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THE COMPOSITION AND BIOSYNTHESIS OF THE GLYCOPROTEINS AND GLYCOLIPIDS OF THE RABBIT SMALL-INTESTINAL BRUSH BORDER

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Summary

1. The glycoprotein and glycolipid composition of isolated rabbit small-intestinal brush borders has been studied.

2. The total glycoprotein fraction contains an average 95 μg carbohydrate per mg protein, composed of mannose, galactose, fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine. Glucose is also present but sialic acid is absent.

3. The isolated glycolipids include ceramide lactoside, ceramide trihexoside and two *N*-acetylglucosamine-containing glycolipids. Sialic acid containing glycolipid (gangliosides) is present only in trace quantities.

4. The biosynthesis of the brush border-bound glycoproteins and glycolipids has been studied following intraperitoneal injection with D-[1-^{14}C]glucosamine and isolation of the brush borders at intervals between 3 and 24 h.

5. The total glycoprotein fraction labels maximally 7.5 h after injection and subsequently exhibits an exponential loss of radioactivity with a half-life of 11.2 h. The labelling kinetics of one of the glucosamine-containing glycolipids is similar to that of the glycoproteins in that it labels maximally between 7.5 and 12 h, but the second glucosamine-containing glycolipid labels later at approximately 18 h. These results indicate that the glycoproteins and glycolipids are actively synthesized and degraded within the mature small intestinal enterocyte and that individual glycolipids turn over independently.

Introduction

A layer of carbohydrate-rich material can be observed upon the surface of many, if not all mammalian cells [1] and this layer may largely be comprised of membrane-bound glycoproteins and glycolipids [2]. Interest was recently

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focused upon the glycoproteins and glycolipids of the plasma membrane because of their possible involvement in cellular interactions and the control of cell growth [2,3].

The small-intestinal brush border, a specialised plasma membrane on the luminal face of the small-intestinal adsorptive cell, exhibits in histochemical studies a very prominant surface coat of carbohydrate-rich substances [1]. Similarly, isolated brush borders from both guinea pig [4] and rat [5] are rich in glycoproteins, some of which may be identical with the membrane-bound digestive enzymes [6]. The brush borders from rat small intestine contain, in addition, considerable quantities of glycolipids [7] and preliminary work on rabbit small intestine by the present authors indicates that this may also be true of brush borders from that source [8].

The purpose of the present work is to study the glycoprotein and glycolipid composition of rabbit small-intestinal brush borders and to observe the interrelationship between the biosynthesis of these membrane components. The advantage of using tissue derived from the rabbit is that, unlike rat, it is possible to study the brush borders from individual animals and hence any idiopathic differences in glycoprotein and glycolipid composition, relating possibly to the antigenic status of the animal, may be revealed.

Experimental

Materials and Methods

Unless otherwise stated materials were analytical grade obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.), Koch-Light Laboratories Ltd. (Colnbrook, Bucks, U.K.) or Sigma (London) Chemical Co. Ltd. (Kingston-upon-Thames, U.K.). All radioactive materials were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.).

The standard gangliosides GM_3, GM_2, GM_1 * were gifts from Dr. R.O. Brady, National Institute of Health, Bethesda, Md., U.S.A.

Isolation of brush borders. Male New Zealand white rabbits (Hylyne Commercial Rabbits, Northwich, Cheshire) of 1.5 kg weight (6—8 weeks old), which had been fed on standard laboratory chow (S.G.I.; Oxoid Ltd., London), were used. The rabbits were fasted for 24 h, but allowed free access to water, and then killed by an intravenous injection of 1.5 ml Nembutol (Pentobarbitone sodium B.P.; Abbot Laboratories Ltd., Queenborough, Kent). At various times before killing (3—24 h) the rabbits were injected intraperitoneally with 50 μCi D-[1-^{14}C]glucosamine hydrochloride (specific activity between 2 and 4 Ci/mol) in 1 ml physiological saline.

Immediately after killing, the abdominal and thoracic cavities were quickly opened and the small intestine was cut six inches from the pyloric sphincter. The next three feet of small intestine was removed, trimmed of adhering fat and mesentery, and washed through with 100 ml ice-cold physiological saline. All subsequent operations were performed at 4°C. The segment of small intes-

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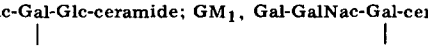
* The ganglioside notation is that of Svennerholm [9]. The following abbreviations are used: Glc, glucose; Gal, galactose; Gal*N*ac, *N*-acetylgalactosamine; AcNeu, *N*-acetylneuraminic acid; GM_3, AcNeu-Gal-Glc-ceramide; GM_2, GalNac-Gal-Glc-ceramide; GM_1, Gal-GalNac-Gal-ceramide; ceramide lactoside, Gal-Glc-



ceramide; ceramide trihexoside, Gal-Gal-Glc-ceramide.

tine was opened longitudinally and mucosa excised by gentle scraping with a glass slide. The mucosal scrapings (approximately 6 g wet weight) were homogenised in 200 ml of 0.3 M sucrose/5 mM EDTA (brought to pH 7.0 with 1 M Tris) with 60 strokes of a glass-metal homogeniser (radial clearance 0.35 mm) and filtered through cheese-cloth. The brush borders were isolated from this homogenate using the procedure of Boyd et al. [10] as illustrated in Fig. 1. All media were adjusted to pH 7.0 with 1 M Tris. The centrifugations were performed in an M.S.E. High Speed 18 centrifuge (Measuring and Scientific Equipment Ltd. London) using the 6 × 100 ml or 8 × 50 ml M.S.E. angle head rotors (cat. no. 69180 and 69181). Successive homogenates were monitored using a Wild M-20 phase contrast microscope (supplier, Microinstruments Ltd., Oxford).

Extraction and analysis of glycolipids. All solvent ratios are given in volumes. The chloroform was redistilled and Aristar methanol (B.D.H.) was used throughout.

The glycolipids were extracted from the tissue using a modification of the classical procedure of Folch et al. [11] (Fig. 1). The brush border fraction (approx. 50 mg dry weight) was suspended in a small volume (final volume usually 2 ml) of 0.01 M aqueous KCl and then in a further 20 volumes of chloroform/methanol (2 : 1, v/v) using a wide-bore Pasteur pipette for dispersal. In the proportions of solvents used, a single liquid phase resulted. The extraction was then performed at 50°C, under reflux, with intermittant shaking for 15 min. After cooling, one fifth of the final volume of methanol (8.4 ml) was added and the suspension was centrifuged in glass centrifuge tubes at 700 × *g* for 15

Mucosal scrapings homogenised (60 strokes) in 200 ml 0.3 M sucrose/5 mM EDTA (pH 7.0) and filtered through cheese-cloth to yield initial homogenate (I), which was then centrifuged. (All centifugations were 700 × *g*, 10 min, 4° C.)

