Biochimica et Biophysica Acta, 690 (1982) 269–281
Elsevier Biomedical Press

BBA 71267

USE OF PERCOLL™ IN THE ISOLATION AND PURIFICATION OF RABBIT SMALL INTESTINAL BRUSH BORDER MEMBRANES

LUELLA M. YAKYMYSHYN, KEITH WALKER and ALAN B.R. THOMSON

Department of Laboratory Medicine and Division of Gastroenterology, University of Alberta and University of Alberta Hospital, 8-104 Clinical Sciences Building, Edmonton, Alberta T6C 2G3 (Canada)

(Received December 16th, 1981)

Key words: Brush border membrane; Percoll; Enzyme marker; Lipid analysis

(1) Intestinal absorption is altered under a variety of circumstances in health and disease and to determine a possible relationship between intestinal absorptive function and intestinal brush border membrane composition, we undertook the isolation and purification of rabbit jejunal and ileal brush borders, to allow further studies of their lipid composition under varied experimental conditions. (2) A modification of an established method (Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112) utilized $CaCl_2$ aggregation and sequential centrifugation followed by purification of the brush border pellet (P_2) at $27000 \times g$ on a Percoll™ (Pharmacia) self-forming gradient. The Percoll™ was removed by ultracentrifugation for 30 min at $100000 \times g$, utilizing a batch rotor in the Beckman airfuge™. (3) Pure brush border membrane vesicles were obtained and characterized by specific marker analysis and electron microscopy. Comparative marker analyses performed on P_2 and final Percoll™ preparations from animals showed that the purification achieved was 8–11-fold greater when compared to the original homogenates. Verification of purity was also demonstrated by the absence of DNA and very low levels of β-gluconridase and $(Na^+ + K^+)$-ATPase in the Percoll™ preparations. (4) Comparative lipid analyses of P_2 and final Percoll™ preparations showed that levels of total phospholipid and free fatty acids were several-fold higher in the Percoll™ preparations on a per mg protein basis. (5) A comparison of the activity of enzyme markers and the levels of total free fatty acids in P_2 pellets obtained after $CaCl_2$ and $MgCl_2$ aggregation showed that $CaCl_2$ aggregation gave the more consistently reproducible results. (6) Although standard procedures of membrane preparations not involving density gradient separation provide membranes of reasonable purity for the estimation of lipid components, we consider the final purification step of density gradient separation using Percoll™ is essential for determining small quantitative changes which might occur in the membrane lipid composition under experimental conditions where intestinal absorptive function is altered.

Introduction

The small intestinal mucosal membrane is involved in the digestion and absorption of luminal contents. However, it is not known whether a change in lipid composition of the brush border membrane may be associated with changes in the intestinal transport of nutrients. Our present interest in membrane function and its possible association with malabsorption and other disease states has led to the investigation of the lipid composition of brush border membranes under defined experimental conditions, where there may be changes in the intestinal uptake of lipids [1]. To achieve this, it was necessary to separate and isolate pure membrane preparations to allow accurate

measurement of their constituent lipids. Currently available methods for the isolation and purification of brush border membranes are necessarily involved and tedious, utilizing sucrose and glycerol gradient ultracentrifugation, resulting in relatively low recoveries. Several procedures were reviewed [2–5] some of which were tried, with the eventual selection of the method of Schmitz et al. [4]; it was rapid, simple, reproducible, gave good yields and was easily applied to handling of large numbers of tissue preparations. The final tedious glycerol gradient purification step was eliminated by the introduction of a Percoll™ self-forming gradient leading to the isolation of highly purified brush border membrane vesicles.

Methods and Materials

Intestinal mucosal samples. Sixteen (16) mature female New Zealand white rabbits (2 to 3 kg weight) were killed by injection of 2 to 3 ml of 7.1% sodium thiopental into a marginal ear vein. Segments of proximal jejunum and distal ileum, 20 cm in length, were rapidly removed, irrigated with ice-cold saline, placed on a prechilled glass plate, and kept on ice throughout the entire procedure. Each segment was slit longitudinally along the mesenteric border, following removal of extraneous fatty tissue; the mucosal surface was blotted with lint-free tissue, then gently scraped with two microscope slides. The scrapings from each segment were placed in a small preweighed vial containing 5.0 ml of 50 mM mannitol in 2 mM Tris-HCl buffer, pH 7.1, and the tissue weight was determined. Small segments of intestine were taken before and after scraping and immersed in Bouins' medium for subsequent light microscopy to determine the depth of removal of intestinal mucosa.

Preparation of brush border vesicles. A homogenate of the mucosal sample was prepared containing 1 g of scrapings in 50 ml of ice-cold 50 mM mannitol in 2 mM Tris-HCl buffer (Fig. 1). A Polytron (Brinkman) Homogenizer using a PT 10ST probe, placed at a setting of '8' for 15 s was found to be most satisfactory for complete homogenization with minimal disruption of lysosomes. The homogenate was then filtered through buffer-soaked sterile gauze, followed by a small pore stainless steel screen (approx. 100 μm mesh size), and was finally filtered through a nylon net of 40 μm mesh (Pharmacia Cat. No. 19-0659-01) to remove cellular debris and mucous. Solid calcium chloride ($CaCl_2$) was added to each homogenate giving a final concentration of 10 mM, and the solution was stirred gently for 10 min to ensure aggregation of the endoplasmic reticulum, basolateral membrane and mitochondria. The mixture was centrifuged at $2000 \times g$ for 10 min at 4°C in a Sorvall HS-4 refrigerated centrifuge. The resultant pellet (P_1) was resuspended in 10 ml 50 mM mannitol in 2 mM Tris-HCl buffer, pH 7.1, and retained for marker analysis. The supernatant (S_1) was centrifuged at $27000 \times g$ for 30 min at 4°C and the volume of resultant supernatant (S_2) was measured and an aliquot was retained for marker analysis. The pellet (P_2) was resuspended in 2.0 ml of 50 mM mannitol in 2 mM Tris-HCl buffer, then sonicated at 4°C for 10 s (5 bursts of 2 s each). The effectiveness of sonication in producing brush border vesicles was verified by placing a small droplet of suspension on a glass slide, then viewing under a microscope. The sonicated suspension was then layered on 40% Percoll™ in 0.12 M NaCl, and was centrifuged for 30 min at $27000 \times g$ and 4°C. The Percoll™ formed a density gradient in which the brush borders layered into a tight band between densities of 1.015 and 1.055 g/ml. The core material sedimented at densities of 1.090 to 1.100 g/ml (Fig. 2). A control tube with known density marker beads in 40% Percoll™ was run with each batch of samples and the density was related to the distance in mm measured from the base of the meniscus. The purified fraction was carefully removed with a Pasteur pipette and was subjected to ultracentrifugation in a Beckman Airfuge™ at $100000 \times g$ for 30 min using a Batch Rotor of 7 ml capacity to remove the Percoll™. The brush border fraction was carefully removed, mixed well, and aliquots put into small tubes for lipid extraction, marker analyses, and electron microscopy. The samples were then stored at −20°C. When calcium was compared to magnesium aggregation [6,7], solid $MgCl_2$ (10 mM) was substituted for $CaCl_2$, and the procedure was as above with the following changes: tissue scrapings were homogenized in 50 mM mannitol, 2 mM Tris, 0.8 mM EGTA buffer, pH 7.1. Aggregation with $MgCl_2$ was achieved by mixing for 10 s with the

