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Preparation and Characterization of the Lateral and Basal Plasma Membranes of the Rat Intestinal Epithelial Cell

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A technique is described for the isolation of a plasma-membrane fraction from the rat intestinal epithelial cell which is distinct from the microvillus membrane of that cell. The isolated fraction contains only about 0.2% of the sucrase activity in the original homogenate and negligible quantities of nuclear and mitochondrial membrane markers. It contains 12% of the total Na^+,K^+-dependent adenosine triphosphatase and 7% of the alkaline phosphatase, with significant increments in specific activity of these enzymes. Multiple membrane preparations were highly reproducible with respect to the specific activities of the markers studied. The small intestine of one rat yields material containing about 1.3mg of protein. In addition an assay is described suitable for determining 5′-nucleotidase in the small intestine.

A landmark in the understanding of intestinal digestive and transport capabilities was the development of methods for the isolation of the brush-border complex of the enterocyte (Miller & Crane, 1961; Forstner *et al.*, 1968*a*). The anatomical arrangement of the enterocyte suggests that there is a distinct polarity in the cell (Trier, 1967). Further, studies of the absorptive mechanisms of many substances such as iron (Manis & Schachter, 1962), Na^+ (Barry, 1967) and Ca^{2+} (Schachter *et al.*, 1966) indicate that there are observable differences between the mechanisms of entry to and exit from the enterocyte. These considerations suggest that there may well be differences between the plasma membrane of the brush border and that of the lateral and basal boundaries of the cell. Since a method has previously been described from this laboratory for preparation of intestinal microvillus membranes (Forstner *et al.*, 1968*a*), we have directed our efforts to the preparation of plasma membranes derived from other parts of the enterocyte. The present paper describes such a method and establishes by microscopic, enzymic and chemical criteria the relative purity and morphological integrity of the resulting membranes. In particular we have concentrated on the elimination from the membrane preparation of material derived from the brush border.

Materials

Female Sprague–Dawley rats (Holtzman Co., Madison, Wis., U.S.A., or Gofmoor Farms, Westboro, Mass., U.S.A.) each weighing approx. 300–350g were used. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Non-radioactive substrates used for enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other chemicals were purchased from standard suppliers and were of the finest grade available.

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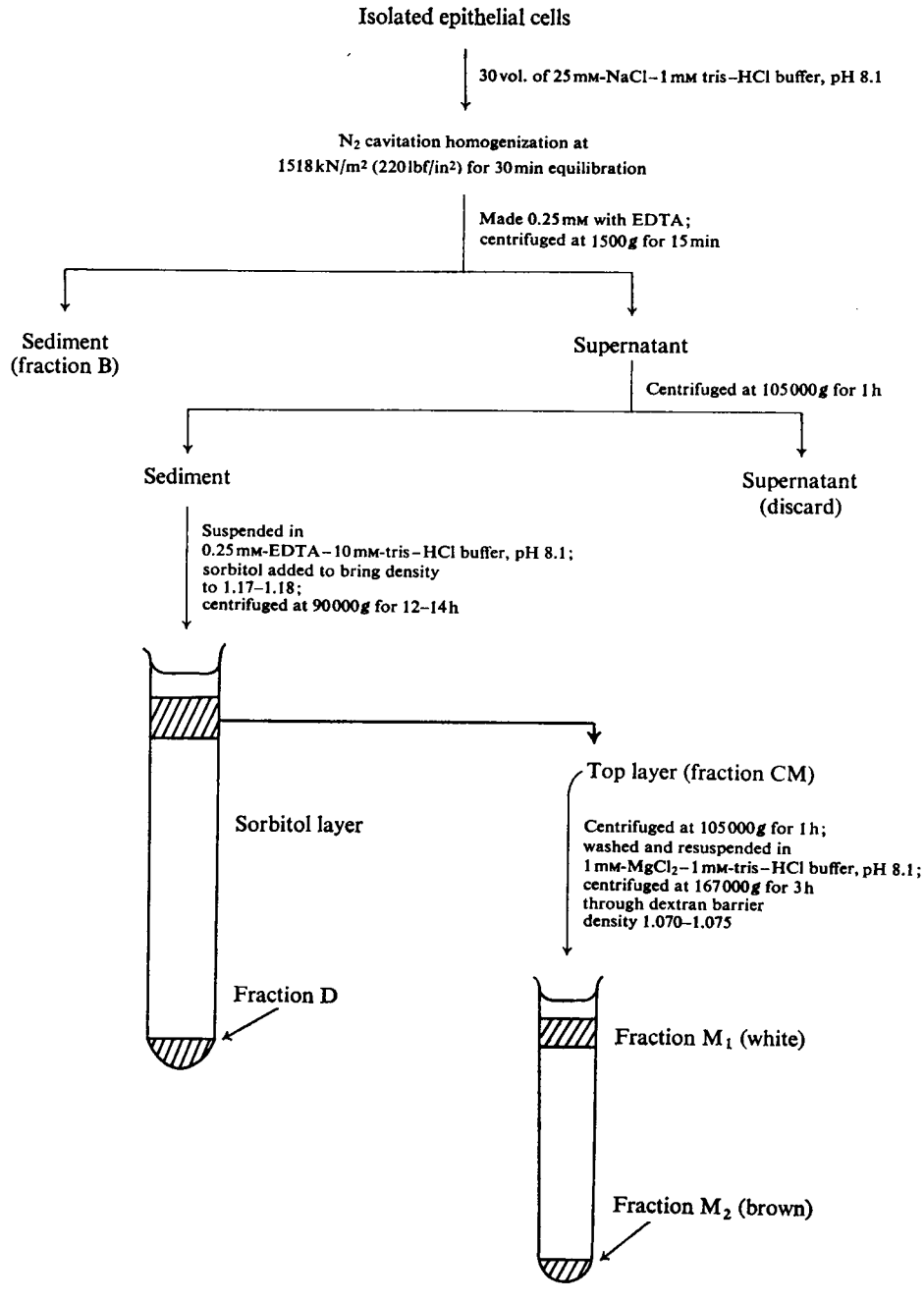
Methods

Preparation of membranes

The procedure is summarized in Scheme 1.

