Glycolipids of peripheral nerve: isolation and characterization of glycolipids from rabbit sciatic nerve

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Abstract Besides cerebroside and sulfatide four other glycolipids were isolated from rabbit sciatic nerve and analyzed by chemical and chromatographic methods. Three of the glycolipids were shown to be fatty acid esters of cerebroside; the fourth was characterized as diacyl glycerol galactoside and its alkyl ether analog. In the ester linkage mainly unsubstituted acids with chain length C_{16} to C_{18} were present. Both hydroxy and unsubstituted acids were found in amide linkage. They varied in chain length from C_{16} to C_{24} and were typical of cerebrosides. The long-chain base fraction contained sphingosine and dihydrosphingosine as the main components.

Supplementary key words fatty acid esters of cerebroside · diacyl glycerol galactoside · alkylacyl glycerol galactoside · cerebroside · sulfatide · fatty acids · hydroxy acids · sphingosine bases · glycerol ethers

THERE HAVE BEEN several recent reports on the lipid composition of peripheral nerves (1–5). Although the phospholipids have been investigated in detail, the glycolipids have been investigated only as a group. The main glycolipids of peripheral nerve are cerebrosides and sulfatides, and the presence and identification of diacyl glycerol galactoside has also been described (3, 6). During the course of our studies on peripheral nerve we observed this latter constituent to represent one of a group of four glycolipid compounds, all of which have a polarity lower than cerebrosides. This communication describes the occurrence, isolation, and characterization of the four glycolipid components from rabbit peripheral nerves, viz., three fatty acid esters of cerebrosides and diacyl glycerol galactoside (monogalactosyl diglyceride) and its alkyl ether analog.

MATERIALS

Sciatic nerves from mature rabbits (1–3 yr old), obtained from Pel-Freez Bio-Animals, Rogers, Ark., were shipped frozen and kept frozen until extracted. Cerebrosides, sulfatides, diacyl glycerol galactoside, and sphingosine were purchased from Applied Science Laboratories, State College, Pa. Phytosphingosine was obtained from Calbiochem, San Diego, Calif. Fatty acid methyl esters were from the Hormel Institute, Austin, Minn., and Applied Science Laboratories; hydroxy fatty acids were from Analabs, North Haven, Conn., and Applied Science Laboratories. Long-chain aldehydes and alkyl glycerols were obtained from Analabs. All other chemicals and solvents were of the highest purity available commercially. All solvents were distilled before use. Precoated stationary phases for GLC were obtained from Applied Science Laboratories or Supelco, Bellefonte, Pa. Sil-Prep, for preparing TMS derivatives of hydroxy acids, was obtained from Applied Science Laboratories; it contained hexamethyldisilazane–trimethylchlorosilane–pyridine 3:1:9 (v/v/v). Unisil for column chromatography was obtained from Clarkson Chemical Co., Williamsport, Pa.

METHODS

Isolation of glycolipids

The rabbit sciatic nerves (avg wt 330–420 mg in lots of 25–200) were cut into small pieces and homogenized in

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl; glycolipid A, fatty acid ester of cerebroside with only unsubstituted acid in amide linkage; glycolipids B and C, fatty acid ester of cerebroside with both hydroxy and unsubstituted acids in amide linkage; glycolipid D, diacyl glycerol galactoside and its alkyl ether analog.

20 vol of CHCl₃–CH₃OH 2:1 (v/v) with a VirTis homogenizer fitted with a Turbo-Shear tissue cutting blade assembly (VirTis Co., cat. no. 16-318). Since these tissues are difficult to grind and much heat is generated, the flask was well cooled during homogenization. After 2–4 hr the homogenate was filtered and the residue was re-extracted with CHCl₃–CH₃OH 2:1 (v/v) for 1 hr. This mixture was filtered and the residue was again extracted with CHCl₃–CH₃OH 1:1 (v/v). The extracts were combined and sufficient CHCl₃ was added to make the volume 2:1 with CHCl₃–CH₃OH. To remove nonlipid impurities the total lipid extract was washed by the procedure of Folch, Lees, and Sloane Stanley (7), using 0.2 vol of 0.74% KCl. The lower CHCl₃ phase was further washed with "Folch upper phase," consisting of CHCl₃–CH₃OH–0.74% KCl 3:48:47. The lower CHCl₃ layer containing the lipids was evaporated and redissolved in 50 ml of CHCl₃. The total lipid was estimated gravimetrically on an aliquot using a Cahn Gram Electrobalance (Ventron Corp., Paramount, Calif.).