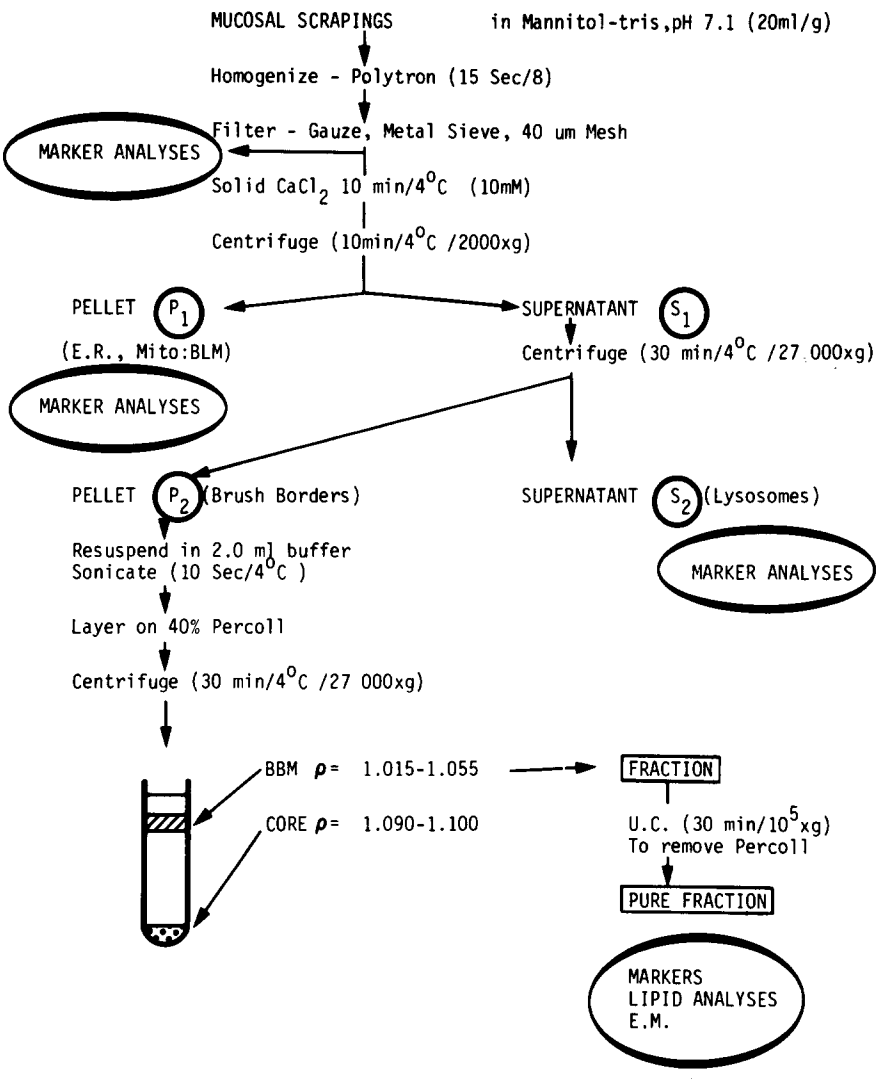

Fig. 1. Scheme of the procedure used to prepare purified jejunal and ileal brush border membrane vesicles described in Methods and Materials.

Polytron homogenizer at a setting of '6'. The homogenate was centrifuged for 5 min at $117000 \times g$ to obtain pellet P_1. The supernatant (S_1) was centrifuged for 20 min at $41000 \times g$ to obtain pellet P_2. The entire procedure was carried out at 4°C.

Protein and enzyme markers. All fractions (P_1, S_2, P_2, and Percoll™ preparation) were assayed for protein according to the method of Lowry et al. [8] using crystalline bovine serum albumin as the standard, and for various enzyme markers of purity. Alkaline phosphatase and sucrase (invertase) were used as brush border membrane markers. Alkaline phosphatase was measured kinetically according to Bowers et al. [9] using *p*-nitrophenyl phosphate as substrate. Sucrase was assayed according to a modification of Dahlqvist [10] whereby the glucose formed was measured using the glucose-specific hexokinase reaction on the Abbott Bichromatic analyzer, ABA-100 [11]. The nuclear marker, DNA, was measured according to [12] the modification of Giles and Myers of the original method of Burton [13].

$(Na^+ + K^+)$-ATPase, chosen as the basolateral

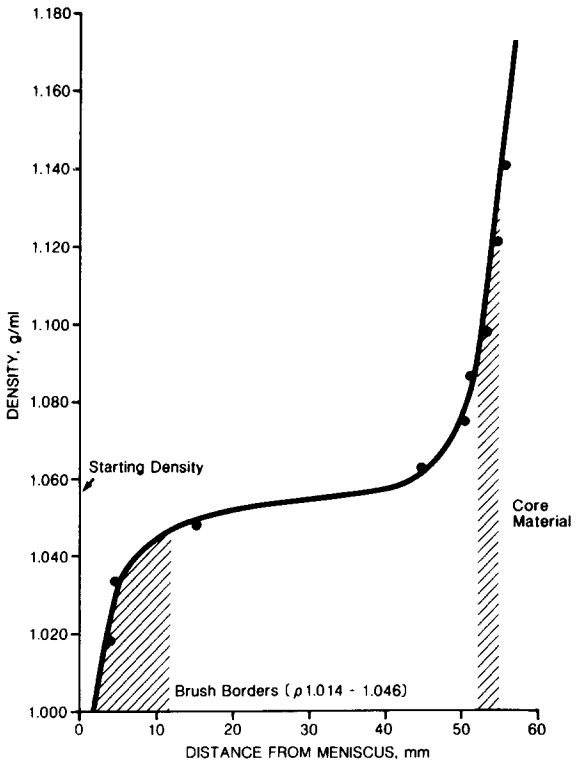

Fig. 2. Standard density marker beads separated on a 40% Percoll™ gradient. Hatched areas indicate density ranges where brush borders and core material are separated.

membrane marker, was measured kinetically by a coupled enzymatic method [14] as the difference between Mg^{2+}-activated ATPase activities in the presence and absence of ouabain. The marker of lysosomal contamination, β-glucuronidase, was measured fluorimetrically according to the method of Bell et al. [15]. Electron microscopy was performed by standard techniques utilizing osmium tetroxide.

