Biochem. J. (1975) **152**, 71–84
Printed in Great Britain

Basolateral Plasma Membranes of Intestinal Epithelial Cells

IDENTIFICATION BY LACTOPEROXIDASE-CATALYSED IODINATION AND ISOLATION AFTER DENSITY PERTURBATION WITH DIGITONIN

By BARBARA A. LEWIS, ALAN ELKIN, ROBERT H. MICHELL and ROGER COLEMAN
Department of Biochemistry, University of Birmingham, P.O. Box 363, *Birmingham B*15 2*TT, U.K.*

(*Received* 29 *April* 1975)

1. Lactoperoxidase-catalysed iodination was used to label intestinal epithelial cell sheets with ^{125}I. The iodination was carried out under conditions that allowed little penetration of lactoperoxidase into the cells and membrane-bound ^{125}I therefore provided an effective marker for following plasma-membrane fragments through subcellular-fractionation procedures. 2. After homogenization and isopycnic zonal centrifugation through sucrose gradients two peaks of membrane-bound ^{125}I were detected. One coincided with brush-border enzymes such as alkaline phosphatase, disaccharidases and L-leucine β-naphthylamidase, whereas the other was coincident with the major peak of $(Na^+ + K^+)$-stimulated ATPase (adenosine triphosphatase), which has been thought to be concentrated in the basolateral plasma membranes of these cells. Neither peak of ^{125}I reflected the distribution of any marker for an intracellular organelle. 3. A large proportion of the $(Na^+ + K^+)$-stimulated ATPase, and thus of the basolateral plasma-membrane material, was found in a crude 'mitochondrial' fraction. It was not readily separated from mitochondria by conventional techniques of subcellular fractionation. 4. Treatment of the 'mitochondrial' fraction with digitonin increased the density of basolateral plasma membrane but had little effect on mitochondrial density. A purified preparation of digitonin-loaded basolateral plasma membranes was isolated at a density of 1.20–1.22 by isopycnic centrifugation. 5. The enzymic composition of this preparation of basolateral plasma membranes is compared with previous preparations isolated from intestinal mucosal 'scrape' materials and from isolated cells.

For many cells in organized tissues the environment to which one part of the cell surface is exposed is different from that seen by other faces of the same cell. This environmental polarity is often reflected by a cellular polarity expressed in terms of metabolism, of responsiveness to stimuli, and of differences in morphology at the cell poles. This cellular differentiation often involves polarity both in terms of the abundance and locations of intracellular organelles and also in distinct differences in the morphology, functional characteristics (enzymes, receptors, antigens) and chemical compositions of the different regions of the plasma membrane.

Initial identification of such regions in isolated subcellular fractions rests on the retention of characteristic morphology, since certain morphological features (e.g. brush borders) survive intact during some preparative procedures. However, during homogenization those regions of the plasma membrane with no distinguishing morphological features often become fragmented into vesicles or membrane sheets, the original subcellular location of which cannot be determined from morphological characteristics.

'Marker' criteria (enzymes, receptor functions etc.) are used to supplement or replace morphological characterization (see De Duve, 1971). This approach is only effective, though, if (i) plasma membranes alone contain the chosen marker, and (ii) this marker is evenly distributed throughout the entire area of the plasma membrane. The first of these assumptions is usually acceptable but the second may not always be true. In particular, distinct regions of the plasma membrane from a cell that shows polarity in its organization seem likely to possess distinct populations of components. A previously effective plasma-membrane marker may therefore only be capable of indicating the progress of one or other of the plasma-membrane regions through the purification scheme.

This problem can be overcome if a covalently linked marker material, detectable at low concentrations, is attached to the outside of the plasma membrane in preparations of intact isolated cells. Since the label should have equal access to all faces of the cell its distribution over the cell surface should be reasonably even. This type of approach, which was pioneered by Maddy (1964), has been reviewed by Wallach (1972).

Many previous studies in cell fractionation using external labelling techniques have used low-molecular-weight labelling reagents, but we decided to use lactoperoxidase-catalysed iodination (Phillips & Morrison, 1970, 1971) since the high molecular weight of the lactoperoxidase should restrict labelling to the cell surface. We chose to study the intestinal epithelial cell and have been able to confirm, refine and extend the information that has been obtained during subcellular fractionations monitored by marker enzymes alone (Quigley & Gotterer, 1969; Fujita *et al.*, 1972, 1973; Douglas *et al.*, 1972; Murer *et al.*, 1974). In particular, we have concentrated on the identification and isolation of the plasma membrane from the basolateral surface of the cell.

Experimental

Materials

Carrier-free ^{125}I (80–140mCi/ml as NaI) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, dicyclohexylcarbodi-imide and digitonin were obtained from BDH, Poole, Dorset, U.K. ATP (disodium salt) was obtained from Boehringer Corp., London W5 2TZ, U.K. Collagenase (type I) and all other fine chemicals were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Other chemicals used were of analytical grade. Lactoperoxidase (EC 1.11.1.7) was made from raw skim milk by the method of Morrison & Hultquist (1963). The final preparation was stored at 11 μM [based on $E^{1\,cm}_{412}$ 1.1×10^5 (Morrison *et al.*, 1957)] in water at −20°C and had a specific activity of 32.5mol of tetraguaicol/min per mg of protein, assayed by the method of Michell *et al.* (1970).

Methods

Preparation of cell sheets and individual cells. The small intestines were taken from young (300–600g) guinea pigs of either sex which had had free access to food and water. Epithelial-cell sheets were then prepared by the method of Evans *et al.* (1971) in which treatment with EDTA releases cells attached to each other at the terminal bar. The cell sheets were washed three times and finally suspended to four times the packed cell volume in ice-cold buffered sucrose [200mM-sucrose, 76mM-Na_2HPO_4, 19mM-KH_2PO_4 gassed with O_2+CO_2 (95:5), pH7.4]. The viability of these cells, assessed by exclusion of eosin or Trypan Blue, was always in excess of 90% and contamination with other cell types was negligible.

When preparations of individual cells were required the cell sheets were incubated with 0.25% collagenase for 3min at 37°C with gentle agitation. The cells were sedimented at 80*g* for 2min, washed twice with cold buffered sucrose and finally suspended to four times the packed cell volume. The viability of these cells was approx. 80% and they had assumed a rounded shape rather than the columnar shape of the cells in sheets.

Lactoperoxidase iodination. This method is based on that of Phillips & Morrison (1970, 1971). A portion (3.4ml) of either the suspension of cell sheets or cells, or an homogenate made from the cell-sheet suspension, was incubated at 25°C in a polypropylene centrifuge tube with 0.2ml of 11 μM-lactoperoxidase and 0.5mCi of carrier-free ^{125}I. Some 30 samples (10μl each) of 1.6mM-H_2O_2 were added at intervals of 30s; the labelling reaction was then terminated by the addition of a large excess of ice-cold buffered sucrose containing 1mM-NaI (non-radioactive). The cells were then washed with this medium by repeated centrifugation at 80*g* for 2min until negligible radioactivity was detected in the supernatant. The viability of the cells was not decreased by the labelling procedure. In later experiments the individual additions of H_2O_2 were replaced by 12 units of glucose oxidase and 3.6mg of glucose which acted as a H_2O_2-generating system (Hubbard & Cohn, 1972).

When intestinal segments were to be labelled only from the luminal surface, 25cm lengths were tied at one end and filled with buffered sucrose. Lactoperoxidase (0.2ml) and ^{125}I (0.5mCi) were introduced and the other end of the segment was tied to include a fine polythene cannula. Portions of H_2O_2 were introduced via an Agla micrometer syringe fitted with fine polyethylene tubing. The labelling reaction was terminated by washing the reagents out of the segment with a large volume of unlabelled 1mM-NaI in buffered sucrose. Cell sheets were obtained by the method of Evans *et al.* (1971) and pooled with untreated cell sheets for washing and homogenization.

The ^{125}I content of the samples was determined by using Gammavials (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) in a Nuclear–Chicago ISOCAP 300 refrigerated liquid-scintillation counter.

Fractionation procedures. Details of homogenization, gradients and centrifugal forces are given in appropriate legends. Centrifugation was carried out at 4°C in MSE '18', '50' and '65' centrifuges in angle (8×50ml), swing-out (3×20ml or 3×40ml) or zonal (B XIV titanium) rotors.

