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# Analytical Isolation of Plasma Membranes of Intestinal Epithelial Cells: Identification of Na, K-ATPase Rich Membranes and the Distribution of Enzyme Activities

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*Summary.* A procedure was developed for the analytical isolation of brush border and basal lateral plasma membranes of intestinal epithelial cells. Brush border fragments were collected by low speed centrifugation, disrupted in hypertonic sorbitol, and subjected to density gradient centrifugation for separation of plasma membranes from nuclei and core material. Sucrase specific activity in the purified brush border plasma membranes was increased fortyfold with respect to the initial homogenate. Basal lateral membrane were harvested from the low speed supernatant and resolved from other subcellular components by equilibrium density gradient centrifugation. Recovery of Na, K-ATPase activity was 94%, and 61% of the recovered activity was present in a single symmetrical peak. The specific activity of Na, K-ATPase was increased twelvefold, and it was purified with respect to sucrase, succinic dehydrogenase, NADPH-cytochrome *c* reductase, nonspecific esterase, β-glucuronidase, DNA, and RNA. The observed purification factors are comparable to results reported for other purification procedures, and the yield of Na, K-ATPase is greater by a factor of two than those reported for other procedures which produce no net increase in the Na, K-ATPase activity.

Na, K-ATPase rich membranes are shown to originate from the basal lateral plasma membranes by the patterns of labeling that were produced when either isolated cells or everted gut sacs were incubated with the slowly permeating reagent $^{35}$S-*p*-(diazonium)-benzenesulfonic acid. In the former case subsequently purified Na, K-ATPase rich and sucrase rich membranes are labeled to the same extent, while in the latter there is a tenfold excess of label in the sucrase rich membranes. The plasma membrane fractions were in both cases more heavily labeled than intracellular protein.

Alkaline phosphatase and calcium-stimulated ATPase were present at comparable levels on the two aspects of the epithelial cell plasma membrane, and 25% of the acid phosphatase activity was present on the basal lateral membrane, while it was absent from the brush border membrane. Less than 6% of the total Na, K-ATPase was present in brush border membranes.

In recent years much progress has been made in study of transport across such epithelia as the intestine, gall bladder, renal tubule and

choroid plexus. Nevertheless, most studies have been limited to "black box" experiments in which the properties of the constituent plasma membranes forming the two boundaries of the epithelium were ignored. To make further significant advances towards the solution of questions posed by the transport of molecules, ions and water across epithelia it is necessary to devise new techniques to study the properties of epithelial cell plasma membranes. There are two approaches that may be taken. The first is the use of electrophysiological techniques to obtain a circuit analysis of the epithelium and to record the electrical manifestations of the transport systems. Already progress has been made in electrophysiological studies of the gall bladder [11], stomach [35] and urinary bladder[1] [27]. The second approach is the use of biochemical techniques to isolate and purify the apical and basal plasma membranes of the epithelium and to characterize the transport systems in the isolated membranes. Brush border membranes from epithelial cells have been available for some time (e.g., [10]) and there have been some steps towards the isolation of basal lateral plasma membranes [26, 13, 15, 7]. Furthermore, there is ample precedent for the successful study of transport reactions in isolated membranes and significant advances have already been made towards understanding transport mechanisms in sarcoplasmic reticulum, bacterial plasma membranes, ascites tumor cell membranes, synaptosomes and red cell ghosts and membrane vesicles. There are also some reports on the mechanisms of sugar and amino acid transport across isolated brush border and basal lateral membranes from the intestine (e.g., [16, 22, 23]).

We have chosen the biochemical approach to the question of mechanisms of epithelial transport because it promises to lead to a molecular understanding of transport processes. The work to be presented here is offered as a prelude to the study of enzymatic and transport activities of the brush border and basal lateral plasma membranes of rat intestinal epithelial cells. The small intestine is well suited for the development and application of biochemical isolation techniques because (1) the epithelium has a relatively simple structure—a single layer of cells in a continuous sheet; (2) large quantities of cells are available from common laboratory animals, and although there is some regional heterogeneity in specific transport systems, the cells in the normal animal are specialized for the absorption of salt, water and nonelectrolytes; (3) much is already known about the mechanisms of absorption in the intact epithelium;

1 Lewis, S.A., Eaton, D.C., Diamond, J.M. 1976. The mechanism of Na transport by rabbit urinary bladder. (*Submitted for publication.*)

(4) methods are available in the literature for the isolation and purification of brush border membranes, and several attempts at the isolation of basal lateral membranes have recently been published; and (5) the enzyme content of the isolated brush border membranes is well established.

We have adopted the criteria urged by DePierre & Karnovsky [5] to devise a procedure which provides high yields of basal lateral and brush border membranes free of contamination by other subcellular components. These authors call for an analytical approach in which a balance sheet is maintained for markers at all stages of the purification procedure, and the use of multiple criteria for the identification of plasma membranes. We chose sucrase as a marker for the brush border membranes and Na, K-ATPase as a provisional marker for the basal lateral membranes, and we kept a balance sheet to ensure that enzymes were neither activated nor inhibited during the isolation procedure. To obtain direct proof that the Na, K-ATPase rich membranes originate from the basal lateral region of the epithelial cell we have used the slowly permeating labeling reagent $^{35}$S-*p*-(diazonium)-benzenesulfonic acid to label the membranes on one or both sides of the epithelium. Our approach has enabled us to draw quantitative conclusions about the distribution of enzymes between the two poles of the intestinal epithelial cell.

Purification of Na, K-ATPase rich membranes from intestinal epithelial cells has been previously reported but these fall short of being definitive plasma membrane isolations for various reasons: yields of Na, K-ATPase were either less than 20% [13, 7] or the total Na, K-ATPase activity increased during the isolation procedure [26]; and no direct independent evidence has been produced to show that Na, K-ATPase rich membranes in fact originate from the basolateral region of epithelial cells.

## Materials and Methods

### *Isolation of Brush Border and Basal Lateral Plasma Membranes*

*Dissection and Harvesting of Intestinal Mucosa.* Male Sprague-Dawley rats (180–200 g) were used in all experiments; one rat provided sufficient material for a conveniently scaled preparation. The animal was killed and the intestine was removed, slit open and washed in ice cold 0.9% NaCl. The intestinal mucosa was harvested by gentle scraping with microscope slides. All subsequent manipulations were performed on ice or in a cold room.

*Homogenization and Differential Centrifugation.* The procedure for preparation of brush border and basal lateral membranes is summarized by the flow sheet in Fig. 1. The initial