A glass column, 4 cm i.d., packed to a height of 10 cm with a slurry of Unisil (60 g, Clarkson Chemical Co.) in CHCl₃ was used to fractionate the lipids. The lipid (1.5 g) dissolved in CHCl₃ was applied to the column and eluted with the following solvents. Neutral lipids were eluted with 1.2 l of CHCl₃ and 250 ml of chloroform–acetone 19:1 (v/v). Acyl cerebrosides and diacyl glycerol galactoside (minor glycolipids) were obtained by elution with 1 l of chloroform–acetone 3:1 (v/v). Cerebrosides were then eluted with 1.5 l of chloroform–acetone 1:1 (v/v) and sulfatides with 1.5 l of acetone. Finally, the phospholipids were eluted with 1.2 l of methanol. This is a slight modification of the procedure of Rumsby (8). The fractions were evaporated at 45°C and made up to a given volume, and the amount of lipid was estimated gravimetrically from an aliquot. The fractions obtained from column chromatography were analyzed for phosphorous (9) and carbohydrate. Carbohydrate was determined colorimetrically with phenol-sulfuric acid (10) after hydrolysis of the lipid with 3 N H₂SO₄ (11). The components of each fraction were monitored by TLC using the solvent system described below.

TLC was done on plates coated with silica gel H 500 μ thick, activated at 120°C. All plates were prewashed with CHCl₃–CH₃OH 1:1 (v/v) and reactivated before use. Chromatography was performed at room temperature in chambers lined with filter paper. The following solvent systems were used: (a) CHCl₃–CH₃OH 90:10 for "minor" glycolipids; (b) hexane–ether–acetic acid 90:10:1 for less polar lipids; (c) CHCl₃–CH₃OH–H₂O 65:25:3 for one-dimensional analysis of polar lipids; (d) for two-dimensional separation of polar lipids, first dimension, CHCl₃–CH₃OH–14 N NH₄OH 65:35:5, and after dry-

ing for 10 min under N₂, chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10 for the second dimension. The various compounds were made visible as described by Singh and Privett (12).

Individual glycolipids were isolated from the crude "minor" glycolipid fraction III (see above and Table 1) by TLC on plates of silica gel H 500 μ thick, developed in CHCl₃–CH₃OH 90:10. The weight of esters applied to each 20-cm-wide plate was approximately 20 mg. The components on TLC plates, after drying under N₂, were made visible by spraying with CH₃OH–H₂O 1:1. The plates were dried under N₂ and the individual bands were scraped off separately and extracted with CHCl₃–CH₃OH–H₂O 7:7:1 (3 × 10 ml). To the combined extracts, water (10 ml) was added to give a biphasic system and the solution was centrifuged. The lipid was recovered from the lower CHCl₃ phase (13). If necessary, the preparations could be further purified by the same procedure. Finally, the purified lipid, which chromatographed as a single component on TLC, was stored in CHCl₃–CH₃OH 2:1 at 4°C. Cerebrosides and sulfatides were isolated from fractions after column chromatography by preparative TLC in chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10 by methods similar to those described above.

Alkaline methanolysis

To 1 mg of the dry lipid sample, 0.5 ml of CHCl₃–0.2 N methanolic KOH 2:1 (v/v) was added and the solution was mixed on a Vortex mixer for 15 min at room temperature. Water (0.1 ml) was added and the solution was centrifuged; the lower CHCl₃ layer was washed by "Folch upper phase." The CHCl₃ phase was then analyzed by TLC in hexane–ether–acetic acid 90:10:1 and chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10. For further analysis of methyl esters (by GLC) and the glycolipid formed, the components were separated by preparative TLC in hexane–ether–acetic acid 90:10:1. The methyl esters were extracted from silica gel with hexane and the glycolipids with CHCl₃–CH₃OH–H₂O 7:7:1 as described above.

Acid hydrolysis of sphingolipids and isolation of sphingosine bases

For acid hydrolysis and sphingosine base analysis, 1-mg samples of sphingolipids were subjected to methanolysis in 1 ml of 1 N HCl–CH₃OH (10 M in H₂O) at 75–80°C for 16 hr, as described by Carter and Hirschberg (14). The hydrolysate was taken to dryness under N₂, 1.8 ml of CH₃OH and 0.2 ml of H₂O were added, and the fatty acid methyl esters were extracted with hexane (3 × 3 ml). The lower phase containing the sugars and long-chain base(s) was taken to dryness under N₂, and 0.5 ml of aqueous 5 N NaOH and 1.5 ml of H₂O were added to

the residue. The free long-chain bases were extracted with 4 ml of ether, the sugar remaining in the lower phase. The sphingosine bases were analyzed by TLC on silica gel H in CHCl₃–CH₃OH–H₂O 100:42:6 and CHCl₃–CH₃OH–2 N NH₄OH 100:25:2.5. Periodate oxidation of long-chain bases was performed as described by Sweeley and Moscatelli (15). The long-chain bases extracted from 1 mg of sphingolipid were taken to dryness under N_2 and dissolved in CHCl₃–CH₃OH 1:1 (v/v); 0.1 ml of freshly prepared 0.2 M sodium metaperiodate was added and the solution was left in the dark for 90 min at room temperature. 1.0 ml of CH₃OH–H₂O 1:1 (v/v) and 1.3 ml of chloroform were then added. The lower phase, containing the aldehydes, was concentrated to a small volume under N_2 and analyzed immediately by GLC on two columns: (a) 15% EGSS-X on Gas-Chrom P (100–120 mesh) at 195 and 175°C; and (b) 3% GC grade SE-30 on Gas-Chrom P (100–120 mesh) at 155°C. The peaks were identified by comparison of retention times with standard aldehydes or aldehydes obtained after oxidation of known sphingosine bases.

