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Laterobasal Membranes from Intestinal Epithelial Cells: Isolation Free of Intracellular Membrane Contaminants

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Summary. A simplified method for isolating highly purified laterobasal membranes (LBM) from enterocytes is based on treatment of membranes with 8 mM $CaCl_2$ concentration in order to aggregate intracellular membrane contaminants. The resultant LBM showed an average 15-fold enrichment and constituted 8% of the original K-stimulated phosphatase in the initial crude homogenate. It showed typical LBM migration on counter-current distribution (CCD) and was essentially free of contamination with endoplasmic reticulum and Golgi membranes. This method is highly efficient and yields sufficient purified LBM to allow comprehensive analysis of enterocyte membrane events.

Key Words small intestine · enterocyte · membrane fractionation · laterobasal membrane · endoplasmic reticulum · Golgi

Introduction

Because they perform highly specialized transport and secretory functions, small intestinal enterocytes and renal tubular cells possess two distinct types of surface membranes at their laterobasal and luminal surfaces [5, 8]. Despite considerable interest in the complimentary specialized roles of these surface membranes, detailed analysis of intracellular membrane protein assembly, glycoprotein sorting and vectorial transport to the respective cell surfaces has been hampered by the absence of efficient membrane separation techniques that yield highly purified endoplasmic reticulum (ER), Golgi, brush border (BB) and laterobasal (LBM) membranes from the same epithelial cell preparation.

Whereas purified BB can be separated from other membranes after the precipitation of contaminating intracellular membranes by low concentrations of $CaCl_2$ or $MgCl_2$, current methods for the purification of LBM rely mainly on differential centrifugation followed by equilibrium density centrifugation in a discontinuous or linear gradient [2, 21], techniques that result in heavy residual contamination of the LBM by ER-Golgi[1] [2, 21]. Although counter-current distribution may allow further separation of the ER-Golgi from LBM [11], this technique requires specialized expensive equipment not generally available. We report here a simplified method for isolating highly purified LBM, uncontaminated by ER or Golgi membranes, that can be readily prepared from either rat small intestinal scrapings or dispersed enterocytes.

Materials and Methods

Enzyme Assays

Enzymes known to be highly enriched in particular membrane fractions were used as markers for these fractions. Aryl esterase (for ER), galactosyl transferase or mannosidase II (for Golgi), potassium-stimulated phosphatase (for LBM) and sucrase (for BB) were assayed as previously described [2, 6, 9, 14]. Mannosidase II was assayed as described by Tulsiani and his colleagues [18, 19]. Recovered membranes from the fractionation scheme (*cf.* Fig. 1) were routinely diluted prior to analysis in 5 mM histidine-imidazole, 50 mM mannitol, 0.5 mM EDTA, pH 7.4; this buffer stabilized the marker enzyme activities.

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[1] ER-Golgi is an operational term designating a membrane fraction enriched in both endoplasmic reticulum and Golgi membranes. The term is used here because these two membrane fractions copurify when the usual subcellular techniques are applied to enterocytes.

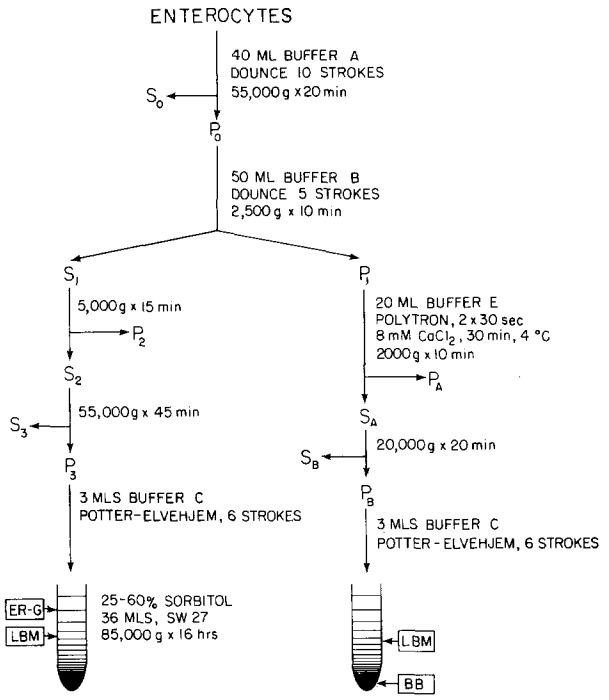

Fig. 1. Intestinal subcellular fractionation scheme (*cf.* Materials and Methods for additional details and composition of buffers). Enterocytes were dissociated from everted intestinal sacs with 2.5 mM EDTA or scraped from intact intestinal segments and homogenized in buffer A. The final pellets, P_B and P_3, were resuspended in 12.5% sorbitol and layered over a 36-ml 25–60% sorbitol gradient and centrifuged to equilibrium. Two-ml fractions were collected and assayed for marker enzymes (*see* Figs. 2 and 3)