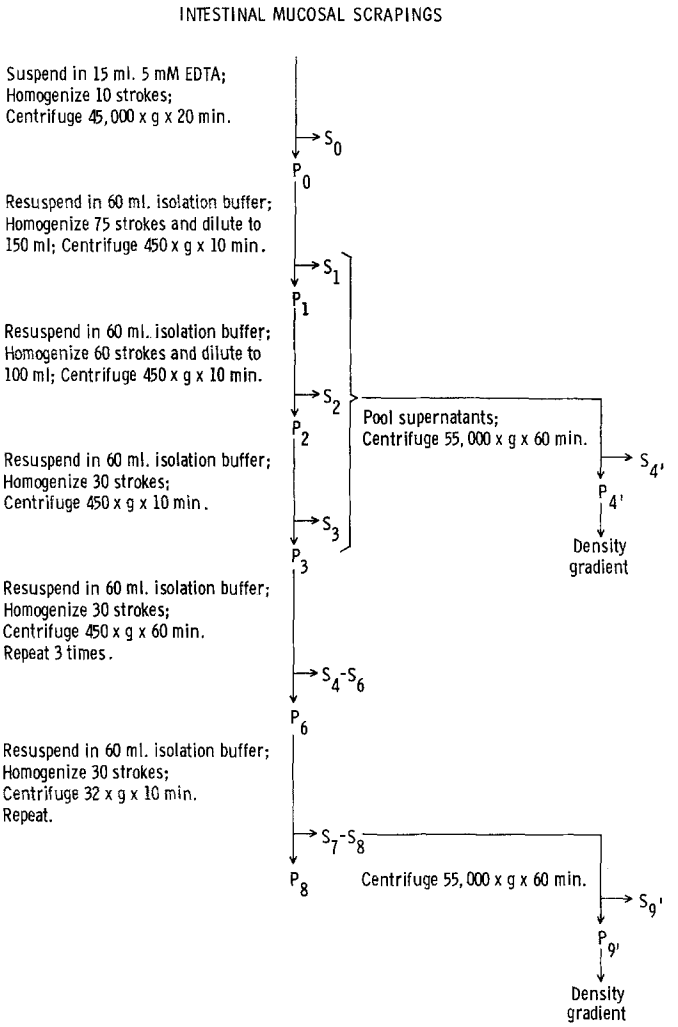


Fig. 1. Procedure for analytical isolation of intestinal epithelial cell plasma membranes. The mucosal scrapings and pellets $P_0$ through $P_8$ were homognized in a Dounce apparatus (Vitro) with the size $B$ pestle supplied by the manufacturer. The isolation buffer contained (in mM): sorbitol, 250; NaCl, 12.5; NaEDTA, 0.5; and pH 7.5 histidine-imidazole buffer, 5. Density gradients constructed by mixing 25% and 65% sorbitol were subjected to centrifugation at 20,000 rpm for 24 hr in a Beckman SW 25.1 rotor. Density gradient centrifugation of $P'_4$ resulted in resolution of basal lateral membranes. Density gradient centrifugation of $P'_9$ separated brush border plasma membranes from nuclei

centrifugation separated soluble protein and light microsomes ($S_0$) from the heavier components ($P_0$) The washed $450 \times g$ pellet ($P_6$) contained, in addition to brush border fragments and nuclei, aggregating particulate material which was removed by subsequent centrifugation at $32 \times g \times 10$ min. The brush border fragments were collected from the $32 \times g$ supernatant by centrifugation and were disrupted by homogenization in 12.5% sorbitol with ten strokes of a motor-driven glass-teflon homogenizer. Brush border plasma membranes were separated

from nuclei by density gradient centrifugation. Basal lateral plasma membranes and mitochondria were collected from the $450 \times g$ supernatant by centrifugation and resuspended for density gradient centrifugation by homogenization in 12.5% sorbitol.

*Density Gradient Centrifugation.* Linear density gradients were prepared by mixing 25% sorbitol and 65% sorbitol in a conventional gradient maker; these solutions were buffered to pH 7.5 with 5 mM histidine-imidazole and 0.5 mM NaEDTA. Fraction 14 contained a cushion of 65% sorbitol, and the linear density gradient spanned fractions 13 through 2. The sample was applied as fraction 1. The interface between the sample layer and the top of the gradient was disrupted, and an overlay of histidine-imidazole-EDTA buffer was applied as necessary for balancing. Density gradients were centrifuged for 24 hr at 20,000 rpm in an SW 25.1 rotor. There was no change in the distributions of markers when centrifugation was prolonged for 48 hr, and density gradient centrifugation for less than 24 hr gave incomplete separation of basal lateral membranes from mitochondria.

Density gradient fractions were collected with a Beckman tube slicer. Fractions 2 through 14 were diluted with one volume of isolation buffer, and the pellet was resuspended in two volumes of isolation buffer. Particulate material in density gradient fractions 2–14 and in the pellet was washed free of the density gradient medium by two centrifugations at $100{,}000 \times g \times 20$ min. Material in fraction 1 could not be harvested by centrifugation without excessive dilution.

### *Labeling with $^{35}$S-p-(Diazonium)-Benzenesulfonic Acid*

In all labeling experiments polyethylene beakers and pipettes were used, Petri dishes and glass slides were ensheathed in parafilm, and all procedures were carried out at 0 °C. Preliminary experiments indicated that the specificity of labeling was greater in borate buffered saline, pH 9.0, than in phosphate buffered saline, pH 7.5.

*Synthesis of $^{35}$S-p-(Diazonium)-Benzenesulfonic Acid.* $^{35}$S-DABS was prepared by the procedure of Hoyer and Trabold [17]. The reaction mixture was diluted with distilled water to a final concentration of 6 mM and 1 ml aliquots were stored frozen until immediately before use.

*Labeling Everted Gut Sacs.* Everted gut sacs were filled with borate buffered saline (BBS: NaCl, 120 mM; KCl, 5 mM; $MgCl_2$, 5 mM; and sodium borate buffer (pH 9.0), 5 mM) and incubated in 20 ml BBS and 2 ml $^{35}$D-DABS. The labeling reaction was allowed to procede for 10 min in an ice water bath with gentle shaking. To remove excess $^{35}$S-DABS, the everted gut sacs were washed extensively with phosphate buffered saline (PBS: NaCl, 120 mM, KCL, 5 mM; and sodium phosphate buffer (pH 7.5), 5 mM); Beef serum albumin (1–5 mg/ml) was present in the initial washes. After two hours the everted gut sacs were slit, drained, and rinsed with 0.9% NaCl. The mucosa was harvested and subjected to the usual membrane isolation procedure.

*Labeling Suspensions of Isolated Cells.* Sheets of intestine were preincubated for 45 min with gentle shaking in the ice-water bath in magnesium-free borate buffered saline (0 Mg-BBS: NaCl, 144 mM; KCl, 5 mM; and sodium borate buffer (pH 9.0) 5 mM). The mucosa was harvested by scraping and dispersed in 20 ml 0 Mg-BBS by several passages through the tip of a polyethylene pipette. The labeling reaction was started by addition of 20 ml 0 Mg-BBS and 2 ml $^{35}$S-DABS and quenched after 5 min by addition of 500 ml of saline containing 60 mM imidazole.

For removal of damaged cells, the quenched reaction mixture was filtered through nylon stocking material. To remove soluble radioactivity, the filtrate was diluted to 1 liter with 60 mM imidazole saline and centrifuged for 10 min at $450 \times g$. The resulting pellet was dispersed and washed twice; a second filtration step was included during the final wash.

The washed pellet was subjected to the usual membrane isolation procedure with the exceptions that (a) 5 mM imidazole was added to trap any diazonium salt that might have remained and (b), brush border membranes were prepared from $P_1$.

In order to determine the covalently bound $^{35}S$ in the differential fractions, aliquots were dialized for 48 hr against two 4 liter changes of a buffer containing NaCl, 12.5 mM; NaEDTA, 0.5 mM; and imidazole-HCl (pH 7.5), 5 mM.

### *Analytical Methods*

*Na, K-ATPase.* Na, K-ATPase was measured in the medium of Fujita *et al.* [12], modified by addition of 3.0 mM NaEDTA; this was the optimal concentration for Na, K-ATPase activity in the presence of 3.0 mM ATP and 5.0 mM $MgCl_2$. Inorganic phosphate was determined by the method of Fiske and SubbaRow [9].

*Alkaline Phosphatase.* Alkaline phosphatase was measured in 1.0 ml of a medium which contained: $MgCl_2$, 5 mM; $CaCl_2$, 0.25 mM; $ZnCl_2$, 0.2 mM; Tris-maleate buffer (pH 9.0), 50 mM; and *p*-nitrophenylphosphate, 5 mM. Samples were preincubated for 15 min before the reaction was started by addition of substrate. The reaction was quenched by addition of 3.0 ml 1 N NaOH; activity is expressed as the rate of change of optical density at 410 nm.

