Long-chain bases in the sphingolipids of atherosclerotic human aorta

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ABSTRACT Long-chain bases were prepared from human aorta sphingomyelin by a combined enzymatic hydrolysis–alkaline hydrolysis procedure and these bases were isolated by thin-layer chromatography. Aldehydes, obtained from the long-chain bases by periodate oxidation, were converted to 1,3-dioxolane derivatives. Dioxolanes were identified and quantified by gas–liquid chromatography before and after catalytic hydrogenation, and before and after separation into saturated, monoene, and diene dioxolane fractions. The monoene dioxolanes were converted to aldehydes by reductive ozonolysis with dimethyl sulfide and these aldehydes were isolated and identified as dioxolane derivatives. The double bond positions in the major diene component were established by reductive ozonolysis and permanganate–periodate oxidation. Sphingenines in the cerebroside–sulfatide and sulfatide fractions of aorta were converted to aldehydes by the reductive ozonolysis of intact sphingolipids and these aldehydes were analyzed as the dioxolanes.

Human aorta sphingomyelin contained significant amounts of 4-hexadecasphingenine, 4-heptadecasphingenine, sphinganine, 4-sphingenine, and 4,*x*14-sphingadienine. Small amounts of hexadecasphinganine, 4-tetradecasphingenine, a sphingadienine isomer, an unknown sphinganine, and two unknown diene long-chain bases were also found in sphingomyelin. The presence of a branched-chain 4-sphingenine was tentatively established and the possible presence of a sphingenine isomer was suggested. The major sphingenines were the same in the sphingomyelin, sulfatide, and cerebroside–sulfatide fractions of human aorta.

SUPPLEMENTARY KEY WORDS sphingomyelin · sulfatide · cerebroside–sulfatide · enzymatic hydrolysis · alkaline hydrolysis · periodate oxidation · reductive ozonolysis · permanganate–periodate oxidation · 1,3-dioxolane · thin-layer · gas–liquid chromatography · sphinganine · sphingenine · sphingadienine · branched-chain sphingenine

Abbreviations: LCB, long-chain base or bases; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DMS, dimethyl sulfide; PTS, *p*-toluenesulfonic acid; EGS, ethylene glycol succinate polyester.

SEVERAL METHODS have been described for the characterization of long-chain bases (LCB) in sphingolipids. Sweeley and Moscatelli (1) analyzed aldehydes prepared from LCB by periodate oxidation. Karlsson (2) prepared the 2,4-dinitrophenyl derivatives of LCB and separated these derivatives by thin-layer chromatography (TLC). Gaver and Sweeley (3), Polito, Akita, and Sweeley (4), and Carter and Gaver (5) prepared the trimethylsilyl derivatives of LCB and analyzed these derivatives by gas–liquid chromatography (GLC) and mass spectrometry (4). Samuelsson and Samuelsson (6) recently analyzed trimethylsilyl derivatives of sphinganine and 4-sphingenine ceramides by GLC. The sphingadienine components of the LCB have been characterized by permanganate–periodate oxidation (2, 4), ozonolysis (7), and mass spectrometry of trimethylsilyl derivatives (4). In the present investigation (Fig. 1), aldehydes obtained from LCB by periodate oxidation were analyzed as their 1,3-dioxolane derivatives (8) and double bonds were located by reductive ozonolysis (9) or permanganate–periodate oxidation.

Approximately one-fifth of the total phospholipid of normal human aorta is sphingolipid. As atherosclerosis increases in extent and severity in the aorta, the proportion of sphingolipids increases to approximately twice that found in the normal aorta, primarily through an increase in sphingomyelin. The high content of sphingomyelin in human atheroma was first reported by Weinhouse and Hirsch (10) and since then this observation has been confirmed in a number of studies (11–14).

Nomenclature is in accordance with the tentative rules of the IUPAC–IUB Commission: the older term "dihydrosphingosine" is called sphinganine; "sphingosine" is called 4-sphingenine; higher and lower homologues are identified by prefixes; "phytosphingosine" is called 4*D*-hydroxysphinganine; unknown geometry is specified by the prefix "*x*-" before the number that indicates the position of the double bond.