Isolated epithelial cells. These were prepared by a modification of the method of Stern (1966). Non-starved rats were killed by cervical dislocation, after which the duodenum was opened and the intestinal contents were flushed with ice-cold 0.15M-NaCl. The intestine between the ligament of Treitz and the ileocoecal valve was removed, again flushed with ice-cold 0.15M-NaCl, and finally filled with the citrate solution described by Stern (1966). Antibiotics were not added to this solution. After a 15min incubation at 37°C, the cells were collected into 0.15M-NaCl adjusted to pH7.4 with 0.15M-$NaHCO_3$ (solution A). In some preparations the cells were harvested by centrifuging for 3–5min at 500*g*. The pellet was resuspended in 40ml of solution A and centrifuged as described above until a clear supernatant resulted (usually three or four washes). In other instances the cell suspension was harvested and washed twice in 40ml of solution A by centrifuging for 15min at 1500*g*. Examination by phase-contrast microscopy revealed that with these procedures, most cells were present in clumps of 10–20 cells. Some single cells, pairs and triplets were found. The brush borders could be clearly delineated and a limiting membrane could be seen around the



Scheme 1. *Flow sheet for the preparation of lateral and basal plasma membranes from the rat enterocyte*

rest of the cell. Occasional free nuclei were observed. The first harvesting procedure removed many bacteria, parasites and ova, which the second did not. Erythrocytes and leucocytes were rarely seen.

Homogenization. The cells were weighed, resuspended in 30 vol. of 25 mM-NaCl–1 mM-tris–HCl buffer, pH 8.1, and placed into a Parr pressure bomb (Parr Instrument Co., Moline, Ill., U.S.A). With

continuous stirring (with a magnetic bar) the pressure within the bomb was raised to 1518kN/m² (220lbf/in²) with N_2 and the cells were allowed to equilibrate for 30min. The contents were then released into a beaker of appropriate size and sufficient 0.25M-EDTA (pH8.1) was added to make the final solution 0.25mM-EDTA. Examination of the homogenate by phase-contrast microscopy revealed free nuclei and intact brush borders. These brush borders differed from those seen when 5mM-EDTA was used as the homogenizing medium (Forstner *et al.*, 1968*a*) in having more of the top of the cell still attached to the terminal web (see the Results section). With the aid of a haemocytometer it was determined that usually more than 95% of the cell nuclei were liberated intact from the cells.

Subcellular fractionation. The homogenate was centrifuged at 1500*g* for 15min and the resulting pellet consisted mainly of brush borders and nuclei. This fraction is subsequently referred to as the B fraction. The supernatant was then centrifuged at 105000*g* for 1h in the type 30 Spinco rotor. The pellet, which contained mitochondria, endoplasmic reticulum and plasma membrane, was resuspended in 0.25mM-EDTA–10mM-tris–HCl buffer, pH8.1, with the aid of a plastic disposable syringe and needle. The final suspension could be passed easily through a 22-gauge needle. Sorbitol solution (in the same buffer) of density about 1.3 was added to the suspension to make its density 1.17–1.18, and a 25ml sample was placed in a Spinco SW 25.1 cellulose nitrate tube. It was overlaid with 5ml of 0.25mM-EDTA–10mM-tris–HCl buffer, pH 8.1, and centrifuged for 12–14h at 90000*g* resulting in the banding pattern shown in Scheme 1. (Results identical with those described have been obtained with a Spinco SW27 rotor.) After centrifugation the interfacial band was carefully removed with a Pasteur pipette, diluted 4-fold with 10mM-tris–HCl buffer, pH8.1, and the membrane fragments were sedimented at 105000*g* for 1h. The microsomal pellet was freed of adsorbed and entrapped soluble proteins by resuspension in 1mM-tris–HCl buffer, pH8.1, and re-centrifugation. The resultant osmotically shocked pellet was finally resuspended in 1mM-$MgCl_2$–1mM-tris–HCl buffer, pH8.1. For these latter two suspensions the final solution could be passed easily through a 25-gauge needle. Completeness of dispersion was most important, since aggregation of particles appeared to limit the success of the next stage of the procedure.

Separation of plasma membranes. The suspension, which contained a mixture of plasma membrane and endoplasmic reticulum, was layered on to approx. 1.5vol. of Dextran 110 (Pharmacia, Uppsala, Sweden) solution, density 1.070–1.075, in 1mM-$MgCl_2$–1mM-tris–HCl buffer, pH8.1. This discontinuous gradient was centrifuged in a Spinco SW41 rotor for 3h at 40000rev./min (approx. $3.01 \times 10^7 g$-min at the barrier). The banding pattern that resulted from this centrifugation procedure is shown in Scheme 1. The contents of the gradient were collected in two fractions by aspiration from the top of the tube with a Pasteur pipette: (1) the matieral above and at the interface (M_1); (2) the material in the dextran and the pellet (M_2). These fractions were diluted with 10mM-tris–HCl buffer, pH8.1, centrifuged at 105000*g* for 1h and resuspended in 10mM-tris–HCl buffer, pH8.1. All fractions were stored at −20°C until required.

Analytical methods and enzyme assays

Protein was determined by the method of Bramhall *et al.* (1969). Preliminary studies showed that neither sorbitol nor dextran interfered with protein determination by this method. Crystalline bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was used as a standard. RNA was measured by the method of Fleck & Munro (1962). An extinction of 1.000 at 260nm was presumed to be equivalent to 32μg of RNA/ml. DNA was measured fluorimetrically with ethidium bromide by the method of Le Pecq & Paoletti (1966). Highly purified calf thymus DNA (Sigma, type I) was used as standard. For the determination of phospholipid and cholesterol the tissue lipids were extracted by the method of Folch *et al.* (1957), by using 0.74% KCl. Phospholipids were determined by measuring P_i after hydrolysis in 70% (v/v) $HClO_4$ by the method of Bartlett (1959) as modified by Marinetti (1962). Cholesterol was determined by a modification of the method of Zlatkis *et al.* (1953) in which all volumes were decreased to one-tenth of those described. To minimize decay of colours the addition of $FeCl_3$–H_2SO_4 was carried out in a darkened room and readings of E_{560} were made after a uniform time-interval. Total lipids were measured by dichromate oxidation by using a modification of the method of Skipski & Barclay (1969) in which the total reaction volume was decreased to one-tenth of that described and centrifugation was omitted. Lipid hexose was determined with anthrone (Spiro, 1966) and total glycolipid was calculated from the hexose content on the assumption that mixed glycolipids have a molecular weight of 846 (Autilio *et al.*, 1964).

Densities were measured by pycnometry in a 200μl constriction micropipette calibrated with water.