Counter-Current Distribution

This technique, essentially a sequential extraction based on the principle of liquid chromatography that separates membrane populations by virtue of their differential partitioning between an upper and lower phase, has been described in detail [1, 4]. The counter-current apparatus consists of two juxtaposed circular plates, each having 120 half-chambers that accommodate the upper and lower aqueous phase [3]. The extraction process includes a series of steps: (i) vigorous agitation of the contents of the aligned upper and lower chambers; (ii) a pause to allow phase reformation, and (iii) transfer of each upper chamber's contents by rotation of the upper plate until its half-chamber is realigned over the next half-chamber in the lower plate. In our experiments, the upper half-chambers were loaded with 600-μl aliquots of 3.5% polyethylene glycol 6000, 5% sorbitol, 10 mM sodium EDTA, 8.3 mM imidazole-HCl, pH 7.0, and the lower half-chambers with 600 μl of 5% dextran T500, 5% sorbitol, 10 mM sodium EDTA and 8.3 mM imidazole-HCl, pH 7.0. Membranes were taken up in 4 ml of upper phase buffer, loaded into the first six upper half-chambers and subjected to 54 transfer steps generating a total of 60 fractions. Six successive fractions were then pooled and assayed for protein and the appropriate marker enzymes.

Membrane Fractionation

Jejunum and proximal ileum (~90 cm length), obtained from fasted, anesthetized, male Sprague-Dawley rats (usually 300–350 g) was rinsed with 1.4% NaCl, and the enterocytes were dispersed and harvested by a method modified from Mitjavila et al. [13]. Similar results were obtained with younger animals (~80 days of age) weighing 250 g or less. Intestinal segments were everted over a glass rod, and sacs, made by tying the everted segment at both ends, were filled with 2% NaCl, and agitated in 1.4% NaCl, 2.5 mM EDTA for 1 hr on ice with a chain stirrer. The stirrer was fashioned from two 9-cm lengths of steel chain (6 mm diameter) that had been covered with polyvinyl tubing (o.d. 8 mm) attached at each end to the top and bottom of a variable speed steel rod with the rheostat set at 60 (Cole Palmer no. 4335-00). The resulting cell suspension was then centrifuged (500 × *g*, 5 min). In some experiments, the enterocytes were also scraped off directly from the everted intestine by use of microscopic slides, as described [6].

The subcellular fractionation scheme, modified from that previously described [2], is outlined in Fig. 1. The enterocytes were homogenized (Dounce, pestle B, ~10 strokes) in 40 ml of 5 mM EDTA, 5 mM histidine-imidazole, pH 7.4 (buffer A) until no intact cells remained, as monitored by phase microscopy. The homogenate was centrifuged at 55,000 × *g* for 20 min to yield pellet P_0 which was resuspended in 30 ml of 5 mM histidine-imidazole, pH 7.4, 0.5 mM EDTA, 250 mM sorbitol, 12.5 mM NaCl (buffer B) and rehomogenized (Dounce, pestle B, ~5 strokes) until only brush border membranes, nuclei and membrane fragments were seen by phase microscopy. The resuspended material was then brought up to 50 ml with buffer B and centrifuged at 2,500 × *g* for 10 min to yield the P_1 pellet (containing 85–90% of the BB and 80% of the LBM of the original P_0). The S_1 supernatant (enriched in ER-Golgi and LBM) was then centrifuged at 5,000 × *g* for 15 min to precipitate dense contaminating membranes (P_2). The membranes in the S_2 supernatant were harvested by centrifugation at 55,000 × *g* for 45 min to yield pellet P_3.

P_1 was resuspended in 10 ml of 2 mM Tris-HCl, pH 7.1, 50 mM mannitol (buffer E) and homogenized with two bursts of 30 sec each in a Polytron homogenizer (Brinkman) at setting 4. The membrane suspension was brought up to 20 ml with buffer E, and $CaCl_2$ from 100 mM stock was added to achieve a final concentration of 5, 8 or 10 mM; the suspension was agitated for 30 min at 4°C on a rotatory shaker at 30 rpm. Careful control of the $CaCl_2$ concentration was found to aggregate ER-Golgi membranes, which could be removed by low-speed centrifugation (2000 × *g* for 10 min, pellet P_A). BB and LBM remained in the S_A supernatant and were harvested by centrifugation at 20,000 × *g* for 20 min to yield pellet P_B.