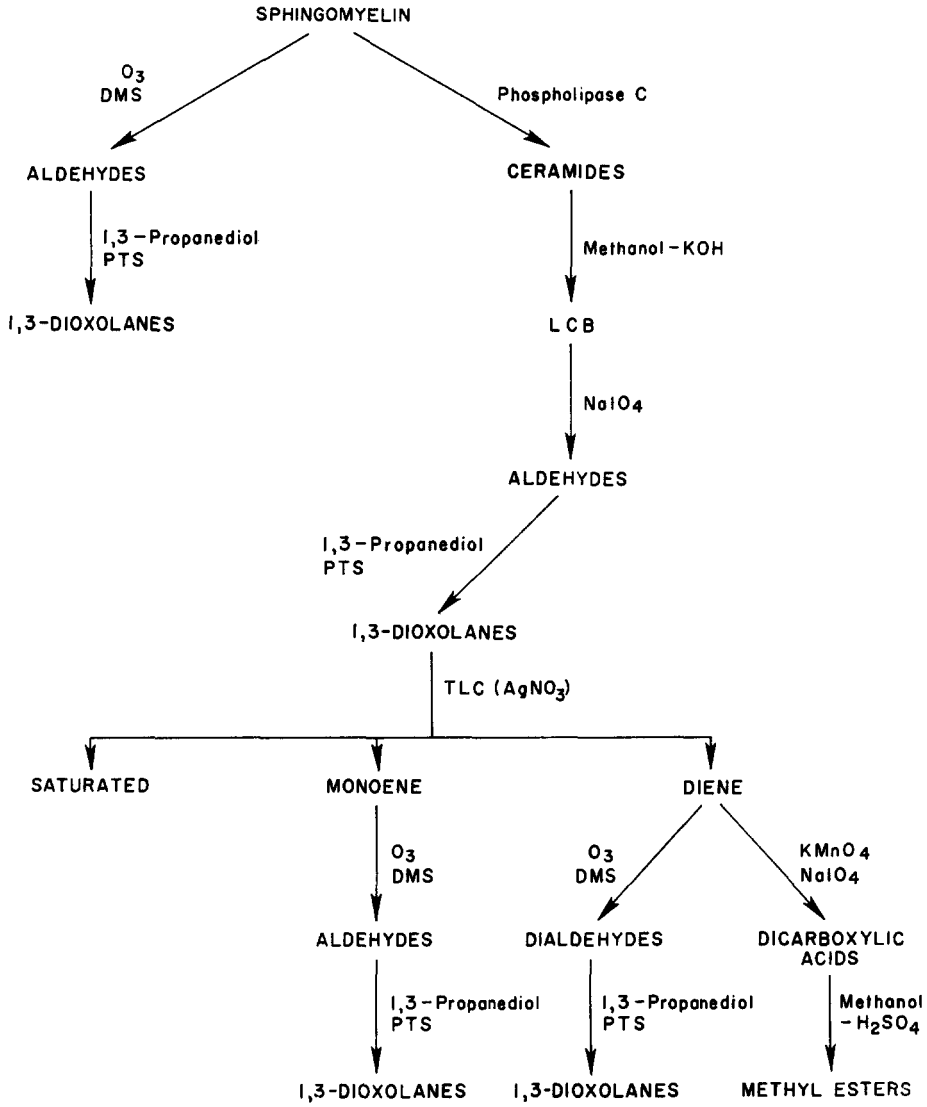

FIG. 1. Isolation of LCB and the preparation of derivatives for analysis. PTS, *p*-toluene sulfonic acid.

The significance of sphingomyelin in the pathogenesis of atherosclerosis is unknown. Although the fatty acid composition of human aorta sphingomyelins has been reported (14), the LCB composition of human aorta sphingomyelins has not been investigated. In the present study, only aortas with extensive and severe atherosclerosis were analyzed in order that sufficient lipid be available for validation of the methods used in the isolation and characterization of LCB. The LCB compositions of human aortas representing increasing age and severity of lesions will be compared in a subsequent investigation.

MATERIALS AND METHODS

Materials

All reagents were analytical grade. Commercial hexane was washed three times with concentrated sulfuric acid, once with water, and finally with a 10% sodium carbonate solution until neutral. The washed hexane was distilled at 68°C. Platinum oxide was purchased from Baker Catalyst, Inc. (Newark, N. J.). 1,3-Propanediol and dimethyl sulfide (DMS) were purchased from Matheson, Coleman and Bell (Cincinnati, Ohio). *p*-Toluenesulfonic acid (PTS) was purchased from Eastman Organic Chemicals (Rochester, N. Y.). Silicic acid (Unisil) was purchased from Clarkson Chemical Co. (Williamsport, Pa.). Silica Gel H (Brinkmann Instruments, Inc., Westbury, N. Y.) was extracted with acetone and then with chloroform–methanol 2:1 (v/v), dried at room temperature, and activated at 110°C for 24 hr. Beef brain sulfatides and ceramides, ethylene glycol succinate polyester (Hi-EFF-2BP), 80–100 mesh Gas-Chrom P, and the methyl esters of dibasic acids were purchased from Applied Science Laboratories, Inc. (State College, Pa.). Beef brain sphingomyelin was pur-