Alkaline phosphatase (EC 3.1.3.1) was determined with β-glycerophosphate as substrate. The assay system consisted of 25μmol of potassium barbital buffer, pH9.0, 10μmol of $MgCl_2$, 10μmol of substrate and enzyme (20–50μg of protein) in a total volume of 1.0ml. The reaction was stopped with 0.2ml of 30% (w/v) trichloroacetic acid and the P_i released was measured by the method of Chen *et al.*

(1956). ATPase* (EC 3.6.1.3) was assayed by the method of Avruch & Wallach (1971). Preliminary studies showed there was no significant difference between what was measured as Na^+,K^+-dependent ATPase and what was measured as ouabain-sensitive ATPase with 1mM-ouabain. As a routine, therefore, assays were only performed with and without Na^+ and K^+. Sucrase (EC 3.2.1.26) was assayed by the two-step method of Dahlquist (1964), succinate dehydrogenase (EC 1.3.99.1) by that of Pennington (1961) and monoamine oxidase (EC 1.4.3.4) by that of Otsuka & Kobayashi (1964). The NADPH–cytochrome *c* reductase activity (EC 1.6.2.3) was assayed by the method of Clark *et al.* (1969) by using a Gilford recording spectrophotometer to record the change in E_{550}.

Because of the large amount of alkaline phosphatase present in intestinal epithelium, standard methods for the assay of 5′-nucleotidase (EC 3.1.3.5) were found inappropriate, since 5′-AMP is readily hydrolysed by non-specific phosphohydrolases. After attempting to inhibit alkaline phosphatase in numerous ways we have eventually used an assay for 5′-nucleotidase based on that of Belfield *et al.* (1970). Potassium barbital buffer (25μmol), pH9.0, 10μmol of $MgCl_2$, 10μmol of β-glycerophosphate, 0.1μmol of AMP and enzyme (20–50μg of protein) are contained in 1.0ml. About 30000d.p.m. of [^{3}H]AMP was added. After 10min incubation, with shaking, at 37°C the reaction was stopped with 0.2ml of 30% (w/v) trichloroacetic acid and samples were taken for determination of liberated adenosine and phosphate. P_i was measured by the method of Chen *et al.* (1956). Free adenosine was measured by adding 0.2ml each of 0.25M-$ZnSO_4$ and 0.25M-$Ba(OH)_2$, immediately mixing and centrifuging. Without disturbing the first precipitate, the same quantities of $ZnSO_4$ and $Ba(OH)_2$ were again added and the specimen was centrifuged. A portion of the supernatant was then counted for radioactivity in a Packard liquid-scintillation spectrophotometer by using a scintillant solution of 1vol. of Triton X-100 and 2vol. of toluene containing 4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre. Appropriate blanks and a standard were also counted. All determinations were made in duplicate. The activity of 5′-nucleotidase is expressed relative to the simultaneously derived alkaline phosphatase activity.

The principles that make separation of the two enzyme activities possible are: (i) alkaline phosphatase has a low and fairly constant affinity for its substrates ($K_m \cong 10^{-4}$M); (ii) the rates of hydrolysis of AMP and β-glycerophosphate by alkaline phosphatase are approximately equal; (iii) 5′-nucleotidase has high affinity for its substrates ($K_m \cong 10^{-6}$M) and only hydrolyses 5′-nucleotides. Thus in the presence of 10mM-β-glycerophosphate and 0.1mM-AMP (as described above) (ratio 100:1), one would expect that pure alkaline phosphatase should hydrolyse about 100 molecules of β-glycerophosphate per molecule of AMP. In a mixture of the two enzymes, 5′-nucleotidase would hydrolyse additional AMP.

Preliminary studies (A. P. Douglas, R. Kerley & K. J. Isselbacher, unpublished work) with purified calf intestinal alkaline phosphatase (Sigma, type VII), a preparation which is apparently homogeneous by isoelectric focusing (Latner *et al.*, 1971), showed that with the assay described 2nmol of adenosine was liberated for every 100nmol of P_i. Free glycerol was not determined. When the AMP was replaced with 0.1mM-*p*-nitrophenyl phosphate, 2 parts of *p*-nitrophenol were liberated per 100 parts of P_i.

By using 5′-nucleotidase from *Crotalus adamanteus* (Sigma, grade II), we found that equimolar amounts of adenosine and P_i were liberated. The initial rate of hydrolysis was the same at both 0.1mM- and 1.0mM-AMP and was not altered by the omission of β-glycerophosphate.

Mixtures of these purified enzymes behaved predictably when more than 10% of liberated P_i came from hydrolysed AMP. Below this value, the assay gave only qualitative information. Homogenates of rat, guinea-pig, hamster and rabbit small intestine were assayed by using the system described. All these homogenates liberated less than 2nmol of adenosine for every 100nmol of P_i liberated. This implies either no 5′-nucleotidase or too little to measure. A homogenate of calf intestinal mucosa, from which 5′-nucleotidase had previously been isolated (Center & Behal, 1966), released 19nmol of adenosine for every 100nmol of P_i liberated. Similarly a homogenate of rat liver, a well authenticated source of 5′-nucleotidase, liberated about 80nmol of adenosine per 100nmol of P_i.

A unit of activity corresponded to the hydrolysis of 1nmol of substrate/h at 37°C for all enzymes listed except NADPH–cytochrome *c* reductase. A unit of the latter is defined as unit change in E_{550}/min at 25°C.

Microscopy

All fractions were examined by phase-contrast microscopy during the various stages of the fractionation procedure. For electron microscopy, cells and pellets of the various subcellular fractions were fixed for 2h at 4°C in 3% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.4, and then rinsed for about 18h in 0.1M-sodium cacodylate buffer, pH7.4. The specimens were then further fixed for 1h in Dalton's chrome osmium and divided into fragments by cutting with a razor blade in a plane perpendicular to the surface of the pellets. They were then stained *en bloc* with 0.5% uranyl acetate for 1.5h, de-

* Abbreviation: ATPase, adenosine triphosphatase.

hydrated in a graded series of ethanol and propylene oxide, and embedded in Epon–Araldite in such a way as to permit thin sections to be cut through the entire depth of the pellet parallel to the axis of sedimentation. Thin sections were stained with 1.3% (w/v) lead citrate and saturated uranyl acetate in 50% (v/v) ethanol, and the entire depth of the pellets was examined in a Philips EM200 electron microscope.

Results

Preparation of plasma membrane

Various methods were investigated for the preparation of plasma membranes. The procedure described in the Methods section gave the highest yield of plasma membrane with the lowest degree of contamination by other subcellular components. N_2 cavitation was chosen for homogenization, because of its controlled reproducibility in yielding apparently small semi-permeable vesicles of plasma membrane which behave as microsomes in fractionation procedures. The many factors involved in this choice have been reviewed by Steck & Wallach (1970). The presence of 0.25M-sucrose during the disruption of the tissue and the initial stages of fractionation (Wallach & Kamat, 1966) was not desirable, since we intended to use the disaccharidase assay to monitor for contamination with microvillus membranes. Replacement with 0.25M-mannitol was abandoned because of difficulties with frothing after release of the cells from the pressure bomb. Because the intestine differs from most tissues in having a very high intracellular content of Mg^{2+}, the addition of extrinsic Mg^{2+} to the homogenization medium to prevent rupture of nuclei was unnecessary. To prevent bivalent-cation-induced aggregation of membrane vesicles, EDTA was added as described, after homogenization.