↓⟶ supernatant S_1

pellet (P_1) homogenised (10 strokes) in 200 ml 0.3 M sucrose/5 mM EDTA (pH 7.0) and centrifuged

↓⟶ supernatant S_2

pellet (P_2) homogenised (10 strokes) in 150 ml 225 mM sucrose/5 mM EDTA (pH 7.0) and centrifuged

↓⟶ supernatant S_3

pellet (P_3) homogenised (10 strokes) in 100 ml of 150 mM sucrose/5 mM EDTA (pH 7.0) and centrifuged

↓⟶ supernatant S_4

pellet (P_4) homogenised (10 strokes) in 50 ml 75 mM sucrose/5 mM EDTA (pH 7.0) and centrifuged

↓⟶ supernatant S_5

pellet (P_5) homogenised (10 strokes) in 50 ml 37.5 mM sucrose/5 mM EDTA (pH 7.0) and centrifuged

↓⟶ supernatant S_6

pellet - brush border fraction (BB)

Fig. 1. Preparation of rabbit small-intestinal brush borders.

min at room temperature. The supernatant was carefully removed and its composition was restored to that of a chloroform/methanol (2 : 1, v/v) mixture by the addition of chloroform. The extraction was repeated on the residual protein pellet using 10 volumes (20 ml) of chloroform/methanol (1 : 2, v/v). This suspension was centrifuged without any further addition of methanol and the resulting supernatant was evaporated to dryness at 40°C under a stream of nitrogen. The residue was dissolved in the first supernatant to give the total lipid extract. The remaining protein pellet was termed the "lipid-free brush border".

The total lipid extract was mixed thoroughly for 5 min with one fifth of its volume of water and centrifuged to obtain two phases. The aqueous upper phase was withdrawn and retained. The procedure was repeated on the lower phase by addition of two fifths of its volume of chloroform/methanol/0.74% aqueous KCl (3 : 48 : 47, v/v) [12]. The combined upper phases were concentrated by rotary evaporation to approx. 5 ml and dialysed against 5 l distilled water at 4°C and the retentate was lyophilised to yield the hydrophilic glycolipid fraction.

The glycosphingolipids present in the lower (chloroform) phase were freed of some contaminating phospholipids by saponification [12]. The dried (40°C/N_2) chloroform phase was redissolved in 2 ml of 0.2 M methanolic sodium hydroxide and shaken at room temperature for 1 h. The solution was neutralised by dropwise addition of 1 M aqueous acetic acid, the pH being tested by spotting aliquots on to pre-wetted indicator paper. Then 15 ml chloroform/methanol (2 : 1) was added and the solution partitioned, as described above, by addition of one fifth of its volume of water. The resultant lower phase was similarly treated with two-fifths of its volume of chloroform/methanol/water (3 : 48 : 47, v/v). The resultant lower phase was termed the hydrophobic glycolipid fraction.

Thin-layer chromatography (TLC). Ascending thin-layer chromatography was performed on 20 cm square glass plates spread to a thickness of 400 μm with silica gel G (E. Merck. Darmstadt, G.F.R.). using chloroform/methanol/2.5 M (aq.) ammonia (60 : 35 : 8, v/v) as solvent [13]. Prior to use the spread plates were pre-washed in the solvent, air dried, and then activated by being heated at 80°C for 1 h. The entire hydrophobic and hydrophilic glycolipid fractions from the brush borders of each rabbit and standard materials were chromatographed in parallel on the same plate.

The glycolipids were detected by placing the dry chromatograms in a tightly sealed tank containing iodine crystals. The iodine-positive areas were marked and the iodine allowed to sublime off. In some experiments the gangliosides were visualised using the resorcinol-HCl reagent [14]. The dry chromatograms were lightly sprayed with the reagent, covered with a clean glass plate, and heated to 80°C until the blue, ganglioside-positive areas developed.

Elution of glycosphingolipids from thin-layer chromatograms. Iodine-positive areas, together with an equivalent area of blank gel as a control, were scraped into 10-ml tapered centrifuge tubes. The neutral glycosphingolipids were eluted by intermittantly vortex mixing the gel with 5 ml chloroform for 5 min followed by the centrifugation and removal of the supernatant. The procedure was repeated using two 5-ml portions of chloroform/methanol (1 . 1)

containing 5% water [15] and then with two 5-ml portions of chloroform/methanol/water (100 : 50 : 10) [16]. The combined supernatants were concentrated (40°C under N_2) to approximately 5 ml. The procedure for extracting the gangliosides was identical except that the initial extraction was performed with 5 ml methanol. Aliquots (1—2 ml) of the concentrated samples were withdrawn and dried down (40°C/N_2) in scintillation vials for determination of radioactivity and the remainders of the samples were similarly dried in 10-ml glass hydrolysis tubes for gas-liquid chromatographic analysis of their monosaccharide components.

Gas-liquid chromatographic analysis of sugars. Samples of dry glycolipids (approx. 100—200 μg) and protein (approx. 1 mg) were mixed with a known amount of mannitol (0.1 or 0.05 μmol) in aqueous solution. The samples were then rigorously dried in vacuo over phosphorus pentoxide and hydrolysed under nitrogen in 1 ml 1 M methanolic hydrogen chloride in sealed glass tubes for 24 h at 80°C. Following hydrolysis the tubes were cooled, opened and the contents extracted twice with equal volumes of dry spectroscopic-grade *n*-hexane. The methyl glycosides in the methanolic phase were then analysed as their trimethylsilyl derivatives as described by Bhatti et al. [17] using a Pye 104 dual flame chromatograph (W.G. Pye and Co. Ltd., Cambridge) with twin columns of 3% OV-I on diatomite CQ, 85—100 mesh, programmed between 120 and 200°C at 2°C per min.

Chemical determinations

Protein estimation. Samples of material (2—3 mg) were solubilized by treatment with 2 ml of 3% (w/v) aqueous sodium hydroxide on a boiling-water bath for 10 min. Protein determinations were made on aliquots of the solubilized material using the method of Lowry et al. [18].