The density of fractions from sucrose gradients was determined by measurements of refractive index by using an Abbé refractometer and by reference to standard conversion tables (Weast, 1964; Dawson *et al.*, 1959). Fractions were stored at 0–2°C. All assays were performed within 3 days; for each activity the interval chosen was such that no significant loss of activity occurred before assay.

Chemical determinations. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Cholesterol was determined as described by Crawford (1958) and DNA by

the method of Burton (1956). Phosphate was measured by the method of King (1932), by the more sensitive method of Bartlett (1959), or, during ATPase (adenosine triphosphatase) and glucose 6-phosphatase determinations, by the method of Baginski *et al.* (1967).

Enzyme assays. The following enzyme assays were used under conditions in which reaction rates were proportional to protein concentration and were essentially independent of small changes in substrate concentration: alkaline phosphodiesterase I, EC 3.1.4.1 (Brightwell & Tappel, 1968); L-leucine β-naphthylamidase, EC 3.4.11.1 (Goldbarg & Rutenberg, 1958); monoamine oxidase, EC 1.4.3.4 (Brdickza *et al.*, 1968); aryl esterase, EC 3.1.1.2 (Ravin & Seligman, 1953); β-glucuronidase, EC 3.2.1.31 (Gianetto & De Duve, 1955); succinate dehydrogenase, EC 1.3.99.1 [Pennington (1961) as modified by Porteous & Clark (1965)]; β-galactosidase, EC 3.2.1.23 [Seidman & Link (1950) as modified by Hübscher *et al.* (1965)]; arylsulphatase, EC 3.1.6.1 [Roy (1953) as modified by Shephard & Hübscher (1969)]; glucose 6-phosphatase, EC 3.1.3.9 [Hübscher & West (1965) after activation by the procedure of Stetten & Burnett (1966)]; sucrase, maltase, trehalase, EC 3.2.1.26 [Dahlqvist (1968) after extensive dialysis of sucrose-containing samples against 0.1M-sodium maleate, pH6.0]. Alkaline phosphatase, EC 3.1.3.1, was measured by a modification of the method of Hübscher & West (1965), by using 1-naphthyl phosphate (Connock *et al.*, 1971); 0.1M-NaF was added to inhibit acid phosphatase activity and 0.2mM-Zn^{2+} was essential for assays of preparations that had been exposed to EDTA.

All assays for ATPase (EC 3.6.1.3) contained 30mM-Tris–HCl buffer, pH7.5, 5mM-ATP (disodium salt), 7.5mM-$MgCl_2$ and 5nmol of dicyclohexylcarbodi-imide per mg of protein. In later experiments, i.e. those after Fig. 2 and Table 1, 2mM-EDTA was included in all assays. ATPase activity was measured under three conditions, (i) in the presence of 20mM-KCl and 120mM-NaCl, (ii) in the presence of 20mM-KCl, 120mM-NaCl and 1mM-ouabain and (iii) in the presence of 60mM-Tris–HCl buffer. Appropriate blanks were used to allow for P_i present in, or released from, subcellular fractions or substrate. After incubation for 1h at 37°C the reactions were terminated with one-half of their own volume of 20% (w/v) trichloroacetic acid. After centrifugation, P_i was measured by the method of King (1932) or Baginski *et al.* (1967); the latter method alleviates the problems that arise from non-enzymic breakdown of ATP under acidic conditions. The presence of dicyclohexylcarbodi-imide in the ATPase assays eliminated a contribution from mitochondrial ATPase, which is sensitive to dicyclohexylcarbodi-imide (Beechey *et al.*, 1967). Preliminary studies showed that in the absence of dicyclohexylcarbodi-imide the values obtained in assays (ii) and (iii) often differed significantly. It was shown that this was due to the partial inhibition of mitochondrial ATPase by Na^+ in assay method (ii). This mitochondrial ATPase was also more labile than the dicyclohexylcarbodi-imide-insensitive Mg^{2+}-ATPase. These factors caused an apparent variation in the activity of (Na^++K^+)-stimulated ATPase depending on whether it was calculated as assay (i) minus assay (ii) or as assay (i) minus assay (iii). When dicyclohexylcarbodi-imide was added to all assays, this variability was removed. When a direct measure of dicyclohexylcarbodi-imide-sensitive ATPase was required as an additional mitochondrial marker, assay (iii) was carried out in the presence and absence of dicyclohexylcarbodi-imide and the difference was taken as mitochondrial ATPase. (Na^++K^+)-stimulated ATPase was calculated as assay (i) minus assay (ii) or assay (i) minus assay (iii), and Mg^{2+}-stimulated ATPase (dicyclohexylcarbodi-imide-insensitive) was obtained from assay (iii).

Effects of digitonin on enzyme activities. At 3mg of digitonin per mg of protein, the concentration that was used as a routine in the final method, monoamine oxidase, sucrase, trehalase, esterase, glucose 6-phosphatase, β-galactosidase or arylsulphatase activities were neither stimulated nor inhibited. Alkaline phosphatase and alkaline phosphodiesterase I activities were increased 20–30% on exposure to digitonin; this factor was allowed for in calculations of recovery.

Succinate dehydrogenase was completely inhibited by digitonin at the above concentration and therefore was replaced by dicyclohexylcarbodi-imide-sensitive ATPase for determination of mitochondrial membrane distribution. This mitochondrial ATPase was activated by digitonin in 'mitochondrial' fractions; the maximum effect was seen at a ratio of 3mg of digitonin/mg of protein; thereafter the activity decreased. This increase appeared to be due to an effect of digitonin on mitochondrial permeability since the digitonin effect was only seen in 'mitochondrial' fractions prepared under iso-osmotic conditions.

(Na^++K^+)-stimulated ATPase was partially inhibited by digitonin, the effect being more dependent on the digitonin/protein ratio than the total concentration of digitonin. Inhibition at 3mg of digitonin/mg of protein was about 80% (Fig. 1).

Dicyclohexylcarbodi-imide-insensitive Mg^{2+}-stimulated ATPase was activated approx. 250% by digitonin, this effect reaching a maximum at approx. 3mg of digitonin/mg of protein. Control experiments indicated that this effect was due to increased permeability caused by the digitonin since comparable effects were produced either by Triton X-100 or by sonication in the presence of 1mM-EDTA–300mM-sucrose. The stimulatory effect of digitonin was not

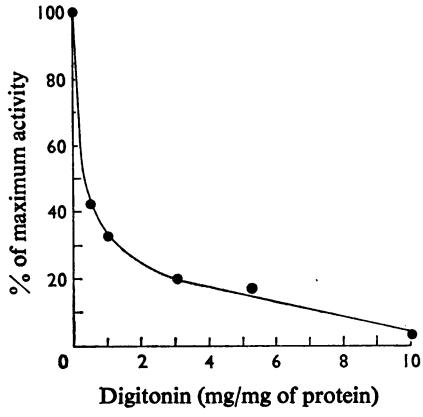


Fig. 1. *Effect of digitonin on the activity of* ($Na^+ + K^+$)*-stimulated ATPase*

Appropriate amounts of digitonin were deposited as thin films on the walls of test tubes by evaporation of an ethanol solution. Samples of a 'mitochondrial' fraction (see legend to Table 2) were added and the tubes agitated to bring the digitonin into suspension. After incubation for 15min at 4°C samples were withdrawn and their ATPase activities determined.

observed in 'mitochondrial' preparations that had been prepared in hypo-osmotic media.

The permeability changes shown by both dicyclohexylcarbodi-imide-sensitive and -insensitive ATPases had no effect on the determination of ($Na^+ + K^+$)-stimulated ATPase since this activity was measured in 'mitochondrial' material after it had been exposed to 3 mg of digitonin/mg of protein at which point these activities were maximally activated.

There was no evidence that passage through the gradient altered the effect of digitonin on the various activities, and recoveries, relative to the activities of the digitonin-treated fractions loaded on the gradients, were usually close to 100%. Thus, by relating activities recovered from gradients to activities in the digitonin-treated 'mitochondrial' fraction, a meaningful distribution profile for all markers could be obtained from the gradients. Combination of this information with the proportion of the total activity of the original homogenate that was found in the untreated 'mitochondrial' fraction allowed the calculation of meaningful values for the recovery of all markers in relation to the original homogenate.