We experimented with a variety of different N_2 pressures in the bomb, but higher pressures than that finally used caused complete breakage of the brush-border complex and lower pressures failed to disrupt most of the cells. Use of a 5mM-EDTA solution such as is used to prepare isolated brush borders (Forstner *et al.*, 1968*a*) was satisfactory but, because EDTA interfered with many enzyme assays, notably that for alkaline phosphatase, we used the buffer described. The presence of 25mM-NaCl was needed to preserve intact brush borders. At the pressure used, NaCl concentrations greater than 50mM prevented total breakage of cells.

After experiments with several different dextran solutions, the one having a density of 1.075 was chosen. The results related to these experiments are discussed below in the section on 'Purification of plasma membranes'.

Characterization of subcellular fractions

The distribution of the various enzyme markers is shown in Table 1.

About 70% of the sucrase activity in the homogenate was removed by the initial low-speed centrifugation. Most of the remainder was detected in the pellet beneath the sorbitol gradient or remained in the sorbitol layer. Thus there was only a trace amount

Table 1. *Distribution of enzyme activities in the sorbitol gradient*

Specific activities are expressed as nmol of substrate hydrolysed/h at 37°C per mg of protein, except for NADPH–cytochrome *c* reductase, which is the change in E_{550}/min at 25°C. n.d. indicates no detectable activity. The percentage activity in each fraction is given in parentheses. Results represent means±S.E.M. of seven separate experiments. For further details see the text.

	Enzyme activities					
Cell fraction	Na^+,K^+-dependent ATPase	Sucrase	Alkaline phosphatase	NADPH–cytochrome reductase	Succinate dehydrogenase	Monoamine oxidase
Homogenate	8.9±0.4 (100)	6.4±0.2 (100)	23.3±2.7 (100)	0.11±0.01 (100)	0.44±0.01 (100)	0.99±0.01 (100)
Fraction B	11.0±1.1 (17)	17.8±1.1 (69)	85.4±7.7 (86)	n.d.	0.15±0.02 (8)	0.68±0.04 (17)
Sorbitol layer	7.9±0.7 (12)	1.1±0.1 (2)	1.7±0.4 (1)	0.55±0.06 (63)	0.24±0.02 (8)	1.14±0.08 (15)
Fraction D	32.8±2.3 (37)	13.0±1.3 (21)	27.4±2.3 (10)	0.12±0.02 (11)	3.44±0.24 (81)	6.09±0.47 (2)
Fraction CM	35.6±1.9 (17)	0.5±0.1 (0.4)	39.6±5.3 (9)	0.67±0.06 (26)	n.d.	0.36±0.02 (2)
Recovery (%)	96.0±1.9	91.9±1.9	105.3±3.2	100.5±2.2	98.6±1.3	98.6±1.3

Table 2. *Distribution of protein and nucleic acids in the sorbitol gradient*

The percentage of nucleic acid in each fraction is given in parentheses; n.d. indicates not detectable. For further details see the text.

	Protein (%)	RNA (μg/mg of protein)	DNA (μg/mg of protein)
Homogenate	100	64.6 (100)	133.0 (100)
Fraction B	25	24.7 (10)	319 (51)
Sorbitol layer	14	96.6 (25)	111.3 (16)
Fraction D	11	16.9 (4)	303 (31)
Fraction CM	4	6.4 (0.5)	n.d.
Recovery (%)	96.2 ± 1.9	100.3 ± 6.8	102.1 ± 11.1
No. of expts.	7	3	4

of this brush-border marker in the interfacial fraction CM.

The major quantities of mitochondrial marker enzymes (Ernster & Kuylenstierna, 1970) were associated with fraction D at the bottom of the gradient. Succinate dehydrogenase, an enzyme associated with the inner matrix of mitochondria, was not found in fraction CM, and this fraction contained negligible quantities of the marker for the outer membrane of the mitochondria (monoamine oxidase).

The distribution of smooth endoplasmic reticulum is denoted by that of NADPH–cytochrome *c* reductase. About two-thirds of this membrane remained within the sorbitol, presumably having a density in this medium of about 1.17, and about 25% of the total activity was associated with fraction CM.

The distribution of two plasma-membrane markers, alkaline phosphatase and Na^+,K^+-dependent ATPase, showed that although plasma membrane was present in all fractions, the CM fraction contained about 17% of the total homogenate ATPase and 9% of the alkaline phosphatase. Further, the specific activities of these two enzymes were 5- and 2-fold greater respectively than in the starting homogenate.

Table 2 shows the distribution of protein and nucleic acids. Fraction CM contained about 4% of the total protein, less than 1% of the total RNA and no detectable DNA.

The results indicated that at this stage the preparation designated CM consisted of plasma membranes and smooth endoplasmic reticulum and that probably only about 40% of this fraction was derived from plasma membrane.

