Free ceramide, sphingomyelin, and glucosylceramide of isolated rat intestinal cells

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Abstract Free ceramide, glucosylceramide, and sphingomyelin were isolated from mature cells of adult rat small intestine. Free ceramide and ceramide cleaved from sphingomyelin by enzymatic hydrolysis were fractionated by thin-layer chromatography on borate-impregnated silica gel plates. Sphingoid bases were characterized by gas-liquid chromatography of aldehydes formed upon periodate oxidation. Fatty acids were quantified as methyl esters. Ceramide structures were confirmed by direct-inlet mass spectrometry. Free ceramide was found to contain two major long-chain bases in nearly equal quantity: sphingosine, mainly linked to palmitic acid, and 4*D*-hydroxysphinganine associated with C_{20} to C_{24} fatty acids, 22% being hydroxylated. Sphinganine occurred as a minor component linked to nonhydroxy fatty acids. Sphingomyelin contained the three long-chain bases and 63% of its ceramide was *N*-palmitoylsphingosine. Mass spectrometry of glucosylceramide confirmed 4*D*-hydroxysphinganine as the major sphingoid base associated preferentially with longer chain hydroxy fatty acids.—**Bouhours, J-F., and H. Guignard.** Free ceramide, sphingomyelin, and glucosylceramide of isolated rat intestinal cells. *J. Lipid Res.* 1979. **20:** 897–907.

Supplementary key words cerebroside · hydroxy fatty acids · mass spectrometry

Free ceramide has been found to occur in numerous mammalian organs. The nature of its components, long-chain bases and fatty acids, is characteristic of the tissue and subject to change with development and aging (1). Extensive studies have been dedicated to brain and liver ceramide and its key role as an intermediate in the synthesis of higher sphingolipids in vitro (2, 3) as well as in vivo (4, 5). Detailed structural analyses have been carried out on ceramide from other origins: human platelets (6) and bovine (7, 8) and equine kidney (9). Although glycosphingolipids of small intestine have been investigated by several laboratories (10–13), little attention has been paid to the important amount of ceramide occurring in this tissue.

In previous studies (14, 15), glycolipid and ceramide distributions were determined in rat intestinal cells isolated according to a villus–crypt gradient. This procedure was shown to yield cells at different stages of differentiation and maturation (16). The cellular concentration and the rate of synthesis of the three major glycolipids, glucosylceramide, globotrialosylceramide, and sialosyllactosylceramide, were modified during differentiation. However the structures of these compounds were not different in villus or in crypt cells (17). They contained hydroxylated and nonhydroxylated fatty acids, and the major long-chain base was 4*D*-hydroxysphinganine, formerly known as phytosphingosine.

Ceramide concentration as well as glycosphingolipid concentration was also modified during differentiation: free ceramide was more abundant in crypt (undifferentiated) cells than in villus cells. It was felt to be of interest to know the structure of free ceramide in order to investigate its role as a precursor in the synthesis of other intestinal sphingolipids. However, in previous attempts, unknown contaminants hindered isolation of free ceramide of rat intestine. In the present study, the different molecular species of free ceramide were isolated from mature cells of rat intestine and were purified by improved techniques. Their structures were determined and compared with the ceramide backbone of the two most closely related sphingolipids, glucosylceramide and sphingomyelin.

MATERIALS

Solvents of Pro analysis grade (E. Merck, Darmstadt, W. Germany) were used without further purification. Chloroform contained 1% ethanol as stabilizer. Standards of LCB were obtained from Supelco Inc., Bellefonte, PA (sphingosine), from Calbiochem, San Diego, CA (4*D*-hydroxysphinganine), and from Miles-Yeda, Rehovot, Israel (sphinganine).

Abbreviations: LCB, long-chain base; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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METHODS

Isolation of intestinal epithelial cells

Male Wistar rats weighing 250 g were fed ad libitum and killed by cervical dislocation. Small intestine was removed and thoroughly washed with saline, and villus cells were collected through the five first steps of the method of Weiser (16).

Extraction and fractionation of lipids

Lipids were extracted in chloroform–methanol mixtures of increasing polarity according to a procedure described earlier (15). Lipid extracts were desalted by the procedure of Folch, Lees, and Sloane Stanley (18). Lipids of the lower phase were concentrated to dryness, dissolved in a small volume of chloroform–methanol 2:1, filtered, and dried in a rotary evaporator. Lipids dissolved in chloroform were applied to a silicic acid column (Bio-Sil A from BioRad Labs. Richmond, CA). The elution diagram is shown in **Table 1.**

Thin-layer chromatography

Silica gel 60 precoated plates (E. Merck), 10 cm in height, were developed with chloroform–methanol 90:10. This solvent system was also used to develop 20 cm × 20 cm borate-impregnated plates made with a slurry containing 30 g of silicic acid G (E. Merck) suspended in an aqueous solution of 1% sodium tetraborate (19). Ceramides were made visible by spraying plates with a solution of 20% ammonium sulfate in 4% sulfuric acid and charring at 200°C for 30 min. Glycolipids were made visible by successive sprays of a 2% ethanolic solution of 1-naphthol and a 50% aqueous solution of concentrated sulfuric acid. Plates were heated 5 min at 120°C.

Isolation of the ceramide moiety of sphingomyelin

Phospholipids (fraction 6 from Bio-Sil A column) were submitted to a mild alkaline methanolysis (20). After neutralization and partition, the organic phase was dried under nitrogen. Sphingomyelin was enzymatically hydrolyzed by phospholipase C from *Clostridium welchii* (Worthing Biochemical Corp., Freehold, NJ) according to the method of Karlsson (21). Ceramides were extracted with chloroform from the incubation medium and purified by column chromatography. A column, 10 mm in diameter, containing 3 g of Bio-Sil A was eluted successively with 50 ml of chloroform and 50 ml of chloroform–methanol 97:3. The chloroform–methanol eluate contained ceramides, which were further analyzed by TLC.

TABLE 1. Elution diagram of a Bio-Sil A column

Eluate	Eluents	Volume	Eluted Lipids
		ml	
1	Chloroform	100	Neutral lipids
2	Chloroform	100	Unknown
3	Chloroform–methanol 97:3	100	Ceramides
4	Acetone	150	Glucosylceramide
5	Acetone–methanol 90:10	300	Other glycolipids
6	Methanol	300	Phospholipids

Solvent volumes are given for a column, 14 mm in diameter, containing 10 g of Bio-Sil A initially suspended in chloroform. This column was used to fractionate lipid extracts of villus intestinal cells isolated from 10 rats.