Results

Assessment of the extent of penetration of ^{125}I into various intestinal preparations

In preliminary experiments an intestinal homogenate was subjected to lactoperoxidase-catalysed iodination and then fractionated. A low-speed pellet, containing principally nuclei and brush borders, had a specific radioactivity of ^{125}I-labelling 2.6 times that of a high-speed pellet containing the remaining particulate material. Much of this labelling proved to be present in the nuclei (Table 1). Nuclear labelling was therefore chosen as a sensitive indicator of the labelling of intracellular material.

The extent of cell surface and intracellular labelling was then assessed for various types of cell preparation after lactoperoxidase-catalysed iodination. Homogenates of the labelled preparations were made in 0.3 M-sucrose and brush-border (cell-surface) and nuclear (intracellular) fractions were then isolated (washed three times) from a 'large particulate' fraction by the method of Miller & Crane (1961) as modified by Limbrick & Finean (1970). Cross-contamination was assessed by measurement of alkaline phosphatase (brush-border) and DNA (nuclei).

When an homogenate was iodinated, nuclei were much more intensely labelled than brush borders (Table 1). Suspensions of single cells isolated by collagenase treatment were only slightly less permeable to the labelling system than the homogenate, even though 80% appeared to be impermeable to eosin. In the cell-sheet preparation, on the other hand, the predominant labelling was in the brush-border fraction and much of the labelling in the nuclear fraction appeared to be due to contamination with brush borders; little penetration of label into the interior of the cell had therefore occurred in this preparation. The results of the labelling of cells in intestinal segments *in situ* were almost identical with those obtained with cell sheets.

Cells labelled *in situ* in an intestinal segment are unlikely to carry label on their basolateral membranes, but cell sheets, which showed the same degree of localization of the label at the cell surface, do have the basolateral surface exposed to the labelling medium, since light-microscopy shows that there is distinct separation of the basolateral surfaces of the neighbouring cells in the sheets (Evans *et al.*, 1971). The preparation of cell sheets was therefore selected for subsequent studies.

Control experiments carried out with cell sheets showed that omission of lactoperoxidase from the iodination system decreased the labelling by 97%, indicating that the cell surface possessed specifically attached radioactive iodine rather than adsorbed or accumulated iodine.

Fractionation of homogenates of labelled epithelial cell sheets by isopycnic zonal centrifugation

^{125}I-labelled cell sheets were homogenized in approx. 10vol. of 5mM-EDTA (buffered with Tris to pH7.4). The homogenate was loaded above a gradient of 30–50% (w/w) sucrose (overlaid with 15% sucrose) and was centrifuged for 5h at 110000*g*

Table 1. *Distribution of ^{125}I, alkaline phosphatase and DNA between brush-border and nuclear fractions isolated from a large-particulate fraction derived from intestinal epithelial material*

Experimental details are given in the text. Protein-bound ^{125}I was defined as that precipitated by 7.5% (w/v) trichloroacetic acid. Recoveries are expressed relative to the protein-bound ^{125}I present in the samples loaded on to the gradients.

Type of cell preparations	Isolated material	Trichloroacetic acid-pptd. ^{125}I (%)	DNA (%)	Alkaline phosphatase (%)
Homogenate	Brush-border fraction	13	2	88
	Nuclear fraction	87	98	12
Separated cells	Brush-border fraction	19	4	85
	Nuclear fraction	81	96	15
Cell sheets	Brush-border fraction	67	0	75
	Nuclear fraction	33	100	25
Intestinal segments	Brush-border fraction	67	0	80
	Nuclear fraction	33	100	20

in a B XIV titanium zonal rotor. Several experiments were carried out in which homogenates, prepared by increased exposure to a rotating-blade blender, were compared. The homogenates were examined by phase-contrast microscopy and ranged from those containing a few unbroken cells through a series of stages in which the brush borders were progressively stripped of subjacent cytoplasm. Fig. 2 represents the results of one such experiment in which homogenization had been taken to the stage were most of the apical cytoplasm and basolateral membrane appeared to have been removed from the brush borders.

The first few fractions at and above the low-density end of the gradient contained ^{125}I-labelled membranes (which could be sedimented at 16×10^6g-min), ^{125}I-labelled soluble proteins (which remained in solution after centrifugation for 16×10^6g-min, but could be precipitated with trichloroacetic acid) and ^{125}I-labelled non-protein material (which remained in solution after precipitation with trichloroacetic acid). These two latter materials dominated the radioactivity profile at the low-density end of the gradient and samples of each zonal fraction were therefore centrifuged at 16×10^6g-min and the radioactivity of the pellets was assessed to obtain a correct estimate of the distribution of the radioactive membranes in the gradient.

The peak of ^{125}I at higher densities was coincident with peaks of alkaline phosphatase and of L-leucine β-naphthylamidase, both of which are known to be concentrated in brush borders. Other marker enzymes showed minor peaks in this area also. The peak of ^{125}I at lower densities occurred in a region of the gradient where there was no significant concentration of alkaline phosphatase or L-leucine β-naphthylamidase. In this region of the gradient the distribution of the ^{125}I was very similar to that of $(Na^+ + K^+)$-stimulated ATPase; it did not resemble either the protein distribution or the distribution of succinate dehydrogenase (mitochondrial inner membrane), esterase (endoplasmic reticulum) or DNA (nuclear material, results not shown). There was partial coincidence with some of the activity of monoamine oxidase, a mitochondrial outer-membrane marker, but this enzyme appeared to be rather widely distributed across the gradient.

In other experiments conducted with homogenates prepared with various extents of homogenization the main findings were that increasing the severity of homogenization decreased the proportion of ^{125}I-labelled membranes, $(Na^+ + K^+)$-stimulated ATPase, succinate dehydrogenase, esterase and monoamine oxidase in the high-density regions of the gradient. This progressive transfer of some of the ^{125}I label and enzyme markers from the heavy to the lighter end of the gradient probably reflected the loss of apical cytoplasm (including attached tags of basolateral plasma membrane) from below the brush borders; this loss was also observed by light-microscopy. In all of these experiments the low-density ^{125}I peak consistently coincided with the peak of $(Na^+ + K^+)$-stimulated ATPase. In contrast, the changes in the distributions of markers for intracellular components and the changes in distribution of particulate ^{125}I appeared independent. This indicated that no significant penetration of label to intracellular membranes had occurred.

Thus, under conditions in which little intracellular labelling had occurred, two peaks of ^{125}I-labelled material were obtained, one of which always coincided with brush-border markers. The other peak always coincided with the low-density peak of $(Na^+ + K^+)$-stimulated ATPase activity and contained little brush-border material. Both of these peaks were accompanied by variable amounts of marker enzymes for intracellular organelles, but there was no correlation between these variations and the amount of ^{125}I present. These observations suggested that the

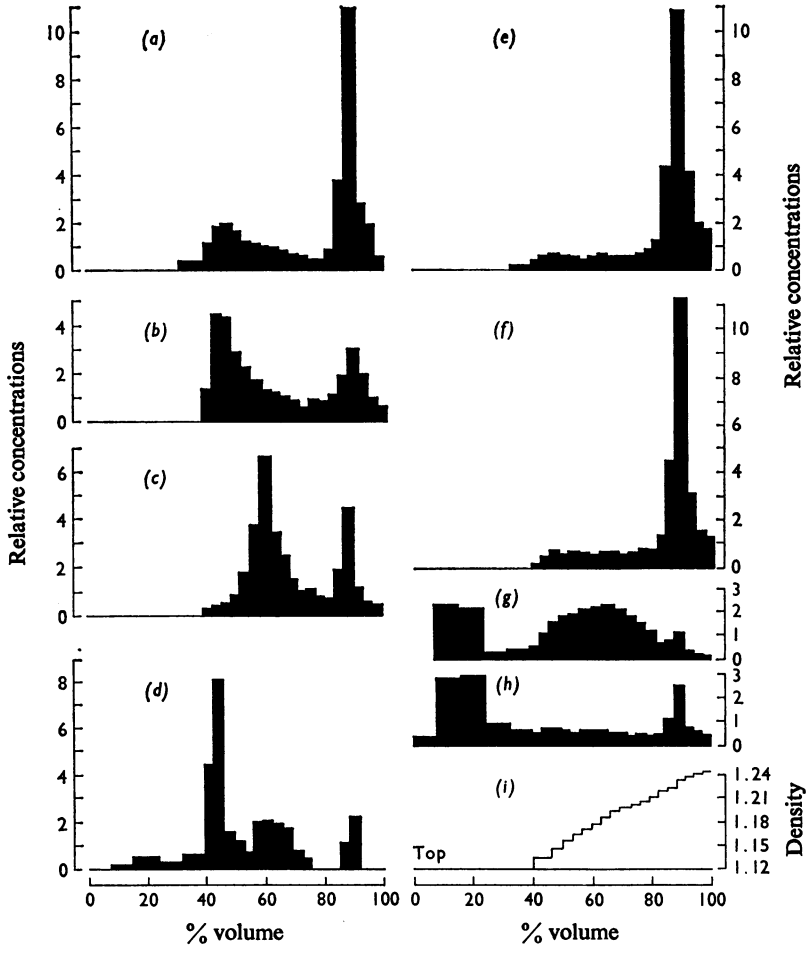


Fig. 2. *Isopycnic zonal separation of an homogenate prepared from* ^{125}*I-labelled cell sheets*