The P_3 and P_B pellets were resuspended in 3 ml of 5 mM histidine-imidazole, 12.5% sorbitol, 0.5 mM EDTA (buffer C) and treated with six vertical strokes in a Potter-Elvehjem homogenizer with a Teflon pestle. Aliquots (2.5 ml) from each membrane suspension were layered on a 36-ml linear sorbitol gradient (25–60% wt/vol in 5 mM histidine-imidazole, 0.5 mM EDTA) and centrifuged in an SW 27 rotor at 85,000 × *g* for 16 hr to equilibrium. Fractions (2 ml) were collected from the top of the gradient by a Buchler Auto Densi Flow II C apparatus and diluted with 2 ml of 5 mM histidine-imidazole, pH 7.4, containing 1 mM $MgCl_2$, 50 mM NaCl and 0.5 mM EDTA (buffer D). Assays for enzymes known to be enriched in various membrane fractions were performed on all fractions (*cf.* enzyme assays above). The relevant membranes were then recovered by pooling the appropriate fractions and centrifuging at 40,000 × *g* overnight.

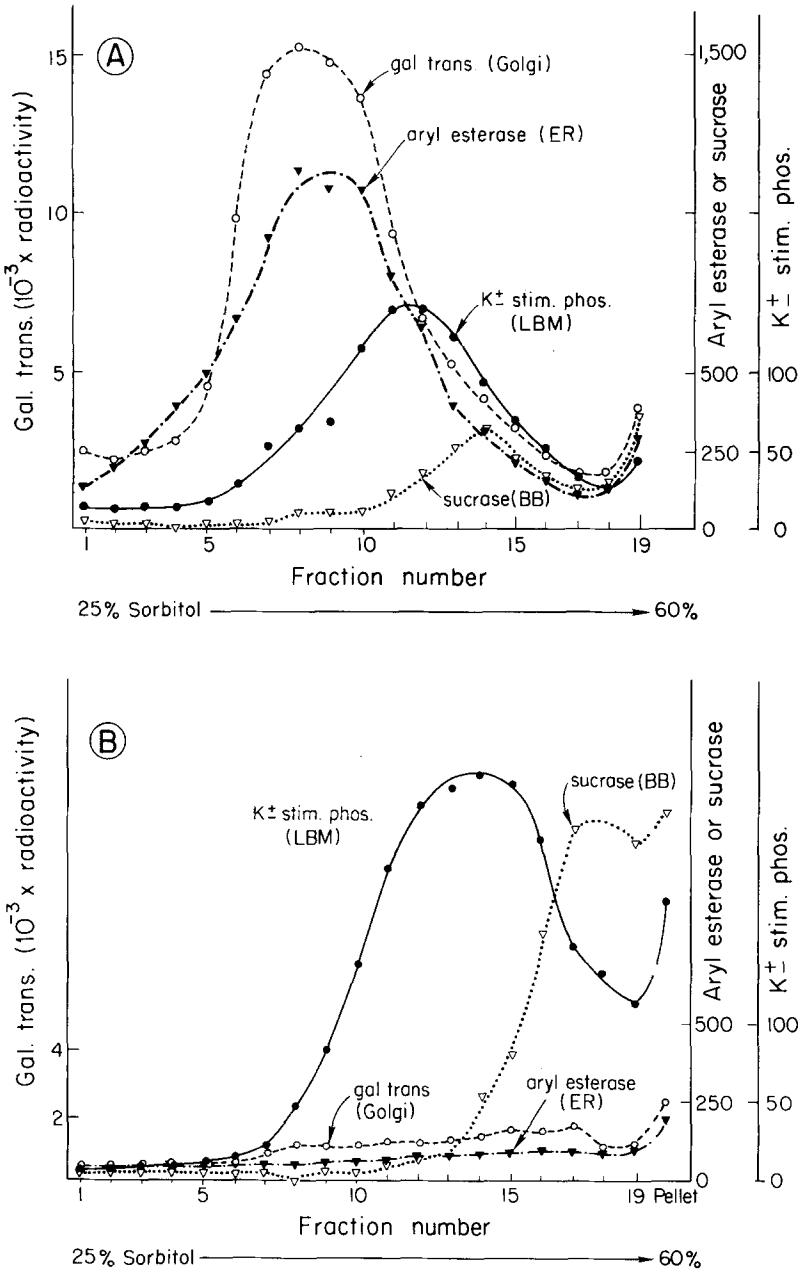

Fig. 2. Sorbitol equilibrium gradient separation of intestinal subcellular organelles from dispersed enterocytes. Pellets P_3 (Panel *A*) and P_B (Panel *B*), obtained as described in Materials and Methods and Fig. 1, were taken up in 3 ml of buffer C, layered over a 36-ml 25–60% linear sorbitol gradient and centrifuged overnight to equilibrium. Fractions (2 ml) were collected and assayed for the appropriate marker enzymes for each membrane species (given in parentheses). Comparisons for a particular enzyme activity can be made between panels *A* and *B*. Ordinate scales have been arbitrarily selected for clarity of marker localizations and actual cross-contamination is provided in Tables 2 and 3. These patterns represent typical results obtained from a single centrifugation experiment

Results

Preliminary Experiments with $CaCl_2$ in the Preparation of Intestinal Membranes

Treatment of crude intestinal membranes with 10 mM calcium is used routinely as a means of aggregating intracellular membranes and laterobasal surface membrane vesicles while leaving brush border vesicles in suspension. In preliminary experiments, we found that reduction of $CaCl_2$ concentration from 10 to 5–8 mM regularly enhanced recovery of LBM in the low speed supernatant (*cf.* Fig. 1, Treatment of Pellet P_1 to yield Pellet P_B). As considered further below, this simple preparative change prior to equilibrium centrifugation was highly beneficial in removing contaminating ER and Golgi membranes from the LBM.

Isolation of LBM from Dispersed Enterocytes

Figure 2 reveals typical membrane distributions in sorbitol equilibrium gradients of the membrane fractions obtained from enterocytes dispersed from everted gut sacs. When the P_3 pellet, the usual source of LBM and ER-Golgi (*cf.* Fig. 1), was