Fatty acid analysis

Ceramides were hydrolyzed in anhydrous methanol–HCl (0.75 N) at 85°C for 16 hr. Fatty acid methyl esters were extracted in *n*-hexane, dried, and dissolved in 1 ml of *n*-hexane–diethyl ether 95:5. The separation of nonhydroxylated and hydroxylated fatty acid methyl esters was accomplished by chromatography on a column, 5 mm in diameter, containing 0.5 g of Florisil. The sample was introduced onto the column. Elution with 6 ml of *n*-hexane–diethyl ether 95:5 yielded nonhydroxy fatty esters and further elution with 6 ml of ethyl acetate yielded hydroxy fatty esters. A rationale of the choice of column and solvents was described in detail elsewhere (22). Fatty acid methyl esters were analyzed by GLC according to previously published procedures (17).

LCB analysis

Ceramides were hydrolyzed in methanol–concentrated hydrochloric acid–water 83:8.6:9.4 at 70°C for 18 hr (23). For qualitative determinations long-chain bases were extracted and submitted to periodate oxidation as described by Sweeley and Moscatelli (24). Aldehydes were separated by GLC on a 6-ft column packed with 10% EGSS-X on Gas-Chrom P (Applied Science Laboratories, State College, PA). Analyses were performed on a gas chromatograph equipped with a double flame-ionization detector. Injector and detector temperatures were set at 200 and 250°C, respectively. Oven temperature was increased linearly from 150 to 220°C at a rate of 2°C/min. Base line was kept horizontal by using the detector in a "dual" mode with a similar column used as reference.

Quantitative determinations of long-chain bases were made by fluorescence measurements after reaction with fluorescamine (Fluram from F. Hoffman-La Roche and Co., Basel, Switzerland) (25).

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Mass spectrometry

Mass spectra were recorded on a high-resolution instrument MS 902 (AEI Ltd, Manchester, Great Britain). Twenty to forty μg of product were dissolved in 50 μl of hexamethyldisilazane–trimethylchlorosilane–pyridine 3:1:15 (by vol) in a conical 2-ml tube. Solvents and reagents were evaporated under a stream of dry nitrogen. Derivatives were dissolved in n-hexane and transferred onto the probe. Solvent was evaporated with hot air. Electron energy was 70 eV, acceleration voltage was 8 KV (6 KV for glucosylceramide), and ionization current was set at 100 μA (500 μA for fractions 3A, 6A, and 3E). The ion source temperature was 200°C for the analysis of ceramide and 250°C for the analysis of glucosylceramide. The probe temperature has been indicated for each spectrum. Mass spectra were recorded at the maximal total ionization current.

RESULTS

Purification of free ceramide

Intestinal cell glycolipids were previously isolated by chromatography of the acetylated total lipid extract on a Florisil column (15). Upon deacetylation and thin-layer chromatography, this fraction was found to contain free ceramide as evidenced by its chromatographic characteristics and a positive response of the hydrolysate with the reagent of primary amines, fluorescamine. When such an analytical procedure was adapted for preparative purposes, the free ceramide was isolated together with unknown nonpolar lipids.

In this work, lipid fractionation was accomplished by chromatography on a Bio-Sil A silicic acid column (Table 1). Neutral lipids were eluted with chloroform and ceramide was selectively eluted with chloroform–methanol 97:3. As shown in **Fig. 1,** most of the neutral lipids (lane 1) were eluted with chloroform (10 ml/g of Bio-Sil A). However, an additional equal volume of chloroform eluted fraction 2 which contained slowly moving products on TLC (lane 2). Fraction 2 had the same TLC characteristics as ceramide eluted in fraction 3 (Fig. 1, lane 4) but did not react with fluorescamine after hydrolysis. Fraction 3 contained long-chain bases and had a mobility similar to that of ceramide containing nonhydroxy fatty acids (Fig. 1, lane 3). Elution with acetone yielded fraction 4 containing chiefly glucosylceramide (Fig. 1, lane 6). Rat intestine contained 0.16 μmol of free ceramide and 0.39 μmol of glucosylceramide per ml of packed isolated cells.

In order to compare fraction 2 and fraction 3, both were chromatographed on borate-impregnated plates.

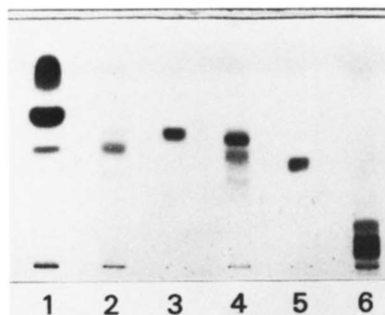

Fig. 1. TLC of rat intestinal lipids fractionated by chromatography on a Bio-Sil A column. Conditions of column chromatography are indicated in Table 1, and the following fraction numbers refer to eluate numbers in the table: fraction 1 (lane 1), fraction 2 (lane 2), nonhydroxy fatty acid ceramide standard (lane 3), fraction 3 (lane 4), hydroxy fatty acid ceramide standard (lane 5), fraction 4 (lane 6). The silica gel 60 precoated TLC plates were developed with chloroform–methanol 90:10.

Fraction 2, (**Fig. 2,** lane 1) gave two spots, neither of which had the same mobility as the two major spots of fraction 3 (Fig. 2, lane 2). The chromatographic pattern of fraction 3 was divided into five areas, A, B, C, D, and E according to the diagram on Fig. 2.

Amounts of ceramide contained in these areas were determined by assay of LCB with fluorescamine. A large proportion of the LCB content of fraction 3 was found in the two major spots (subfractions 3B and 3D) and only a small percentage of the total LCB was detected in subfractions 3A, 3C, and 3E (**Table 2**). For structural analyses (GLC and mass spectrometry) subfractions 3A, 3B, and 3E were prepared by TLC on borate-impregnated plates. Only subfraction 3B was further purified by an additional TLC on silica

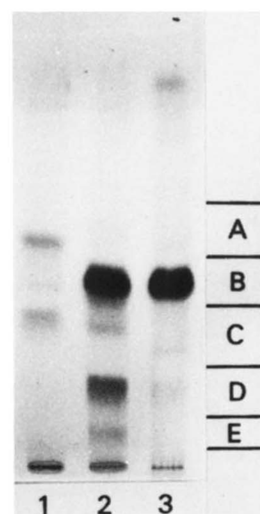

Fig. 2. Ceramide subfractionation on a borate-impregnated TLC plate. Lane 1, fraction 2 from Bio-Sil A column; lane 2, free ceramide (fraction 3); lane 3, ceramide from sphingomyelin (fraction 6).