*Calcium-Stimulated ATPase.* Calcium-stimulated ATPase was estimated as the difference between the rates of release of phosphate from ATP in the presence and absence of 2 mM $CaCl_2$ in a medium containing: $MgCl_2$, 5 mM; NaATP, 3 mM; and Tris-maleate buffer (pH 8.2), 50 mM. The final volume was 1 ml. The reaction was initiated by addition of ATP to the otherwise complete incubation, and it was quenched by addition of 1 ml 5% trichloroacetic acid.

*Other Enzyme Activities.* Sucrase was measured by the method of Dahlqvist [4]. Succinic Dehydrogenase was measured as succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase by the method of Pennington [25]. β-glucuronidase was measured according to the method of Michell *et al.* [20]. Acid phosphatase was measured in the same medium as β-glucuronidase, modified by the inclusion of 10 mM *p*-nitrophenyl-phosphate as substrate and, as suggested by Hubscher and West [18], 4 mM NaEDTA to inhibit alkaline phosphatase. Nonspecific esterase was measured by the procedure of Seligman and Nachlas [32] as modified by Pelichova *et al.* [24]. NADPH-cytochrome *c* reductase was measured according to the procedure of Sottocasa *et al.* [34].

*Nucleic Acids.* Acid soluble material was extracted with ice-cold trichloroacetic acid, and nucleic acids were extracted by hydrolysis in 5% trichloroacetic acid. RNA was determined with orcinol reagent by the method of Schneider [30], and DNA was determined by the method of Croft and Lubran [3].

*Protein.* Protein was determined by the method of Lowry *et al.* [19]. All enzyme assays and chemical determinations were performed at 22–23 °C.

## Results

### *Analytical Isolation of Brush Border and Basal Lateral Plasma Membranes*

*Differential Centrifugation.* From the outset it was considered most advantageous to achieve by differential centrifugation as complete a separa-

tion as possible of Na, K-ATPase rich-membranes from the brush borders; this would reduce the possibility of cross contamination during subsequent density gradient centrifugation, and it would minimize the amount of time basal lateral membrane fragments were exposed to the hydrolytic enzyme of the brush border surface.

The distributions of all markers followed during the sequence of homogenizations and differential centrifugations are presented in Table 1. The first three centrifugations at $450 \times g$ separate 75% of the initial Na, K-ATPase and succinic dehydrogenase activities from 65% of the sucrase and 58% of the DNA.

*Density Gradient Purification of Basal Lateral Membranes.* The Na, K-ATPase activity in the pooled supernatants $S_1$–$S_3$ was further separated from sucrase and DNA and resolved from succinic dehydrogenase, RNA, nonspecific esterase, and β-glucuronidase by equilibrium density gradient centrifugation on linear gradients of 25% to 65% sorbitol. In contrast to the results of Quigley and Gotterer [26], no aging process was necessary for this separation, and the separation entailed no net

Table 1. The distribution of markers among the differential centrifugation fractions obtained during analytical isolation of brush border and basal lateral plasma membranes[a]

| | $S_0$ | $P_0$ | $S_1$–$S_3$ | $S_4'$ | $P_4'$ | $P_3$ | $S_4$–$S_6$ | $S_7$–$S_8$ | $P_8$ | $S_9'$ | $P_9'$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Na,K-ATPase | 1.2 | 99.1 | 75.9 | 3.9 | 64.2 | 14.6 | 5.7 | 10.0 | 12.8 | 2.7 | 6.7 |
| Alkaline phosphatase | 8.8 | 91.2 | 39.0 | 4.7 | 38.2 | 65.8 | 5.8 | — | 8.1 | — | 25.0 |
| Sucrase | 4.2 | 95.9 | 22.2 | 6.0 | 16.6 | 63.3 | 8.7 | 67.6 | 13.0 | 1.0 | 65.3 |
| Succinic dehydrogenase | 5.5 | 94.6 | 74.5 | 3.4 | 56.9 | 7.4 | 10.3 | 6.0 | 4.3 | 5.0 | 2.3 |
| Acid phosphatase | 31.2 | 68.8 | 45.3 | 13.3 | 34.4 | 13.7 | — | — | — | — | 1.1 |
| β-Glucuronidase | 40.8 | 59.2 | 42.8 | 29.6 | 6.0 | 1.4 | — | — | — | — | — |
| Esterase, eserine-sensitive | 9.5 | 90.5 | 99.0 | 79.6 | 23.9 | 2.4 | n.d. | n.d. | — | — | — |
| Esterase, eserine-insensitive | 22.1 | 78.0 | 62.5 | 38.8 | 36.7 | 5.4 | 13.7 | 6.0 | — | — | — |
| NADPH-cytochrome *c* reductase | 16.2 | 83.8 | — | 9.4 | 77.7 | 11.7 | — | — | — | — | — |
| RNA | 47.9 | 52.1 | 50.4 | 31.4 | 15.5 | 3.3 | — | — | — | — | — |
| DNA | 0.5 | 98.8 | 35.6 | 6.8 | 29.9 | 58.2 | 7.6 | 43.8 | 16.7 | 0.8 | 25.8 |
| Protein | 36.0 | 64.0 | 50.0 | 18.1 | 21.5 | 13.5 | 5.4 | 3.5 | 5.7 | n.d. | 4.7 |

[a] Values presented are percentages of the total initial amounts; since the homogenate was viscous and difficult to sample, initial quantities were taken as the sums of the amounts present in $S_0$ and $P_0$. Recoveries of succinic dehydrogenase and β-glucuronidase were 73% and 78%, respectively. Recoveries of all other markers were greater than 80%. Samples lacking detectible activity are indicated by the abbreviation n.d.

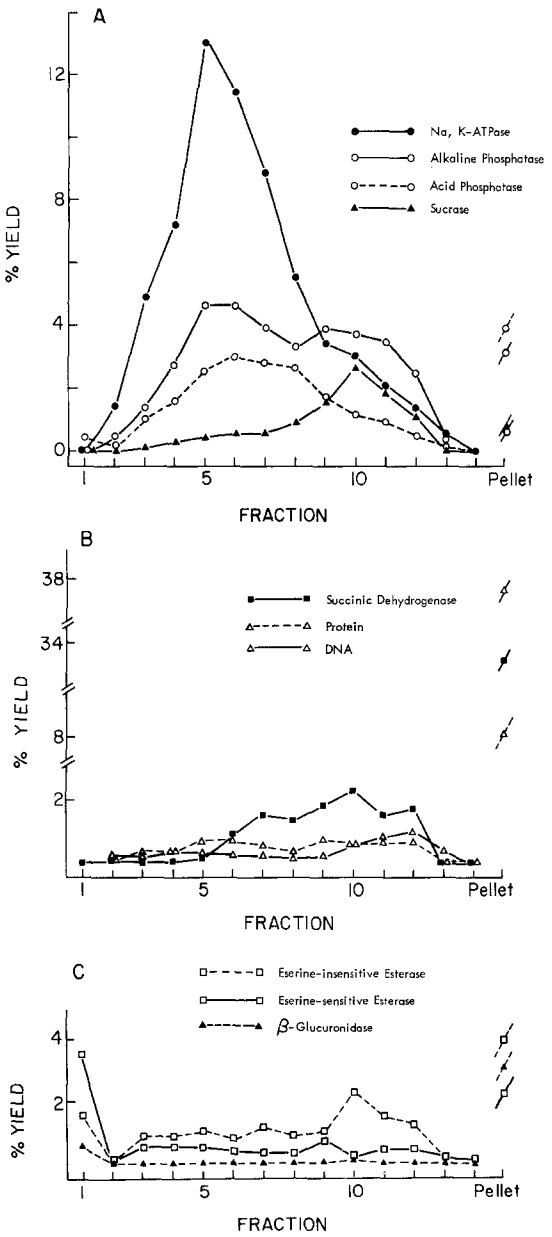


Fig. 2. Distribution of markers following equilibrium density gradient centrifugation of $P_4'$. Yields are expressed as percentages of the total initial marker activities represented by the individual density gradient fractions. (*a*), (*b*), and (*c*) were obtained from the same density gradient. RNA, not shown, was concentrated in the pellet

increase of Na, K-ATPase activity. The distributions of enzyme activities, protein and DNA obtained in one preparation are presented in Fig. 2.