Total free fatty acids were measured in the Percoll™ and P_2 preparations according to the method of Falholt et al. [16]. Total phospholipids were measured fluorimetrically [17] utilizing the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). A standard was prepared from a pooled Percoll™ preparation and its phospholipid content was determined from 10 replicate measurements of inorganic phosphorus by the classical acid digestion, phosphomolybdate reaction [18] following Folch lipid extraction [19].

Results

Preparation and isolation of brush border membrane vesicles

The depth of removal of the intestinal mucosal surface is important in providing selective isolation of mucosal surface with a high relative concentration of brush border membrane. We ascertained that gentle scraping with fine long strokes gave the best results, removing the tips of the villi and leaving the crypt cells virtually intact (Figs. 3A and 3B). Similar results were obtained for jejunum and ileum.

The use of a Polytron homogenizer gave complete homogenization of mucosal samples with minimal disruption of lysosomes, confirmed by the low β-glucuronidase levels. Filtration of the homogenate through buffer-soaked sterile gauze and a steel screen prior to the small pore 40 μm nylon mesh, eliminated plugging of the mesh and provided an easy means of eliminating mucous and cellular debris.

The use of $CaCl_2$ to aggregate nuclei, mitochondria, basolateral membranes, and microsomal material followed by centrifugation at low speeds was first described by Crane and co-workers in 1973 [4], thereby eliminating much of the contamination of brush border preparations with these organelles. By modifying centrifugation conditions to 30 min at $27000 \times g$, we were able to obtain a higher yield of P_2 pellets. The introduction of a buffer wash step resulted in pure white P_2 pellets with minimal loss of specific enzyme markers instead of the brownish pellets which resulted when no wash was used. The buffer wash step should also remove some of the calcium bound to the brush border and any residual calcium from the aggregation step. Sonication of resuspended P_2 pellets was sufficient to disrupt the membranes allowing them to form vesicles without disrupting lysosomes, as confirmed by the low concentration of the lysosomal marker β-glucuronidase in the final preparations (Tables I and II). The measurement of sucrase and alkaline phosphatase in unsonicated and sonicated P_2 preparations showed no difference for sucrase but a 3-fold increase of alkaline phosphatase in sonicated samples.

We initially tried using Tris disruption of brush borders prior to glycerol gradient ultracentrifuga-

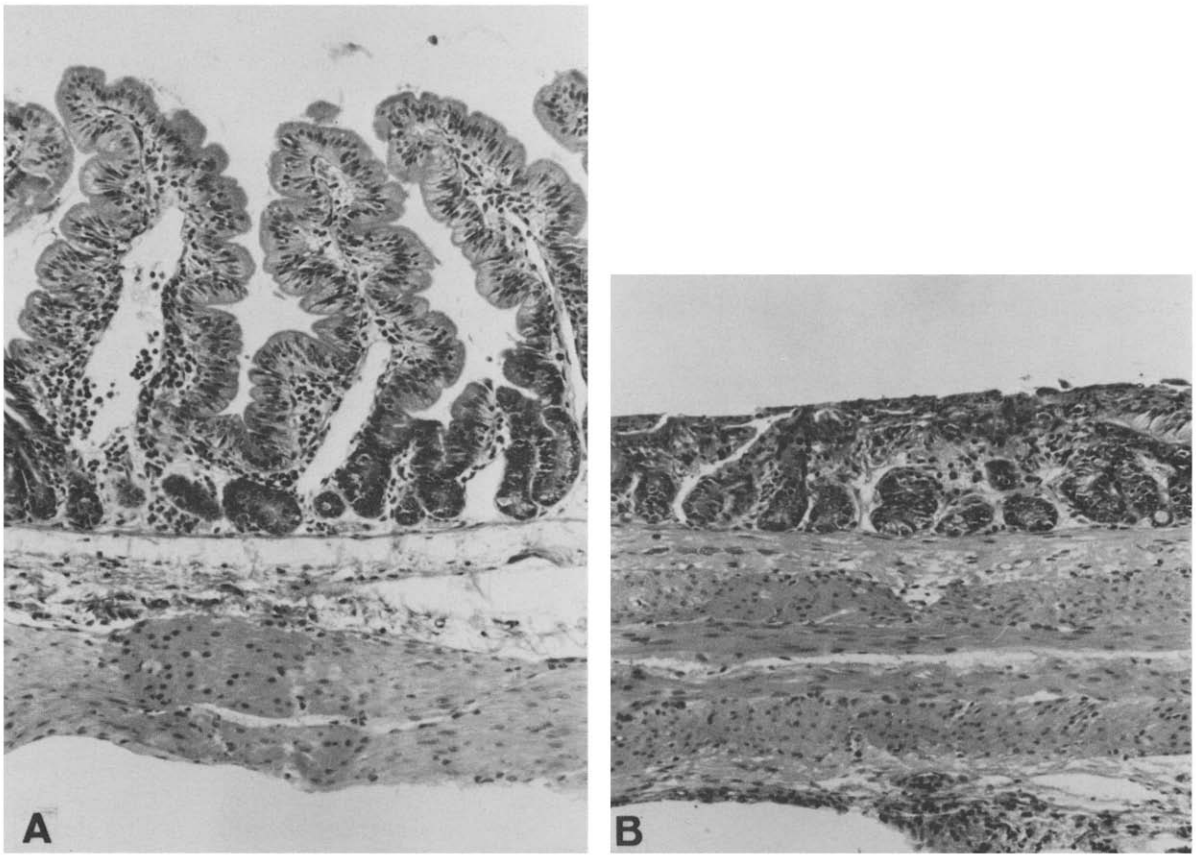

Fig. 3. Cross-section of rabbit jejunum before scraping (A) and after scraping (B) of the mucosal layer, showing that intestinal villi have been harvested above the level of crypts of Lierberkühn. Hematoxylin and eosin stain. Magnification ×135.

tion, but were dissatisfied with the poor yields obtained and the need to remove the Tris/glycerol to prevent interference with the biochemical analyses. Sonication was introduced, successfully eliminating the problem of sample dilution and variability in the disruption of prepared membranes.