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TABLE 2. LCB content of ceramide subfractions isolated by TLC on borate-impregnated plates

Subfractions	Fraction 3 Free Ceramide	Fraction 6 Sphingomyelin Ceramide
	LCB mol%	
A	4.5	6.4
B	45.2	77.4
C	5.4	4.3
D	37.9	10.7
E	6.9	1.2

Free ceramide and ceramide obtained from sphingomyelin by phospholipase C hydrolysis were purified by silicic acid column chromatography and subfractionated by TLC on borate-impregnated silica gel plates as indicated in Fig. 2.

gel 60 plates in order to remove remaining traces of a fraction 2-type contaminant.

Purification of ceramide from sphingomyelin

Rat intestine contained 0.76 μmol of sphingomyelin per ml of packed isolated cells. Ceramide was cleaved from sphingomyelin by phospholipase C and purified by chromatography on a Bio-Sil A column. Analysis of the product by TLC on borate-impregnated silica gel plates gave a major spot in the B area and two weak ones in the C and D areas (Fig. 2, lane 3). This finding was confirmed by quantitative determination of LCB (Table 2, fraction 6). Subfractions 6A, 6B, and 6D were prepared for structural analyses by TLC on borate-impregnated plates.

Characterization of long-chain bases

The LCB of free ceramides and glucosylceramide were identified by analysis of the aldehydes formed by periodate oxidation of the free bases. The chromatographic behavior of aldehydes derived from standard sphinganine, sphingosine, and 4*D*-hydroxysphinganine are shown on **Fig. 3*a*.** The free ceramide (fraction 3) yielded two major peaks of pentadecanal and hexadecenal corresponding to the parent bases 4*D*-hydroxysphinganine and sphingosine (Fig. 3*b*). A small peak of hexadecanal, coming from sphinganine, was also seen. Fraction 6 contained chiefly sphingosine which gave a peak of hexadecenal (Fig. 3*c*). The smallest peaks on both profiles were tentatively identified as tetradecanal (retention time 3.90 min), heptadecanal (retention time 8.70 min), and heptadecenal (retention time 13.14 min). Glucosylceramide gave a major peak of pentadecanal corresponding to the parent base 4*D*-hydroxysphinganine (Fig. 3*d*). Smaller peaks of hexadecanal and hexadecenal accounted respectively for 1.8 and 6.5% of the total peak area. A peak that was tentatively identified as heptadecanal accounted for 4.7% of the total peak area.

Ceramide subfractions that were obtained by chromatography on borate-impregnated silica gel plates gave simple profiles of aldehydes (**Fig. 4**). Subfractions 3A and 6A gave a major aldehyde peak of hexadecanal, indicating that sphinganine-containing ceramide was the major parent structure. Occurrence of hexadecenal on the profiles was explained by the proximity on the plates of the much more important subfraction B. Subfractions 3B and 6B were sphingosine-containing ceramides, and subfractions 3D and 6D were 4*D*-hydroxysphinganine-containing ceramides. Subfraction 3E gave the same aldehyde profile as subfraction 3D. The subfractions C were considered as a tailing portion of subfractions B because their LCB content was low and gave aldehyde profiles like those of the subfractions B.

These results (Table 2, Figs. 3 and 4) demonstrated that, in mature rat intestinal cells, sphingosine and 4*D*-hydroxysphinganine were the major LCB in sphingolipids. In free ceramides, they occurred in nearly equal proportions. In sphingomyelin, sphingosine was the major LCB (77.4% of LCB). A reverse

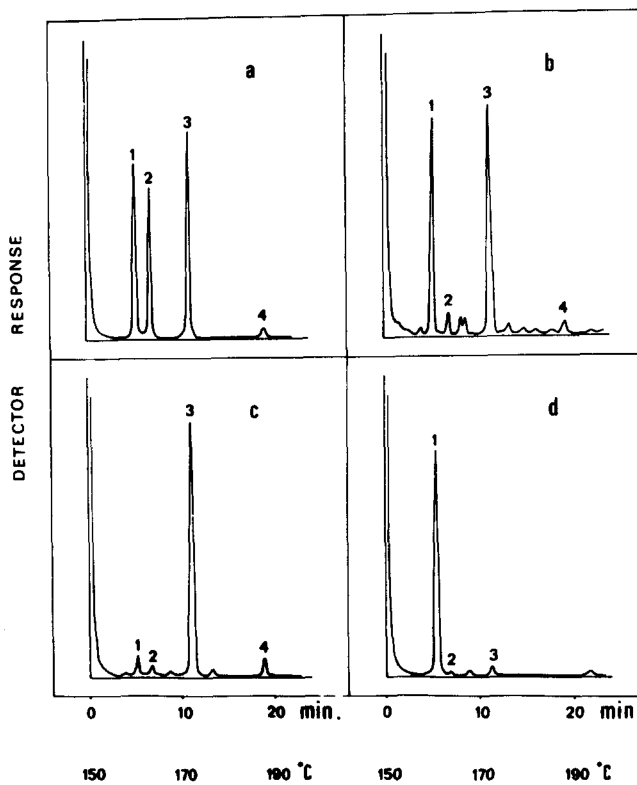

Fig. 3. Gas–liquid chromatographic analyses of aldehydes derived from LCB. *a*) Standard LCB: 4*D*-hydroxysphinganine (*1*), sphinganine (*2*), sphingosine (*3*), and 3-*O*-methylsphingosine (*4*). *b*) Free ceramide (fraction 3 from Bio-Sil A column). *c*) Sphingomyelin ceramide (fraction 6 from Bio-Sil A column). *d*) Glucosylceramide. Aldehydes were analyzed with temperature programming at 2°C/min from 150 to 220°C.