Purification of plasma membranes

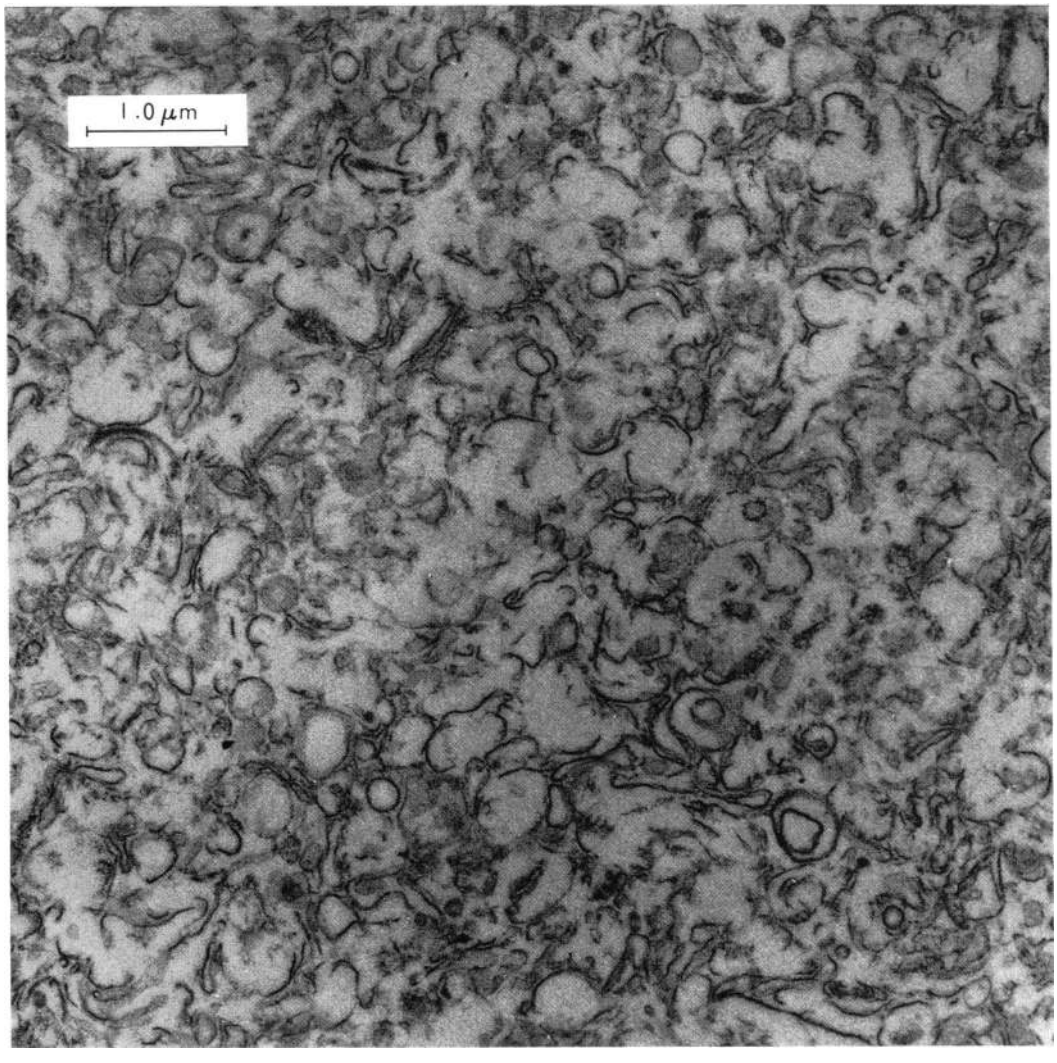
The effect of further centrifugation in dextran solutions of various densities on the distribution of Na^+,K^+-dependent ATPase and NADPH–cytochrome *c* reductase is shown in Table 3. Under all conditions tested when fraction CM was placed over a dextran barrier more of the endoplasmic-reticulum marker than of the plasma-membrane marker passed through the barrier. Optimum separation was achieved in discontinuous gradients containing 1 mM-$MgCl_2$–1 mM-tris–HCl, pH 8.1, with a density barrier at 1.075. Under these conditions over 90% of the NADPH–cytochrome *c* reductase passed through the interface, whereas approx. 65% of the applied Na^+,K^+-dependent ATPase was retained at or above the interface.

The distribution of other marker enzymes under these optimum conditions is shown in Table 4. Thus the final M_1 fraction contained a small amount of the endoplasmic-reticulum marker, had negligible quantities of sucrase and monoamine oxidase, and contained 12 and 7% respectively of Na^+,K^+-dependent ATPase and alkaline phosphatase. The specific activities of these enzymes were increased above those in the homogenate by 12- and 4-fold respectively.

5′-Nucleotidase activity was measured as described in the Methods section. In the initial homogenate there were 1.47±0.08 units/100 units of alkaline phosphatase determined simultaneously (mean ±S.E.M. of 7 expts.). The activities in fractions B, M_1 and M_2 were 1.17±0.06, 11.32±0.53 and 6.53±0.68 respectively. This demonstrates at least a 5-fold increase in activity in the M_1 fraction compared with the original homogenate. When the cells from one rat were used, fraction M_1 was found to contain about 1.3 mg of protein.

Membrane lipids

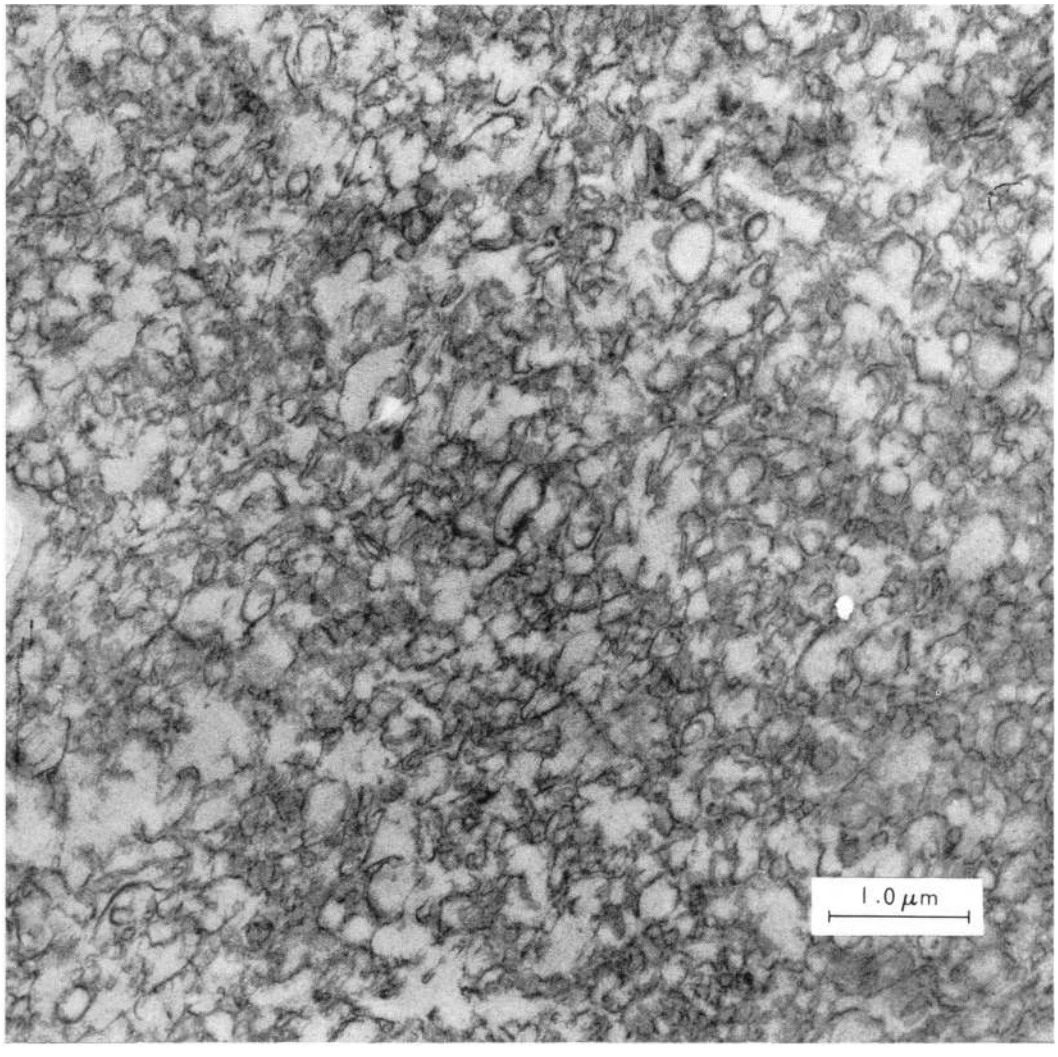
Purified membranes (fraction M_1) were assayed for total lipid, glycolipid, cholesterol and phospholipid.