The use of Percoll™ (Pharmacia), eliminating the need for tedious preparation of glycerol or sucrose gradients, resulted in a much higher yield of membranes which formed a tight heavy band between densities of 1.015 and 1.055, while the denser villus core and fibrous material sedimented at densities of 1.090 or more (Fig. 2). Percoll™ was tested at various concentrations ranging from 25% to 50%; 40% Percoll™ gave the best separation. Similar results were obtained for ileum and jejunum. At concentrations below 35% the brush border fraction separated as two diffuse bands, each having similarly high specific activities of alkaline phosphatase and sucrase (Table III). Thus it was assumed that several populations of brush border vesicles were present, having slightly different densities according to size and did not require subfractionation. Electron micrographs of our P_2 preparations (Figs. 4A and 4B) showed relatively pure preparations of bursh border membrane vesicles of variable sizes but similar shapes.

Removal of Percoll™ from purified preparations was easily accomplished by ultracentrifugation of the fraction whereby the heavy polymeric beads pelleted tightly leaving the brush borders in suspension. An alternative method of diluting the fraction with buffer prior to centrifugation was

TABLE I

PURIFICATION OF RABBIT JEJUNUM BRUSH BORDER MEMBRANE VESICLES

Mean ± S.D. The number of different tissues studied is given in parenthesis. The specific activity (spec. act.) is expressed as I.U./g protein.

Marker	Homogenate (H)	Pellet (P_1)	Supernatant (S_2)	Pellet (P_2)	Percoll prepn. (PP)	P_2/H	PP/H
Protein							
mg/g wet wt.	76.4 ± 16.1	33.5 ± 8.0	39.9 ± 9.6	6.9 ± 5.4	1.85 ± 0.58	–	–
%	100	42.0 ± 7.3	49.7 ± 4.7	7.5 ± 4.5	2.6 ± 0.9		
	(16)	(16)	(16)	(16)	(16)		
Alkaline phosphatase							
Spec. act.	136 ± 44	170 ± 90	89 ± 37	1204 ± 348	1414 ± 462	8.2 ± 5.4	11.4 ± 5.2
%	100	25.4 ± 8.2	16.0 ± 5.6	58.7 ± 10.2	25.7 ± 7.1	–	–
	(16)	(13)	(13)	(13)	(16)	(4)	(15)
Sucrase							
Spec. act.	106 ± 48	82 ± 55	47 ± 39	813 ± 312	995 ± 286	9.1 ± 2.5	10.8 ± 4.4
%	100	29.6 ± 7.5	23.2 ± 9.7	46.0 ± 13.2	25.6 ± 8.1	–	–
	(16)	(13)	(13)	(13)	(16)	(5)	(16)
Sucrase/alkaline phosphatase							
Ratio	0.81 ± 0.33	0.59 ± 0.42	0.75 ± 0.71	0.66 ± 0.20	0.77 ± 0.30	–	–
	(18)	(13)	(11)	(12)	(16)		
$(Na^+ + K^+)$-ATPase							
Spec. act.	20.1 ± 24.3	74	65	4.8 ± 5.4	0	–	–
%	100	41	54	4.8 ± 4.8	0		
	(8)	(2)	(2)	(4)	(12)		
β-Glucuronidase							
Spec. act.	3.2 ± 0.3	5.4 ± 2.7	7.0 ± 3.5	1.8 ± 1.4	2.2 ± 0.8	–	–
%	100	35.0 ± 12.9	60.2 ± 16.2	3.3 ± 3.3	2.9 ± 1.8		
	(7)	(11)	(12)	(12)	(16)		
Deoxyribonucluic acid							
mg/g protein	8.0 ± 6.0	34.4 ± 23.0	2.3 ± 5.4	4.7 ± 7.3	0	–	–
%	100	87.0 ± 24.0	11.9 ± 22.3	1.5 ± 2.0	0		
	(7)	(12)	(12)	(12)	(8)		

TABLE II

PURIFICATION OF RABBIT ILEUM BRUSH BORDER MEMBRANE VESICLES

Mean ± S.D. The number of different tissues studied is given in parenthesis. The specific activity (spec. act.) is expressed as I.U./g protein.

Marker	Homogenate (H)	Pellet (P_1)	Supernatant (S_2)	Pellet (P_2)	Percoll prepn. (PP)	P_2/H	PP/H
Protein							
mg/g wet wt.	71.4 ± 19.9	27.2 ± 11.8	41.2 ± 10.6	5.44 ± 1.97	3.28 ± 1.07	–	–
%	100	36.2 ± 7.9	56.2 ± 6.3	7.0 ± 2.2	4.4 ± 1.4		
	(16)	(12)	(12)	(11)	(16)		
Alkaline phosphatase							
Spec. act.	180 ± 66	126 ± 84	102 ± 36	1264 ± 492	1234 ± 702	7.0 ± 1.7	8.4 ± 6.4
%	100	17.9 ± 9.7	24.6 ± 10.8	57.0 ± 14.9	28.2 ± 14.9		
	(14)	(9)	(8)	(9)	(16)	(9)	(14)
Sucrase							
Spec. act.	143 ± 130	6.9 ± 5.9	48.4 ± 35.7	654 ± 364	789 ± 339	8.4 ± 2.2	8.6 ± 4.5
%	100	22.5 ± 12.1	29.3 ± 10.7	49.8 ± 15.8	32.3 ± 9.1		
	(16)	(12)	(12)	(12)	(15)	(9)	(15)
Sucrase/alkaline phosphatase							
Ratio	0.59 ± 0.25	0.50 ± 0.41	0.56 ± 0.37	0.77 ± 0.39	0.72 ± 0.54	–	–
	(14)	(9)	(8)	(8)	(15)		
$(Na^+ + K^+)$-ATPase							
Spec. act.	25.3 ± 30.8	–	–	0	0		
%	100	–	–	0	0		
	(7)	–	–	(4)	(5)		
β-Glucuronidase							
Spec. act.	3.2 ± 0.6	5.2 ± 3.2	9.2 ± 5.4	2.1 ± 1.1	2.0 ± 0.7	–	–
%	100	25.6 ± 11.9	75.5 ± 6.5	2.4 ± 1.7	3.2 ± 2.2		
	(3)	(11)	(9)	(11)	(16)		
Deoxyribonucleic acid							
mg/g protein	27	38.2 ± 22.8	0	4.0 ± 7.0	0	–	–
%	100	97.9 ± 3.8	0	2.9 ± 4.3	0		
	(2)	(10)	(12)	(11)	(16)		