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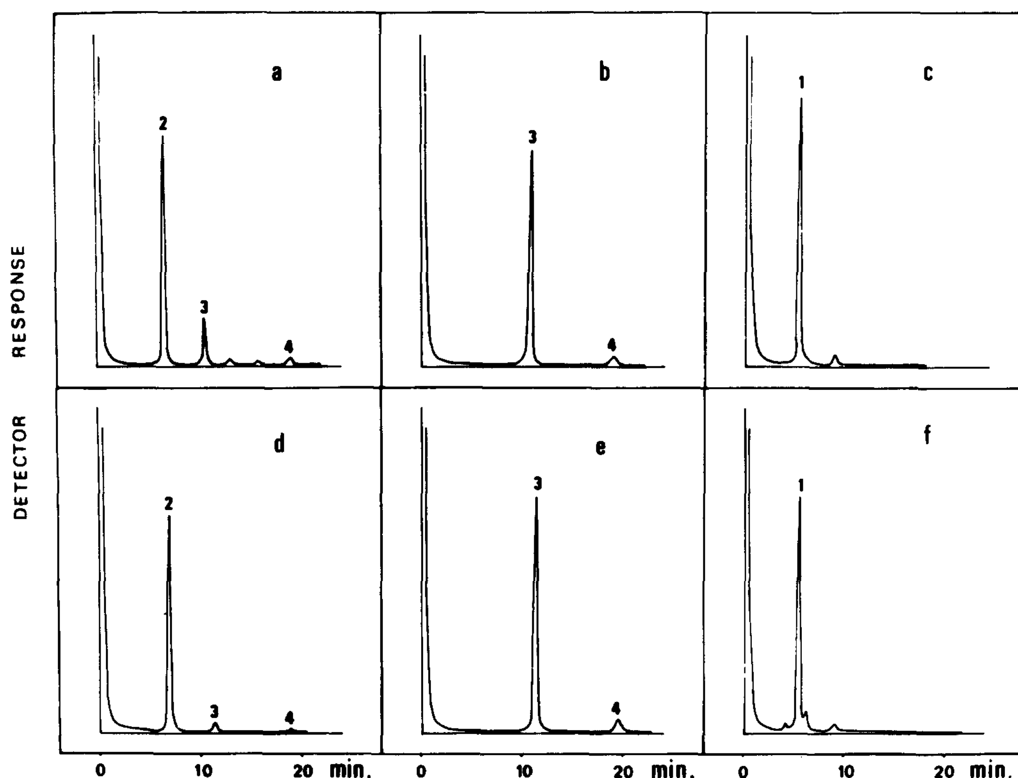

Fig. 4. Gas–liquid chromatographic analyses of aldehydes derived from LCB of intestinal ceramide subfractions. Free ceramides (fraction 3) and sphingomyelin (fraction 6) were obtained by Bio-Sil A column chromatography according to Table 1. Ceramides were subfractionated on borate-impregnated TLC plates according to the scheme in Fig. 2. Aldehydes from free ceramide subfractions 3A (*a*), 3B (*b*), 3D (*c*) and from sphingomyelin ceramide subfractions 6A (*d*), 6B (*e*), and 6D (*f*). Chromatographic conditions and peak identifications were as in Fig. 3.

proportion was found in glucosylceramide where 4*D*-hydroxysphinganine was about 6 times more abundant than sphingosine. A low level of sphinganine was also found in the three products.

Fatty acid analysis

In rat intestinal glucosylceramide, fatty acids had 16, 20, 22, 23, and 24 carbon atoms, and 70% of them were hydroxylated (17). All the free ceramide subfractions but one, subfraction 3E, contained only normal fatty acids. Hydroxy fatty acid content of subfraction 3E had a chain length distribution closely related to that of glucosylceramide: 24:0 (43%), 24:1 (15%), 23:0 (13%), 22:0 (20%), and 20:0 (9%). In sphingomyelin ceramide no hydroxylated fatty acids were found by GLC analysis.

Long-chain fatty acids (arachidic to lignoceric) were found in higher proportion in free ceramide than in sphingomyelin (**Table 3**). The subfractions A had more long-chain fatty acids than the subfractions B and less than the subfractions D in free ceramide as well as in sphingomyelin. In all subfractions but one, subfraction D, palmitic acid was the major fatty acid

TABLE 3. Fatty acid composition of free ceramide and sphingomyelin ceramide subfractions

Fatty Acids	Free Ceramide			Sphingomyelin Ceramide		
	3A	3B	3D	6A	6B	6D
	%	%	%	%	%	%
n16:0	35.0	71.5	13.7	61.0	82.4	42.9
n16:1	5.3	0.5	2.7	4.0	0.7	6.0
n18:0	10.0	5.8	2.7	10.2	4.6	16.1
n18:1	14.0	1.0	4.0	6.2	1.4	5.7
n18:2	2.3		1.2		0.9	
n20:0	5.0	6.8	10.1	4.9	4.5	6.5
n21:0	0.1	0.2	0.3	2.2	1.0	0.5
n22:0	7.1	5.6	15.3	5.1	2.3	6.6
n23:0	2.5	1.2	5.7	1.4	0.3	1.1
n24:0	13.7	6.7	30.2	2.4	1.4	7.3
n24:1	5.0	0.7	8.7	2.2	0.5	4.5
n25:0			1.3			
n25:1			1.2			1.7
n26:0			2.3			
n26:1			1.3			1.2

Subfractions A contained sphinganine as LCB, subfractions B contained sphingosine, and subfractions D contained 4D-hydroxysphinganine (Fig. 4). Results are expressed as percentage by weight of fatty acid methyl esters determined by GLC.