EXPLANATION OF PLATES 1–4

Electron micrographs of CM, M_1 and M_2 fractions

(1) Low-power electron micrograph of sectioned membrane preparation CM. The pellet consists of a homogeneous array of membranous sheets and vesicles. There is no adherent material such as glycocalyx or ribosomes, and no structures recognizable as mitochondria. Magnification ×18000. (2) and (3) Electron micrographs of sectioned membrane preparations M_1 and M_2 respectively. The two preparations consist solely of membranous material and are not morphologically distinguishable. Magnification ×18000. (4) Membrane preparation of fraction M_1 at high magnification. The vesicles are surrounded by well-preserved trilaminar membranes. Magnification ×48600.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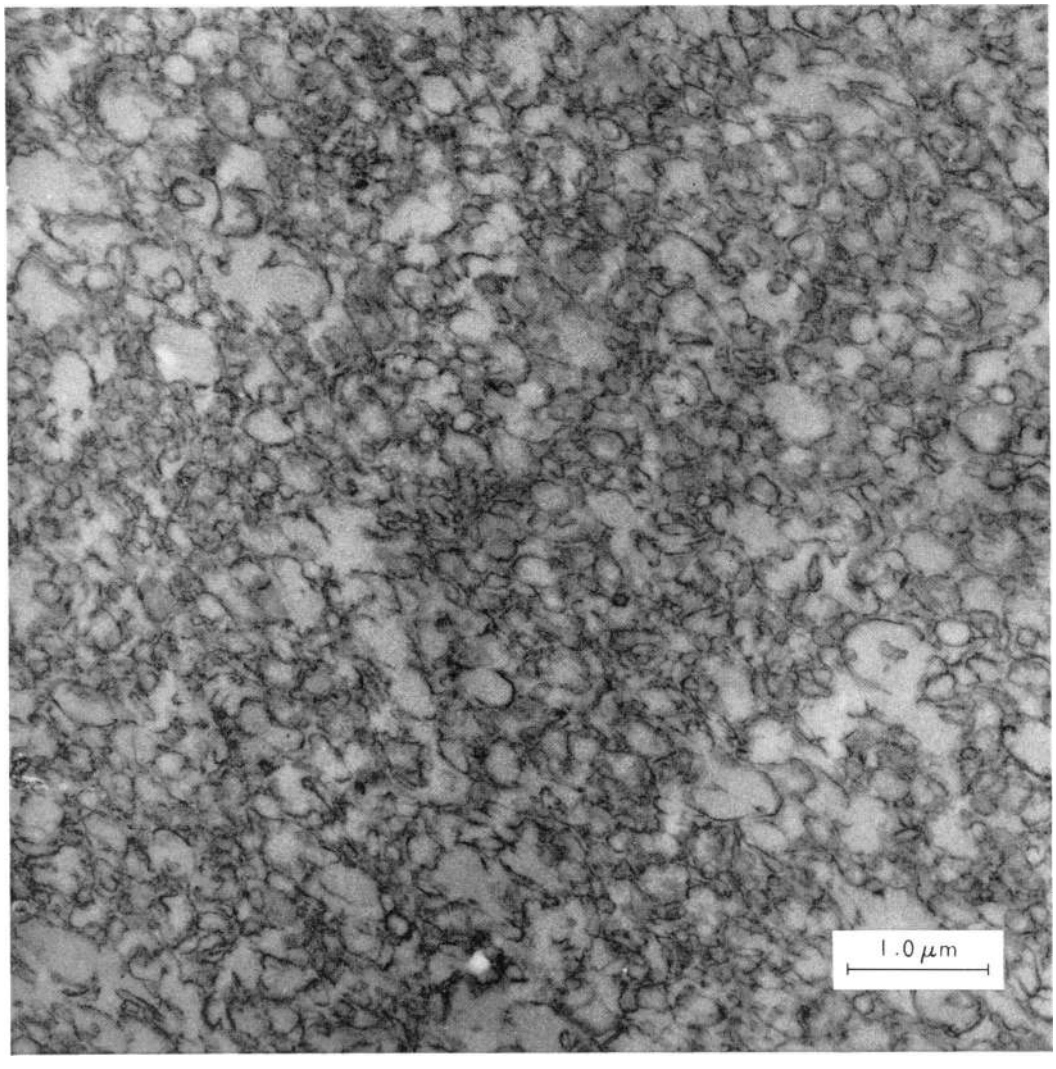
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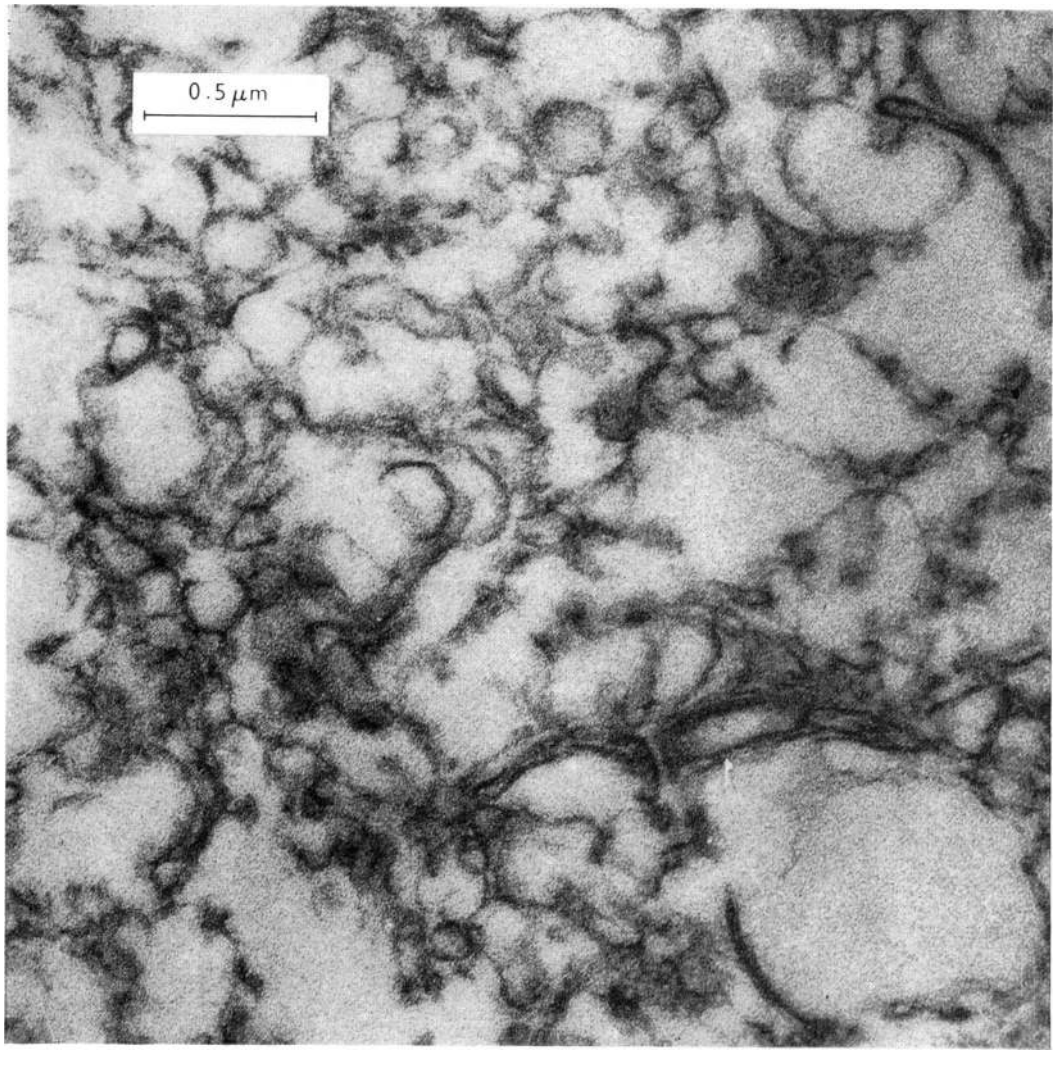
0.5 μm