TABLE III

ENZYME MARKERS IN BANDS OBTAINED FROM JEJUNAL PREPARATIONS USING THREE CONCENTRATIONS OF PERCOLL™

Percoll (%)	Density (g/ml)	Sucrase		Alkaline phosphatase	
		I.U./fraction	Spec. act. [a]	I.U./fraction	Spec. act. [a]
Jejunum					
25% band I	1.012–1.048	5.44	907	20.8	3467
band II [b]	1.110–1.137	6.40	1600	26.3	6575
40% band I	1.019–1.055	5.88	1176	6.7	1950
band II [c]	1.090–1.100	Low	–	Low	–
50% band I	1.019–1.055	6.02	669	53.0	6575
band II [c]	1.090–1.100	Low	–	Low	–

[a] Spec. act., specific activity in I.U./g protein.
[b] Band II in 25% Percoll is an intermediate band.
[c] Band II in 40% and 50% Percoll is core material.

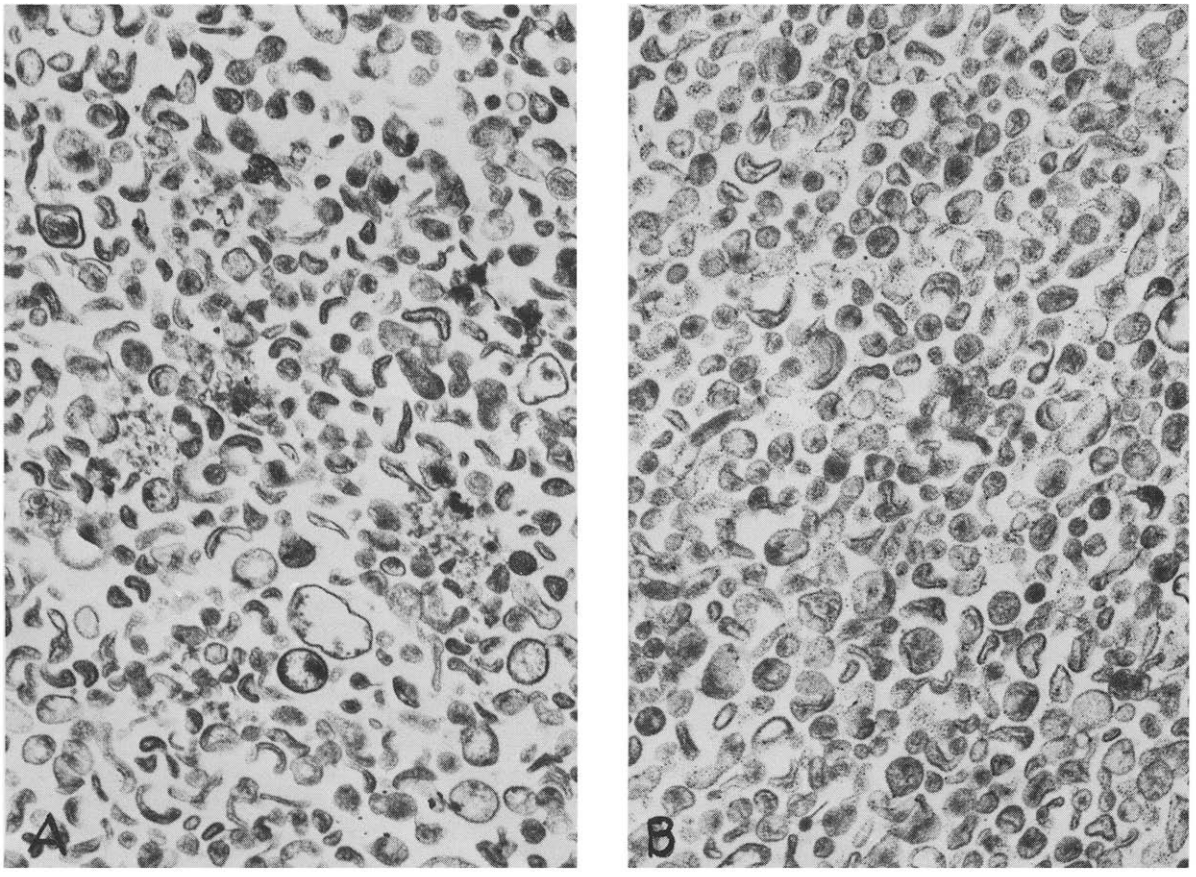

Fig. 4. Electron micrographs of brush border membrane vesicles in P_2 pellets of (A) jejunum and (B) ileum. Micrographs prepared by standard techniques involving the use of osmium tetroxide following fixation of P_2 pellets in 4% glutaraldehyde. Magnification ×37500. (Courtesy of Dr. T.K. Shnitka, Professor of Pathology, Faculty of Medicine, University of Alberta).

found to be unsatisfactory for effective removal of Percoll™. The vesicles in the purified Percoll™ preparations were similar to those in the P_2 preparations but less densely packed because of volume dilution. (Figs. 5A and 5B) contamination with Percoll™ was sometimes noticed because of dense granules in the electron microscopic preparations and interference with the protein and alkaline phosphatase assays. Further removal of the Percoll™ was achieved by a subsequent centrifugation for 30 min at $100000 \times g$.

Preparations made from fresh or frozen tissue scrapings stored at $-70°C$ for periods of up to two months gave similar results for the activity of the enzymatic marker assays which were conducted within two days of the purification procedure.