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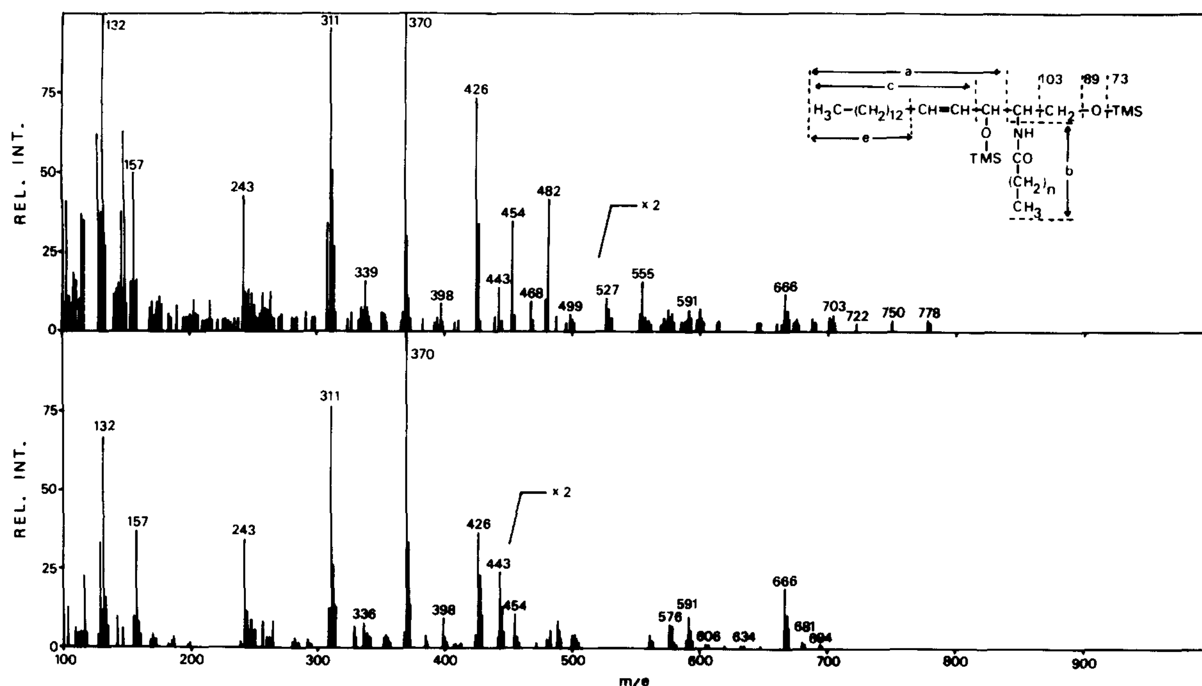

Fig. 5. Mass spectra of *N*-acylsphingosines. Upper part, subfraction 3B (probe temperature 190°C). Lower part, subfraction 6B (probe temperature 200°C). Molecular weights for TMS derivatives of *N*-palmitoylsphingosine and *N*-lignocerylsphingosine are, respectively, 681 and 793. Major peaks on the spectra came from ceramide fragments M − 15, M − 73, M − 103 and from fatty acid fragments M − a, M − a + 73. Peaks at *m/e* 311, 426, and 243 came from sphingosine fragments: a, M − b − 1, and M − b − 1 − e, respectively.

component. In subfraction 3D, 4*D*-hydroxysphinganine-containing free ceramide, lignoceric acid accounted for 30% of the fatty acid weight.

Mass spectrometry of sphingosine-containing ceramides

Major cleavages of *N*-acylsphingosine are indicated in the upper right corner of **Fig. 5.** Mass spectra of subfractions 3B and 6B were interpreted according to Samuelsson and Samuelsson (26). The sphingosine fragments were easily recognized by intense peaks at *m/e* 243, 311, and 426. Peaks originating from *N*-palmitoylsphingosine fragmentation were major on both spectra. A molecular ion of low intensity was seen at *m/e* 681 on the 6B spectrum. Ceramide fragments gave peaks at *m/e* (M − 15), (M − 90), and (M − 103) corresponding, respectively, to the molecular weight minus a methyl group, a trimethylsilanol, or the terminal group $CH_2{=}O{-}Si(CH_3)_3$. The ion at *m/e* 666, (M − 15) for *N*-palmitoylsphingosine was predominant in the high mass range. The major peak was seen for the ion at *m/e* 370 (M − a).

Differences between spectra of Fig. 5 were related to the observed differences in the fatty acid compositions of subfractions 3B and 6B. Occurrence of C_{24}, C_{23}, C_{22}, and C_{20} fatty acids in subfraction 3B was confirmed by related peaks of the ions at *m/e* (M − 15) and (M − a) on the spectrum.

Mass spectrometry of sphinganine-containing ceramide

The subfractions A have been characterized as *N*-acylsphinganines by analyses of their fatty acid and LCB components. Mass spectra of intact molecules were obtained (**Fig. 6**). Cleavage between C-2 and C-3 gave rise to an intense peak at *m/e* 313. Complementary fragments (M − a), (M − a − 89), and (M − a + 73) were found at *m/e* 370, 281, and 443 for the dominant molecular species, *N*-palmitoylsphinganine. Ceramide fragments of the same molecule were detected at *m/e* 668 (M − 15), 593 (M − 90), and 580 (M − 103).

Ions at *m/e* 311 (M − 372), 328 (M − 355), and 353 (M − 330) were mentioned as characteristic of sphinganine derivatives by Samuelsson and Samuelsson (26) although the mechanisms of formation of these ions were not elucidated. The peak observed at *m/e* 426 and part of the peak at *m/e* 311 were likely to come from a contamination by *N*-acylsphingosine. The ions coming from long-chain fatty acid fragments and related ceramides were found on the mass spectrum of subfraction 3A as expected by the GLC analysis of fatty acids. However mass spectrometry did not confirm the presence of *N*-oleylsphinganine in the proportion found by GLC analysis; part of this oleic acid might have come from a contamination by residual fraction 2 products. Mass spectrometry of subfraction 6A yielded chiefly ions derived from the fragmenta-