This pattern was highly reproducible. 75% of the Na, K-ATPase activity recovered from density gradient centrifugation was contained in a single symmetrical peak scanning fractions 3 through 8; 6% of the recovered activity was in the pellet; most of the remaining Na, K-ATPase was present in a broad shoulder from the high density end of the main peak. The Na, K-ATPase activity of fractions 3–8 was purified 12-fold with respect to protein, 18-fold with respect to sucrase, and 19-fold with respect to succinic dehydrogenase. The Na, ATPase activity of the lower density half of the main peak, fractions 3–5, representing 31% of the recovered Na, K-ATPase, was purified 12-fold with respect to protein, 23-fold with respect to sucrase, and 70-fold with respect to succinic dehydrogenase. The constant 12-fold increase of Na, K-ATPase specific activity over the entire peak suggests that contamination of the basal lateral membranes by mitochondria represented an insignificant fraction of the total protein in the peak. Succinic dehydrogenase activity was divided between the pellet formed beneath the cushion of 65% sorbitol and a broad peak extending from fraction 6 to fraction 13. RNA (not shown) and DNA were concentrated in the pellet.

The distribution of NADPH-cytochrome *c* reductase was also followed during differential centrifugation and density gradient purification of basal lateral membranes. Recovery of NADPH-cytochrome *c* reductase during differential centrifugation was 115%. The NADPH-cytochrome *c* reductase activity of fraction $P'_4$ decreased by 42% from the time this fraction was first prepared until the time the density gradient fractions were collected and assayed; the total recovery of NADPH-cytochrome *c* reductase from density gradient centrifugation was 99% of the activity remaining in fraction $P'_4$. Since recovery of protein and most markers from density gradient centrifugation was usually about 80%, it appears that, superimposed upon a time-dependent decrease of NADPH-cytochrome *c* reductase activity, there had been activation by the manipulations involved in density gradient centrifugation and washing of density gradient fractions. Therefore, in order to permit evaluation of the results of this experiment, all activities are referred to the total recovered activity rather than the measured initial activity. These results are presented in Fig. 3..The density gradient distribution of NADPH-cytochrome *c* reductase was more complex than the distributions of other markers. There were peaks of activity in fraction 1, in the pellet, and distributed through the gradient in parallel with the combined plasma membrane markers, Na, K-ATPase and sucrase. The distribution of NADPH-cytochrome *c* reductase was independent of the distribution

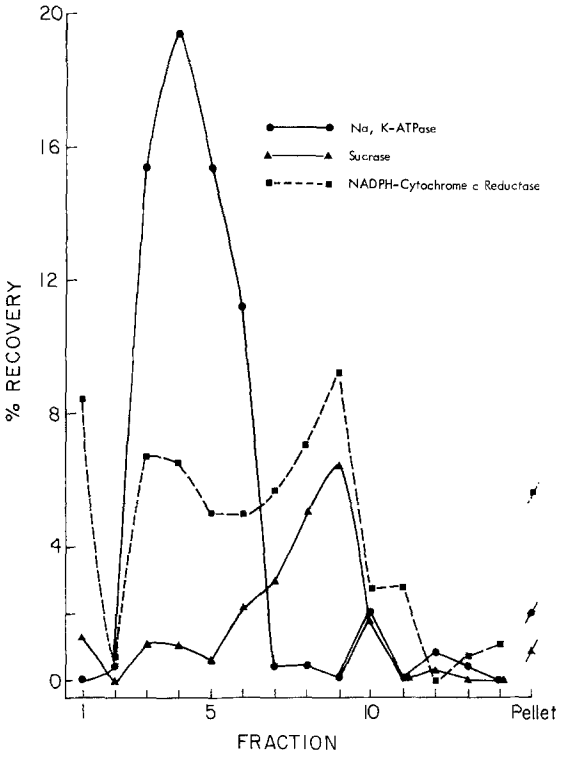


Fig. 3. Distribution of NADPH-cytochrome *c* reductase and plasma membrane markers following equilibrium density gradient centrifugation of $P'_4$. To compensate for activation of NADPH-cytochrome *c* reductase, the marker contents of the density gradient fractions are expressed as percent recoveries, i.e., as percentages of the total amount of the markers recovered rather than the percentage yields

of protein, so that it was, apparently, nonrandom. In this preparation the purification of Na, K-ATPase activity was 11-fold with respect to protein and three-fold with respect of NADPH-cytochrome *c* reductase.

The pattern of enzyme distributions presented in Fig. 2 suggests that acid phosphatase and alkaline phosphatase activities are associated specifically, but not exclusively, with the basal lateral plasma membranes. There was a close parallel between the density gradient distributions of Na, K-ATPase and acid phosphatase. This parallel was highly reproducible, and it was not possible to achieve any dissociation of the two activities, either by prolonging the density gradient centrifugation to forty eight hours or by resuspending $P'_4$ in 65% sorbitol and loading it onto the density gradient as fraction 14. Since acid phosphatase is conventionally assumed to be a lysosomal marker, it is of interest to note the distributions of other enzyme activities believed to be at least

partially associated with lysosomes, β-glucuronidase and nonspecific esterase. Acid phosphatase and β-glucuronidase were distributed independently of each other during differential centrifugation, and there was no detectible β-glucuronidase activity in density fractions 3 through 8. Furthermore, there was no correlation between the density gradient distribution of acid phosphatase and the distributions of either eserine-sensitive or eserine-insensitive esterase activities. These results suggest that the fraction of the total acid phosphatase activity present in purified basal lateral membranes cannot be attributed to contamination by lysosomes.

Alkaline phosphatase followed a bimodal distribution during density gradient centrifugation; there was a peak of activity subsumed by the main peak of Na, K-ATPase activity and a second peak which paralleled the distribution of sucrase activity. Since there was little sucrase activity in the basal lateral membrane region of the density gradient, it is unlikely that the alkaline phosphatase activity there is the result of contamination by fragments of the brush border plasma membranes. It appears, then, that alkaline phosphatase is a native activity of both the brush border and the basal lateral plasma membranes.

*Density Gradient Purification of Brush Border Plasma Membranes.* The highly reproducible appearance of a peak of sucrase activity within the linear regions of the density gradients used for purification of basal lateral plasma membranes suggested that brush border plasma membranes from fraction $P_3$ would be separated from DNA by the same procedure. In a preliminary experiment large, particulate material was removed from $P_3$ by centrifugation at $32 \times g \times 10$ min, and the material in the resulting supernatant was harvested by centrifugation and subjected to equilibrium centrifugation on linear 25% to 65% sorbitol density gradients. 80% of the recovered sucrase activity occurred in a sharp, symmetrical peak between fractions 10 and 13. About two-thirds of the recovered Na, K-ATPase activity was found to have come to equilibrium in fractions characteristic of basal lateral membranes, and one-sixth of the recovered activity was distributed in parallel with the sucrase activity.