Preparation of methyl esters of glycolipids

For fatty acid analysis, lipid samples were interesterified with 14% BF₃ in CH₃OH at 100°C for 90 min (16). Methyl esters were then extracted with ether (3 × 3 ml). Samples containing hydroxy and unsubstituted acids were separated by TLC, using benzene as the solvent. After spraying the plates with a solution of 0.5% rhodamine 6G and visualization of the lipids under UV light, the methyl esters of unsubstituted and hydroxy acids were scraped separately and extracted from silica gel with ether (3 × 3 ml). Unsubstituted acids were taken to dryness under N_2 and dissolved in hexane and analyzed by GLC. Hydroxy acids were converted to TMS derivatives before analysis by GLC. TMS derivatives were prepared at room temperature for 30 min with Sil-Prep (0.5 ml) added to the dry hydroxy acid ester. The solvent was removed under a stream of N_2 and the TMS derivative was dissolved in carbon disulfide. GLC of methyl esters of unsubstituted acids and TMS derivatives of hydroxy acids was done on a column of 15% EGSS-X on Gas-Chrom P (100–120 mesh) at 195°C. The peaks were identified by comparison with standard fatty acid methyl esters or by equivalent chain length. The linearity of the detector response was checked by NIH mixtures D and F.

Analysis of carbohydrate moieties of glycolipids

Glycolipid (0.5–1 mg) was dissolved in 2 ml of freshly prepared, dry 0.5 M (approx.) HCl in CH₃OH and was subjected to methanolysis at 80°C for 24 hr (17). After methanolysis the solutions were cooled to 40°C and concentrated under N_2 to 0.5 ml. The fatty acid methyl

esters liberated during the methanolysis were extracted with hexane (0.5 ml × 3). The methanolic solution was taken to dryness under N_2 in a Reacti-Vial (Pierce Chemical Co., Rockford, Ill.) and dried over solid KOH for 1 hr in a vacuum desiccator. To obtain trimethylsilyl derivatives of sugars for analysis by GLC, the dry residues were treated with 100 μl of Sil-Prep. After 1 hr at room temperature, the solvent was removed under N_2 and the residue was dissolved in a small volume of hexane. The solution was centrifuged and the supernatant was injected (1–4-μl aliquots) into a gas chromatograph. The peaks were identified by comparison of the retention times with standard glucose, galactose, cerebroside, and digalactosyl diglyceride carried through the same procedure.

GLC of trimethylsilyl derivatives of carbohydrates was carried out on glass columns (6 ft × 0.25 inch I.D.) packed with 3% GC grade SE-30 on Gas-Chrom P (100–120 mesh) at 165°C.

Analysis of O-alkyl moieties in glycolipid D

Glycolipid D (0.5 mg) was heated under reflux for 2 hr with 2 ml of methanolic HCl, prepared by adding acetyl chloride 10% (v/v) to reagent grade CH₃OH (18). Water (0.5 ml) was added and the solution was extracted with ether (3 × 3 ml). The ether extract was taken to dryness under N_2 and analyzed by TLC in hexane–ether–acetic acid 90:10:1 and CHCl₃–CH₃OH 95:5. For isolation of alkyl glycerols, preparative TLC was done in CHCl₃–CH₃OH 95:5. Lipids were located under UV light after spraying the plates with rhodamine 6G (0.5% in methanol) and alkyl ether was (R_F 0.48) identified by reference to a standard 1-hexadecyl glycerol chromatographed simultaneously. Lipids were eluted from the adsorbent with anhydrous ether. The alkyl glycerols were also isolated by preparative TLC from the products of BF₃/CH₃OH treatment used for the preparation of methyl esters from this glycolipid.