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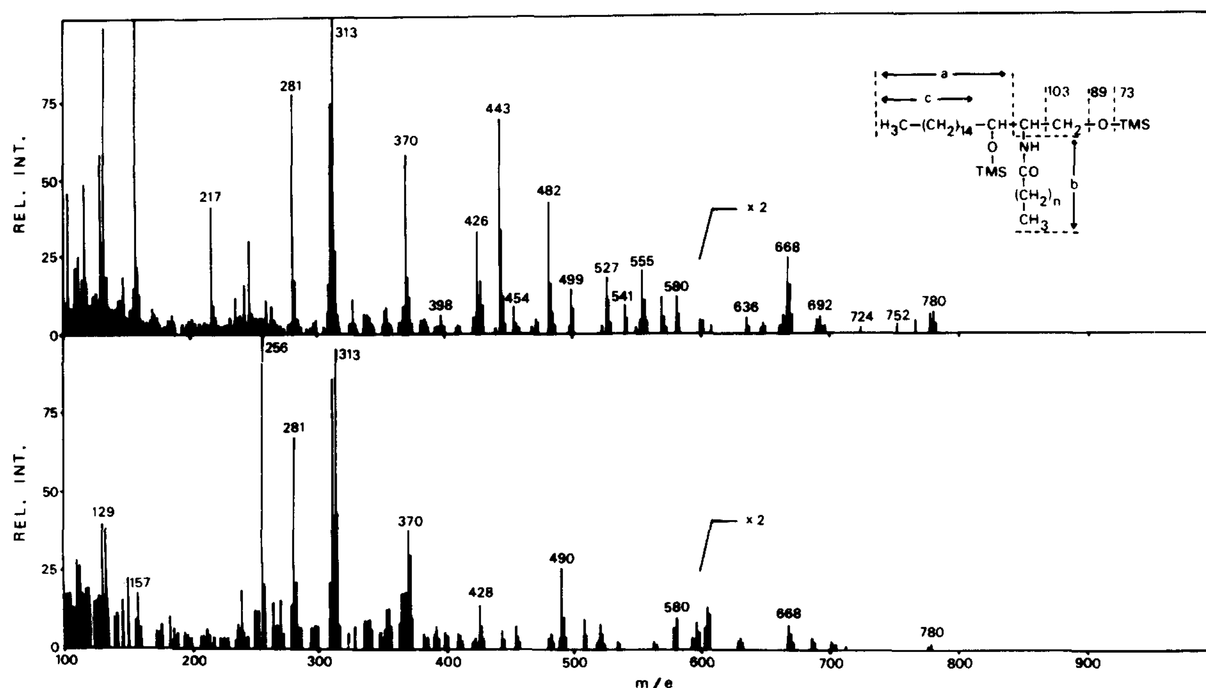

Fig. 6. Mass spectra of *N*-acylsphinganines. Upper part, subfraction 3A (probe temperature 210°C). Lower part, subfraction 6A (probe temperature 220°C). M for the TMS derivative of the predominant species, *N*-palmitoylsphinganine, is 683. Fragmentations were the same as those indicated under Fig. 5. Peaks at *m/e* 313, 428, and 217 originated from the characteristic fragments of sphinganine: a, M − b − 1, and M − b − 1 − c, respectively.

tion of *N*-palmitoylsphinganine. However the origin of intense peaks at *m/e* 256 and 490 remained unclear.

Mass spectrometry of 4*D*-hydroxysphinganine-containing ceramide

Trihydroxy bases undergo similar fragmentations as dihydroxy bases but additional cleavage occurs between carbons C-3 and C-4 bearing hydroxyl groups substituted with trimethylsilane (27). This cleavage gave rise to a prominent peak (fragment c) at *m/e* 299 (**Fig. 7**) and to complementary peaks (M − c). Other typical ions of 4*D*-hydroxysphinganine were found at *m/e* 218 (M − b − c) and 401 (fragment a). Besides fragments (M − a) and (M − c), nonhydroxy fatty acids, when associated with trihydroxy bases, gave stable ions at (b + 1 + 73) and (b + 2). These ions derived from C_{20}, C_{22}, C_{23}, and C_{24} fatty acids gave intense peaks on the spectrum of subfraction 3D and those derived from C_{16} fatty acid were major on the spectrum of subfraction 6D.

Predominant molecular weight ions were observed at *m/e* (M − 15) and at *m/e* (M − 103 − 90). This latter ion was formed by cleavage of a trimethylsilanol in addition to the elimination of the terminal group $CH_2{=}O{-}Si(CH_3)_3$. Major peaks at *m/e* 868 (M − 15), 780 (M − 90), 793 (M − 103), and 690 (M − 103 − 90) were seen on the spectrum of subfraction 3D, accounting for the large contribution of lignoceric acid. Peaks at *m/e* (M − 15) and (M − 103 − 90) were also clearly visible for C_{25}, C_{26}, and C_{27} fatty acid-containing ceramide, but they were more intense than one would expect from the GLC analysis of fatty acids. A distillation effect in the source was likely to be the origin of these oversized peaks. In the subfraction 6D of sphingomyelin ceramide, major molecular weight ions were found at *m/e* 756 and 578, (M − 15) and (M − 103 − 90) fragments of palmitic acid-containing ceramide.

Molecular weight fragments (M − 103 − 90) of ceramide containing 4*D*-hydroxysphinganine and C_{20}, C_{22}, C_{23}, C_{24}, and $C_{24:1}$ hydroxy fatty acids were obvious on the mass spectrum of ceramide subfraction 3E, (**Fig. 8**). In addition to previously described fragmentations of ceramides, 2-hydroxy fatty acids gave rise to a fragment f (28). Some important ions appearing in the middle range of mass *m/e* 368, 384, 412, and 439 have not been characterized. Contamination of this subfraction cannot be ruled out. Material from subfraction 6E was too scarce to make a mass spectrum.

Mass spectrometry of glucosylceramide

The ceramide part of glucosylceramide undergoes the same fragmentation and rearrangements as free ceramide molecules. Typical ions were found at *m/e* 299, 311, or 313 whether the LCB was 4*D*-hydroxysphinganine, sphingosine, or sphinganine. The glucose