Protein and enzyme markers

Marker methods with sufficient sensitivity to detect small amounts of contaminating materials in small volumes of sample were essential to confirm the purity of the preparations. Of the marker methods used by Schmitz et al. [4], we adapted those for DNA, protein and sucrase, modifying the procedure for measuring glucose formed after Dahlqvist's classical incubation reaction for sucrase, by utilizing a hexokinase glucose-specific reaction on the automated Abbott ABA-100 bichromatic analyzer. A sensitive method for β-glucuronidase was chosen using the fluorophore methylumbelliferone enabling us to detect levels of enzyme not picked up by others using less sensitive methods [4,7]. The ATPase method suggested by Schmitz and co-workers [4] lacked the sensitiv-

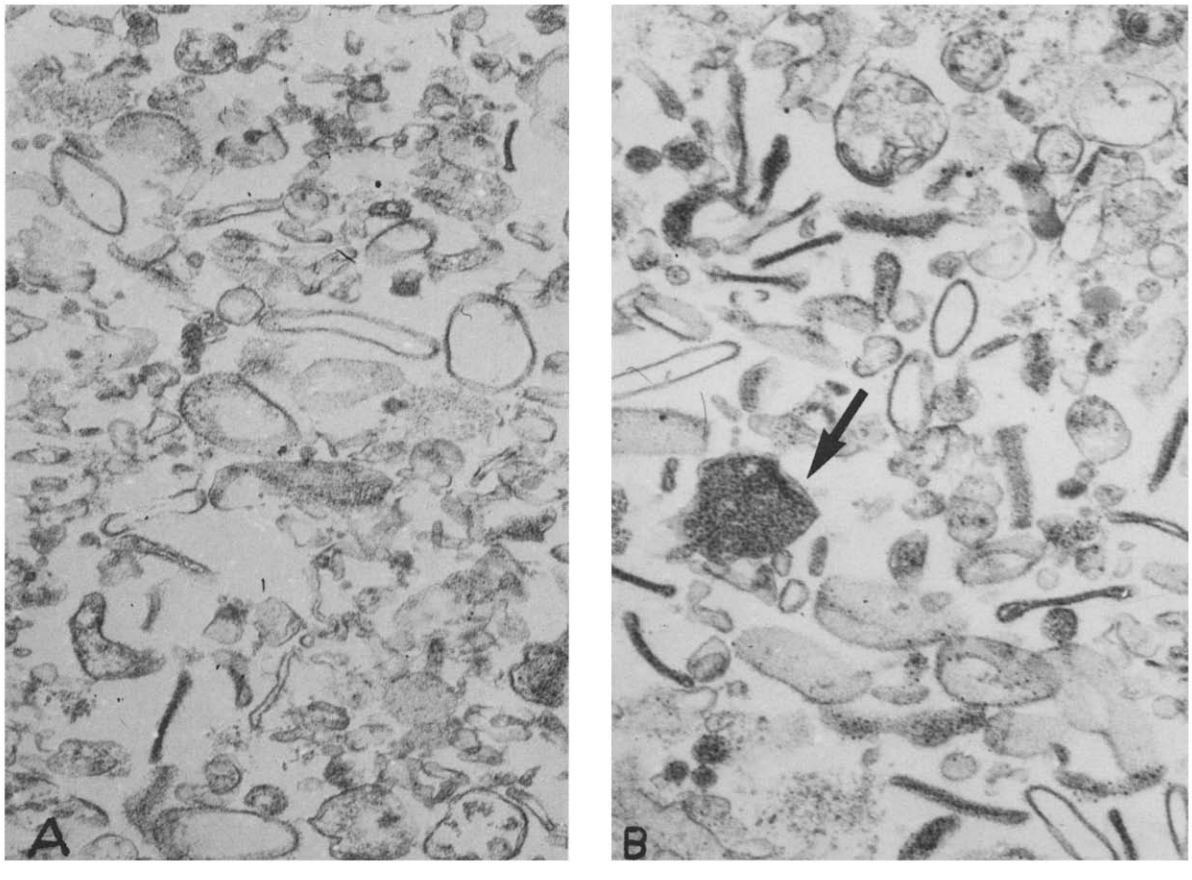

Fig. 5. Electron micrographs of Percoll™ fractions of brush border membrane vesicles prepared from (A) jejunum and (B) ileum. Magnification ×39000. The arrow points to coarse dark granular material though to represent residual Percoll™. (Courtesy of Dr. T.K. Shnitka, Professor of Pathology, Faculty of Medicine, University of Alberta).

ity and reproducibility and was also subject to interference, which often resulted in negative result for $(Na^+ + K^+)$-ATPase. The method adopted in our study involved a coupled enzyme assay of proven specificity, sensitivity, and reliability, subject to few interferences [14].

The distribution of the various protein and enzyme markers in the individual fractions obtained throughout the course of the brush border membrane preparation procedures are indicated in Tables I and II, respectively. Fraction P_1 contained essentially all of the DNA nuclear marker, and about one half of the $(Na^+ + K^+)$-ATPase activity, whereas fraction S_2 contained the major proportion of the β-glucuronidase and the remainder of the $(Na^+ + K^+)$-ATPase activity. Some DNA was invariably found in the P_2 pellets, with small amounts of β-glucuronidase and $(Na^+ + K^+)$-ATPase, indicating varying degrees of nuclear, lysosomal and basolateral membrane contamination, respectively.

The Percoll™ purification step successfully eliminated nuclear (DNA) and basolateral membrane $(Na^+ + K^+)$-ATPase contamination while making only a slight difference to lysosomal (β-glucuronidase) contamination. We have since been able to reduce the latter to less than 0.5% of the total β-glucuronidase activity by decreasing the period of sonication to less than five seconds thus preventing lysosomal disruption, and by modifying conditions of the first centrifugation step from $2000 \times g$ to $3000 \times g$, eliminating any residual P_1 contaminating material left in suspension. The level of purification according to specific activity, expressed as International Units per g protein, in both P_2 and Percoll™ preparations, was 8–11-fold for alkaline phosphatase and 9–11-fold for sucrase, compared to the original homogenate of jejunum. The levels of purification were slightly less for the ileum (Table II).

Comparison of $CaCl_2$- and $MgCl_2$-treated preparations

A comparison of P_2 pellets of brush border membrane vesicles obtained after $CaCl_2$ and $MgCl_2$ aggregation of portions of the same tissue samples from four different animals (Table IV) showed that $CaCl_2$ aggregation gave a slightly better yield in terms of protein, and a slightly lower but not significant total fatty acid and total phospholipid content, than was achieved by aggregation with $MgCl_2$. Lysophosphatidylcholine was not detected in either type of preparation by thin-layer chromatography. Immediate lipid extraction of purified preparations should avoid the problem of calcium activated phospholipase breakdown of phospholipids with concommitantly increased fatty acid levels, as reported by Hauser et al. [7] on

TABLE IV

$CaCl_2$ AGGREGATION VERSUS $MgCl_2$ AGGREGATION IN THE PREPARATION OF JEJUNAL BRUSH BORDER MEMBRANES

Mean ± S.E. The number of different tissues studied is given in parenthesis.