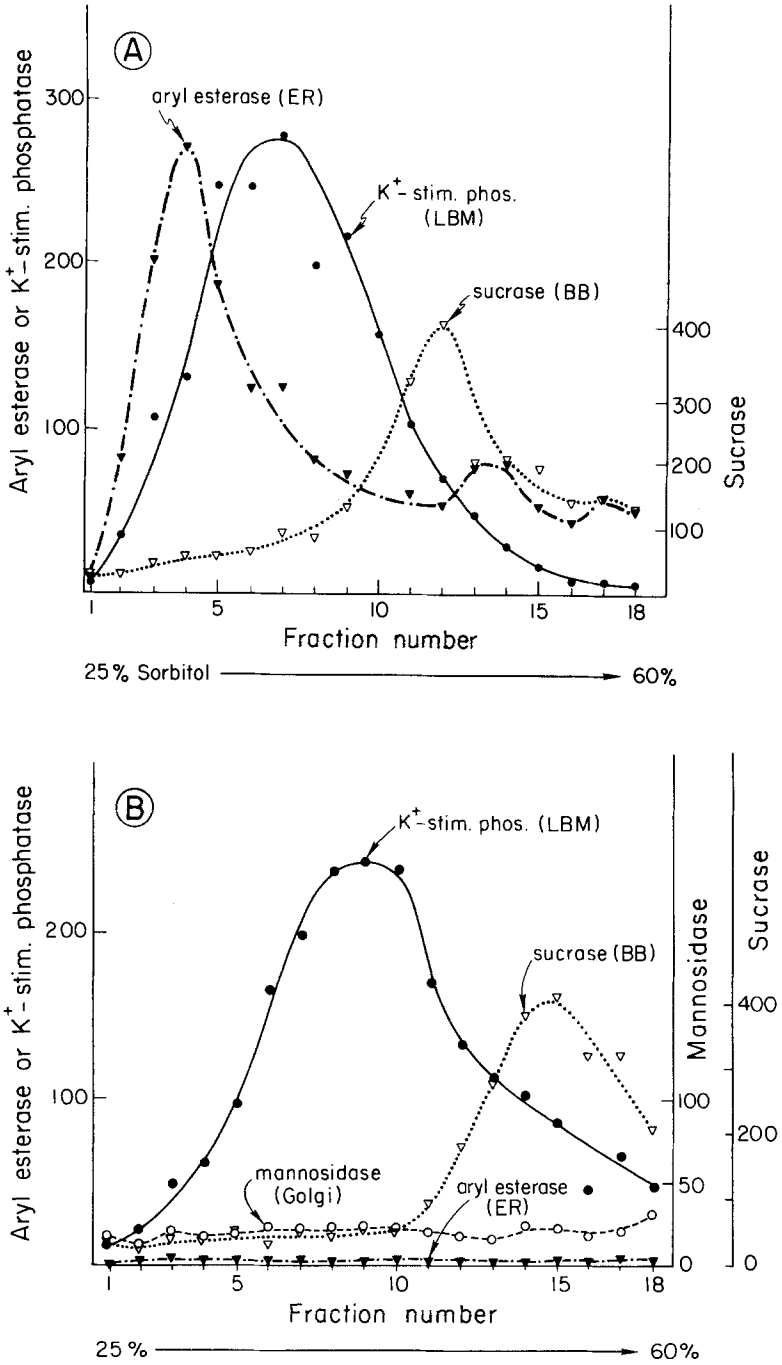

Fig. 3. Sorbitol equilibrium gradient separation of intestinal subcellular organelles obtained from scraped cells. Enterocytes were scraped off rat intestines using microscopic slides and subjected to membrane fractionation as outlined in Materials and Methods and Fig. 1. The resulting P_3 and P_B pellets were loaded on top of 25–60% sorbitol gradients and centrifuged to equilibrium. Other details are given in the legend to Fig. 2

placed on the top of the gradient and centrifuged to equilibrium, ER and Golgi (identified by aryl esterase and galactosyl transferase, respectively) comigrated in a single peak at fraction 8 (Fig. 2*A*). Laterobasal membranes (LBM), monitored by K-stimulated phosphatase, displayed maximal activity at fractions 11-12. As previously noted [2], there was incomplete separation of the ER-Golgi and LBM peaks. A small peak containing sucrase actually was also detected at fraction 14.

As shown in Fig. 2*B*, equilibrium centrifugation of the P_B fraction (isolated after 8 mM $CaCl_2$ treatment, *cf.* Fig. 1) produced a distinctly different pattern of membrane distribution than that found for P_3. The predominant markers identified were those for LBM (K-stimulated phosphatase, fractions 12-14) and BB (sucrase, fraction 17, 18); no peaks of aryl esterase or galactosyl transferase were identified. This suggested that treatment with 8 mM $CaCl_2$ selectively enriched the LBM component of P_B