Alkyl glycerols were converted to TMS derivatives by treatment with Sil-Prep at room temperature for 30 min. Solvent was then removed under N_2 and TMS derivatives were dissolved in hexane and analyzed by GLC. The GLC analyses were done on a 3% GC grade SE-30 column (6 ft × 0.25 inch I.D.) on Gas-Chrom P (100–120 mesh) at 220°C. Peaks were identified by comparison of retention times of TMS derivatives of synthetic 14:0, 16:0, and 18:0 alkyl glycerols. TMS derivatives of alkyl glycerols were also analyzed by GLC–mass spectrometry, using a Varian MAT 111. Direct on-column injections of the silylation reaction mixture (0.5–2 μl) were employed using a 3% GC grade SE-30 on Gas-Chrom P (100–120 mesh) in glass columns (6 ft × 4 mm I.D.) at 230° and a helium flow rate of 15 cm³/min. The injection port of the gas chromato-

TABLE 1. Fractionation of lipids of rabbit sciatic nerve on columns of Unisil[a]

Fraction	Eluting Solvent	Eluate Volume per Column	P in Fraction	Hexose	Weight of Fraction	Main Constituents
		ml	% of total	% of total	% of total	
I	Chloroform	1200			50.53 ± 3.18^b	Less polar lipids
II	Chloroform–acetone 19:1	250		1.3	0.12 ± 0.04	Less polar lipids
III	Chloroform–acetone 3:1	1000		8.8	0.99 ± 0.26	"Minor" glycolipids[c]
IV	Chloroform–acetone 1:1	1500	1.7	61.2	8.91 ± 0.53	Cerebroside
V	Acetone	1500	0.29	21.2	3.96 ± 0.36	Sulfatide + some more polar cerebroside
VI	Methanol	1200	98.1	7.4	35.45 ± 3.22	Phospholipids

[a] In a typical experiment, 1.5 g of lipid dissolved in 10 ml of $CHCl_3$ was chromatographed on a 60-g column of Unisil (4 cm I.D.; column height 10 cm).

[b] Averages ± SEM of four different runs.

[c] Acyl cerebroside and diacyl glycerol galactoside fraction.

graph was maintained at 250°C, the molecular separator at 250°C. Spectra were recorded at ionizing energy of 70 ev.

Gas–liquid chromatography

Gas–liquid chromatographic analyses were performed on a Packard 805 gas chromatograph with U-shaped glass columns (6 ft × 0.25 inch I.D.) and a flame ionization detector. Helium was the carrier gas at a flow rate of 45 cm³/min. Depending on the compound to be analyzed, different stationary phases and column temperatures were used (see above). In general, the injection port and the detector had a temperature 20°C higher than the column. The peaks were identified by comparison of the retention times with appropriate standards. The areas under the peaks were estimated by integration using an electronic integrator (Infotronics Corp., Houston, Tex.).

RESULTS

Isolation

Rabbit sciatic nerves contained about 20% lipid based on wet tissue weight, 84 μg-atoms of lipid P/g of nerve, and 25 μmoles of galactolipid/g of nerve. These values are higher than those reported by Sheltawy and Dawson (2), who reported 63 μg-atoms of lipid P/g of nerve and 15.5 μmoles of galactolipid/g of nerve for rabbit sciatic nerve.

Chromatography of the total lipids on Unisil columns gave six fractions (Table 1). The amounts of lipid phosphorus and hexose in various fractions are also given in Table 1. Fraction III, eluted with chloroform–acetone 3:1 (v/v), contained most of the acyl cerebroside and diacyl glycerol galactolipids ("minor" glycolipids, Fig. 1). TLC analysis showed that fraction IV, eluted with chloroform–acetone 1:1 (v/v), contained trace amounts of these glycolipids.
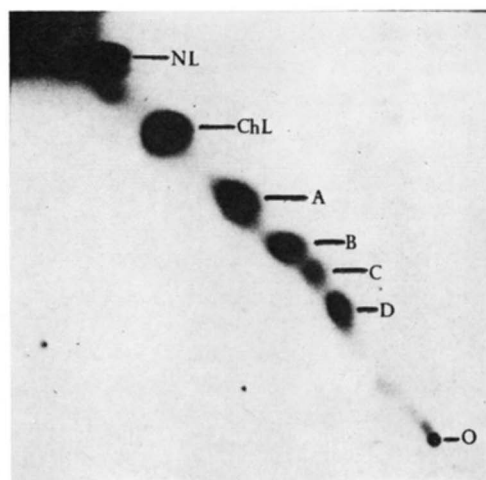

FIG. 1. Two-dimensional TLC of minor glycolipid fraction of rabbit sciatic nerve (fraction III from Unisil column, see Table 1). Components were visualized by charring after spraying with $K_2Cr_2O_7$–H_2SO_4. TLC was done on layers of silica gel H 300 μ thick and activated at 110°C for 1 hr. The chromatograms were developed in the vertical direction with $CHCl_3$–CH_3OH 90:10, dried for 10 min under nitrogen, and developed from right to left with the same solvent. Chl, cholesterol; NL, neutral lipids; A, acyl cerebroside containing unsubstituted acids in amide linkage; B, acyl cerebroside containing hydroxy acids as major component in amide linkage; C, acyl cerebroside; D, galactosyl diglyceride and its alkyl ether analog; O, origin.