The membrane isolation procedure described under *Materials and Methods* and outlined in Fig. 1. achieves a virtually complete separation of Na, K-ATPase activity from the purified brush border membranes. For analytical purposes the recovery of Na, K-ATPase activity following density gradient centrifugation of $P_9$ was unacceptably low, about 50%. However, if 1 mM iodoacetamide was included in the isolation and density gradient media it increased the recovery of Na, K-ATPase activity from

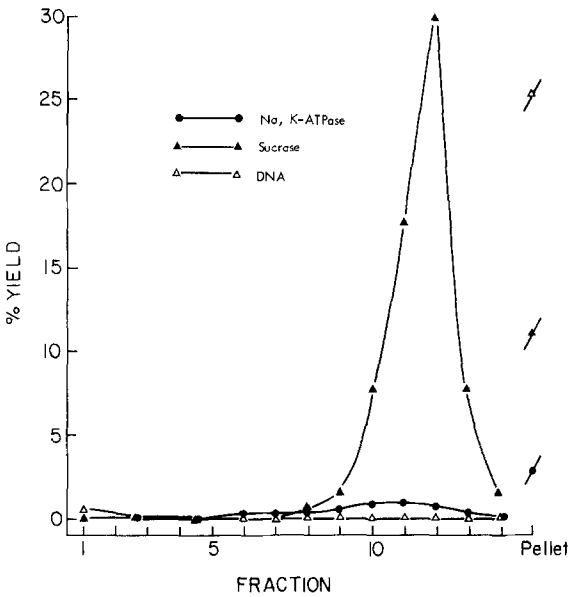


Fig. 4. Distributions of plasma membrane markers and DNA during purification of brush border plasma membranes by density gradient centrifugation of $P'_9$. Yields are expressed as percentages of the total initial marker contents. Sucrase specific activity in fractions 11 and 12 was increased 40-fold compared to the initial homogenate

differential centrifugation to 104%, and it decreased recovery during density gradient purification of brush borders to 75%. The distributions of Na, K-ATPase, sucrase, and DNA following preparation and density gradient centrifugation of $P'_9$ in the presence of iodoacetamide are presented in Fig. 4. In a separate experiment performed under the same conditions, the particulate material in pooled supernatants $S_4$–$S_6$ was harvested by centrifugation and subjected to sorbitol density gradient centrifugation. These experiments indicated that purified brush border membranes account for 5% of the total initial Na, K-ATPase activity, and that much of the Na, K-ATPase activity present in $P_3$ may be attributed to residual basal lateral membranes. The combined basal lateral membranes resolved by density gradient centrifugation of fractions $P'_4$, $P'_9$, and $S_4$–$S_6$ account for 59% of the total initial Na, K-ATPase activity, and the pellets formed under the 65% sorbitol cushions of these density gradients account for an additional 12% of the initial Na, K-ATPase activity.

*Presence of Calcium-Stimulated ATPase Activity in Purified Brush Border and Basal Lateral Plasma Membranes.* Since there is a body of circumstantial evidence relating calcium-stimulated ATPase activity to

alkaline phosphatase [14, 28], the analytical isolation results presented in Fig. 2 suggested that calcium-stimulated ATPase activity might be detected in both the brush border and the basal lateral plasma membranes. Optimal conditions of pH and calcium concentration were determined in a preparation of crude brush borders corresponding to $P_3$ in the present work (A.K. Mircheff, R. Freedman, and K.M. Ng, *unpublished*). Under these assay conditions, described in detail under *Materials and Methods*, there is no detectable calcium-stimulated ATPase activity in $P_0$, and calcium strongly inhibits ATP hydrolysis in fractions which are enriched in succinic dehydrogenase. In three preparations the distribution of Ca-ATPase was followed after density gradient centrifugation of $P'_4$; calcium-stimulated ATPase activity was found to closely parallel the distribution of Na, K-ATPase activity. In other experiments, the distributions of calcium-stimulated ATPase, alkaline phosphatase, and

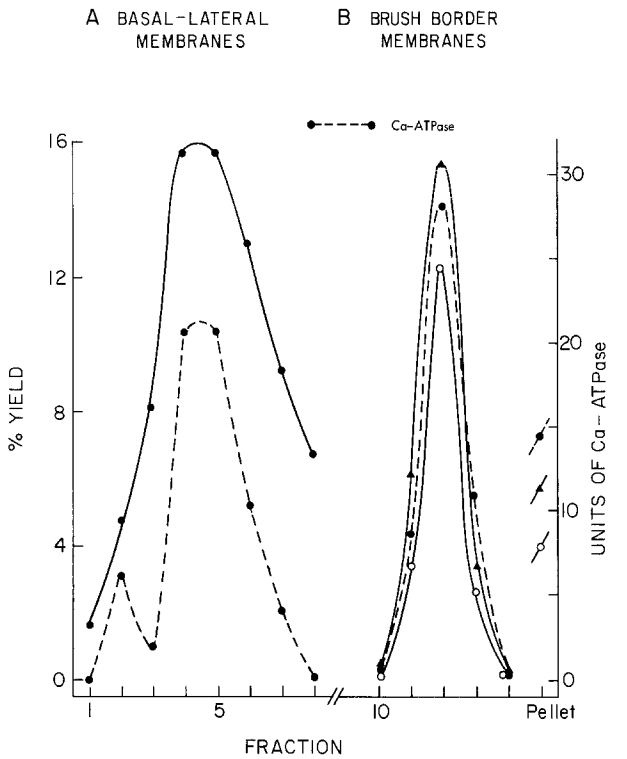


Fig. 5. Parallel distributions of calcium-stimulated ATPase activity and plasma membrane markers during density gradient centrifugation of $P'_4$ (*A*) and $P'_5$ (*B*). Yields of Na, K-ATPase, sucrase, and alkaline phosphatase are expressed as percentages of the total initial amounts; calcium-stimulated ATPase activity is expressed in units of activity, μmoles of phosphate liberated/hr. The key of the figure is identical to that given in Fig. 2 (Na, K-ATPase ●—●, Alkaline phosphatase ○—○, Sucrase ▲—▲)

sucrase were determined after density gradient purification of brush border membranes; there was a close parallel between all three activities. Representative density gradient patterns are presented in Fig. 5. These results demonstrate calcium-stimulated ATPase to be a native activity of both the brush border and basal lateral plasma membranes.

### *Use of a Slowly Permeating Labeling Reagent to Verify the Plasma Membrane Origins of Na, K-ATPase-Rich and Sucrase-Rich Membranes*

*Labeling Everted Gut Sacs with $^{35}$S-DABS.* The distributions of $^{35}$S and sucrase in differential fractions and purified brush border membranes after everted gut sacs were reacted with $^{35}$S-DABS are presented in Table 2, and distributions of $^{35}$S, sucrase, Na, K-ATPase, and protein after density gradient centrifugation of $P'_4$ from the same experiment are presented in Fig. 6. It is clear that the brush border membranes were preferentially labeled with $^{35}$S; these results were reproduced quantitatively in three experiments. $^{35}$S was present in all fractions, but the specific content of radioactivity (cpm/mg protein) in purified brush border membranes was at least 18-fold greater than in the initial supernatant, $S_0$, and ninefold greater than in the initial homogenate. The Na, K-ATPase-rich membranes showed no enrichment of $^{35}$S specific content compared to the initial homogenate and only a twofold increase with respect to $S_0$.[2] The localization of sucrase activity to the brush border

Table 2. Distributions of sucrase activity and $^{35}$S in differential centrifugation fractions and purified brush border membranes after everted gut sacs were reacted with $^{35}$S-DABS[a]

| | | $S_0$ | $P_0$ | $S_1$–$S_3$ | $P_8$ | $S_9$ | $P_9$ | Purified brush border membranes |
|---|---|---|---|---|---|---|---|---|
| Sucrase | A | 8.6 | 91.4 | 30.8 | 0.2 | n.d. | 44.6 | 26.3 |
| | B | 0.17 | 1.17 | 0.53 | 0.03 | n.d. | – | 35.09 |
| $^{35}$S | A | 19.7 | 80.0 | 55.1 | 8.3 | 1.7 | 11.8 | 5.3 |
| | C | 1,671 | 4,370 | 4,007 | 5,871 | 3,000 | – | 30,310 |

[a] Values presented are: *A*, percentage of the total initial activity; *B*, sucrase specific activity, relative to its specific activity in the initial homogenate; and *C*, $^{35}$S specific content, cpm/mg protein. The high concentration of sorbitol in fraction $P_9$ prevented determination of protein content.