while eliminating all but traces of ER-Golgi membranes. This enrichment could not be explained by activation of the marker enzyme, there actually being a 5–15% loss of K-stimulated phosphatase during the membrane fractionation procedure. However, because the possibility of selective inactivation or destruction of the ER or Golgi marker enzymes remained, further studies were done, as described below.

Absence of ER-Golgi Membranes from the P_B Fraction Is Not an Artifact

Unlike the P_3 fraction, the P_B pellet is prepared by exposure of membranes to 8 mM $CaCl_2$ for 30 min, a treatment that could have either aggregated ER-Golgi membranes or preferentially inactivated the aryl esterase and galactosyl transferase markers while maintaining sucrase and K-stimulated phosphatase. Notably, however, when fractions from the P_B gradient were assayed for mannosidase II, another marker enzyme localized to the middle Golgi stacks, no peak of activity was identified (*data not shown*). Furthermore, the overall recoveries of the marker enzymes for ER (aryl esterase), Golgi (mannosidase II) and brush border (sucrase) in the terminal fractions (S_0, P_2, S_3, P_3, P_A, S_B, P_B, *cf.* Fig. 1) were between 85 and 95%, the same as the total recovery of K^+-stimulated phosphatase and of protein. Ca^{2+} treatment neither enhanced nor inhibited any of the marker enzymes. Thus, the 8 mM $CaCl_2$ treatment appears to selectively aggregate these membranes while leaving the LBM in the S_A supernatant for subsequent recovery in the P_B pellet. Also of note was the absence of activation of K-stimulated phosphatase.