Fractions containing the "minor" glycolipid constituents (~ 0.01% of total lipid) from several column chromatographic analyses were combined and the individual components were isolated by preparative TLC (Fig. 2). They were designated glycolipids A, B, C, and D in order of decreasing R_F values. Glycolipids A, B, and D were isolated as single homogeneous constituents and gave one spot on TLC in $CHCl_3$–CH_3OH 90:10 and $CHCl_3$–CH_3OH–H_2O 65:25:3. The glycolipid C contained smaller amounts of glycolipid B or D. Based on the yields from preparative TLC, the composition of the group of glycolipids was 24.2% A, 34.9% B, 15.6% C, and 25.1% D.
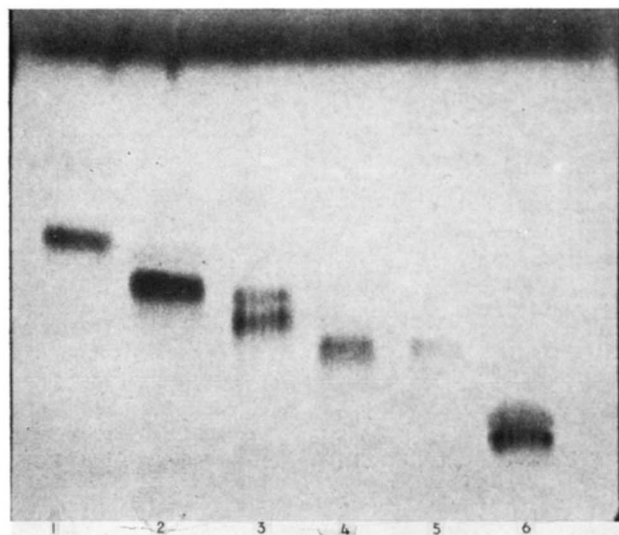
FIG. 2. TLC of minor glycolipids of rabbit sciatic nerve isolated after preparative TLC in CHCl₃–CH₃OH 90:10 (for details see Fig. 1). 1, glycolipid A; 2, glycolipid B; 3, glycolipid C containing some glycolipid B; 4, glycolipid D; 5, standard diacyl glycerol galactoside; 6, beef brain cerebroside.
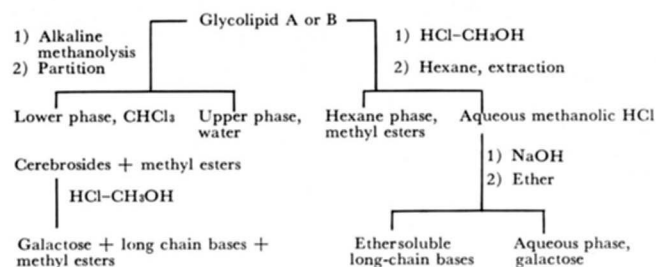

FIG. 3. Flow diagram of the reactions used in the characterization of glycolipids A and B.

and cerebrosides were isolated by preparative TLC. The methyl esters were analyzed by GLC, and their compositions are given in Table 2.

Acid hydrolysis or BF_3–CH_3OH treatment (16) of the cerebrosides isolated after alkaline methanolysis of glycolipid A gave two fractions (this procedure cleaves the ester, the amide, and the glycosidic linkages). The fatty acid moiety contained only unsubstituted acids (TLC in benzene), and the composition of amide-linked fatty acids of cerebrosides isolated after alkaline methanolysis of glycolipid A is given in Table 2. The sphingosine moiety showed three compounds, corresponding to *threo*, *erythro*, and *O*-methyl sphingosines, by TLC. During acid hydrolysis of sphingolipids, the *erythro* isomer of sphingosine, which occurs naturally, is partially isomerized to the *threo* isomer and also gives *O*-methyl sphingosine as a rearrangement product. Thus, it is reasonable to assume that glycolipid A contains only the *erythro* isomer and that *threo* and *O*-methyl sphingosines are formed during acid hydrolysis. This, however, does not exclude the possibility of a natural occurrence of the *threo* isomer in glycolipid A. The sphingosine bases

Glycolipid A

The degradative reactions employed in the characterization of glycolipid A are summarized in Fig. 3.

Alkaline methanolysis of glycolipid A (this procedure cleaves only the ester-linked fatty acids) followed by TLC showed two products. One of them was identified as methyl esters of unsubstituted acid (TLC in hexane–ether–acetic acid 90:10:1). The other was similar to the less polar beef brain cerebrosides (TLC in CHCl₃–CH₃OH 90:10) and, presumably, contained only unsubstituted acids (Fig. 4). The fatty acid methyl esters

TABLE 2. Fatty acid compositions of glycolipids of rabbit sciatic nerve[a]