The components whose distributions are shown are: (*a*) particulate ^{125}I, (*b*) (Na^++K^+)-stimulated ATPase, (*c*) succinate dehydrogenase, (*d*) monoamine oxidase, (*e*) alkaline phosphatase, (*f*) L-leucine β-naphthylamidase, (*g*) esterase (versus naphthyl acetate), (*h*) protein and (*i*) density. The volumes of the fractions are plotted cumulatively along the abscissa and the relative concentration of each component is plotted on the ordinate. Relative concentration is defined as the concentration in the fraction divided by the concentration that would have been present if the recovered material had been evenly distributed throughout all of the fractions. After iodination and washing (see the Experimental section) cell sheets were suspended in 10vol. of 5mM-EDTA–Tris buffer, pH7.4, and were homogenized as described in the legend to Table 2. The homogenate was loaded above a gradient of 30–50% (w/w) sucrose (overlaid with 15% sucrose) in a B XIV titanium rotor and centrifuged for 5h at 110000*g*. The gradient was collected in fractions and a sample of each was taken, diluted with 3vol. of 5mM-EDTA–Tris buffer, pH7.4, and centrifuged for 1.5h at 200000*g*. The pellets were resuspended and used for determination of particulate ^{125}I (*a*). The information shown is taken from a typical experiment in which recoveries of all enzymes and of protein were in the range 85–110%.

labelling of membranous materials was largely restricted to the cell surface and that both brush borders and also a second type of plasma-membrane material, presumably basolateral, could be identified; the latter was characterized by a high activity of (Na^++K^+)-stimulated ATPase and was deficient in the characteristic enzymes of brush borders. However, the density at which the ^{125}I peak and its coincident (Na^++K^+)-stimulated ATPase were found varied from experiment to experiment. In the experi-

ment depicted in Fig 2 the modal density was 1.14; modal densities up to 1.165 were observed in other experiments. This meant that the extent of contamination of the membranes containing (Na^++K^+)-stimulated ATPase with other organelles was also variable and a simple zonal separation could not reproducibly yield highly purified preparations of basolateral plasma membranes. Attempts to identify the factor(s) responsible for this variability (pH, extent of homogenization, content of medium) were largely unsuccessful. Purification of the basolateral plasma membranes was therefore sought by alternative procedures.

(Na^++K^+)-stimulated ATPase in mitochondrial fractions prepared by differential centrifugation

When cell sheets were homogenized and then fractionated by an established procedure developed originally for intestinal mucosal 'scrape' material (Hübscher *et al.*, 1965) a considerable proportion of the (Na^++K^+)-stimulated ATPase activity was found in the 'mitochondrial' fraction. However, it proved difficult to homogenize cell sheets effectively and consistently with the Potter–Elvehjem homogenizer used in that method. Homogenization with a blade-type blender was therefore substituted and routine homogenization was continued until all cells were broken and the brush borders were, by light-microscopy, largely freed of subjacent cytoplasm and attached tags of basolateral membrane. Most of the (Na^++K^+)-stimulated ATPase and mitochondrial marker enzymes could be sedimented by somewhat lower gravitational fields than were used by Hübscher *et al.* (1965) and a 'mitochondrial' fraction prepared in this way had a decreased content of markers for brush borders, endoplasmic reticulum and lysosomes. 'Mitochondrial' fractions of this type, whose composition is described in Table 2, were used as the starting material for the remaining experiments, all of which were aimed at the isolation of a highly purified preparation of basolateral plasma membranes.

When these 'mitochondrial' fractions were subfractionated in various types of sucrose density gradients, no consistent relationship between (Na^++K^+)-stimulated ATPase and the mitochondrial enzymes was maintained on the gradients indicating that they were not common to the same organelle. No complete separation of the (Na^++K^+)-stimulated ATPase from the mitochondrial activities could be achieved on any type of gradient tested and the highest purification of (Na^++K^+)-stimulated ATPase relative to succinate dehydrogenase was only about twofold. Since these methods alone did not yield a fraction highly enriched with basolateral plasma membranes and largely devoid of intracellular organelles, we decided to try to modify selectively the density of the basolateral membrane and thereby separate it from other components of the 'mitochondrial' fraction.

Effect of digitonin on the density of basolateral membranes

Portions (5ml) of a mitochondrial fraction (4mg of protein/ml in 0.3M-sucrose, pH7.4) were put into

Table 2. *Composition of 'mitochondrial' fraction*

Cell sheets were homogenized in 9 vol. of buffered sucrose (0.3M-sucrose, buffered to pH7.4 with 5mM-Tris–HCl) at 0°C for about 1min with an MSE top-drive homogenizer in a 100ml vortex beaker. Homogenization was stopped when all the cells were broken and the released brush borders appeared, by light-microscopy, to be free of subjacent cytoplasm and tags of basolateral membrane. The homogenate was centrifuged at 800*g* for 10min and the pellet suspended and washed twice with 5vol. of buffered sucrose. The combined supernatants were centrifuged at 12000*g* for 15min and the pellet was suspended gently in buffered sucrose to a concentration of approx. 4mg of protein/ml; this suspension was the 'mitochondrial' fraction. The percentage of the total activity of the homogenate that was recovered in this fraction is recorded for each component as a mean ± S.E.M. (numbers of experiments in parentheses).

Components	Subcellular localization	Percentage of component in 'mitochondrial' fraction
(Na^++K^+)-stimulated ATPase	Basolateral plasma membrane(?)	45.0±6.3 (9)
Dicyclohexylcarbodi-imide-sensitive ATPase	Mitochondrial inner membrane	64.3±3.6 (9)
Monoamine oxidase	Mitochondrial outer membrane	62.3±4.9 (3)
Alkaline phosphatase	Brush-border plasma membranes	15.8±2.7 (8)
Phosphodiesterase I		20.5±3.8 (7)
Sucrase		24.1±7.0 (2)
Trehalase		20.0±5.3 (3)
L-Leucine β-naphthylamidase		20.1±8.8 (2)
Esterase (versus naphthyl acetate)	Endoplasmic reticulum	15.0±0.7 (2)
Glucose 6-phosphatase		12.8±0.8 (2)
β-Galactosidase	Lysosomes	11.6±1.0 (2)
Arylsulphatase		13.5±3.2 (2)
Protein		23.0±1.2 (9)