Hexosamine estimation. Protein samples (2–5 mg) were hydrolysed in 1 ml 4 M aqueous hydrochloric acid under nitrogen in sealed glass tubes on a boiling-water bath for 6 h. The hydrolysate was concentrated to dryness by rotary evaporation and then in vacuo over potassium hydroxide pellets overnight. The dried residue was dissolved in 1 ml water and applied to columns (0.57 × 5 cm) of Dowex 50W × 8 (H^+ form), 200–400 mesh prepared as described by Spiro [19]. The hydrolysate was run into the column, the column was washed through with 10 ml water and then the hexosamines were eluted with 10 ml 2 M hydrochloric acid. The hexosamine fraction was dried, dissolved in a small volume of water (usually 1.1 ml), a sample removed for determination of radioactivity (0.1 or 0.2 ml) and the remainder assayed for hexosamine by the method of Elson and Morgan as described by Davidson [20].

Sialic acid. Dried glycoprotein samples (2—5 mg) were hydrolysed in 1 ml 0.1 M H_2SO_4 at 80°C under nitrogen in sealed glass tubes. The liberated sialic acid was isolated by chromatography on Dowex 1 X 8 [19] and quantified by the thiobarbituric acid method [21].

Nucleic acids. These were extracted from samples as described by Schneider [22]. DNA was assayed by the method of Burton [23] using ex-calf thymus DNA (Koch-Light) as standard and RNA was assayed by the method of Schneider [22] using D-ribose (Koch-Light) as standard.

Enzyme determinations. Sucrase and glucose-6-phosphatase assays were per-

formed on mucosal samples which had been dialysed against 5 l distilled water at 4°C for 24 h. Succinate dehydrogenase and aryl sulphatase were estimated directly on fractions of the mucosal homogenate (Fig. 1).

Sucrase (EC 3.2.1.26). Samples were incubated for 1 h at 37°C in 0.04 M sodium phosphate buffer (pH 6.7) containing 0.05 M sucrose. The glucose liberated was estimated as described by Dahlqvist [24] using glucose oxidase (EC 1.1.3.4, Sigma type V), horse-radish peroxidase (EC 1.11.1.7, Worthington Biochem. Co., U.S.A.) and *o*-dianisidine (Sigma).

Succinate dehydrogenase (EC 1.3.99.1). This was assayed by the method of Zeijlemaker et al. [25] using ferricyanide as the electron acceptor.

Glucose-6-phosphatase (EC 3.1.3.9). This was assayed by the method of Hubscher and West [26] using glucose 6-phosphate (disodium salt; B.D.H.) as substrate. The phosphate released was estimated using the method of Chen et al. [27].

Aryl sulphatases A and B. These were determined by the procedure of Dodgson and Spencer [28] using *p*-nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate; Koch-Light) as substrate.

Determination of radioactivity. Radioactive samples were counted by a Beckman LS 200B liquid-scintillation counting system. The composition of the scintillant used was: butyl PBD (2-(4-*t*-butylphenyl)-5-(4^{11}-biphenyl)-2,3,4-oxadiazole; Koch-Light), 8 g; naphthalene (scintillation grade; Koch-Light), 80 g; 2-methoxyethanol, 400 ml; toluene (scintillation grade, BDH) 600 ml. Aqueous samples (0.1 ml) and dried lipid samples were dissolved in 7 ml scintillant and counted to within 5%.

Results

Preparation of brush borders. Brush borders were prepared from rabbit mucosal scrapings by the relatively mild method of Boyd et al. [10]. This method was superior to other procedures used for other species (involving either filtration through glass wool [29] or flocculation with citrate-phosphate solutions [30]) on account of better recoveries of brush borders (greater than 10% by sucrase activity). The efficacy of the method of preparing brush borders was determined using the marker enzymes, sucrase (brush border), succinate dehydrogenase (mitochondria), aryl sulphatases A and B (lysosomes) and glucose-6-phosphatase (microsomes). The results are shown in Table I and indicate that brush borders were prepared from the whole mucosal homogenate in 37% yield with 11.4-fold enrichment. Contamination of the brush borders by mitochondria and lysosomes as measured by enzyme activity is negligible. The glucose-6-phosphatase activity suggests the possibility of some microsomal contamination. However the enzyme activity in the brush border preparation was, unlike the total mucosal activity and the microsomal enzyme [29], not inhibited by glucose (Table II). Also, the isolated brush borders contained no detectable amounts of RNA as determined by the method of Schneider [22]. This indicates that the brush border glucose-6-phosphatase activity is not due to the microsomal contamination and is most likely due to the action of non-specific digestive phosphatases.

Chemical assay by the diphenylamine method [23] indicates the presence of

TABLE I

ANALYSIS OF THE METHOD OF BRUSH BORDER PREPARATION FROM RABBIT SMALL INTESTINE

The results from a typical experiment are shown below. The symbols S_1-S_6 and P_1-P_5 refer to the fractions of the small intestinal mucosal homogenate isolated as described in Fig. 1. The enzyme units are expressed as μmol substrate hydrolysed or reduced per min and the specific activities are expressed as enzyme units per mg protein.

Fraction of the mucosal homogenate	Total protein (mg)	Sucrase		Aryl sulphatase		Succinate dehydrogenase		Glucose-6-phosphatase		DNA	
		Units	Spec. act.	Units	Spec. act.	Units	Spec. act.	Units	Spec. act.	Total (mg)	μg/mg protein
Initial homogenate	918.88	73.81	0.08	0.51	0.00056	7.98	0.0087	31.03	0.0034	20.48	22.3
S_1	—	11.91	—	0.39	—	6.58	—	13.74	—	7.59	—
P_1	297.1	64.36	0.22	0.15	0.00051	1.23	0.0041	19.02	0.064	11.83	39.82
S_2	—	7.42	—	0.086	—	0.94	—	8.05	—	3.55	—
P_2	155.8	58.21	0.37	0.046	0.00029	0.52	0.0033	14.05	0.09	10.46	67.16
S_3	—	6.33	—	0.036	—	0.17	—	7.63	—	2.63	—
P_3	107.1	53.74	0.50	n.d.	n.d.	n.d.	n.d.	8.99	0.084	6.87	64.20
S_4	—	9.36	—	n.d.	n.d.	n.d.	n.d.	3.09	—	1.66	—
P_4	67.38	45.31	0.67	n.d.	n.d.	n.d.	n.d.	6.62	0.098	5.19	77.02
S_5	—	13.02	—	n.d.	n.d.	n.d.	n.d.	4.58	—	1.20	—
P_5	39.13	29.73	0.76	n.d.	n.d.	n.d.	n.d.	3.73	0.095	4.44	113.5
S_6	—	6.26	—	n.d.	n.d.	n.d.	n.d.	3.17	—	3.27	—
Brush border	29.62	27.24	0.92	n.d.	n.d.	0.017	0.00058	1.96	0.066	3.79	128.16
Total recovery		110.5%		99.72%		96.58%		136.1%		115.8%	
Recovery in brush border fraction		36.9%		0.00		0.22%		6.27%		18.53%	
Enrichment in brush border fraction *		11.44		0.00		0.067		2		5.75	

—, not determined.

n.d., not detectable.