Table 3. *Comparison of the distribution of plasma-membrane and endoplasmic-reticulum markers in various discontinuous dextran gradients*

Specific activities are expressed relative to those observed in the starting homogenate, which was given the value of 1.00. The percentage values given in parentheses refer to the original total activities found in the starting homogenate. All gradients were 1 mM-$MgCl_2$–1 mM-tris–HCl buffer, pH 8.1, and were centrifuged under identical conditions as described in the Methods section. M_1, Membrane fraction at and above the barrier; M_2, membrane fraction below the barrier.

Barrier density	Fraction	Na^+,K^+-dependent ATPase (nmol of substrate hydrolysed/h per mg of protein)	NADPH–cytochrome *c* reductase (ΔE_{550}/min per mg of protein)	Protein (mg)
1.063	M_1	12.1	6.9	0.2
		(5)	(1)	(0.2)
	M_2	4.9	8.1	2.9
		(15)	(25)	(3)
1.070	M_1	9.7	3.8	0.3
		(3)	(1)	(0.3)
	M_2	3.1	9.8	2.6
		(9)	(30)	(3)
1.075*	M_1	11.7±1.8	3.3±0.6	1.3±0.1
		(12)	(3)	(1.1)
	M_2	2.6±0.4	12.5±0.9	2.6±0.2
		(6)	(27)	(2.2)
1.081*	M_1	6.2±0.6	6.0±0.4	1.6±0.2
		(9)	(6)	(1.6)
	M_2	3.7±0.5	6.7±0.9	2.6±0.4
		(9)	(16)	(2.5)
1.92	M_1	7.0	9.3	2.0
		(9)	(12)	(1.3)
	M_2	55.3	10.8	1.4
		(5)	(10)	(0.9)

* Mean±S.E.M. of three experiments.

Table 4. *Distribution of some marker enzymes in a discontinuous dextran gradient (d 1.075)*

The gradient was prepared and centrifuged as described in Table 3. Relative specific activities±S.E.M from three experiments are shown. The percentage of original total homogenate enzyme activity present in each fraction is shown in parentheses.

	Enzyme activities		
Fraction	Sucrase	Alkaline phosphatase	Monoamine oxidase
M_1	0.16±0.02 (0.2)	4.3±1.0 (7)	0.05±0.04 (0.1)
M_2	0.02±0.01 (0.1)	1.0±0.2 (4)	0.61±0.02 (1.3)

The results from four preparations (±S.D.) are shown in Table 5. Glycolipid constituted 41.9±4.0%, cholesterol 15.5±1.4% and phospholipid 47.5±4.0% of the total lipid in these preparations. The cholesterol/phospholipid molar ratio was 0.52±0.03. Working with purified brush borders, Forstner *et al.* (1968*a*) found a cholesterol/phospholipid molar ratio of 1.00, a value significantly higher than that in fraction M_1. In our hands the cholesterol/phospholipid molar ratio of purified brush borders was 0.89.

Electron microscopy

Plates 1–4 show the appearance of CM, M_1 and M_2 fractions. These fractions are morphologically indistinguishable and consist almost exclusively of membrane material in both closed and open vesicular form. The membranes have the typical trilaminar 'unit membrane' structure and are approx. 9nm (90 Å) in width. The membranes have no adherent material, such as glycocalyx or ribosomes. Generally no electron-dense material was present in the numerous vesicles.

Examination of the intestine after removal of the cells showed that the basement membrane remained as an integral layer over the villus cores of lamina propria.

Table 5. *Lipid composition of fraction* M_1

Experimental details are given in the text.

Membrane preparation	Total lipid (mg/mg of protein)	Glycolipid (mg/mg of protein)	Phospholipid (mg/mg of protein)	Cholesterol (mg/mg of protein)	Cholesterol/ phospholipid molar ratio
1	1.34	0.68	0.79	0.20	0.49
2	1.38	0.52	0.68	0.17	0.50
3	1.15	0.50	0.64	0.16	0.50
4	1.23	0.44	0.69	0.20	0.57

Discussion

A technique for the isolation of a non-microvillus-derived plasma membrane from the small-intestinal epithelial cell has been described. We believe that the plasma membrane thus isolated is derived from the sides and base of the enterocyte. Because information on the subcellular localization of membrane markers, their histological distribution and stability during fractionation are incomplete, it is necessary to use multiple criteria for identity and purity.