2 Labeling of the Na, K-ATPase-rich membranes in these experiments may, at least in part, be due to leakage of the reagent across the tight junctions into the lateral intercellular spaces.

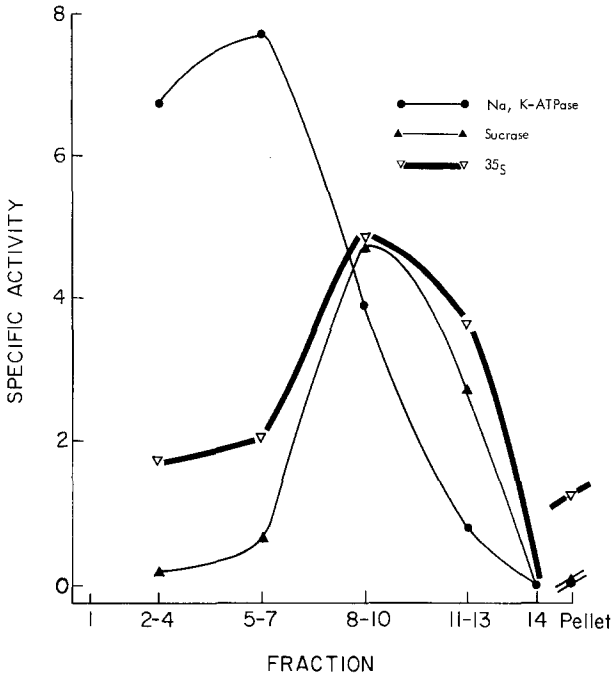


Fig. 6. Reaction of everted gut sacs with $^{35}$S-DABS. Distributions of Na, K-ATPase, sucrase, and $^{35}$S specific activities following density gradient centrifugation of $P'_4$. Enzyme specific activities are expressed as μmoles of product liberated/mg protein × hr; $^{35}$S specific activity is the $^{35}$S specific content (cpm/mg protein) relative to the $^{35}$S specific content of the intracellular fraction, $S_0$. Note that the actual distribution of all markers was similar to that shown in Fig. 2

membranes of intestinal epithelial cells is well established (e.g., [21]), so the result that purified sucrase-rich membranes became preferentially labeled, compared to an intracellular fractions, $S_0$, when the brush border surface of the intestinal epithelium was exposed to $^{35}$S-DABS confirms the validity of the use of this compound as a slowly permeating labeling reagent. This result complements the results already presented on the analytical isolation of basal lateral plasma membranes: analytical isolation established the existance of a population of particles which accounts for 60% of the total initial Na, K-ATPase and which is free of sucrase activity, and reacting everted gut sacs with a slowly permeating labeling reagent confirms that the Na, K-ATPase-rich and the sucrase-rich membranes have different subcellular origins.

*Labeling Isolated Intestinal Epithelial Cells with $^{35}$S-DABS.* When suspensions of isolated intestinal epithelial cells were reacted with $^{35}$S-DABS, i.e., when both the brush border and the basal lateral plasma membranes were exposed to the labeling reagent, the subsequently

purified sucrase-rich and Na, K-ATPase-rich membranes were labeled to nearly the same extent, more than fourfold greater than the extent of labeling of the intracellular protein of fraction $S_0$. Brush border membranes isolated by density gradient centrifugation of $P_1$ had a specific $^{35}S$ content greater than the specific content $S_0$ by a factor of 4.1. The distributions of Na, K-ATPase specific activity, sucrase specific activity, and relative $^{35}S$ specific content obtained by density gradient centrifugation of the $450 \times g$ supernatant are presented in Fig. 7. There is a clear parallel between the distributions of $^{35}S$ and the combined plasma membrane markers Na, K-ATPase and sucrase; the average $^{35}S$ specific content of the pooled Na, K-ATPase-rich fractions, fractions 2–7, is greater than the specific content of $S_0$ by a factor of 4.6. Similar results were obtained when damaged cells were removed from the suspension of isolated, labeled cells by centrifugation on a 25% to 35% linear gradient of beef serum albumin in the low pH medium described by Shortman *et al.* [33].

When purified basal lateral membranes, brush border membranes, succinic dehydrogenase rich density gradient fractions and $S_0$ were

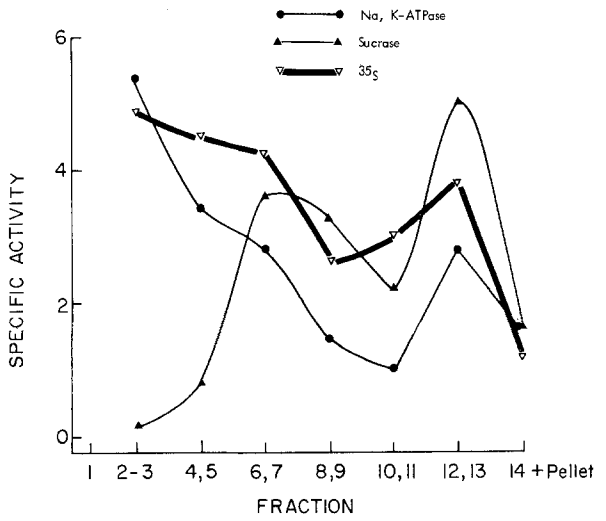


Fig. 7. Reaction of a suspension of isolated intestinal epithelial cells with $^{35}S$-DABS. Distributions of Na, ATPase, sucrase, and $^{35}S$ specific activities after fraction $S_1$ was prepared as described in the text and subjected to density gradient centrifugation for resolution of basal lateral membranes. Units of specific activity are described in the legend to Fig. 6. Relative to Fig. 6, the specific activity curves were distorted owing to a shift of mitochondrial and nuclear protein from the pellet to fractions 7–11. This phenomenon was also observed when membranes were purified from isolated cells that had not been labeled or exposed to high pH

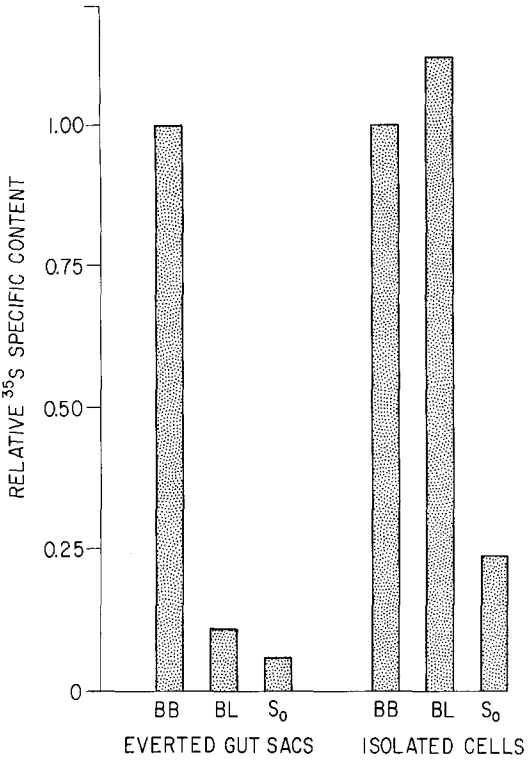


Fig. 8. The labeling patterns obtained when everted gut sacs and isolated epithelial cells were reacted with $^{35}$DABS. The relative $^{35}S$ specific content of the basal lateral cell membranes and intracellular protein ($S_0$) in both experiments is normalized to the specific content of the brush border membranes. In everted sacs only the brush border surface of the epithelium is exposed to the reaction mixture

reacted with $^{35}S$-DABS at constant protein and $^{35}S$-DABS concentrations, the relative $^{35}S$ specific contents after removal of unreacted label were, respectively, 1.08, 0.76, 0.92, and 1.0. This result indicates that the patterns of labeling in Table 2 and Figs. 6 and 7 do not result from differences in the capacities of the various subcellular fractions to react with DABS.