* Specific activity in brush border fraction/specific activity in initial homogenate.

TABLE II

THE EFFECT OF GLUCOSE ON INTESTINAL GLUCOSE-6-PHOSPHATASE

Samples of the initial mucosal homogenate and the brush border fraction (Fig. 1) were assayed for glucose-6-phosphatase activity in the absence or presence of D-glucose (0.4 M final concentration) as described in the text. Duplicate determinations of the enzyme activity are given.

Tissue fraction	Enzyme units (mol glucose-6-phosphatase hydrolysed/min per mg protein)		
	— Glucose	+ Glucose	% Inhibition
Initial homogenate	0.0335	0.0272	18.8
	0.0335	0.0264	21.2
Brush border	0.0661	0.0661	0.0
	0.0658	0.0661	—1.2

DNA (123 μg/mg protein) in the brush border preparation, sialic acid being absent. However, spectroscopic analysis by the method of Warburg and Christian [31] yielded values for the DNA content of the brush border of 80 μg/mg protein. The reason for this difference is uncertain. However, diligent monitoring by phase contrast microscopy failed to show any intact nuclei in the brush border fraction and it is, therefore, unlikely that there is any appreciable contamination by nuclear membranes.

Carbohydrate composition of brush border glycoproteins

Following extraction of the brush border preparations with chloroform/methanol (2 : 1), the residual insoluble material represents the gross glycoprotein and protein components of the membrane. The component sugars of this fraction were analysed by gas-liquid chromatography and the results are

TABLE III

CARBOHYDRATE COMPOSITION OF THE GLYCOPROTEIN FRACTION FROM RABBIT SMALL-INTESTINAL BRUSH BORDERS

The sugars were analysed by gas-liquid chromatography as described in the text. Independent determinations from the brush borders of three individual New Zealand white male rabbits are given together with analyses of two preparations each from the pooled brush borders of two Blue Bevron X Chinchilla rabbits.

	Molar ratios (galactose taken as unity)					μg Hexosamine/mg protein		Total sugar (μg/mg protein) *
	Mannose	Glucose	Fucose	Glucosamine	Galactosamine	Gas-liquid chromatography	Elson-Morgan	
1. New Zealand Whites	1.79	0.89	1.02	0.84	0.39	17.5	23.8	78.1
	1.29	>4	0.59	0.93	0.40	37.8	33.2	113.5
	0.94	>4	1.08	0.81	0.70	31.9	27.2	93.5
2. Blue Bevron X Chinchilla	0.89	3.8	0.58	0.80	0.58	41.9	—	123.4
	0.85	>4	0.61	0.80	0.54	39.9	—	113.3

—, not determined.

* These values exclude the contribution from glucose.

shown in Table III. The sugars present are glucose, galactose, mannose, fucose, glucosamine, and galactosamine, but sialic acid could not be detected either by gas-liquid chromatography or the thiobarbituric acid assay [21]. The amounts of total hexosamine were estimated by gas-liquid chromatography and by the Elson-Morgan procedure and generally the values given by the two methods are in agreement (Table III).

Comparisons between different strains of rabbit are of interest and data are also presented (Table III) for the molar ratios of sugar present in the brush border glycoprotein fraction obtained from male Blue Bevron X Chinchilla rabbits (1.5 kg wt., Oxford University Farm). These latter results were obtained from two different preparations of brush borders each from two rabbits. The other experimental details, including diet, were identical to those described for the New Zealand white rabbits used in the remainder of this work. No significant differences exist between the carbohydrate compositions of the brush border glycoprotein fraction from these two different strains.

Analysis of brush border glycolipids. In this section the term hydrophobic glycolipid is used to denote a glycolipid preferentially partitioning into the chloroform-rich phase during the extraction procedure described previously and similarly the term, hydrophilic glycolipid, denotes a glycolipid preferentially partitioning into the water-methanol phase. In animal tissues, the hydrophobic glycolipid fraction is comprised mainly of neutral glycolipids and the hydrophilic fraction of the sialic acid-containing glycosphingolipids (gangliosides).

A typical chromatographic separation of the brush border glycolipids, stained with iodine vapour, is shown in Fig. 2. Both hydrophobic glycolipid

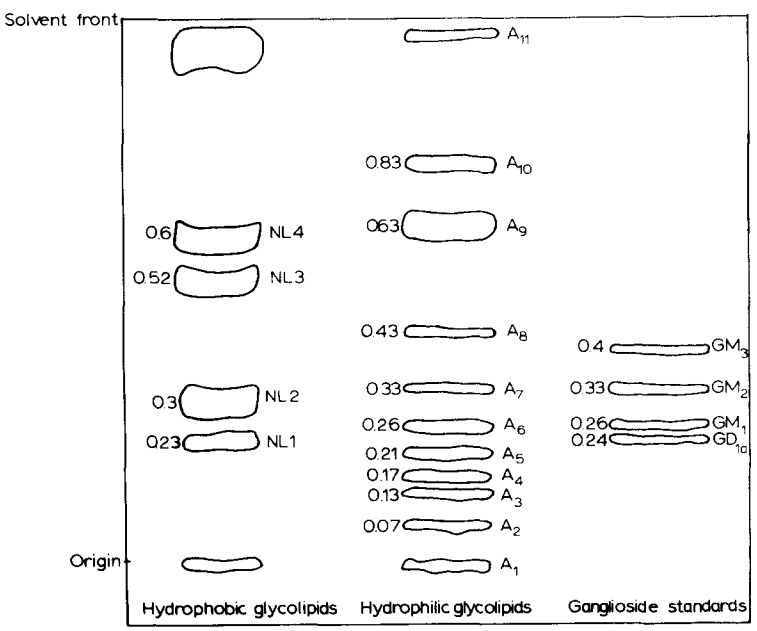


Fig. 2. Chromatographic separation of the glycolipids from the rabbit small-intestinal brush border. The medium used was silica gel G and the chromatogram was developed in chloroform/methanol/2.5 M aqueous ammonia (60 : 35 : 8, v/v). The R_F values are given to the left of each component.

and hydrophilic glycolipid fractions are shown chromatographed side by side.