Isolation of LBM from Intestinal Scrapings

Cell scrapings rather than dispersed cells are commonly used as the starting material for enterocyte subcellular fractionation. The data presented above were derived from cells dislodged by shaking for 60 min, a condition that may promote diffusion of membrane proteins between the LBM and BB, thereby creating an LBM-BB hybrid artifact. Although this seemed unlikely because of the separation of LBM and BBM markers when the P_B fraction was subjected to equilibrium centrifugation (*cf.* Fig. 2*B*), we examined this possibility by using enterocytes scraped from freshly prepared small intestine. Equilibrium centrifugation of the P_3 pellet in a sorbitol gradient produced only partial separation of LBM from ER-Golgi (Fig. 3*A*); in contrast, the P_B pellet yielded LBM (identified by K-stimulated phosphatase) that was clearly separated from ER or Golgi (monitored by aryl esterase and mannosidase II, respectively) (Fig. 3*B*). The slight difference in migration between the membrane fractions derived from scraped and dispersed cells may be produced by a change in the lipid composition that is known to be associated with the scraping technique [20]. But notably, the enhanced isolation of LBM after 8 mM $CaCl_2$ treatment occurred with either dispersed or scraped enterocytes as the starting material (*compare* Figs. 2*B* and 3*B*).

Table 1. Comparison of distribution of the LBM from P_3 and P_B by counter-current distribution

Fraction no.	K-stimulated phosphatase activity recovered (%)	
	P_3	P_B
1	3	5
2	13	22
3	21	24
4	28	26
5	23	16
6	9	5
7	2	1
8	1	1
9	0	0
10	0	0

The LBM from P_3 and P_B, harvested from pooled fractions containing the K-stimulated phosphatase peak (*cf.* Fig. 2*A* and *B*), were subjected to counter-current distribution as outlined in Materials and Methods. The K-stimulated phosphatase activity in the resulting fractions was then expressed as the percentage of the total enzyme activity present in the P_3 or P_B starting material.

Comparison of LBM from P_3 and P_B Membrane Fractions by Counter-Current Distribution

To establish that the K-stimulated phosphatase-rich membrane fraction isolated from P_B constituted typical LBM, we compared the behavior of the LBM derived from P_B (P_BLBM) with that of LBM derived from P_3 (P_3LBM) by counter-current distribution. This technique has been shown to separate epithelial cell membranes into several subpopulations on the basis of relative partitioning between polyethylene glycol- and dextran-rich phases [11]. As shown in Table 1, both the P_BLBM fraction and the P_3LBM fraction were distributed primarily in fractions 3–5 where the highest specific activity (normalized for protein) of the K-stimulated phosphatase marker enzyme was also identified (*data not shown*). Some contamination amounting to ap-

Table 2. Distribution of the K-stimulated phosphatase and aryl esterase in the pooled ER-Golgi, P_3LBM and P_BLBM membrane fractions

	ER-Golgi		P_3LBM		P_BLBM	
	K-Phos.	Aryl Est.	K-Phos.	Aryl Est.	K-Phos.	Aryl Est.
Yield						
Mean	2.6%	14%	4.8%	4.9%	8.0%	0.63%
Range	(0.72–11)	(5.4–21)	(1.0–19)	(0.85–9.5)	(4.1–15)	(0.20–1.2)
SD					(3.25)	
Enrichment						
Mean	×1.8	×9.1	×3.6	×4.5	×15	×1.1
Range	(0.43–7.6)	(4.8–20)	(1.3–7.8)	(1.7–8.0)	(10–23)	(0.56–1.3)

The ER-Golgi, P_3LBM and P_BLBM were harvested by high speed centrifugation of the pooled fractions containing the appropriate enzyme marker peak (*cf.* Fig. 2*A* and *B*). These membranes and the whole homogenate were resuspended in buffer D at a concentration of 0.5 mg/ml and the K-stimulated phosphatase and aryl esterase activities determined. The yield in a purified fraction is the total activity expressed as a percentage of the total activity in the original whole cell homogenate. Enrichment is the ratio of the enzyme activity per unit protein present in the purified fraction divided by the enzyme activity per unit protein present in the whole cell homogenate.

proximately 25% of the membrane protein of the purified P_BLBM migrated in the first two fractions on CCD, apparently reflecting contamination with BB and possibly other membranes (*data not shown*). Notably, the comparable distribution pattern of the marker enzyme for LBM in the P_3 and P_B fractions indicates that both P_3 and P_B fractions contain a similar population of LBM membranes.