Fatty Acid	Ester-linked Acids			Amide-linked Unsubstituted Acids				Amide-linked Hydroxy Acids		
	A	B	D	Cerebroside	Sulfatide	A	B[b]	Cerebroside	Sulfatide	B
				% of total fatty acid						
14:0[c]			4.3							
15:0			0.4							
16:0	39.4	28.1	76.4	1.0	1.5	1.9	6.5			
16:1		0.3	1.8							
18:0	26.0	22.1	9.4	2.9	3.6	4.7	10.8		0.7	
18:1	35.0	44.0	7.1	0.3	1.1	1.6	11.3			
18:?[d]		1.5					0.4			
20:0		2.1	0.6	5.5	5.8	6.4	7.0	2.3	2.9	1.4
				0.4	0.2	0.2		0.3		
22:0		1.7		18.8	21.7	23.0	16.0	26.3	28.9	31.1
23:0				8.1	6.0	7.1	5.0	11.5	9.9	11.4
24:0				34.6	41.4	40.3	29.0	49.6	51.4	54.0
24:1				26.4	18.9	14.3	13.9	9.5	4.0	2.1
25:0				0.5				0.4		
25:1				1.5						

[a] The values are averages of at least two determinations on two separate preparations.
[b] Unsubstituted acids constituted 33% of the total acids in this fraction as determined by GLC.
[c] Number of carbon atoms:number of double bonds.
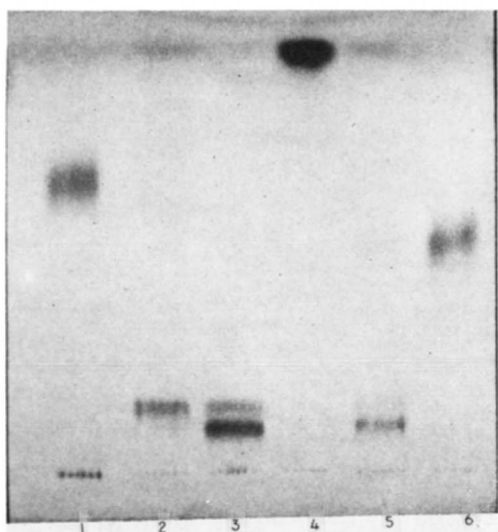[d] Uncharacterized.

Fig. 4. TLC of alkaline methanolysis products of glycolipids A and B in CHCl₃–CH₃OH 90:10. *1*, glycolipid A; *2*, alkaline methanolysis product of glycolipid A; *3*, beef brain cerebrosides; *4*, unsubstituted fatty acid methyl esters; *5*, alkaline methanolysis product of glycolipid B. The lower two components corresponding to cerebrosides are designated fraction E; *6*, glycolipid B.

isolated after acid hydrolysis were oxidized by NaIO₄, and the aldehydes obtained were analyzed by GLC. The compositions of aldehydes are given in Table 3. Acid hydrolysis of glycolipid A under anhydrous conditions followed by analysis of the TMS derivatives of carbohydrate moieties by GLC showed that galactose was the only sugar present. From the experiments described above it is concluded that glycolipid A is a fatty acid ester of cerebroside with only unsubstituted acids in the amide linkage.

Glycolipid B

Glycolipid B was analyzed by the same methods as used for glycolipid A (Fig. 3), and the following results were obtained. Alkaline methanolysis of glycolipid B gave methyl esters of unsubstituted fatty acid (TLC in hexane–ether–acetic acid 90:10:1) and two other components (fraction E) with chromatographic properties similar to beef brain cerebrosides (Fig. 4). Further acid hydrolysis or treatment with BF₃–CH₃OH of fraction E and analysis (TLC in benzene) of the fatty acid moiety showed both unsubstituted and hydroxy acid methyl esters. GLC analysis showed that fraction E contained 33% and 66% of unsubstituted and hydroxy acids, respectively. The fatty acid compositions of the ester- and amide-linked fatty acids are given in Table 2. The sphingosine bases of glycolipid B were similar to those of glycolipid A by TLC, and their compositions are given in Table 3. The carbohydrate moiety in this glycolipid was shown by GLC to be galactose. Thus, glycolipid B is a fatty acid ester of cerebrosides with both unsubstituted and hydroxy acids in amide linkage.

Glycolipid C

Alkaline methanolysis of glycolipid C (containing small amounts of glycolipid B or D) and analysis of the products gave results similar to those obtained for glycolipid B. It is thus reasonable to assume that glycolipid C is also a fatty acid ester of cerebrosides.

Glycolipid D

This compound had an R_F value similar to that of diacyl glycerol galactoside in two solvent systems (CHCl₃–CH₃OH 90:10 and CHCl₃–CH₃OH–H₂O 65:25:3).