(a) Hydrophobic glycolipids. The major iodine-positive areas were all in the hydrophobic glycolipid fraction and the carbohydrate compositions of these lipids are given in Table IV. The results shown are from several experiments and in each case the glycolipids were isolated from the brush borders of individual rabbits. All four lipids detected in the hydrophobic glycolipid fraction contain carbohydrate; two (NL2 and NL4) contain glucose and galactose and two (NL1 and NL3) contain glucosamine in addition to both of these sugars. From one rabbit of the five analysed the chromatographically slowest glucosamine-containing glycolipid contained an additional sugar residue (fucose; Table IV) and has been designated NL1a. From these results it is concluded that NL2 represents ceramide trihexoside (ceramide-glucose-galactose-galactose), NL4 represents ceramide lactoside (ceramide-glucose-galactose) and that NL1/1a and NL3 are glucosamine-containing glycolipids.

(b) Hydrophilic glycolipids. On chromatographic separation of the individual hydrophilic glycolipid fractions from seven rabbits, eleven components were routinely observed after staining with iodine (Fig. 2). In each case components (A_{6-8}) co-chromatographed with GM_3, GM_2 and GM_1 standard gangliosides. Carbohydrate analyses of the separated components (Table V) were hampered by the small amounts of individual sugars present which made their absolute identification difficult. However, components A_{4-9} contain glucose and galactose, indicating that they are glycolipids.

Confirmation that gangliosides were present was obtained by separating the hydrophilic glycolipid fraction from the pooled brush borders of four rabbits.

TABLE IV

CARBOHYDRATE COMPOSITION OF COMPONENTS OF THE HYDROPHOBIC GLYCOLIPID FRACTION

The carbohydrate compositions of the glycolipids isolated by TLC were determined by gas-liquid chromatography as described in the text. All of the isolated glycolipids contain glucose and the values for the nmol glycolipid present assume that each glycolipid contains 1 mol of this sugar. The amounts of glycolipid separated by TLC and analysed correspond to 16.1 mg brush border protein for experiment A and 13.95 mg for experiment B.

Glycolipid	Expt.	R_F	Molar ratios (glucose taken as unity)			nmol glycolipid/mg brush border protein
			Galactose	Glucosamine	Fucose	
NL1a	A	0.23	2.1	0.9	1.1	4.3
NL1	B	0.27	2.0	0.6	—	2.25
NL2	A	0.32	2.0	—	—	13.04
	B	0.37	2.1	—	—	10.75
NL3	A	0.52	2.3	1.9	—	8.45
	B	0.50	1.4	1.7	—	3.37
NL4	A	0.60	1.0	—	—	12.42
	B	0.60	1.0	—	—	12.90
Data on glycolipid NL1 as isolated independently from three other rabbits						
		0.28	1.7	0.6	—	
		0.28	1.8	0.6	—	
		0.28	2.0	0.9	—	

—, Absent.

TABLE V

CARBOHYDRATE COMPOSITIONS OF THE COMPONENTS OF THE HYDROPHILIC GLYCOLIPID FRACTION

The hydrophilic glycolipids were separated by TLC and analysed by gas-liquid chromatography as described in the text. The results given are for a typical experiment and components A_{1-11} are from the hydrophilic glycolipid fraction corresponding to experiment A in Table IV. The hydrophilic glycolipids were isolated from 16.9 mg brush border protein. No sugars other than those shown below were detected. The theoretical glucose to galactose ratios of gangliosides GM_3, GM_2 and GM_1 are given for comparison because of the co-chromatography of these gangliosides with components A_8, A_7 and A_6 respectively (Fig. 2). The amounts of components A_{1-11} isolated in other experiments were similar to those reported below.

Component	R_F	Glucose		Galactose		Glucosamine		Theoretical glucose to galactose ratios
		Molar ratio	Quantity (nmol/mg brush border protein)	Molar ratio	Quantity (nmol/mg brush border protein)	Molar ratio	Quantity (nmol/mg brush border protein)	
A_1	origin	1	0.52	—	—	3.77	2.95	
A_2	0.07	n.d.	75	—	—	n.d.	2.25	
A_3	0.13	n.d.	0.55	—	—	trace		
A_4	0.17	1	1.18	0.96	1.12	—	—	
A_5	0.21	1	0.83	0.72	0.59	—	—	
A_6	0.26	1	1.72	1.41	2.43	—	—	1 : 2 (GM_1)
A_7	0.33	1	0.77	0.44	0.33	—	—	1 : 1 (GM_2)
A_8	0.43	1	1.72	0.71	1.24	—	—	1 : 1 (GM_3)
A_9	0.63	1	3.25	0.59	1.89	0.49	1.60	
A_{10}	0.83	—	—	—	—	—	—	
A_{11}	solvent front		trace		trace		trace	

—, absent.
n.d., not determined.

After staining with resorcinol-HCl reagent [14] two blue, ganglioside-positive areas with the chromatographic mobilities of GM_3 and GM_1 were observed. The areas A_{1-3} stained a golden-brown indicative of non-ganglioside contaminants.

From the sugar composition, the staining reactions and the chromatographic mobilities it is concluded that the components A_{4-9} are glycolipids and these include the gangliosides GM_3 (as A_8), GM_2 (as A_7) and GM_1 (as A_6).

The low amounts of ganglioside detected during these experiments was not due to degradation or incomplete extraction since identical experiments on rabbit brain extracts yielded quantitative results [32].

The distribution of total carbohydrate between the hydrophobic glycolipids, hydrophilic glycolipids and glycoproteins of the brush border as determined in these experiments are given in Table VI.

Incorporation of [1-^{14}C]glucosamine into the fractions of the small intestinal brush border. In the above experiments the carbohydrate compositions of the glycoprotein fraction and of the various glycolipids of the small intestinal brush border have been characterised. Experiments were then undertaken to determine the turnover of these carbohydrate-containing components.

The experimental procedures used were devised to minimise any possible ambiguities introduced by randomization of the radio label to sugars other than hexosamine.

Rabbits were injected with 50 μCi of D-[1-^{14}C]glucosamine and, at various intervals afterwards (3—24 h), were killed. The brush borders were then prepared as before and the glycoprotein, hydrophobic glycolipid and hydrophilic glycolipid fractions were isolated. The incorporation of radioactivity into the glycoprotein fraction was determined by liberating the sugars by acid hydrolysis, isolation of the hexosamine by ion-exchange chromatography and determination of its specific activity. The isolated radioactive hexosamine contained between 0.1 and 0.3 μmol of amino sugar and between 350 and 3550 dpm depending upon the amount of glycoprotein analysed, the rabbit from which it was derived and the time following administration of the radioisotope. The incorporation of label into the brush border hexosamine is shown in Fig. 3 and indicates that maximum labelling occurs approximately 7.5 h following injection of radioactivity. The subsequent loss of radioactivity from this fraction follows an exponential function (Fig. 4) with a half-life of 11.2 h.