Quantitative Comparison of LBM Preparations

The relative purification and yield as compared to the original homogenate for 10 separate membrane separation analyses are given in Table 2. Of particular note is the strikingly greater enrichment of LBM in the fractions collected from the sorbitol gradient when the P_B was the starting material (15-fold over homogenate) rather than P_3 (3.6-fold). Also, the recovery of LBM in P_B was two times greater than in the P_3 fraction. The additional purification afforded by application of the CCD technique to P_BLBM was approximately 1.4, presumably reflecting removal of residual contamination including BB. The relatively minor residual contamination of the P_BLBM fraction with ER-Golgi membranes can be appreciated from examination of the very low yield (0.6%) and absence of enrichment (×1.1) of the aryl esterase marker and mannosidase II (yield 0.6%, enrichment × 1.0). By contrast, the P_3LBM fraction was heavily contaminated with ER-Golgi membranes (aryl esterase enriched 4.4-fold); indeed there was about 16 times more aryl esterase contaminating P_3LBM than P_BLBM.

Discussion

Despite the successful isolation of purified intestinal brush borders more than 25 years ago [10] and subsequent fruitful efforts to further purify the surface membrane as brush border membrane vesicles, isolation of highly purified enterocyte LBM fractions has continued to be elusive. The fact that LBM copurifies with ER-Golgi in conventional enterocyte subcellular fractionation procedures [2, 21], has made it difficult and even treacherous to examine the kinetics and structural dynamics of intracellular membrane protein assembly that is initiated in ER-Golgi and eventually terminates in preferential insertion into one or the other of the surface membranes. Established methods for intestinal subcellular fractionation, utilizing equilibrium centrifugation with discontinuous or linear gradients, have produced appreciable copurification or cross-contamination of ER-Golgi and laterobasal membranes [2, 11, 21]. Although galactosyl transferase is highly concentrated in Golgi membranes and is generally considered to be a suitable marker for these membranes, it is possible that some of the apparent contamination of LBM with Golgi may be related to actual residence of a portion of the enterocyte's galactosyl transferase in the LBM [11, 21]. Also, recent immunocytochemical studies have localized some galactosyl transferase to the BB [17]. Yet we now find that, for purpose of fractionation of enterocyte membranes, the relative quantity of galactosyl transferase associated with BB or LBM is very low (*cf.* Figs. 1 and 2) making it an excellent marker enzyme for Golgi membranes. We

have also examined another Golgi enzyme, mannosidase II, which can be assayed much more efficiently and less expensively than galactosyl transferase (S.M. Najjar and G.M. Gray, *unpublished data*). At times a minor peak for mannosidase II can also be detected in BB; the major peak is coincident with that for galactosyl transferase when either P_3 or P_B fractions are analyzed on sorbitol gradients. Indeed, mannosidase II is a very useful enzyme marker for routine localization of Golgi in enterocytes. The considerable elimination of membranes harboring galactosyl transferase, aryl esterase and mannosidase II in the P_BLBM fraction (Figs. 1 and 2 and Table 2) is in sharp contrast to the apparent copurification of LBM and ER-Golgi in previous studies. This is considered further below and in Table 3.

Table 3 compares our method for LBM purification with those of others that have been published over the last several years. In addition to the conventional purification and recovery factors, data are expressed, whenever possible, as the ratio of enrichment or yield of the marker enzyme for LBM to that for ER-Golgi or BB. This serves as a measure of the purification of LBM in excess of that for the other potentially contaminating membranes. Notably, despite the high apparent purification of LBM in all previous reports, LBM/ER-Golgi enzyme marker ratios in purified LBM were only 2.1–2.8, reflecting the fact that the ER-Golgi marker copurified with LBM to an appreciable extent. Although this could reflect actual residence of the galactosyl transferase or aryl esterase markers in the LBM, we obtained an LBM/ER-Golgi purification ratio of 15, an observation more consistent with the concept that the appreciable copurification of LBM and ER-Golgi in previous reports represented cross-contamination. In the most recent study of Mircheff and coworkers in 1985 where a simplified differential centrifugation scheme was used, the enrichment of LBM was 15-fold and the recovery greater than 25% [11]; yet, as estimated from LBM/ER-Golgi ratios (Table 3), cross-contamination with ER-Golgi was still appreciable. Our new method provides LBM at reasonable yield that is purified at least fivefold greater relative to ER-Golgi than achieved by any previously detailed enterocyte subcellular membrane fractionation technique (Table 3).