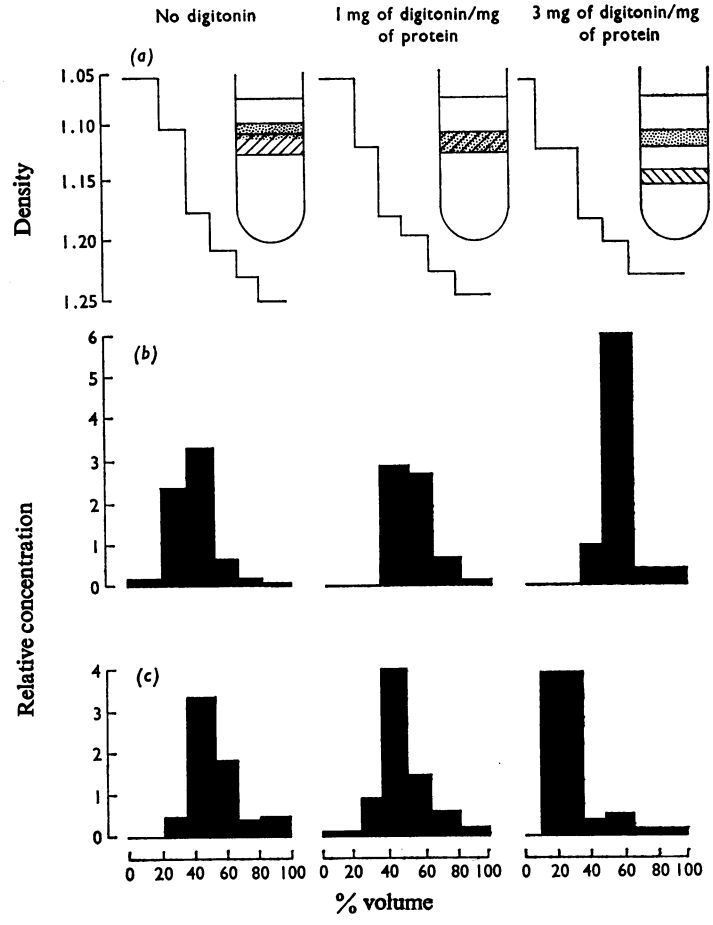


Fig. 3. *Effects of digitonin on the densities of the components of a 'mitochondrial' fraction*

Appropriate amounts of digitonin were deposited as thin films in tubes by evaporation of an ethanol solution, and a 5ml sample of a 'mitochondrial' fraction was added to each tube. The tubes were agitated to bring the digitonin into suspension and were incubated at 4°C for 15min. Their contents were loaded on linear gradients of sucrose (36–56% by weight, pH7.4) and centrifuged at 30000*g* for 16h. Fractions were obtained with a syringe by using a right-angled needle. The information shown is: (*a*) the appearance and density of the gradients, (*b*) (Na^++K^+)-stimulated ATPase and (*c*) dicyclohexylcarbodi-imide-sensitive ATPase. Presentation of results is as in Fig. 2 and recoveries, relative to the digitonin-treated material loaded on the gradients, were in the range 88–135%.

tubes in which appropriate amounts of digitonin had been deposited as a thin film on the walls by evaporation of an ethanol solution. The tubes were agitated to bring the digitonin into suspension and incubated for 15min at 4°C. The digitonin-treated mitochondrial fractions were then loaded on to linear gradients of 36–56% (w/w) sucrose (pH7.4) in 40ml centrifuge tubes and centrifuged at 30000*g* for 16h. The appearance and distribution of components in the tubes is shown in Fig. 3.

In the untreated fractions a single wide band of material was observed. (Na^++K^+)-stimulated ATPase activity was found in the upper part of this broad band and overlapped with the mitochondrial enzyme dicyclohexylcarbodi-imide-sensitive ATPase which was mainly in the lower part of the band. In the presence of 1mg of digitonin/mg of protein the density of membranes containing (Na^++K^+)-stimulated ATPase had increased and become equal to that of mitochondrial material containing dicyclohexylcarbodi-imide-sensitive ATPase. At the higher digitonin concentration the density of (Na^++K^+)-stimulated ATPase had greatly increased and its activity appeared in a discrete band with a density of

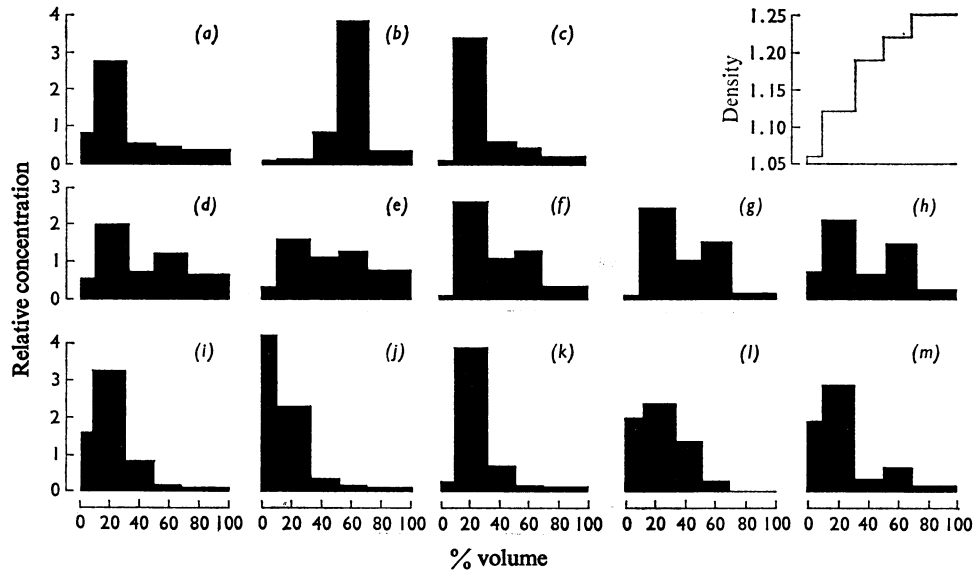


Fig. 4. *Isopycnic centrifugation in a swing-out rotor of a 'mitochondrial' fraction*

Treatment with digitonin, separation and presentation of results are all the same as in Fig. 3. The components shown are: (*a*) protein, (*b*) $(Na^+ + K^+)$-stimulated ATPase, (*c*) dicyclohexylcarbodi-imide-sensitive ATPase, (*d*) alkaline phosphatase, (*e*) phosphodiesterase I, (*f*) dicyclohexylcarbodi-imide-insensitive Mg^{2+}-stimulated ATPase, (*g*) sucrase, (*h*) trehalase, (*i*) glucose 6-phosphatase, (*j*) esterase (versus naphthyl acetate), (*k*) monoamine oxidase, (*l*) arylsulphatase, (*m*) β-galactosidase.

around 1.21. The density of the mitochondrial material had slightly decreased to about 1.11.

Further experiments indicated that 3mg of digitonin/mg of 'mitochondrial' protein was the minimum quantity that reproducibly shifted the density of $(Na^+ + K^+)$-stimulated ATPase to a value that freed it from dicyclohexylcarbodi-imide-sensitive ATPase activity. At higher ratios of digitonin/membrane protein the inhibitory effects of digitonin on enzyme activities outweighed its advantages.

Isolation of a basolateral plasma-membrane fraction from digitonin-treated mitochondrial fractions in a swing-out rotor

The detailed enzyme profiles of subfractions isolated from digitonin-treated 'mitochondrial' fractions (3mg of digitonin/mg of protein) are given in Fig. 4.

Most of the activity of $(Na^+ + K^+)$-stimulated ATPase was found in the fraction that had a density of about 1.21, whereas most of the enzymes representing intracellular membranes were found at a density of about 1.12. The enzymes of the brush border showed a dual localization, with most around 1.12 but a small amount at about 1.21.

Isolation of a basolateral plasma-membrane fraction from digitonin-treated mitochondrial fractions in a zonal rotor

Digitonin-treated mitochondrial fractions were applied to a gradient in a B XIV zonal rotor and separated by isopycnic centrifugation ($1 \times 10^9 g$-min). Several experiments were carried out, some with 'mitochondrial' fractions that had been prepared from homogenates in 0.3M-sucrose–5mM-Tris–HCl buffer, pH7.4, and others with 'mitochondrial' fractions prepared from homogenates in 5mM-EDTA–Tris buffer, pH7.4. In some of these experiments the cell sheets were iodinated with the lactoperoxidase–^{125}I system before their homogenization. Two representative experiments are depicted in Fig. 5.

In all experiments a major peak of $(Na^+ + K^+)$-stimulated ATPase activity was observed within the density range 1.20–1.22; this was the same as the modal density of $(Na^+ + K^+)$-stimulated ATPase in the tube gradients (Figs. 3 and 4). When homogenization was in 5mM-EDTA–Tris, but not if in 0.3M-sucrose–Tris, a second peak of $(Na^+ + K^+)$-stimulated ATPase activity was evident at a modal density of 1.18.