TABLE 3. Relative retention times and compositions of aldehydes prepared from long-chain bases of rabbit sciatic nerve glycolipids

Aldehyde	Relative Retention Time[a]	Composition of Aldehydes[b]				Corresponding Long-chain Base
		A	B	Cerebroside	Sulfatide	
		% of total aldehydes				
2-Tetradecenal	0.517	1.7	4.6	t	1.3	C_{16}-Sphingosine
Tetradecanal	0.400	0.7	2.5	0.8	1.8	C_{16}-Dihydrosphingosine
2-Pentadecenal	0.829	0.8	1.3	0.9	0.7	C_{17}-Sphingosine
Pentadecanal	0.633	t	0.5	0.6	1.0	C_{17}-Dihydrosphingosine
2-Hexadecenal	1.303	74.3	69.8	79.8	74.5	C_{18}-Sphingosine
Hexadecanal	1	22.5	21.2	17.8	20.5	C_{18}-Dihydrosphingosine
4-Methoxy-2-pentadecenal	2.686					5-*O*-Methyl-C_{16}-sphingosine
4-Methoxy-2-heptadecenal	3.337					5-*O*-Methyl-C_{18}-sphingosine

[a] Relative retention time is given with respect to hexadecenal on an SE-30 column. The values are averages for six chromatograms. GLC analyses were done on a 3% SE-30 column at 155°C and on a 15% EGSS-X column at 195 and 175°C (for details see Methods).

[b] Composition is calculated to total 100% on the basis of aldehyde (formed after NaIO₄ oxidation of sphingosines) peak area without considering components present in amounts < 0.5%; t indicates aldehyde was present < 0.5%. The amounts of 5-*O*-methyl sphingosines formed during acid hydrolysis are given in the original bases they were derived from, e.g., the average value for 4-methoxy-2-heptadecenal was ∼ 10% of total aldehydes and was added to values obtained for 2-hexadecenal to give the amount of C_{18}-sphingosine.

Alkaline methanolysis of glycolipid D followed by extraction with hexane gave two products (Fig. 5). One of them was identical with methyl esters of unsubstituted acids (TLC in benzene). The other component (R_F 0.4) had a mobility lower than glycolipid D (R_F 0.85) and beef brain cerebrosides (R_F 0.74; TLC in $CHCl_3$–CH_3OH–H_2O 65:25:3). This compound appears to be lysoglycolipid D. Treatment of the glycolipid D with BF_3–CH_3OH or HCl–CH_3OH followed by analysis of ether-soluble components showed the presence of: (a) methyl esters of unsubstituted acids (TLC in hexane–ether–acetic acid 90:10:1) and (b) alkyl glycerols (TLC in hexane–ether–acetic acid 90:10:1, R_F 0.05, and $CHCl_3$–CH_3OH 95:5, R_F 0.48). The alkyl glycerols were isolated by preparative TLC and analyzed by GLC as their TMS derivatives. The major component ($> 85\%$) had a retention time identical with the TMS derivative of synthetic 1-hexadecyl glycerol ether. The identity of the major naturally occurring alkyl glycerol with 1-hexadecyl glycerol ether was further confirmed by GLC–mass spectrometry of the TMS derivatives. Both the natural and the synthetic compounds gave identical retention times and mass fragmentation patterns. The compositions of fatty acids of glycolipid D, isolated after preparative TLC, are given in Table 2.

Acid hydrolysis with dry methanolic HCl followed by analysis of the carbohydrate moieties as their TMS derivatives showed galactose to be the only sugar present in glycolipid D. The evidence presented above suggests that glycolipid D is a mixture of alkylacyl and diacyl glycerol galactoside.

Cerebrosides and sulfatides

There have been only a few studies on the sphingosine base composition of sphingolipids of the peripheral nervous system, and for direct comparison with acyl cerebrosides, sphingosine bases and fatty acid compositions of rabbit nerve cerebrosides and sulfatides were also determined. The results are given in Tables 2 and 3.

Fatty acid composition

The ester-linked fatty acids of glycolipids A and B were mainly 16:0, 18:0, and 18:1 (Table 2). The ester-linked fatty acids of compound D contained 76% 16:0; 14:0, 18:0, and 18:1 formed the remainder.

```
            HCl–CH₃OH
Glycolipid D───────────→Galactose + methyl esters + alkyl glycerols
          │    1) Alkaline methanolysis
          │    2) Hexane, partition
          │
     ┌────┴───────────────────┐
Lower phase,            Hexane phase (upper),
water-soluble          methyl esters + lysoglycolipid D
compound
```
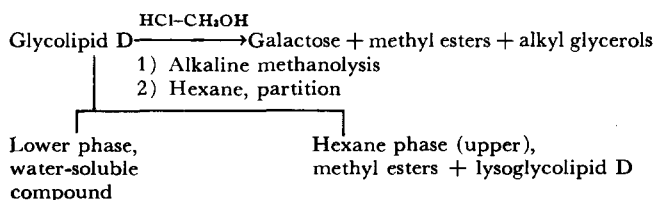
Fig. 5. Flow diagram of the reactions used in the characterization of glycolipid D.