The incorporation of radioactivity into the glycolipids (Fig. 3) was determined by TLC separation of the individual glycolipids, their elution from the gel and quantification of radioactivity by liquid scintillation counting and of hexosamine by gas-liquid chromatography. The isolated glycolipids NL1 and NL3 contained between 0.019 and 0.12 μmol of hexosamine and between zero and 1500 dpm of radioactivity. No radioactivity was found associated with either the isolated hydrophilic glycolipids or with the glycolipids lacking hexosamine (NL2 and NL4; Table IV). The incorporation of label into glycolipid NL1 shows a maximum between 7.5 and 12 h following injection of radioactivity (Fig. 3) and generally appears to be similar to the labelling kinetics of the brush border glycoprotein. However, the glycolipid NL3 is distinct in that it labels maximally at approximately 18 h.

Confirmation that glycolipids NL1 and NL3 have different time scales for

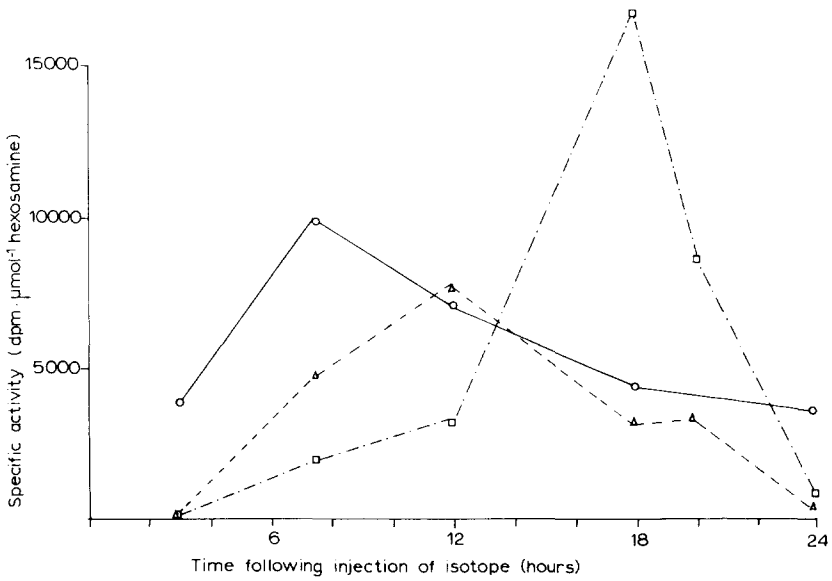


Fig. 3. The incorporation of D-[1-^{14}C]glucosamine into fractions of the rabbit small-intestinal brush border. The specific activities are shown of the glycoprotein hexosamine (○———○), the hexosamine of glycolipid NL3 (□———□) and the hexosamine of glycolipid NL1 (△- - - - -△) at various time intervals (3—24 h), following injection of rabbits with 50 µCi of D-[1-^{14}C]glucosamine

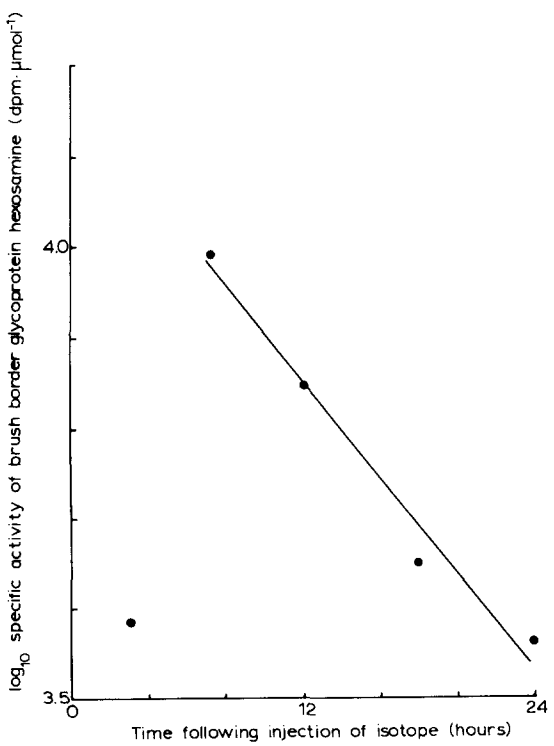


Fig. 4. Semi-logarithmic graph of the specific activity of the brush border hexosamine versus time following injection of D-[1-^{14}C]glucosamine. The data are derived from Fig. 3 and the line was fitted by the method of least squares.

TABLE VI

THE DISTRIBUTION OF CARBOHYDRATE BETWEEN THE FRACTIONS OF THE BRUSH BORDER

	Carbohydrate (μg/mg protein)	
	Expt. A	Expt. B
Hydrophobic glycolipid *	17.75	14.9
Hydrophilic glycolipid **	3.43	<2
Glycoprotein ***	95 μg/mg protein	

* Summation from Table IV.
** Summation of components A_{4-9} from Table V.
*** Average of results from New Zealand White rabbits given in Table III.

TABLE VII

THE INCORPORATION OF [^{14}C]GLUCOSAMINE AND [^{3}H]GLUCOSAMINE INTO THE COMPONENTS OF THE BRUSH BORDER

Brush borders were isolated from a rabbit following injections of [^{3}H]glucosamine 7.5 h previously and [^{14}C]glucosamine 18 h previously. The glycoprotein hexosamine and the glucosamine-containing glycolipids (NL1 and NL3; Table IV) were then separated and their $^{3}H/^{14}C$ ratios determined as described in the text.

Membrane fraction	$^{3}H/^{14}C$ ratio
Glycolipid NL3	0.33
Glycolipid NL1	1.12
Glycoprotein hexosamine	1.21

their incorporation of radioactivity was obtained using the dual label procedure of Arias et al. [33]. A rabbit was injected with 50 μCi of [1-^{14}C]glucosamine and then 10.5 h later was injected with 100 μCi [^{3}H]glucosamine; 7.5 h following this later injection the rabbit was killed. Therefore, at the time of death the [^{14}C]glucosamine has been present for 18 h and the [^{3}H]glucosamine for 7.5 h. The brush borders were then prepared, the various glycolipids and the glycoprotein fraction isolated and the ratios of the incorporated ^{3}H and ^{14}C determined. The results are shown in Table VII. As expected, the brush border glycoprotein and the glycolipid NL1 exhibit very similar $^{3}H/^{14}C$ ratios indicating similar rates of turnover. However, the glycolipid NL3 has a markedly different and lower $^{3}H/^{14}C$ indicating that its time of maximal labelling is later than for either glycolipid NL1 or the brush border glycoprotein. These results are entirely consistent with the time course reported in Fig. 3.