The contrasting patterns of labeling obtained when everted gut sacs and isolated cells were reacted with $^{35}S$-DABS are summarized in Fig. 8. The result that Na, K-ATPase-rich membranes become specifically labeled when isolated intestinal epithelial cells are reacted with $^{35}S$-DABS comprises direct evidence that these membranes originate from the plasma membranes, and the possibility that they arise from a subfractionation of the brush border plasma membranes is excluded by the results obtained when everted gut sacs are reacted with the slowly permeating labeling reagent.

## Discussion

Using sucrase activity as a marker for the brush border plasma membrane and Na, K-ATPase activity as a provisional marker for the basal lateral plasma membrane, we have developed an analytical isolation procedure which separated the two aspects of the intestinal epithelial cell plasma membrane from each other and from other subcellular components.

### *Analytical Isolation of Basal Lateral Plasma Membranes*

*Yield of Na, K-ATPase Activity.* 50% of the total initial Na, K-ATPase activity was readily recovered in a single population of membrane fragments defined by density gradient centrifugation. A further 9% of the initial activity was attributed to the same membrane population by density gradient centrifugation of low speed supernatants arising during purification of brush border plasma membranes. Since the overall recovery of Na, K-ATPase activity was 80%, the unique population of Na, K-ATPase-rich membranes accounts for 74% of the recovered Na, K-ATPase activity. The purified brush border plasma membranes account for an additional 6% of the recovered Na, K-ATPase activity.

*Direct Evidence that Na, K-ATPase-Rich Membranes Originate from the Basal Lateral Plasma Membranes.* The distinct patterns of labeling that resulted when everted gut sacs or suspensions of isolated epithelial cells were reacted with $^{35}$S-DABS comprise direct evidence that the Na, K-ATPase-rich membranes resolved by density gradient centrifugation arise from the basal lateral plasma membranes. That is, Na, K-ATPase-rich membranes were more heavily labeled than intracellular protein—i.e., fraction $S_0$ and mitochondria rich fractions, and they were labeled to the same extent as brush border membranes, when suspensions of isolated cells were reacted with $^{35}$S-DABS, while only brush border membranes were preferentially labeled when everted gut sacs were reacted with the diazonium salt. This is precisely the result expected if the Na, K-ATPase-rich membranes arise from the basal lateral plasma membrane and if $^{35}$S-DABS is slowly permeating labeling reagent. The results obtained from labeling isolated subcellular fractions with $^{35}$S-DABS and, the observation that brush border membranes were more heavily labeled than fractions rich in soluble protein, succinic dehydrogenase, and DNA when either everted gut sacs or isolated cells were reacted with $^{35}$S-DABS,

confirm the validity of using this compound as a slowly permeating labeling reagent.

Previous applications of $^{35}$S-DABS in studies of membrane surface architecture of erythrocytes [1, 2], mitochondrial inner membranes [29] and chloroplast thylakoid membranes [6] have already established the general validity of $^{35}$S-DABS as a slowly permeating, and therefore surface-specific, labeling reagent.

The conclusion drawn from the results of labeling everted gut sacs and isolated epithelial cells with $^{35}$S-DABS are straightforward, and these results are the first direct evidence that the Na, K-ATPase-rich membranes which are resolved by density gradient centrifugation originate from the basal lateral plasma membranes of intestinal epithelial cells.[3] The previously adduced evidence for the identity of such purified membranes has been of a circumstantial nature, including analogy to the distribution of Na, K-ATPase in more thoroughly characterized cell types, i.e., erythrocytes and liver cells; predictions of models of epithelial transport mechanisms, which require that sodium pump sites be localized on the basal lateral membranes; and an autoradiographic demonstration of specific ouabain binding sites on the basal lateral membranes of rabbit intestinal epithelial cells [34].

*Other Procedures for Isolation of Basal Lateral Plasma Membranes.* The analytical isolation procedure presented here is the first to be reported which succeeds in isolating the major fraction of the total initial Na, K-ATPase activity without a concomitant increase in the measured activity. The purification of Na, K-ATPase activity is similar to the purifications obtained by two methods which produce smaller yields. In the procedure of Fujita *et al.* [13], the yield of Na, K-ATPase activity in the purified basal lateral membranes was only 17% of the total initial activity; in that procedure, the major portion of the Na, K-ATPase activity was lost to the differential fractions which resulted from preliminary subfractionation of the initial homogenate into brush border, nuclear, mitochondrial, and microsomal fractions. The yield of Na, K-ATPase activity in basal lateral membranes purified by the procedure of Douglas *et al.* [7] was 12% of the total initial activity;

3 After this manuscript was prepared for publication a report by Lewis *et al.* (Basolateral Plasma Membranes of Intestinal Epithelial cells: Identification by lactoperoxidase catalyzed iodination and isolation after density pertubation with digitonin. *Biochem. J.* **152**:71, 1975) appeared describing the preparation of plasma membranes from guinea pig intestinal epithelial cells. These workers obtained Na, K-ATPase-rich membranes by a combination of differential and density gradient centrifugation similar in principle to that described here, but they found it necessary to treat mitochondrial fractions with digitonin to permit separation of Na, K-ATPase activity from sucrase. They used lactoperoxidase catalyzed iodination to demonstrate the plasma membrane origins of Na, K-ATPase-rich density gradient fractions defined by zonal rotor centrifugation of crude epithelial cell homogenates. In rat intestine we were unable to demonstrate lactoperoxidase catalyzed iodination of plasma membranes.

in that work, most of the activity was lost during low speed removal of brush borders and preliminary separation of mitochondria by density gradient centrifugation.

The yield of Na, K-ATPase activity in the analytical isolation procedure presented here is comparable to that obtained by Quigley and Gotterer [26], and the purification factors in these two procedures are comparable if they are expressed as the ratios of the percentage recovery of Na, K-ATPase to the percentage recovery of protein. The absolute purification of Na, K-ATPase activity obtained by Quigley and Gotterer [26] was twice that produced in the present work, but it seems likely that this difference is attributable to alterations in the organization of the basal lateral membrane fragments which are produced during the aging procedure required in the earlier procedure. The basic strategy of the separation of Quigley and Gotterer [26] is similar to the strategy described here, and the inability of that procedure to separate basal lateral membranes from mitochondria without preliminary aging may result from differences in the homogenization procedures employed (Waring blender homogenization in contrast to Dounce homogenization) or the compositions of the isolation buffers (2.5 mM NaEDTA in contrast to 250 mM sorbitol, 12.5 mM NaCl, 5 mM histidine-imidazole buffer, and 0.5 mM NaEDTA).