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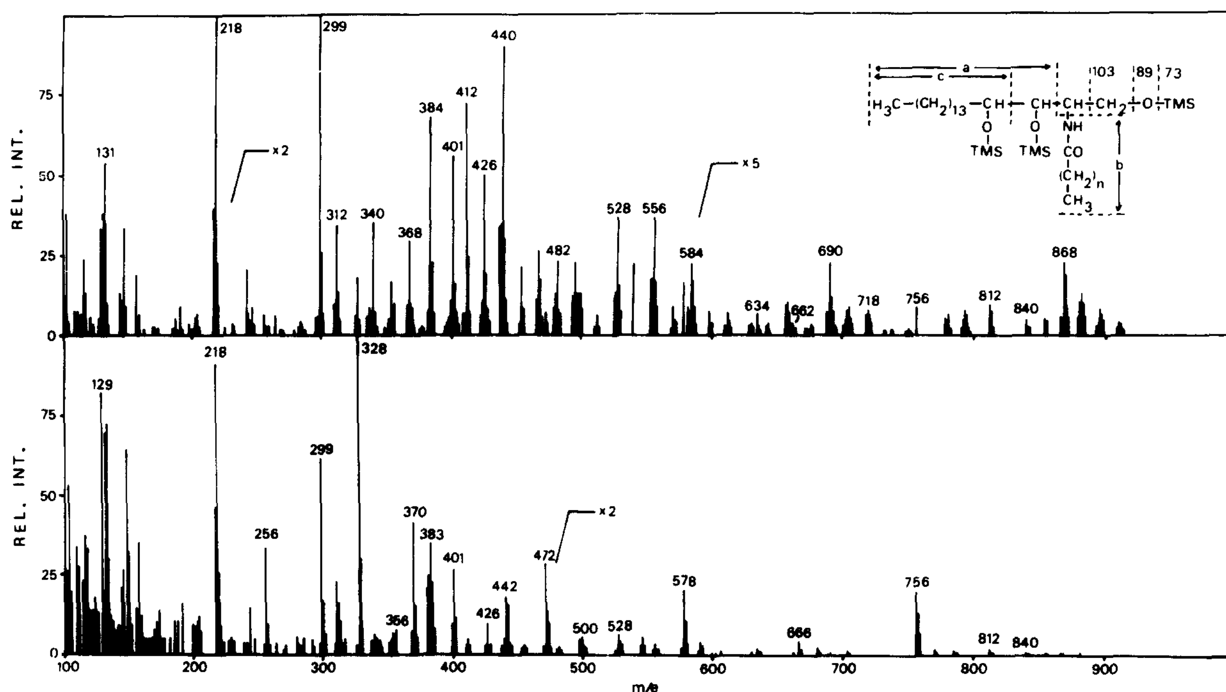

Fig. 7. Mass spectra of ceramides containing 4*D*-hydroxysphinganine and nonhydroxy fatty acids. Upper part, subfraction 3D (probe temperature 260°C). Lower part, subfraction 6D (probe temperature 240°C). Molecular weights for TMS derivatives of *N*-palmitoyl 4*D*-hydroxysphinganine and of *N*-lignoceryl 4*D*-hydroxysphinganine are 771 and 883, respectively. Major peaks on the spectra originated from ceramide fragments M − 15, M − 103 − 90 and from fatty acid fragments M − a, M − c, b + 1 + 73, and b + 2. Peaks at *m/e* 218, 299, and 401 came from 4*D*-hydroxysphinganine fragments M − b − c, c, and a, respectively.

moiety was cleaved and gave rise to ions with one or two lacking TMS groups at *m/e* 361 and 271 (29, 30). Although the mass spectrum of rat intestinal glucosylceramide was complex (**Fig. 9**), most of the ions were identifiable.

The peak at *m/e* 299 (fragment c of 4*D*-hydroxysphinganine) was much higher than the peak at *m/e* 311 (fragment a of spingosine). Major peaks were derived from 4*D*-hydroxysphinganine associated with C_{20} to C_{24} normal or hydroxy fatty acids. C_{16} hydroxy acid associated with sphingosine was also detected by ions at *m/e* 458 (M − a − b + 73), 680 (M − a − 16), and 798 (M − a + 102); this latter ion was also interpreted as (M − d − 16) derived from glucosylceramide containing C_{16} hydroxy fatty acid and 4*D*-hydroxysphinganine.

DISCUSSION

Free ceramide was isolated from a lipid extract of rat intestinal cells. This fraction was shown to be hetero-

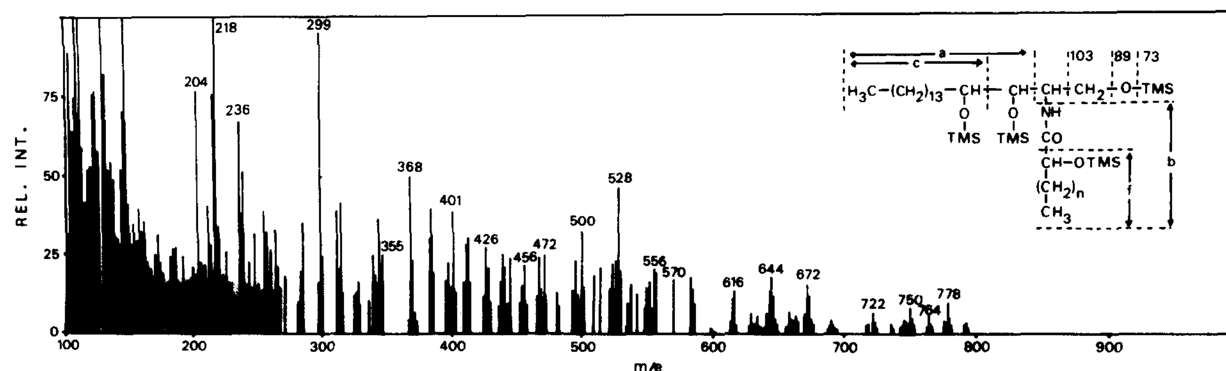

Fig. 8. Mass spectrum of ceramides containing 4*D*-hydroxysphinganine and hydroxy fatty acids, subfraction 3E (probe temperature 260°C). Molecular weights of TMS derivatives are 915 for the hydroxy C_{20} and 971 for the hydroxy C_{24} fatty acid containing ceramides. In addition to the fragmentations already indicated under Fig. 7, fragments f coming from hydroxy fatty acids gave rise to peaks of intermediate intensity.