Discussion

Significant quantities of both lipid and protein-bound carbohydrate are present in the rabbit small intestinal brush border. The protein fraction from the brush border contains mannose, glucose, galactose, fucose, glucosamine and galactosamine, glucose being the predominant sugar. All of these sugars, with the exception of glucose, are commonly found associated with glycoproteins. However, in agreement with the present study Kim and Perdomo [5] have

found considerable amounts of glucose in the glycoprotein fraction from rat small intestinal brush borders. It is not known whether the glucose is covalently- or non-covalently bound to the glycoproteins. However, hamster small-intestinal brush borders contain a glucose-binding protein [34] suggesting that non-covalent binding of this sugar to the membrane may occur.

The brush border glycolipids contain 12–15% of the total carbohydrate present in the brush border. The major glycolipids all contain glucose and galactose but lack sialic acid. Two of the glycolipids present, ceramide lactoside and ceramide trihexoside contain only these sugars and the other two glycolipids contain in addition glucosamine. The chromatographically faster glucosamine-containing glycolipid contains glucose, galactose and glucosamine in the approximate molar ratios of 1 : 2 : 2 and, hence, it may be a ceramide pentasaccharide. However, its relatively fast chromatographic behaviour is inconsistent with this interpretation and it is possible that this lipid is either a novel glycolipid with a more hydrophobic non-sugar moiety than ceramide or, as is more likely, represents an unresolved mixture of sphingolipids each containing one or two sugars. The chromatographically slower glucosamine-containing glycolipid from the majority of the brush border samples analysed has the chromatographic mobility and sugar composition expected of a ceramide tetrasaccharide and contains glucose, galactose and glucosamine in the molar proportions of 1 : 2 : 1. The brush border glycolipids from one rabbit (cf. Smith et al. [51]) however, lacked the above ceramide tetrasaccharide and contained a chromatographically similar ceramide pentasaccharide having glucose, galactose, glucosamine and fucose in the molar ratios of 1 : 2 : 1 : 1. A possible explanation for this may be that the ceramide tetrasaccharide and the ceramide pentasaccharide are related glycolipids differing only in the presence or absence of fucose and that a minority of rabbits possess a specific fucosyl transferase capable of adding a fucose residue to the ceramide tetrasaccharide. The situation may therefore be analogous to the blood group systems in humans where the various blood groups determinants are specified by sequential action of specific sugar transferases adding sugars to a common oligosaccharide "core" [35]. Indeed this ceramide tetrasaccharide has a carbohydrate composition closely resembling that of the oligosaccharide "core" common to many blood group active glycolipids (e.g. glycolipids bearing Lewis blood group a [36] and b [37] activity). This observation is of interest in the light of the uncertainty surrounding the site of biosynthesis of erythrocyte-bound Lewis blood group active glycolipids [52] in humans and that it has been suggested that these are not made in the bone marrow, but at some other site [35]. The active synthesis (see later) of the ceramide tetrasaccharide in rabbit small-intestinal mucosa described in the present study raises the possibility that this tissue may be an important site of biosynthesis of Lewis blood group active glycolipids in humans. An association of such glycolipids with the chylomicrons would provide a pathway for their transfer into the blood stream. Indeed, a transfer at Lewis blood group active glycolipids from serum low density lipoproteins to erythrocytes has previously been observed in vitro [38].

The sialic acid-containing glycolipids (gangliosides) constitute at most only a minor fraction (approx. 10%) of the total glycolipid and include the monosialogangliosides GM_3, GM_2 and GM_1. Generally, as no sialic acid could be

detected in the glycoprotein, it appears that the rabbit small-intestinal brush borders lack any significant quantities of this sugar. A similar, although less marked, situation exists in rat small-intestinal brush borders in which gangliosides are a minor component of the total glycolipid [7] and the glycoprotein carbohydrate contains only 2.2% of its sugar as sialic acid [5]. This contrasts with observations on the rabbit kidney brush border glycoproteins which contain 20% of their carbohydrate as sialic acid [39] and other plasma membranes, for example those from liver cells and lymphocytes, are similarly enriched in this sugar [40]. Therefore, a low sialic acid content may be a unique characteristic of the small intestinal brush border.

Various hypotheses have been proposed to explain the presence of considerable quantities of carbohydrate in the brush border. For example the glycocalyx may reduce diffusion of the products of the membrane-bound hydrolyases and hence facilitate their adsorption by the epithelial cell [41]. Bound carbohydrate may also serve to inhibit degradation of the brush border by the soluble intestinal proteolytic enzymes [42] and the glycolipids, specifically, have been suggested to play a role in the passage of solutes across the membrane [7]. However, we wish to suggest that since many of the brush border-bound enzymes are glycoproteins [6], the carbohydrate may be involved in the binding of these digestive enzymes to the brush border.

The labelling studies on the brush border glycoprotein and both glucosamine -containing glycolipids show that they turn over during the time period studied (3—24 h). It is unlikely that turnover of cells is responsible for these observations as the life time of the small intestinal enterocyte is 42 h in mice [43], 36—40 h in rat [44] and approximately 5 days in man [45]. Also, there is no evidence for a plateau in the specific activity of the glycoproteins and glycolipids. This would be expected if these membrane components were synthesized solely during the maturation of cells in the crypts of Lieberkuhn and remained metabolically inert during the passage of the cell up the villus to its eventual loss at the villus tip. Therefore, there is an active synthesis and degradation of both the brush border glycoprotein and the glucosamine-containing glycolipid during the life-time of the mature rabbit small-intestinal enterocyte. Similarly, the protein components of rat small intestinal brush borders [46] and the protein and glycoprotein components of rabbit kidney brush borders [47] also turn over during the life-time of the mature cell.

The significance of this membrane turnover may be that it reflects a continuing need for the cell to replace denatured or damaged components of the cell membrane. Such an explanation has attractions when the small intestinal brush border is considered in that the plethora of digestive enzymes in the gut may continually erode the membrane [42]. However, an alternative, and perhaps more general, explanation for membrane turnover may be that it is a mechanism whereby the cell can quickly regulate the composition and structure of its surface membrane in response to environmental stimuli. This latter hypothesis may be more consistent with the observation that the plasma membrane components of cultured mammalian cells also turn over [48].