*Aspects of the Analytical Isolation Procedure.* The details of the procedure described in this work represent a synthesis of procedures developed by earlier workers. The strategy of initially separating basal lateral membranes from brush borders by low speed differential centrifugations is common to all three previously published methods. Dounce homogenization, suggested by Fujita [12], was chosen because it promised to be both convenient and mild. 5 mM EDTA comprised the homogenization and isolation medium in several early procedures for isolation of brush borders [10, 21], and it was adopted for the initial homogenization in the present work after preliminary experiments showed that it gave the best yields of brush borders in the $450 \times g$ pellet. Preliminary harvesting of both the brush border and basal lateral membranes by centrifugation at $45{,}000 \times g$ was inspired by the brush border isolation of Russell *et al.* [28]; it was included because, at the outset, the effects of 5 mM EDTA on the integrity of the basal lateral membranes were unknown, and because it would minimize the amount of time that the plasma membranes would be exposed to soluble hydrolytic enzymes that might have been released from lysosomes. The isolation buffer used in this analytical isolation procedure is similar in composition to the histidine-imidazole buffered sucrose employed by Fujita *et al.* [12, 13]; the presence of a polyhydroxyl alcohol was desirable since such compounds frequently stabilize the native structures of proteins, and sucrose was replaced by sorbitol to permit direct assay of the sucrase contents of differential and density gradient fractions. Low concentrations of NaCl helped to preserve the structure of brush border fragments during cell disruption by nitrogen cavitation [7], and in preliminary experiments 12.5 mM was found to be an optimal concentration for preserving brush border structure and minimizing aggregation of basal lateral membranes with the brush border fragments. Similarly, preliminary experiments indicated that the presence of 0.5 mM NaEDTA in the isolation buffer favors preservation of large brush border fragments.

*Purity of the Basal Lateral Plasma Membranes.* The distribution patterns of succinic dehydrogenase, sucrase, and DNA presented in Table 1 and Fig. 2 indicate that the purified basal lateral membranes are free of significant contamination by mitochondria, brush border plasma membranes, and nuclei.

Several enzyme activities classically assumed to be associated with lysosomes were followed, and a dissociation of acid phosphatase from

nonspecific esterase and β-glucuronidase is apparent from the results presented in Table 1 and Fig. 2. Roughly 25% of the acid phosphatase was distributed in parallel with Na, K-ATPase during density gradient centrifugation of fraction $P'_4$. While portions of the inital β-glucuronidase and nonspecific esterase activities also remained in $P'_4$, these were distributed completely independently of Na, K-ATPase during density gradient centrifugation. This result suggests that basal lateral membranes are free of lysosomal contamination and that a fraction of the total cellular acid phosphatase activity is a native component of the basal lateral membrane.

The problem of possible contamination by elements of the endoplasmic reticulum is complex. Only 16% of the initial RNA was present in fraction $P'_4$, the remainder being distributed between $S_0$ and $S'_4$. This behavior is consistent with the properties of rough microsomes from other preparations [36], but it is complicated in the present case by the possibility that ribosomes have become dissociated due to the presence of EDTA in the homogenization and isolation media. A firmer indication that the basal lateral membranes are free of contamination by elements of the rough endoplasmic reticulum is provided by the behavior of that fraction of the RNA which remains in $P'_4$; on density gradient centrifugation this RNA was sedimented to the pellet, i.e., it was distributed independently of Na, K-ATPase.

The smooth endoplasmic reticulum has historically presented the most difficult problems during purification of plasma membranes [36]. In the analytical isolation described here, NADPH-cytochrome *c* reductase had a complex distribution among differential and density gradient fractions; in the purified basal lateral membranes, Na, K-ATPase activity was purified with respect to NADPH-cytochrome *c* reductase by a factor of 3. This is comparable to the factor of 4 obtained by Douglas *et al.* [7]. That two different purification methods yield such similar purification factors may be indicative of a specific relationship between NADPH-cytochrome *c* reductase and the basal lateral membrane.

*Purification of Brush Border Membranes.* The 40-fold enrichment of sucrase activity in purified brush border plasma membranes is comparable to the purifications obtained by Eichholz [8] and by Forstner *et al.* [10]. Although procedures for the isolation of brush border membranes have been available for several years, the method developed here is novel and deserves some brief note. Our purification procedure is different from most previous methods of isolating brush border membranes in that it employs density gradient centrifugation to remove nuclear

contamination. Electron micrographs of the purified brush border membranes (*unpublished*) reveal a mixture of membrane sheets and vesicles completely free of densely staining or filamentous material; since the brush border fragments must have remained intact to be sedimented by centrifugation at $450 \times g \times 10$ min, it appears that resuspension and homogenization of the brush border fragments in 12.5% sorbitol prior to density gradient centrifugation caused their disruption, separating the brush border plasma membranes from the underlying core material.

*Distribution of Activities Between the Brush Border and Basal Lateral Membranes.* Because the isolation procedure we developed is analytical, and because it produces high yields of brush border and basal lateral plasma membranes from the same initial homogenate, it permits us to make quantitative statements about the distributions of enzyme activities between the two surfaces of the intestinal epithelial cell.

Sucrase activity has long been known to be concentrated in the brush border plasma membrane, and the present work shows it to be absent from the basal lateral plasma membrane. This result is consistent with the supposition that the physiological role of sucrase is to release glucose from intraluminal sucrose, making the hexose available for specific sugar transport mechanisms. Na, K-ATPase activity is localized on the basal lateral plasma membranes—only 6% of the activity is associated with the brush border membranes; this is the result predicted from the current model of intestinal sodium absorption [31], and, as such, it complements the biophysical evidence for that model by demonstrating that the enzymatic apparatus for sodium active transport is present and arrayed in the epithelial cell plasma membrane in the proper fashion to produce sodium absorption. About 25% of the cellular acid phosphatase activity is present in the basal lateral plasma membranes, but none is present in the brush border membrane; the physiological significance of this observation is obscure.

Alkaline phosphatase and calcium-stimulated ATPase activities are distributed between the brush border and basal lateral plasma membranes. In a single preparation in which both calcium-stimulated ATPase and alkaline phosphatase were followed during density gradient purification of brush border and basal lateral membranes, equal amounts of calcium-stimulated ATPase were found on the two membranes, while there was a 40% excess of alkaline phosphatase in the brush border membranes. Haussler *et al.* [14] and Russell *et al.* [28] have presented evidence that a single enzyme is responsible for both alkaline phosphatase and calcium-stimulated ATPase activities of brush borders. The simulta-

neous presence of these activities in the basal lateral membrane suggests that this hypothesis may be generalized to the basal lateral membrane as well; in fact, three separate preparations of basal lateral membranes had a constant ratio of alkaline phosphatase to calcium-stimulated ATPase activity. The disparate ratios of alkaline phosphatase to calcium-stimulated ATPase in brush border and basal lateral membranes, however, suggest that some molecular heterogeneity exists. It should be noted that the observation of alkaline phosphatase activity in basal lateral membranes contradicts much of the previous literature, in which alkaline phosphatase was frequently employed as a specific marker for the brush border plasma membrane [13, 23]; the present result is consistent with that obtained by Douglas *et al.* [7]. It is not likely that the alkaline phosphatase activity present in purified basal lateral membranes is the result of contamination by brush border membranes, since sucrase activity was distributed completely independently of the basal lateral membranes (Fig. 2). The discrepancy between this observation and the earlier literature may arise from differences in the conditions various workers have employed for the assay of alkaline phosphatase; the assay medium used in the present work is unique. The results presented here are of special interest because calcium stimulated ATPase activity may be an enzymatic manifestation of a calcium active transport system.

Recent work performed in this laboratory is further demonstration of the power of the present analytical isolation procedure for resolving questions about the subcellular localization of enzyme activities. In this work (van Os, Mircheff & Wright, *manuscript in preparation*) bicarbonate stimulated ATPase activity was found to be absent from brush border and basal lateral membranes of the rat intestine, and all of the $HCO_3$ stimulated ATPase of cell homogenates was found to be associated with mitochondria.

The goal of future work in our laboratory is to understand the physiological significance of the distinct enzymatic composition of the brush border and basal lateral plasma membranes, and in particular to understand relationships between enzyme activities and intestinal transport. Towards this end we are adapting the analytical isolation procedure described here for the preparative scale isolation of plasma membranes with high capacity zonal rotors.

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