The amide-linked unsubstituted acids of glycolipid A, cerebrosides, and sulfatides, and the hydroxy acids of glycolipid B, cerebrosides, and sulfatides were similar in composition and contained mostly long-chain fatty acids (above C_{20}), which constituted more than 95% of the total acids. The main components were 22:0, 23:0, 24:0, and 24:1. The amounts of 24:1 were different in the unsubstituted and hydroxy acids. It is of interest to note that the unsubstituted acids of compound B contained a smaller amount of long-chain fatty acids as compared with compound A, cerebrosides or sulfatides.

Long-chain base composition

Like fatty acids, the long-chain base compositions of glycolipids A and B, cerebrosides, and sulfatides, were similar (Table 3). The main components were C_{18}-sphingosine and C_{18}-dihydrosphingosine; they constituted 75% and 20%, respectively.

DISCUSSION

The glycolipid components of peripheral nerve were shown to contain four other components in addition to cerebrosides and sulfatides. Alkaline methanolysis of isolated components showed that three of these components were long-chain fatty acid esters of cerebrosides. The fatty acids, long-chain bases, and sugar moieties in acyl cerebrosides were analyzed by GLC. The fourth component was shown to consist of diacyl and alkylacyl glycerol galactoside. We have found no previous reports on the occurrence of acyl cerebrosides or the alkylacyl galactosyl diglyceride in the peripheral nervous system, although they have been shown to be present in the central nervous system (19–25). The relative amounts of these glycolipids present in rabbit sciatic nerve are different from those reported by Kishimoto, Wajda, and Radin for pig brain (23). Diacyl glycerol galactoside has been characterized in rat sciatic nerve by Inoue, Deshmukh, and Pieringer (6); however, the occurrence of an alkylacyl glycerol galactoside was not reported because their method detects by GLC only glycerol galactoside, formed on mild alkaline hydrolysis, as the TMS derivative. Further, it is interesting to note that a convenient procedure for isolation of sphingolipids, which involves an initial treatment with alkali to degrade ester-linked lipids, will not detect fatty acid esters of cerebrosides because they are converted to cerebrosides (26) during this treatment. We have also isolated from human and rabbit femoral nerves glycolipids similar to those present in rabbit sciatic nerve.[1]

The experiments described demonstrate that amide-linked fatty acids of glycolipid A contained only un-

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[1] Singh, H. Unpublished results.

substituted acids, while glycolipid B had both the unsubstituted and hydroxy acids, with the latter predominating. The unsubstituted acids of compound A and the hydroxy acids of compound B had chain length distributions similar to those of cerebrosides isolated from rabbit sciatic nerve. However, the unsubstituted acids present in compound B contained smaller amounts of longer-chain fatty acids as compared with compound A, and this may determine the TLC behavior of unsubstituted acids containing acyl cerebrosides in compound B.

The amount of ester-linked glycolipids and their relative compositions in peripheral nerve are different than in the central nervous system (23). Further, there are significant differences in the distribution of long-chain moieties in these glycolipids. Thus, the amounts of major unsubstituted and hydroxy fatty acids, viz., 22:0, 24:0, 24:1, differ from the amounts reported by Kishimoto et al. (23) for similar compounds from pig brain. It is interesting to note that although we do not observe any amide-linked hydroxy acid in glycolipid A, Kishimoto et al. (23) found high levels of 26:1 hydroxy acids in this fraction (compound KE). As mentioned above, glycolipid B contained rather higher amounts of shorter-chain unsubstituted fatty acids in amide linkage, and this resembles the data of Kishimoto et al. (23) (glycolipid B corresponds to their compound CE). The long-chain base compositions of various sphingolipids isolated resemble those of cerebrosides and sulfatides reported in peripheral nerve (27). However, the compositions differ from sphingolipids in the central nervous system where sphingosine is the only major base (more than 97%) (27, 28). In comparison, peripheral nerve contains 75% sphingosine, about 20% dihydrosphingosine, and 5% minor constituents.

This report thus establishes that both the central and the peripheral nervous systems contain similar types of glycolipid constituents.

Glycolipids A and B differ mainly in the nature of the amide-linked fatty acids; how glycolipid C differs from A and B is not known at present. In this communication only the partial structures could be elucidated because of the small amounts of sample available. The number and positions of long-chain fatty acid moieties and the stereochemistry must be defined before the complete structure is elucidated.

Recent investigations have shown diacyl glycerol galactoside to be a component of central nervous system myelin (6). Our preliminary experiments have shown that it is also present in peripheral nerve myelin isolated from human femoral nerves.[1] However, little is known about the levels, subcellular distribution, and changes that take place during development, and in disease, of these components in the peripheral nervous system.

Some of these components might be markers for various pathological states. Little is known about the biosynthesis of acyl cerebrosides in the central nervous or peripheral nervous systems. However, the similarities in the compositions of amide-linked fatty acids and long-chain bases in cerebrosides and acyl cerebrosides suggest that the latter might be formed by acylation of preformed cerebrosides.

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