	P_2 pellets		Percoll prepn. (PP)	
	$CaCl_2$	$MgCl_2$	$CaCl_2$	$MgCl_2$
Protein (mg/g wet wt.)	4.27 ± 1.38 (10)	3.00 ± 1.17 (8)	3.04 ± 0.75 (4)	0.97 ± 0.33 (4)
Total free fatty acids (nmol/mg protein)	65.2 ± 20.7 (4)	91.1 ± 17.9 (6)	204 ± 20.6 (4)	221 ± 74.6 (6)
Total phospholipids (nmol/mg protein)	57.1 ± 8.7 (4)	65.3 ± 7.4 (4)	64.7 ± 9.1 (4)	94.6 ± 26.2 (10) [a]
$(Na^+ + K^+)$-ATPase (I.U./g protein)	21.0 ± 21.8 (6)	329 ± 139 (10) [a]	111 ± 38 (4)	271 ± 135 (10)
β-Glucuronidase (I.U./g protein)	0.52 ± 0.16 (6)	3.71 ± 1.60 (10) [a]	0.63 ± 0.02 (4)	4.41 ± 1.18 (10) [a]

[a] Mean values for $CaCl_2$ and $MgCl_2$ treatments were significantly different ($P < 0.05$).

storage of membranes prepared with $CaCl_2$ microsomal aggregation, for periods of 24 h at room temperature. The extremely high levels of ($Na^+ + K^+$)-ATPase observed in $MgCl_2$-treated preparations indicate considerable basolateral contamination. There was also significant lysosomal contamination in these preparations. The yield of brush border membranes in terms of protein and size of pellet obtained in magnesium preparations was less, and the Percoll™ separation was not as satisfactory as with the calcium prepared membranes. Whether this is related to the calcium aggregation of brush borders thus aiding with the centrifugation of a tight band as opposed to non-aggregation with magnesium is not presently known.

Comparison of P_2 pellet and Percoll™ preparation

On comparing P_2 pellets and Percoll preparations prepared form jejunal scrapings with $CaCl_2$ and $MgCl_2$ aggregation it was obvious that this final step using Percoll™ was in fact required. Mean values obtained for total free fatty acids and phospholipids, were considerably higher on a per mg protein basis in the Percoll™ preparations (Table IV) than in either $CaCl_2$- or $MgCl_2$-prepared P_2 pellets.

Discussion

Procedures which have been tried for the preparation of brush borders are tedious, requiring large numbers of wash steps which can lead to the lack of precision, and give low recoveries with insufficient sample quantities for subsequent lipid analyses [2,3]. Less tedious procedures have been described [5,7]. However, they are considered unsatisfactory due to the presence of substantial amounts of DNA, (nuclear contamination), or ($Na^+ + K^+$)-ATPase and β-glucuronidase (basolateral membrane and lysosomal contamination, respectively).

We have described a simple and precise method for the rapid preparation of highly purified intestinal brush border membranes from rabbit jejunum and ileum, utilizing a Percoll™ self-forming gradient. Results were practically identical for enzyme markers in preparations made from fresh or frozen tissue scrapings, a finding reported by others for human intestine [4,7] and rabbit kidney [6]. Electron microscopy verified the absence of foreign contaminating material (Fig. 5).

We originally anticipated that the P_2 pellet would have been a satisfactory preparation, rather than the final Percoll™ purification. However, it was decided that the presence of substantial amounts of nuclear material and basolateral membrane in our P_2 pellets (Tables I and II) in addition to the different levels of lipids (Table IV) when compared to Percoll™ preparations, necessitated the final Percoll™ purification step. The use of Percoll™ is simple, not requiring layering of various concentrations of gradient material. One might conclude from this data that there is a relatively higher lipid content in brush borders than in basolateral membrane, since the P_2 pellet contains membrane contaminants other than brush borders. More important, however, is the fact that such a discrepancy in relative lipid composition of P_2 and Percoll™ preparations from the same animals clearly demonstrates the need for a final purification step, enabling the subsequent detection of small differences in membrane chemical composition.

Despite a loss of approx. 50% of the protein following purification of P_2 pellets with Percoll™, the recovery of protein in the Percoll™ preparations was of the order of that obtained by others with partially purified P_2 preparations [4,20], and somewhat better than similar purified preparations obtained by tedious gradient separations involving ultracentrifugation [4,21].

Several markers were used to assess the purity of our membrane preparations. The procedures used for the various marker analyses were chosen for simplicity, specificity, reproducibility and sensitivity. Thus one could work with very small amounts of material, usually a volume of less than 1.0 ml out of a total volume of 6 ml of Pecoll™ preparation, leaving the remainder for lipid analyses requiring larger volumes of material. Purification levels of approx. 10-fold for alkaline phosphatase and sucrase were obtained. These values were slightly lower than those obtained with human intestine [4,22] but similar to those reported previously for rabbit intestine [23]. The discrepancy might be explained by the different experimental conditions of the alkaline phos-

phatase procedure [9,24]. Our experience with the use of two different buffer systems for alkaline phosphatase showed that this could result in 2-fold differences in activity. From this preliminary observation, we decided to use the method established in our laboratory for routine patient serum alkaline phosphatase, using 2-amino-2-methyl-1-propanol buffer, and standardized our results according to an already established quality control procedure.

Sucrase is considered to be the best specific enzyme marker for brush borders [3], and perhaps the slightly lower sucrase levels observed in our preparations could be due to species differences, or the presence of Tris buffer which has been reported to have an inhibitory effect on sucrase activity [21].

Others have reported the adequacy of P_2 pellet preparations for subsequent lipid analysis [7]. We considered it necessary to continue the purification one step further, as was demonstrated by the removal of nuclear material (DNA), basolateral membrane, ($(Na^+ + K^+)$-ATPase), along with a slight increase in sucrase to alkaline phosphatase ratio, and thus the achievement of a higher purity for the membrane preparation.

Kessler and coworkers [20] have reported the presence of sucrase only on the luminal side of prepared membrane vesicles and suggested that closed vesicles are oriented luminal side out. However, the majority of vesicles in our preparations are open-ended as shown by electron microscopy (Figs. 4A and 4B). The 3-fold increase of alkaline phosphatase activity observed in sonicated versus unsonicated preparations would suggest that alkaline phosphatase on both inner and outer vesicle surfaces is accessible to substrate following sonication. This difference was not seen with sucrase, as the enzymically active sites are totally confined to the outer membrane surface [20] and one would not expect to see a difference between sonicated and unsonicated preparations if both surfaces are accessible following sonication.