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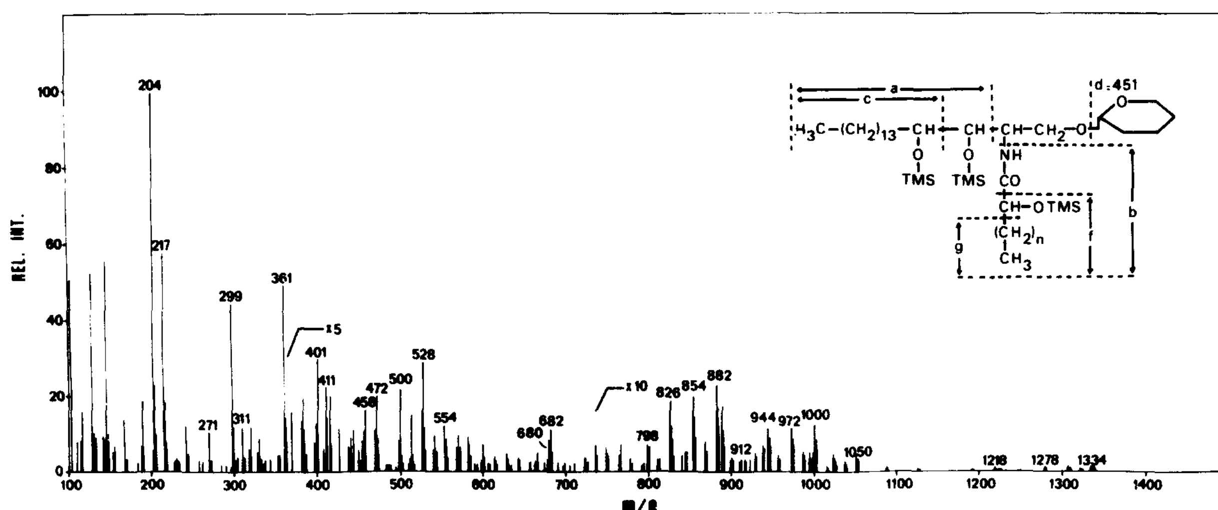

Fig. 9. Mass spectrum of glucosylceramide. The spectrum was recorded when the probe temperature had reached 270°C. Intense peaks were observed for the following fragments: M − 15, M − d + 102, M − d − 16, M − d − a + 73, M − d − a + 31, M − d − a + 1 and M − c. The major fatty acids, hydroxy and nonhydroxy C_{16}, C_{20}, C_{22}, C_{23}, C_{24}, and $C_{24:1}$, were associated with 4*D*-hydroxysphinganine (ref. 17). M for the TMS derivative of glucosylceramide containing hydroxy C_{24} fatty acid and 4*D*-hydroxysphinganine is 1349.

geneous by chromatography on borate-impregnated thin-layer plates. It was composed of two major ceramide species, *N*-acylsphingosine and *N*-acyl-4*D*-hydroxysphinganine, both devoid of hydroxy fatty acids. Minor molecular species were identified as ceramide containing sphinganine and nonhydroxy fatty acids, and as ceramide containing 4*D*-hydroxysphinganine and hydroxy fatty acids.

These results were established by analyses of the ceramide components, fatty acids, and LCB. However the presence of other lipids could have influenced the fatty acid distribution determined by GLC of the hydrolyzed products. Furthermore the same aldehydes could be derived from different LCB. The direct-inlet mass spectrometry gave unambiguous answers to these problems.

The occurrence of 4*D*-hydroxysphinganine in rat intestinal ceramide was already suggested by Crossman and Hirschberg (31) who found a small amount of label on this LCB in ceramide and sphingomyelin after in vivo injection of tritiated sphinganine.

The nature and the distribution of the different ceramide species of rat small intestine (Table 2) are remarkably similar to those of equine kidney (9) in all respects but one: *N*-hydroxyacylsphingosine accounted for 4.4% of the total free ceramide in equine kidney but was undetectable in rat intestine.

In order to compare the structure of free ceramide with its most closely related sphingolipids, the molecular species of ceramide obtained from sphingomyelin by phospholipase C hydrolysis were investigated by the same methods. *N*-Acylsphingosine containing mainly palmitic acid was the major ceramide species of rat intestinal sphingomyelin. A few percent of *N*-acylsphinganine and *N*-acyl-4*D*-hydroxysphinganine were also found. Contrary to a previous report (32) the occurrence of the C_{19} homologue of sphinganine was not quantitatively important.

Hydroxy fatty acids were detected by GLC analysis in only one sphingomyelin ceramide subfraction, subfraction 6E, which was not analyzed due to its scarcity but was assumed to contain *N*-hydroxyacyl-4*D*-hydroxysphinganine. Its contribution to the total ceramide of rat intestinal sphingomyelin was small (1.2% on a molar basis) and in the same range as in bovine intestinal sphingomyelin (33, 34).

The structure of glucosylceramide was investigated in a previous study together with other major glycolipids of rat intestine (17). The present results confirmed the major contribution of the LCB 4*D*-hydroxysphinganine associated with hydroxy fatty acids. Such a ceramide backbone was already found in bovine (13, 33), rat and rabbit (35), and dog and human (36) intestine. Mass spectrometry provided evidence for a minor ceramide constituted of sphingosine and hydroxy palmitic acid.

Free ceramides are components of the lipid core of intestinal plasma membranes (37) and they are precursors in the biosynthesis of all sphingolipids (1). Distribution of free ceramide molecular species in rat intestinal cells appeared to reflect their branch-point position in the pathway of biosynthesis leading either to glucosylceramide or to sphingomyelin. Ceramide containing sphingosine and palmitic acid is likely to be the main precursor in the synthesis of intestinal sphingomyelin. However other LCB and fatty acids were

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found to occur in sphingomyelin, reflecting the heterogeneity of the precursor.

Ceramide containing 4*D*-hydroxysphinganine resembled the ceramide part of glycolipids with respect to the high occurrence of long-chain fatty acids but differed in the degree of hydroxylation of these fatty acids. In the free ceramide, only 6.9% of the fatty acids were hydroxylated. This was considerably less than the 30–70% range of hydroxylation occurring in the fatty acids of glycolipids (17). It has also been shown in vitro that hydroxy fatty acid-containing ceramides were better substrates for the synthesis of glucosylceramide than nonhydroxy fatty acid ceramides (15). These findings might indicate that the synthesis of hydroxy fatty acid-containing ceramide proceeds at a slower rate than its glycosylation. Whether the intestinal ceramides are synthesized de novo or are largely submitted to fatty acid replacement as has been observed in the developing rat brain (5) is unknown. If this latter mechanism took place in rat intestinal mucosa, ceramide glycosylation would occur at a faster rate than replacement of nonhydroxy by hydroxy fatty acids. This process would prevent accumulation of the best precursor for glucosylceramide synthesis, *N*-hydroxyacyl-4*D*-hydroxysphinganine, and would allow *N*-acyl-4*D*-hydroxysphinganine to accumulate.

Ceramides of bovine kidney were contaminated by *N*-acylethanolamine (7). In the rat small intestine, two lipids had similar mobility as ceramide upon TLC but were eluted earlier by the Bio-Sil A column chromatography. These lipids yielded mainly C_{16} and $C_{18:1}$ methyl esters when methanolyzed. Elucidation of their structures is under current investigation.

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