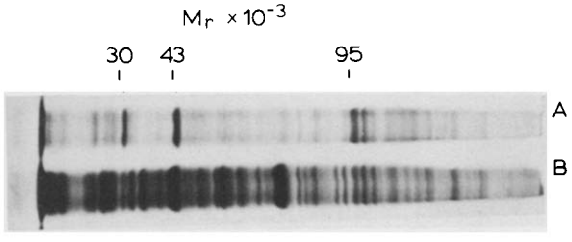


Fig. 6. Electrophoretic separation of the proteins of a homogenate of the colonocytes (B) and of the 30/37.5% membrane fraction (A). SDS-electrophoresis was performed on 8.4% polyacrylamide gels. Each lane contained the same amount of protien (100 µg).

In the present study, alkaline phosphatase in a scraped mucosa of the proximal colon of the guinea pig was 5.5-times less active (0.137 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) than in a scraped mucosa of the small intestine. Interestingly, the specific activity of alkaline phosphatase in the isolated colonocytes was only about 25% of that found in a scraping. Assuming that alkaline phosphatase is exclusively localized in the luminal membrane, this finding might suggest that the isolated cell population is rather inhomogeneous with respect to colonocytes. However, as shown in Fig. 7A, in histochemical studies alkaline phosphatase activity could not be detected in the superficial cell layer (colonocytes) nor in the crypt region of the proximal colon of the guinea pig. Most activity was detected in blood vessels in the lamina propria. This finding, which is in contrast to the reported localization of the alkaline phosphatase in the rat colon [18], indicates that the alkaline phosphatase is absent from the luminal membrane and is not a suitable marker

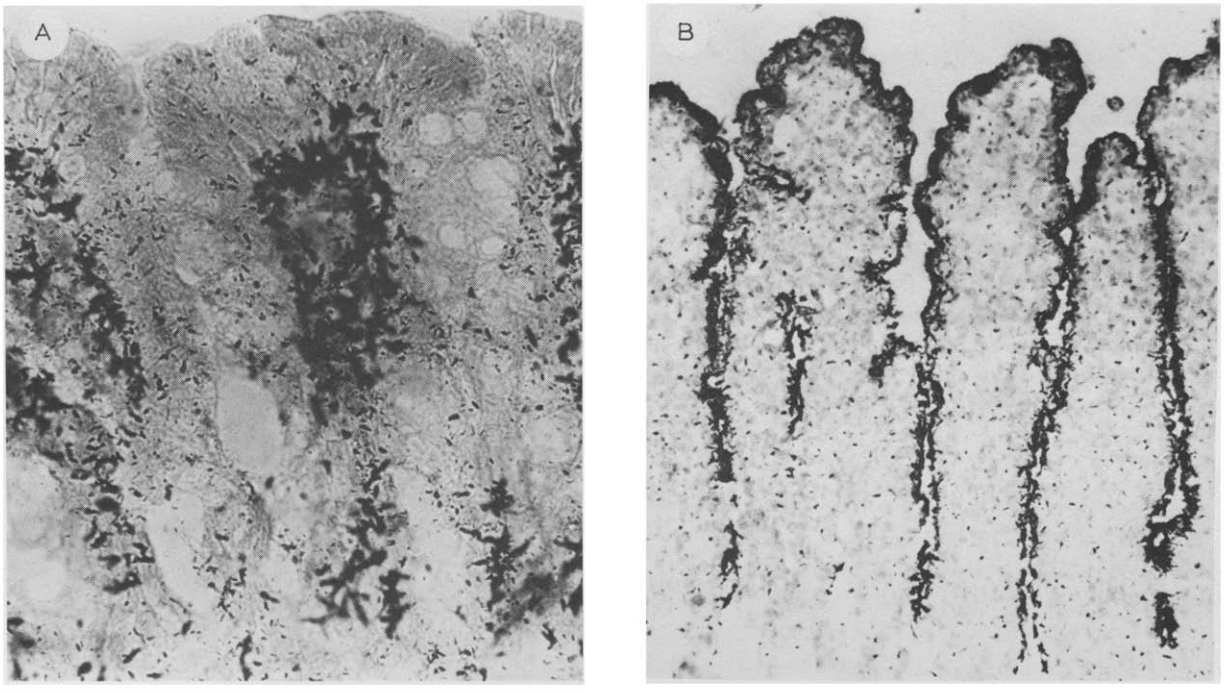


Fig. 7. (A) Photomicrograph demonstrating alkaline phosphatase activity in the proximal colonic epithelium of the guinea pig. The tissue was stained for alkaline phosphatase as described in Materials and Methods. Magnification: 420×. (B) Distribution of alkaline phosphatase in the small intestinal (ileum) epithelium of the guinea pig. Magnification 170.