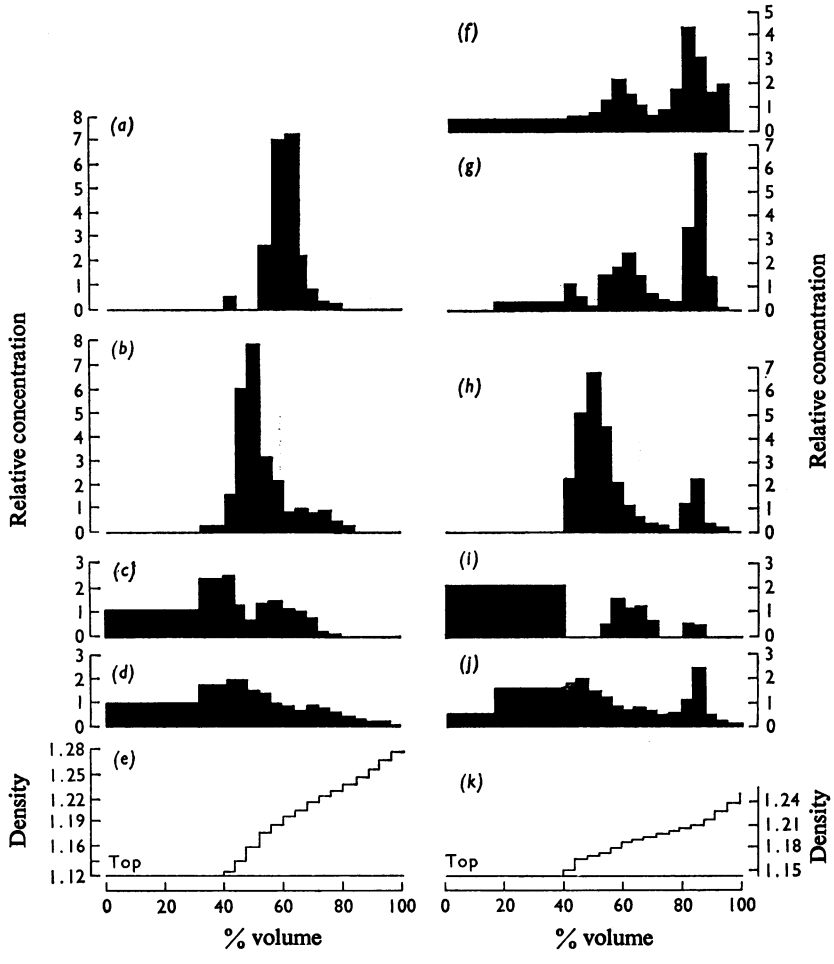


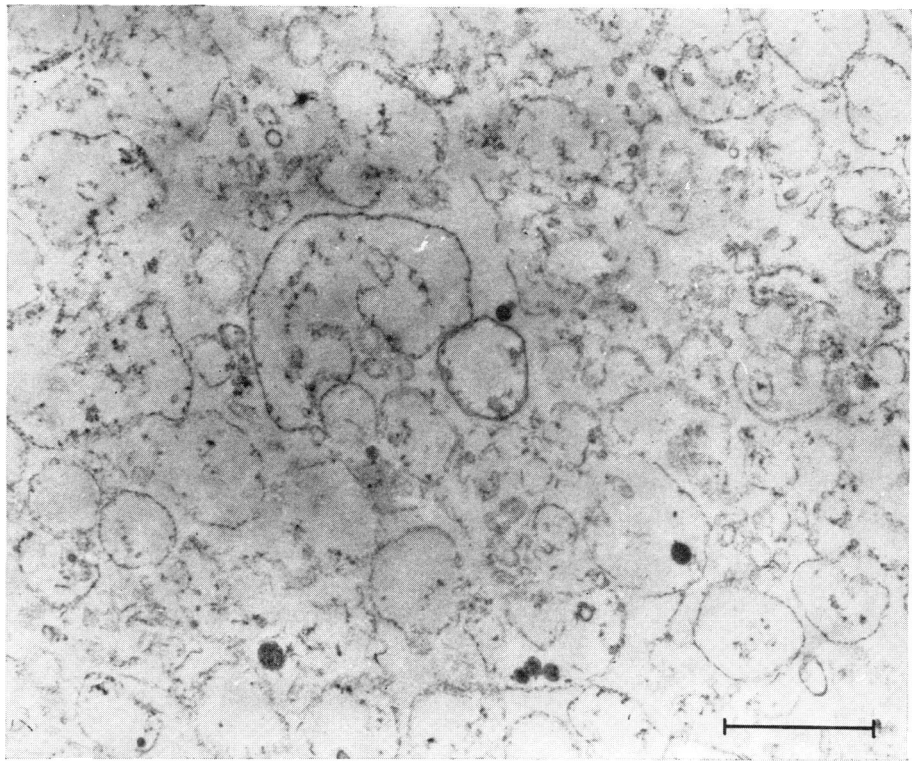
Fig. 5. *Isopycnic centrifugation in a zonal rotor of a 'mitochondrial' fraction treated with 3 mg of digitonin per mg of protein*

Two representative experiments are shown. In that depicted on the left (*a–e*) the 'mitochondrial' fraction was prepared in 0.3 M-sucrose–5 mM-Tris–HCl buffer, pH 7.4, whereas in that on the right (*f–k*) the isolation medium was 5 mM-EDTA–Tris, pH 7.4. Centrifugation of the digitonin-treated 'mitochondrial' fractions was for 5 h or more in a B XIV zonal rotor at 110000*g*. Otherwise the conditions of the experiments were as in Table 2 and Fig. 4. The components whose distributions are shown are: (*a*) and (*g*) (Na^++K^+)-stimulated ATPase, (*b*) and (*h*) dicyclohexylcarbodi-imide-sensitive ATPase, (*c*) L-leucine β-naphthylamidase, (*d*) and (*j*) protein, (*e*) and (*k*) density, (*f*) protein-bound ^{125}I and (*i*) trehalase. Recoveries, relative to the digitonin-treated material loaded on to the gradient, were in the range 81–119%. Protein-bound ^{125}I is defined as that precipitated by 7.5% (w/v) trichloroacetic acid.

Protein-bound ^{125}I (trichloroacetic acid-precipitable) accompanied both peaks of (Na^++K^+)-stimulated ATPase activity. In contrast, the ^{125}I label did not coincide with the distributions of the markers for any intracellular membranes. The fractions containing (Na^++K^+)-stimulated ATPase activity at densities between 1.20 and 1.22 were pooled and formed a larger-scale preparation of basolateral plasma membrane similar to the fraction of density 1.21 obtained from tube gradients.

Digitonin-loaded basolateral plasma-membrane fraction obtained at a density of 1.20–1.22

The composition of this material, isolated with either type of rotor, is presented in Table 3 and its appearance is shown in Plate 1. It was clearly en-



EXPLANATION OF PLATE 1

Electron micrograph of the isolated basolateral plasma-membrane fraction

A sample of the material of density 1.20–1.22 obtained from isopycnic centrifugation of a digitonin-treated 'mitochondrial' fraction was diluted with buffered sucrose and centrifuged at 200000*g* for 90min to yield a pellet. This was fixed with glutaraldehyde and buffered OsO_4, dehydrated, embedded in Araldite, sectioned, stained with uranyl acetate and examined in a Phillips 301 electron microscope. The scale bar represents 1 μm.

Table 3. *Composition of the digitonin-loaded basolateral plasma-membrane fraction isolated at a density of* 1.20–1.22

The values presented are collected from 13 experiments, nine using a swing-out rotor and four a zonal rotor. Calculation of the values presented, which are percentages of the original homogenate activity or protein recovered in the isolated basolateral plasma-membrane (BLPM) fraction, has included a correction to take into account any effect of digitonin on the activity in question. The calculation used was:

$$\text{Percentage in BLPM fraction} = 100 \times \frac{\text{Activity in 'mitochondrial' fraction}}{\text{Activity in homogenate}} \times \frac{\text{Activity in BLPM fraction}}{\text{Activity in digitonin-treated mitochondrial fraction}}$$

Values below 2 have been rounded off to the nearest 0.2 and those above 2 to the nearest integer. The number of experiments considered is shown in parentheses.