The marked difference between the labelling kinetics of the two glucosamine-containing glycolipids indicates that this membrane turnover is not a concerted process and that these different membrane components have widely

different rates of synthesis and degradation. A similar phenomenon is also apparent amongst the proteins of rat small-intestinal brush borders [46] and the proteins [49] and phospholipids [50] of rodent liver plasma membrane. However, the individual protein components of the rabbit kidney brush border membrane are thought to turn over at similar rates [47]. The differences between these observations may indicate that subtle variations exist between different cell types in the mechanism of plasma membrane assembly and its control. The present work at least extends the class of membrane components exhibiting heterogenous rates of turnover to include the glycolipids for the first time.

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References

1 Leblond, C.P. and Bennett, G. (1974) in The Cell Surface in Development (Moscona, A.A., ed.), pp. 29—49, John Wiley and Sons, New York
2 Cook, G.M.W. and Stoddart, R.W. (1973) Surface Carbohydrates of the Eukaryotic Cell, Academic Press, New York
3 Critchley, D.R. (1973) in Membrane Mediated Information (Kent, P.W., ed.), Vol. 1, pp. 20—47, M.T.P., Lancaster U.K.
4 Andre, F., Bouhours, J.F., Guignard, H. and Lambert, R. (1971) CR Soc. Biol. 165, 1665—1667
5 Kim, Y.S. and Perdomo, J.M. (1974) Biochim. Biophys. Acta 342, 111—124
6 Forstner, G.G. (1971) Biochem. J. 121, 781—789
7 Forstner, G.G. and Wherrett, J.R. (1973) Biochim. Biophys. Acta 306, 446—459
8 Cooper, J.R. and Kent, P.W. (1973) Biochem. Soc. Trans. I, 1176
9 Svennerholm, L. (1972) in Methods in Carbohydrate Chemistry (Whistler, R.L. and BeMiller, J.N., eds.), Vol. 6, pp. 464—474, Academic Press, New York
10 Boyd, C.A.R., Parsons, D.S. and Thomas, A.V. (1968) Biochim. Biophys. Acta 150, 723—726
11 Floch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
12 Dijong, I., Mora, P.T. and Brady, R.O. (1971) Biochemistry 10, 4039—4044
13 Wherrett, J.R. and Cumings, J.N. (1963) Biochem. J. 86, 378—382
14 Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604—611
15 Weinstein, D.B., Marsh, J.B., Glick, M.C. and Warren, L. (1970) J. Biol. Chem. 245, 3928—3937
16 Vance, D.E. and Sweeley, C.C. (1967) J. Lipid Res. 8, 621—630
17 Bhatti, T., Chambers, R.E. and Clamp, J.R. (1970) Biochim. Biophys. Acta 222, 339—347
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Spiro, R.G. (1966) Methods Enzymol. 8, 3—26
20 Davidson, E.A. (1966) Methods Enzymol. 8, 52—60
21 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
22 Schneider, W.C. (1957) Methods Enzymol. 3, 680—684
23 Burton, K. (1956) Biochem. J. 62, 315—323
24 Dahlqvist, A. (1964) Anal. Biochem. 7, 18—25
25 Zeijlemaker, W.P., Dervartanian, D.V., Veerger, C. and Slater, E.C. (1969) Biochim. Biophys. Acta 178, 213—224
26 Hubscher, G. and West, G.R. (1965) Nature 205, 799—800
27 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756—1758
28 Dodgson, K.S. and Spencer, B. (1957) Methods Biochem. Anal. 4, 211—255
29 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381—390
30 Porteous, J.W. (1968) FEBS Lett. 1, 46—49
31 Warburg, O. and Christian, W. (1941) Biochem. Z 310, 384—421
32 Cooper, J.R. (1974) D. Phil thesis, University of Oxford, U.K.

33 Arias, I.M., Doyle, D. and Schimke, R.I. (1969) J. Biol. Chem. 244, 3303—3315
34 Eichholz, A., Howell, K.E. and Crane, R.K. (1969) Biochim. Biophys. Acta 193, 179—192
35 Ginsburg, V. (1972) Adv. Enzymol. 36, 131—149
36 Hakomori, S. (1970) Chem. Phys. Lipids 5, 96—115
37 Hakomori, S. and Andrews, H.D. (1970) Biochim. Biophys. Acta 202, 225—228
38 Marcus, D.M. and Cass, L.E. (1969) Science 164, 553—555
39 Quirk, S.J. and Robinson, G.B. (1972) Biochem. J. 128, 1319—1328
40 Glick, M.C. (1976) in Mammalian Cell Membranes (Jamieson, G.A. and Robinson, D.M., eds.), Vol. 2, pp. 45—77, Butterworths, London
41 Hamilton, J.D. and McMichael, H.B. (1968) Lancet ii, 154—156
42 Alpers, D.H. and Kinzie, J.L. (1973) Gastroenterology 64, 471—496
43 Sigdestad, C.P., Hagemann, R.F. and Lesher, S. (1970) Gastroenterology, 58, 47—48
44 Loehry, C.A., Croft, D.N., Singh, A.K. and Creamer, B. (1969) Gut 10, 13—18
45 MacDonald, W.C., Trier, J.S. and Evertt, N.B. (1964) Gastroenterology 46, 405—417
46 James, W.P.T., Alpers, D.H., Gerber, J.E. and Isselbacher, K.J. (1971) Biochim. Biophys. Acta 230, 194—203
47 Quirk, S.J., Byrne, J. and Robinson, G.B. (1973) Biochim. J. 132, 501—508
48 Hughes, R.C., Sanford, B. and Jeanloz, R.W. (1972) Proc. Natl. Acad. Sci. U.S. 69, 942—945
49 Gurd, J.W. and Evans, W.H. (1973) Eur. J. Biochem. 36, 273—279
50 Lee, T., Stephens, N., Moehl, A. and Snyder, F. (1973) Biochim. Biophys. Acta 291, 86—92
51 Smith, E.L., McKibbin, J.M., Karlsson, K.-A., Pascher, I., Samuelsson, B.E., Li, Y.-T. and S-CH (1975) J. Biol. Chem. 250, 6059—6064
52 Koscielak, J. (1978) in New Approaches to Genetics (Kent, P.W., ed.), pp. 206—222, Oriel Press, London