for the brush-border membrane of the proximal colon of the guinea pig. Based on the histochemical localization of alkaline phosphatase, the finding that less activity of this enzyme is present in the isolated cells than in a scraping seems reasonable and might represent a criteria for the purity of the isolated cell fraction. As a control, Fig 7B illustrates alkaline phosphatase activity in the small intestinal (ileum) epithelium of the guinea pig. It is evident that the main activity is localized at the surface of the villous cells.

Along the purification steps, no evidence for an enrichment of alkaline phosphatase was obtained. As shown in Fig. 3, alkaline phosphatase activity did not enter the isokinetic sucrose gradient but showed a similar distribution pattern as lactate dehydrogenase and the KCN-resistant NADH oxidoreductase. As a control experiment, fraction one of the isokinetic gradient was also applied to the sucrose gradient which was centrifuged isopycnically. Again, most of the activity did not enter the gradient (data not shown), suggesting alkaline phosphatase in our colonocyte preparation to be present either in a soluble form or to be bound to structures of very low density.

(ii) Dipeptidylpeptidase IV. The distribution pattern of the activity of the dipeptidylpeptidase IV was very similar to that of the alkaline phosphatase (Fig. 3). As shown by Gossrau [12], dipeptidylpeptidase IV activity in the colon of the guinea pig is present in the cryptic epithelium but is absent from the cells of the superficial epithelium. In agreement with Gossrau [12], we found less activity of dipeptidylpeptidase IV in a scraping of the proximal colon (3.6 nmol · min^{-1} · mg^{-1}) than in a scraping of the small intestinal mucosa (44.4 nmol · min^{-1} · mg^{-1}). As in the case of alkaline phosphatase, the activity of dipeptidylpeptidase IV in a homogenate of the isolated cells was approx. 70% lower compared to the activity of a scraping. Based on the localization of this enzyme in the crypt cells (see Ref. 12), these findings also suggest that by the EDTA method, predominantly surface epithelial cells were harvested.

(iii) K^+-stimulated, ouabain-insensitive phosphatase. Recently, Gustin and Goodman [13] published a method for the purification of brush-border membranes of the rabbit descending colon. It was reported that a K^+-stimulated, ouabain-insensitive phosphatase activity copurifies with the alkaline phosphatase. Therefore, alkaline phosphatase and K^+-stimulated phosphatase could be markers for the brush-border membrane in the rabbit descending colon The specific activity of the K^+-stimulated, ouabain-insensitive phosphatase in a homogenate of the guinea pig colonocytes was found to be similar (0.011 ± 0.005 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) as reported in Ref. 13, but no evidence for enrichment of this enzyme was obtained.

Since on the basis of the enzymatic determinations no evidence for purification of the brush-border membranes could be obtained (see above), we also tried to label the cell surface by ^{125}I using the lactoperoxidase method [21]. The cells were labeled either in situ – by perfusion of the isolated but intact colon (labeling of the luminal membrane) – or in suspension (isolated colonocytes, labeling of the luminal and contraluminal membrane). The results obtained by these labeling experiments (data not shown) indicated a low incorporation of the label into the membranes, yet a rather high incorporation of label into other structures probably representing mucus proteins (see also Ref. 21). Whereas after a labeling of the cells in suspension a small fraction of label was detected only in peak I, no label was detected either in peak I or in peak II after labeling of the cells in the intact tissue. This again suggests that in peak I, basolateral membranes are enriched.

Conclusion

Starting from a reasonably uniform population of surface epithelial cells of the proximal colon of the guinea pig, a purified membrane fraction was obtained which is suggested to correspond with purified basal-lateral plasma membranes. Due to the lack of suitable marker enzymes, no evidence for an enrichment of the brush-border membranes could be obtained. A possible marker enzyme, alkaline phosphatase, could be shown to be absent from the luminal border of the surface cells of the proximal colon of the guinea pig.

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