Component	Percentage in basolateral plasma-membrane fraction	Specific activity of homogenate (μmol/h per mg of protein)
$(Na^+ + K^+)$-stimulated ATPase	29 (13)	3.3
Dicyclohexylcarbodi-imide-sensitive ATPase	5 (13)	4.3*
Monoamine oxidase	2 (3)	14.9
Alkaline phosphatase	3 (11)	36
Phosphodiesterase I	4 (7)	8
Sucrase	6 (2)	3.5
Trehalase	4 (4)	2.7
L-Leucine β-naphthylamidase	1.0 (3)	8.6
Esterase (versus naphthyl acetate)	0.4 (5)	110
Glucose 6-phosphatase	0.4 (5)	3.1
β-Galactosidase	1.0 (2)	1.8
Arylsulphatase	0.8 (2)	133
Protein	1.8 (13)	

* In the presence of 3 mg of digitonin per mg of protein.

riched in $(Na^+ + K^+)$-stimulated ATPase to a very considerable extent and contained only relatively small quantities of marker enzymes indicative of other organelles. Its major morphological constituent appeared to be relatively large vesicles and/or sheets of well-defined smooth membranes.

Discussion

When a 'plasma-membrane' fraction with a new and unique enzyme profile is isolated from an homogenate of a complex tissue such as an intestinal mucosal 'scrape' it is usually represented as a preparation derived from the predominant cell type in the tissue. The properties of the new fraction may, however, be either those of a previously unpurified area of the plasma membrane of the predominant cell type or dominated by plasma membrane derived from other cell types. Since techniques such as lactoperoxidase-catalysed iodination would label the surfaces of all cell-types in any mixture we have studied an homogeneous cell population and have therefore tried to confine our experiments, like those of Douglas *et al.* (1972) and Murer *et al.* (1974), to the columnar epithelial cells of the small intestinal mucosa. Our results can therefore provide a rigorous confirmation and refinement of those of Douglas *et al.* (1972) and Murer *et al.* (1974) and of previous workers who isolated putative basolateral membrane fractions from intestinal 'scrape' material that contains variable proportions of submucosal tissue (e.g. Quigley & Gotterer, 1969, 1972; Fujita *et al.*, 1972, 1973).

In the past, lactoperoxidase-catalysed iodination has been used to impose an externally added label on the plasma membranes of many cells, including erythrocytes (Phillips & Morrison, 1970, 1971; Hubbard & Cohn, 1972), normal and neoplastic lymphocytes (Marchalonis *et al.*, 1971, 1972), platelets (Barber & Jamieson, 1971; Phillips, 1972; Nachman *et al.*, 1973; Taylor & Crawford, 1974), L cells (Poduslo *et al.*, 1972), *Micrococcus lysodeikticus* protoplasts (Salton *et al.*, 1972), HeLa cells (Tsai *et al.*, 1973), thymocytes (Schmidt-Ullrich *et al.*, 1974) and hepatocytes (Evans, 1974). The value of extending this approach and using iodination as a primary criterion for following plasma membranes during fractionation has been clearly shown in the present studies and also in the hands of Taylor & Crawford (1974). We have now shown that its use also allows the simultaneous detection of multiple types of plasma membrane present in the same cell. We have used a cell-sheet preparation; application of the lactoperoxidase technique to a solid tissue will obviously present additional problems. Appropriate choice of conditions may, however, allow the selective

iodination of particular faces of the cell and thus permit their isolation and identification.

Our initial isopycnic zonal separations of homogenates (Fig. 2, and B. A. Lewis, unpublished work) showed that the distribution of all the known brush-border enzymes (alkaline phosphatase, sucrase, maltase, trehalase, L-leucine β-naphthylamidase and phosphodiesterase I) were always coincident. Their activities were concentrated at the high-density end of the gradient. There was considerable variation in the minor extent to which the profiles overlapped with that of the low-density peak of $(Na^+ + K^+)$-stimulated ATPase; in some experiments they were essentially absent from this region of the gradient. It therefore seems that the basolateral plasma membrane of the intestinal epithelial cell is essentially devoid of these enzymes and that the small and variable quantities that appeared in our basolateral plasma membrane fractions reflected a small amount of contamination with brush-border material.

These experiments do not indicate quite as clearly whether any $(Na^+ + K^+)$-stimulated ATPase is present in the brush borders. The major peaks of $(Na^+ + K^+)$-stimulated ATPase and brush-border enzymes were distinct but the minor peak of $(Na^+ + K^+)$-stimulated ATPase usually showed a pattern coincident with that of the brush-border enzymes. This pattern could result either from the presence of tags of basolateral membrane, which remained attached to the brush border throughout homogenization and fractionation, or from the presence of intrinsic $(Na^+ + K^+)$-stimulated ATPase in the brush-border membranes. The former alternative is supported by the observation that decreased amounts of $(Na^+ + K^+)$-stimulated ATPase accompanied the brush borders in the more vigorously homogenized samples. In addition, we have observed that purification of microvillus membranes from brush borders largely eliminated their $(Na^+ + K^+)$-stimulated ATPase activity but markedly purified them in terms of brush-border enzymes (B. A. Lewis, unpublished work).

Unfortunately, our attempts to purify the basolateral plasma membranes by any simple combination of differential rate and isopycnic centrifugation failed. The main difficulty was a persistent association between $(Na^+ + K^+)$-stimulated ATPase and mitochondrial enzymes, as has been observed by Quigley & Gotterer (1969). They achieved separation of membranes containing $(Na^+ + K^+)$-stimulated ATPase from mitochondria only after 'aging' their mitochondrial preparations for 5 days, a treatment whose effects were not understood. We have achieved this separation with fresh 'mitochondrial' fractions by selectively increasing the density of the plasma membranes by treatment with digitonin in the manner

Table 4. *Compositions of basolateral plasma-membrane preparations*

n.m., Not measured; n.d., none detected, i.e. some of these values are recorded by the original authors as zero, but without defining the sensitivity of the assay at the protein concentrations used. Relative specific activity is defined as the specific activity of the fraction divided by the specific activity of the homogenate.

	Relative specific activities of marker enzymes			
References ...	This work	Douglas *et al.* (1972)	Fujita *et al.* (1972, 1973)	Quigley & Gotterer (1969, 1972)
Species ...	Guinea pig	Rat	Mouse and rat	Rat
Starting material ...	Epithelial cell sheets	Epithelial cells	Mucosal scrapings	Mucosal scrapings
Basolateral plasma membrane $(Na^+ + K^+)$-stimulated ATPase	15–20	12	11	25–35
Mitochondrial inner membranes:				
Dicyclohexylcarbodi-imide-sensitive ATPase	3	n.m.	n.m.	n.m.
Succinate–cytochrome *c* reductase	n.m.	n.m.	n.d.	n.m.
Succinate dehydrogenase	n.m.	n.d.	n.m.	n.m.
Cytochrome oxidase	n.m.	n.m.	n.m.	'Negligible'
Mitochondrial outer membranes:				
Monoamine oxidase	1	0.05	n.m.	n.d.
Endoplasmic reticulum:				
Glucose 6-phosphatase	0.3	n.m.	2	'Negligible'
Aryl esterase	0.3	n.m.	n.m.	n.m.
NADPH–cytochrome *c* reductase	n.m.	3.3	n.m.	n.m.
NADH–cytochrome *c* reductase	n.m.	n.m.	0.7	n.m.
Brush borders:				
Alkaline phosphatase	2	4.3	0.5	'Negligible'
Sucrase	n.m.	0.2	0.2	'Negligible'
Trehalase	2	n.m.	n.m.	n.m.

described by Thinès-Sempoux *et al.* (1969) and Amar-Costesec *et al.* (1974). In this technique digitonin becomes associated with membranes approximately in proportion to their cholesterol content; it therefore allows separation of cholesterol-rich plasma membranes from cholesterol-poor mitochondrial.

Use of the density-perturbation technique does, however, present certain problems and has its limitations. In particular, digitonin modifies membrane structure and therefore modifies the activities of some membrane-bound enzymes. The inhibitory effect of digitonin on $(Na^+ + K^+)$-stimulated ATPase may be due to disorganization of the membrane structure in the vicinity of the enzyme since the activity of $(Na^+ + K^+)$-stimulated ATPase is known to be affected by changes in its lipid environment (see for example, Coleman 1973; Wheeler 1975).