Many of the markers used are not necessarily peculiar to the enterocyte. It was therefore important in the isolation procedure to start with a homogenate containing only intestinal epithelial cells. Intestinal scrapings, which are usually used for the isolation of brush borders, were considered unsuitable, since they would undoubtedly contain other cells from the lamina propria and even perhaps the muscle layers. Such heterogeneity is unimportant in the isolation of brush borders, because they have a distinctive and unique morphology. The preservation of the epithelial basement membrane and microscopic observation of the starting material supports the belief that any plasma membrane subsequently isolated was only derived from the enterocyte.

The electron-microscopic appearance of the final products obtained with this separation technique showed that they were smooth membranes of plasmalemma type. Morphologically they did not resemble mitochondria and the distribution of monoamine oxidase was such that any contamination with stripped-off outer mitochondrial membrane was negligible. By similar reasoning, and from observation of the nucleic acid distribution, they did not appear to be nuclear membranes or endoplasmic reticulum. (We have not attempted an assessment of the distribution of the Golgi apparatus.)

In the enterocyte, sucrase appears to be specifically located in the microvillus membrane (Eichholz, 1967; Johnson, 1967; Forstner *et al*, 1968*a*). The observed distribution of this enzyme therefore argues against the brush border being the source of our M_1 preparation. There were also no morphologically recognizable microvillus components in the final product. Alkaline phosphatase, although associated with the brush border, has also been observed in the lateral plasma membrane (Clark, 1961) and it is generally accepted as a plasma-membrane marker (Steck & Wallach, 1970). The preponderance of this enzyme in the brush border may largely relate to the greater surface area of the brush-border-associated plasma membrane. Because so much of the total alkaline phosphatase is in the microvillus membrane, it is not surprising that the M_1 fraction showed only a 4-fold increase in alkaline phosphatase specific activity. The difficulties of assessing 5'-nucleotidase in the presence of so much intestinal alkaline phosphatase has been discussed above. Within the limits thus set we believe that the M_1 fraction contains an enrichment of 5'-nucleotidase and that this provides further evidence for its plasmalemmal origin.

Finally, note should be made of the distribution of Na^+,K^+-dependent ATPase, which is widely recognized as a plasma-membrane marker (Steck & Wallach, 1970). Previous workers have concluded that this enzyme activity is at least associated with the brush border (Taylor, 1962; Berg & Chapman, 1965; Rosenberg & Rosenberg, 1968). However, these workers used crude brush borders in their studies. Our own results (Table 1) would also suggest that Na^+,K^+-dependent ATPase is present in crude brush-border preparations. Quigley & Gotterer (1969) found a high activity of this enzyme in a membrane fraction apparently not derived from brush borders. These workers used a homogenate of mucosal scrapings as their starting material and, as discussed above, their final membrane may have been derived from cell types other than the enterocyte. Fujita *et al.* (1971) have shown that highly purified brush-border membranes from rat contain two-thirds of the original sucrase but only 2–4% of the ouabain-sensitive ATPase activity. The Na^+,K^+-activated ouabain-sensitive ATPase is intimately associated with alkali-ion transport (Glynn, 1968). Barry (1967) has reviewed the evidence that the sodium pump is asymmetrically situated in the enterocyte, as was also proposed for frog skin by Koefoed-Johnsen & Ussing (1958). Thus, as Fujita *et al.* (1971) propose, it seems

likely that this enzyme activity is associated with basal and lateral plasma membranes, as we believe we have demonstrated.

We have used NADPH–cytochrome *c* reductase as a marker for smooth endoplasmic reticulum as suggested by Clark *et al.* (1969). Glucose 6-phosphatase is not suitable because of the difficulty in distinguishing it from non-specific phosphatases (Forstner *et al.*, 1968*a*) and because the rat intestinal mucosa apparently contains a potent inhibitor of the enzyme (Ginsburg & Hers, 1960; Lygre & Nordlie, 1968). If NADPH–cytochrome *c* reductase is not intrinsically present in the plasma membrane, the M_1 fraction is contaminated by about 15% with endoplasmic reticulum. This compared favourably with a 10–20% contamination of liver plasma membranes (Emmelot *et al.*, 1964; Stein *et al.*, 1968; Berman *et al.*, 1969), a 12–32% contamination of Ehrlich-ascites-cell plasma membranes (Steck & Wallach, 1970) and a 27% contamination of fat-cell plasma membranes (Avruch & Wallach, 1971). Because there are no apparent morphological features that distinguish plasma membrane from smooth endoplasmic reticulum, a true estimate of contamination by endoplasmic reticulum remains uncertain.

The plasma membrane that we have isolated from the rat intestine contains about 1.3 mg of lipid/mg of protein. This is about twice as much lipid as Forstner *et al.* (1968*b*) found in microvillus membranes. In fact, more cholesterol and phospholipid/mg of protein were found in the M_1-membrane preparation than in microvillus membranes. However, the major increase was in phospholipid content, so that the cholesterol/phospholipid molar ratio was only half that in the brush border.

The cholesterol/phospholipid molar ratio in our membrane fraction was comparable with that reported for a variety of other plasma membranes (Steck & Wallach, 1970), and was very different from the values (i.e. below 0.1) found in endoplasmic reticulum and mitochondrial inner and outer membranes (Korn, 1969).

Within the limitations of the method for determining glycolipid, a significant proportion (42%) of the total membrane lipid was glycolipid. Although this was less than Forstner *et al.* (1968*b*) found in microvillus membranes, it is significantly more than has been found in other rat plasma membranes. Liver (Skipski *et al.*, 1965), myelin (Autilio *et al.*, 1964) and erythrocytes (deGier & van Deenen, 1961) all have less than 30% glycolipid. It would obviously be worthwhile to study the glycolipid composition of the M_1 fraction and to compare it with that of the microvillus membrane.

Forstner *et al.* (1968*b*) speculated that the cholesterol/polar-lipid ratio was at least as significant in plasma membranes as the cholesterol/phospholipid ratio. They found the value for the former to be 1:3. In our membranes the cholesterol/polar-lipid (i.e. phospholipid and glycolipid) ratio was approximately the same indicating, at least in this regard, a similarity between the two membranes.

The results of the present work indicate that the M_1 fraction has properties consistent with the plasma membrane, which lines the lateral and basal limits of the intestinal epithelial cell. The ability to separate and isolate the microvillus and lateral plasma membranes should be of great help in further studies of the structure and function of these membranes and might perhaps provide further knowledge about the mechanisms involved in the functional polarity of the enterocyte.

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