Removal of mucous in our initial preparations was an important consideration since its presence could interfere with satisfactory purification. Freezing of tissue scrapings prior to processing apparently resulted in a breakdown of mucous material and facilitated its subsequent removal. The technique described for removing small amounts of mucous from the jejunum or ileum consisted of using a gauze screen and a small-pore mesh and was satisfactory for the small intestine. However, an attempt to apply this procedure to colonic mucosal material was unsuccessful because of the excessive amounts of mucous material normally present in this tissue.

Max et al. [21] found small amounts of β-glucuronidase in their preparations, even after spinning on two gradients, which resulted in low yields of purified material, and Schmitz et al. [4] reported less contamination of his P_2 preparations with β-glucuronidase than we discovered. Small amounts of β-glucuronidase persisted even into our Percoll™ preparations.

There are two schools of thought on the use of calcium or magnesium as an aggregating agent. Those favouring magnesium [7] are concerned about possible phospholipase activation by calcium and breakdown of phospholipid to lysophosphatides. On the other hand, those favoring calcium aggregation [6] are concerned about the greater contamination of magnesium preparations with basolateral membranes and lysosomal material. In this study, calcium aggregation of microsomal material was superior to $MgCl_2$; with $CaCl_2$ aggregation, there was a higher yield and greater specific activities of enzyme markers, less contamination with lysosomes and basolateral membranes. The band on Percoll™ is tighter with $CaCl_2$ than $MgCl_2$. The concentration of buffer and time of aggregation is critical with $MgCl_2$, in order to prevent indescriminate aggregation of brush borders and microsomal material in contrast to aggregation with $CaCl_2$. Aggregation with $CaCl_2$ is also more highly selective for lysosomes. Finally, the levels of free fatty acids were lower with calcium than with magnesium. Thus it was decided that $CaCl_2$ was probably the most reliable aggregant to use for our preparations and all future preparations would be extracted immediately following purification for subsequent lipid analysis.

Indeed, we were surprised to find fatty acids in the membrane preparations, as one would not normally expect free fatty acids to be present as structural components of the intestinal brush border. There remains, however, the possibility that fatty acids are in absorptive transit across the

intestinal mucosa at the time of sampling and are not an integral part of the brush border structural architecture. This point awaits further study.

The ileum gave qualitatively similar results to the jejunum in terms of yield and recovery of protein. However, quantitative purification of alkaline phosphatase and sucrase, expressed as ratios of P_2/Homogenate and Percoll™ preparation/Homogenate was slightly less than for the jejunum. This may have been due to slightly different mucosal cell sized populations, resulting in differences in density separation. This suggestion is supported by the finding that the Percoll™ band from the ileum was usually more diffuse than the comparable band seen with the jejunum. Alternatively, there may be actual differences in the mucosal cellular content of sucrase and alkaline phosphatase in the ileal brush borders as compared with the jejunum.

In conclusion, the use of Percoll™ in density gradient purification of brush borders is a significant improvement over other available types of gradients. The removal of Percoll™ is simple and straight forward, facilitating the processing of a number of tissue samples in a very short period of time, thus enabling one to prepare lipid extracts on the same day. Standard methods of membrane preparation not involving density gradient separation may provide membranes of reasonable purity for the approximation of lipid components. However, our results indicate the final purification step involving Percoll™ is essential for determining small changes which might occur in the membrane on exposure to small amounts of lipid materials.

Acknowledgements

We wish to acknowledge and thank Dr. T.K. Shnitka, Professor of Pathology. Faculty of Medicine, University of Alberta and his technical staff Miss Rosemary Harris and Mrs. Ginny Burgess for the electron microscopy and light microscopic work performed on some of our membrane preparations and tissues, respectively. The technical assistance of Miss Monika Hoffmann and Ms. Barbara Philips is gratefully acknowledged. The authors also acknowledge use of facilities within the Department of Laboratory Medicine, University of Alberta Hospital, and wish to personally thank Mrs. Carol Gates for typing of the manuscript. The work was funded in part by the Special Services and Research Committee, University of Alberta Hospital, by the Medical Research Council (Canada) and the Alberta Heritage Foundation for Medical Research.

References

1 Thomson, A.B.R. (1980) J. Lipid Res. 21, 687–698
2 Billington, T. and Nayudu, P.R.V. (1975) J. Membrane Biol. 21, 49–64
3 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381–390
4 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
5 Andersen, K., Vonder Lippe, G., Morkrid, L. and Schjonsby, H. (1975) Biochem. J. 152, 157–159
6 Malathi, P., Preiser, H., Fairclough, P., Mallett, P. and Crane, R.K. (1979) Biochim. Biophys. Acta 554, 259–263
7 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Bowers, G.N., Kelley, M.L. and McComb, R.B. (1967) Clin. Chem. 14, 595–607
10 Dahlqvist, A. (1964) Biochemistry 7, 18–25
11 Bondar, R.J.L. and Mead, D. (1974) Clin. Chem. 20, 586–590
12 Giles, K.W. and Myers, A. (1965) Nature 206, 93
13 Burton, K. (1965) Biochem. J. 62, 315–323
14 Scharschmidt, B.F., Keefe, E.B., Blankenship, N.M. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799
15 Bell, C.E., Jr., Sly, W.S. and Brot, F.E. (1977) J. Clin. Invest. 59, 97–105
16 Falholt, K., Lund, B. and Falholt, W. (1973) Clin. Chim. Acta 46, 105–111
17 Jouanel, P., Motta, C., Delattre, J. and Dastugue, B. (1980) Clin. Chim. Acta 105, 173–181
18 Sunderman, F.W. and Sunderman, R.N., Jr. (1960) Lipids and Steroid Hormones in Clinical Medicine, Ch. 5, p. 28
19 Bowyer, D. and King, J. (1977) J. Chromatogr. 143, 473–490
20 Kesler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
21 Max, E.E., Goodman, D.B.P. and Rasmussen, H. (1978) Biochim. Biophys. Acta 511, 224–239
22 Welsh, J.D., Preiser, H., Woodley, J.F. and Crane, R.K. (1972) Gastroenterology 62, 572–582
23 Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575–581
24 Bowers, Jr., G.N. and McComb, R.B. (1966) Clin. Chem. 12, 70–89