Digitonin did not cause removal of lipid from the membranes present either in our 'mitochondrial' fraction (B. A. Lewis, unpublished work) or in the microsomal fractions used by Amar-Costesec *et al.* (1974). In our experiments it caused an increase in density of the basolateral plasma membranes and not of mitochondrial membranes; this is consistent with the suggestion of Thinès-Sempoux *et al.* (1969) that digitonin binds to membrane cholesterol and therefore only perturbs the density of membranes which are rich in cholesterol. However, in our experiments the quantity of digitonin required for adequate perturbation of the density of the basolateral membranes was far more than would be needed for a simple molecular association; this is at present unexplained.

The overall enzyme composition of our final basolateral plasma-membrane fractions is compared in Table 4 with preparations that have been presented in the past as purified basolateral plasma-membrane fractions. We estimate, from the amounts of marker enzymes and from rough estimates of the contributions of different cell components to cell mass, that basolateral membranes probably constitute about 60–80% of the material present in the purified fraction prepared by the density-perturbation procedure. The major contaminant in the new preparation was of mitochondrial origin, in contrast with previous preparations which were more heavily contaminated with endoplasmic reticulum membranes. When the relative specific activities of $(Na^+ + K^+)$-stimulated ATPase, the primary marker enzyme for basolateral plasma membranes, are to be compared for the different preparations it must be remembered that isolation of purified epithelial cells or cell-sheet preparations removes the submucosal material that contaminates the intestinal 'scrape' preparations used by Fujita *et al.* (1972, 1973) and Quigley & Gotterer (1969, 1972). In principle, therefore, purifications starting from homogeneous cell preparations cannot be as great. When this factor is taken into account it appears that the preparation described here is at least as pure as, and possibly purer than, the purest preparations that have been reported previously. Thus it provides both a novel approach to isolation of epithelial basolateral membranes and independent confirmation of the special characteristics of these membranes. Further studies have shown marked differences between these basolateral membranes and brush-border membranes from the same cell in their complements of polypeptides, glycopeptides, glycolipids and phospholipids. These studies, which further emphasize the specialized nature of the basolateral membranes, have interesting implications in relation to membrane structure (Lewis *et al.*, 1975).

B. A. L. and A. E. are grateful to the Science Research Council and the Medical Research Council respectively for Research Studentships.

References

Amar-Costesec, A., Wibo, M., Thinès-Sempoux, D., Beaufay, H. & Berthet, J. (1974) *J. Cell Biol.* **62**, 717–745
Baginski, E. S., Zak, B. & Foa, P. P. (1967) *Clin. Chem.* **13**, 326–332
Barber, A. J. & Jamieson, G. A. (1971) *Biochemistry* **10**, 4711–4717
Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
Beechey, R. B., Robertson, A. M., Holloway, C. T. & Knight, J. G. (1967) *Biochemistry* **6**, 3867–3879
Brdickza, D., Pette, D., Brunner, G. & Miller, F. (1968) *Eur. J. Biochem.* **5**, 294–304
Brightwell, R. & Tappel, A. L. (1968) *Arch. Biochem. Biophys.* **124**, 325–332
Burton, K. (1956) *Biochem. J.* **62**, 315–323
Coleman, R. (1973) *Biochim. Biophys. Acta* **300**, 1–30
Connock, M. J., Elkin, A. & Pover, W. F. R. (1971) *Histochem. J.* **3**, 11–22
Crawford, N. (1958) *Clin. Chim. Acta* **3**, 357–367
Dahlqvist, A. (1968) *Anal. Biochem.* **22**, 99–107
Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1959) *Data for Biochemical Research*, Oxford
De Duve, C. (1971) *J. Cell Biol.* **50**, 20D–55D
Douglas, A. P., Kerley, R. & Isselbacher, K. J. (1972) *Biochem. J.* **128**, 1329–1338
Evans, E. M., Wrigglesworth, J. M., Burdett, K. & Pover, W. F. R. (1971) *J. Cell Biol.* **51**, 452–464
Evans, W. H. (1974) *Nature (London) New Biol.* **250**, 391–394
Fujita, M., Ohta, H., Kawai, K., Matsui, H. & Nakao, M. (1972) *Biochim. Biophys. Acta* **274**, 336–347
Fujita, M., Kawai, K., Asano, S. & Nakao, M. (1973) *Biochim. Biophys. Acta* **307**, 141–151
Gianetto, R. & De Duve, C. (1955) *Biochem. J.* **59**, 433–438
Goldbarg, J. A. & Rutenberg, A. M. (1958) *Cancer* **11**, 283–291
Hubbard, A. L. & Cohn, Z. A. (1972) *J. Cell Biol.* **55**, 390–400
Hübscher, G. & West, G. R. (1965) *Nature (London)* **205**, 799–800
Hübscher, G., West, G. R. & Brindley, D. N. (1965) *Biochem. J.* **97**, 629–643

King, E. J. (1932) *Biochem. J.* **32**, 292–302
Lewis, B. A., Gray, G. M., Coleman, R. & Michell, R. H. (1975) *Biochem. Soc. Trans.* in the press
Limbrick, A. R. & Finean, J. B. (1970) *J. Cell Sci.* **7**, 373–386
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Maddy, A. H. (1964) *Biochim. Biophys. Acta* **88**, 390–399
Marchalonis, J. J., Cone, R. E. & Santer, V. (1971) *Biochem. J.* **124**, 921–927
Marchalonis, J. J., Cone, R. E. & Atwell, A. (1972) *J. Exp. Med.* **135**, 956–971
Michell, R. H., Karnovsky, M. J. & Karnovsky, M. L. (1970) *Biochem. J.* **116**, 207–216
Miller, D. & Crane, R. K. (1961) *Anal. Biochem.* **2**, 284–286
Morrison, M. & Hultquist, D. E. (1963) *J. Biol. Chem.* **238**, 2847-2849
Morrison, M., Hamilton, H. B. & Stotz, E. (1957) *J. Biol. Chem.* **228**, 767–776
Murer, H., Hopfer, U., Kinne-Saffran, E. & Kinne, R. (1974) *Biochim. Biophys. Acta* **345**, 170–179
Nachman, R. L., Hubbard, A. & Ferris, B. (1973) *J. Biol. Chem.* **248**, 2928–2936
Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
Phillips, D. R. (1972) *Biochemistry* **11**, 4582–4588
Phillips, D. R. & Morrison, M. (1970) *Biochem. Biophys. Res. Commun.* **40**, 284–289
Phillips, D. R. & Morrison, M. (1971) *Biochemistry* **10**, 1766–1771
Poduslo, J. F., Greenberg, C. S. & Glick, M. C. (1972) *Biochemistry* **11**, 2616–2621
Porteous, J. W. & Clark, B. (1965) *Biochem. J.* **96**, 159–172
Quigley, J. P. & Gotterer, G. S. (1969) *Biochim. Biophys. Acta* **173**, 456–468
Quigley, J. P. & Gotterer, G. S. (1972) *Biochim. Biophys. Acta* **255**, 107–113
Ravin, A. A. & Seligman, A. M. (1953) *Arch. Biochem. Biophys.* **42**, 337–354
Roy, A. B. (1953) *Biochem. J.* **53**, 12–15
Salton, M. R., Schor, M. T. & Ng, M. H. (1972) *Biochim. Biophys. Acta* **290**, 408–413
Schmidt-Ullrich, R., Ferber, E., Knüffermann, H., Fischer, H. & Wallach, D. F. H. (1974) *Biochim. Biophys. Acta* **332**, 175–191
Seidman, M. & Link, K. P. (1950) *J. Am. Chem. Soc.* 4324–4334
Shephard, E. H. & Hübscher, G. (1969) *Biochem. J.* **113**, 429–441
Stetten, M. R. & Burnett, F. F. (1966) *Biochim. Biophys. Acta* **128**, 344–350
Taylor, D. G. & Crawford, N. (1974) *FEBS Lett.* **41**, 317–322
Thinès-Sempoux, D., Amar-Costesec, A., Beaufay, H. & Berthet, J. (1969) *J. Cell Biol.* **43**, 189–192
Tsai, C-M., Huang, C-C. & Canellakis, E. S. (1973) *Biochim. Biophys. Acta* **332**, 47–58
Wallach, D. F. H. (1972) *Biochim. Biophys. Acta* **265**, 61–83
Weast, R. C. (1964) *Handbook of Chemistry and Physics*, 45th edn., p. E-107, Chemical Rubber Co. Press, Cleveland.
Wheeler, K. P. (1975) *Biochem. J.* **146**, 729–738