Despite the greatly enhanced separation of LBM and ER-Golgi, our purification factor of 15 is not significantly higher than that in several previous reports where there was considerable contamination with ER-Golgi (*cf.* Table 3). A possible reason for similar purification factor to that in previous studies may relate to the $CaCl_2$ treatment. The use of 8 mM $CaCl_2$ for preparing the P_B fraction, while

Table 3. Comparison of intestinal LBM preparations

Author, year (ref.)	Enrichment	Yield	LBM/ERG[b]	LBM/BB[b]
	[a]	%[a]		
Mircheff & Wright, 1976 [12]	12	38	NA[c]	18
Weiser et al., 1978 [21]	12	6	2.4	NA[c]
Colas & Maroux, 1980 [7]	10	5	NA[c]	9.0
Ahnen et al., 1982 [2]	17	4	2.8	13
Mircheff et al., 1985 [11]	15	28	2.1	14
Nguyen et al., 1987 (this paper)	15	8	15	4.6

[a] Denotes ratio of activity per mg protein (for K^+-stimulated phosphatase or Na^+-K^+ ATPase) in the final membrane fraction to that in the original crude homogenate; % is the total activity recovered in the final fraction expressed as a percentage of that in the original crude homogenate.

[b] Ratio of LBM marker enzyme enrichment or yield (whichever gave the higher ratio) to that for potentially contaminating ER-Golgi (marker enzyme: aryl esterase, galactosyl transferase or mannosidase II) or BB (sucrase or aminopeptidase).

[c] NA—Data not available.

differentially precipitating the ER-Golgi, leaves both LBM and BB in a fluffy suspension. Although the BB sediments to a more dense position in the sorbitol gradient (*cf.* Figs. 2*B* and 3*B*), some residual BB is inevitably present in the LBM fractions. Hence the LBM/BB ratio which was 9-18 in previous reports, is somewhat lower with this new method (Table 3).

The final average yield of 8% in the P_BLBM preparation pooled from the sorbitol gradient compares favorably with the yields reported by Weiser et al. [21], Colas and Maroux [7], and Ahnen et al. [2]. Based on the complex distribution of membrane markers found recently by Mircheff et al., only about 28% of cellular Na^+K^+ phosphatase appears to be associated with the LBM [11]; thus our recovery of 8% actually appears to represent nearly one-third of the available enterocyte LBM fraction. Higher yields by use of simpler methods undoubtedly reflected some Na^+K^+ phosphatase marker actually associated with non-LBM membrane populations (*cf.* Table 3). Considering the necessity of selectively pooling fractions from the final sorbitol gradient (*cf.* Figs. 2*B* and 3*B*), this is obviously an excellent yield.

Because of the difficulties in separation of the intracellular microsomal fractions from LBM, it has remained unsettled whether BB proteins that are

initially synthesized in association with ER and Golgi are transferred via vesicles directly to the surface membrane [1, 2] or, instead, reside temporarily in the LBM before their final vectorial movement to the luminal surface membrane [16]. Our experiments have revealed that a relatively simple change of the previous methods, including reduction of the $CaCl_2$ concentration, greatly enhances separation of LBM from ER-Golgi contamination. The new fractionation scheme which exploits the differential aggregation of essentially all ER-Golgi from LBM and promotes separation of BB from total LBM on equilibrium gradient separation can be completed within a single day and allows a substantial yield of intracellular and surface membranes, particularly the LBM. Glycoprotein hydrolase activity in the brush border fractions and functional VIP receptor in the LBM are preserved [15].

The use of EDTA to facilitate cell lysis may render the vesicles leaky and therefore inadequate for transport studies. However, in other studies not detailed here, we have lysed enterocytes with a Waring blender or Polytron apparatus in buffers devoid of EDTA, and the final gradient pattern in P_B (*cf.* Figs. 2*B* and 3*B*) was still preserved (L.A. Schering and G.M. Gray, *unpublished data*). Although the LBM fraction derived from 8 mM $CaCl_2$ treatment of P_1 displays a slightly higher density on sorbitol gradients as compared to the conventional LBM obtained through the differential and equilibrium centrifugation, comparative examination of these membranes by counter-current distribution, a potent technique for separating numerous subpopulations of intestinal membranes [11], showed essentially identical profiles for the 8 mM $CaCl_2$-treated and the conventional LBM fractions.

We conclude that this new method can be performed efficiently and with sufficient yield of highly purified intestinal LBM to provide the basis for precise examination of the interactive kinetics of macromolecular movement and transport within and between intracellular and specialized surface membranes of the enterocyte. Its use should lead to more refined analysis of synthesis, assembly and transport of the numerous intestinal membrane glycoproteins including digestive hydrolases, transport proteins and receptors for peptide hormones, viruses and bacteria.

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