chased from General Biochemicals, Inc. (Chagrin Falls, Ohio). 1,7-Octadiene and 1,9-decadiene were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Phospholipase C (*Cl. welchii*, type-1) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Cerebrosides, purified gangliosides, and pure LCB (a mixture of sphinganine and 4-spingenine) were kindly supplied by Dr. R. H. McCluer. Octadecanal, 9-octadecenal, and 9,12-octadecadienal were synthesized from the corresponding fatty acids (8).

Five aortas were obtained at necropsy from persons between 57 and 76 yr old. These aortas were selected because of extensive and advanced lesions of atherosclerosis. By terminology recommended by the World Health Organization (15) the aortas were at stage III of atherosclerosis. The aortic intima, from the level of the first intercostal vessels of the descending thoracic segment to the iliac bifurcation, was removed by blunt dissection.

Isolation of Sphingomyelin

The intimal tissue, 25–30 g, was homogenized in a Sorvall Omni-Mixer in 10 volumes of chloroform–methanol 2:1. The filtered residue was homogenized twice more in 10 volumes of chloroform–methanol 1:1 and the filtered residue from this step was then homogenized in 10 volumes of absolute methanol. The combined extracts were evaporated under reduced pressure in a Rinco flash evaporator. The crude lipids were dissolved in chloroform–methanol 2:1 and washed by the Folch partition method (16). The chloroform phase was then evaporated under reduced pressure. The lipids were dissolved in a small volume of chloroform and placed on a 60 g Unisil column (3.5 cm O.D.). Neutral lipids were eluted with 600 ml of chloroform and polar lipids were eluted with 600 ml of methanol.

When the polar lipid fraction contained less than 500 mg, sphingomyelin was isolated by TLC. Plates were developed with chloroform–methanol–water 65:25:4 and bands were viewed under UV radiation after the plates had been sprayed with 2′,7′-dichlorofluorescein. The sphingomyelin fraction (R_f 0.23) was extracted from the Silica Gel H with methanol. Sphingomyelin was isolated from larger polar lipid fractions (500–2000 mg) by a different method. Aldehydes were liberated from plasmalogens by mild acid hydrolysis (17) and the hydrolysate was then placed on a 20 g Unisil column. Aldehydes were eluted from the column with 100 ml of chloroform and polar lipids were eluted with 250 ml of methanol. The acid-stable lipids were then subjected to mild alkaline hydrolysis (18) and the alkali-stable fraction was extracted into chloroform–methanol 2:1. The fraction was placed on a 20 g Unisil column and lipids were eluted successively with 100 ml of chloroform, 200 ml of acetone, and 200 ml of methanol. The methanol eluate contained only sphingomyelin as judged by TLC.

Isolation of Sulfatides

About 500 mg of the polar lipid fraction isolated from aortic intima was applied to TLC plates. Plates were developed with chloroform–methanol–water 65:25:4 and then sprayed with 2′,7′-dichlorofluorescein; the overlapping choline phosphoglyceride (R_f 0.28) and sulfatide (R_f 0.34) band was extracted with methanol. This fraction was incubated with phospholipase C and then placed on a 20 g Unisil column. The diglycerides obtained from the choline phosphoglycerides were eluted with 100 ml of chloroform and the sulfatides were eluted with 250 ml of methanol. Removal of choline phosphoglycerides was judged by the absence of a phosphorus-containing spot (molybdic acid spray) on TLC.

Isolation of Cerebroside–Sulfatide (Glycolipid) Fraction

The polar lipid fraction isolated from aortic intima was applied to a 30 g column of activated Florisil. The column was packed with Florisil suspended in chloroform–methanol 70:30 and the glycolipids were eluted with 200 ml of the same solvent by the method of Rouser, Kritchevsky, and Yamamoto (19) except that 2,3-dimethoxypropane was omitted from the solvent. No glycolipids were detected on TLC when an additional 100 ml of chloroform–methanol 70:30 was passed through the Florisil column. Homogeneity was judged by the absence of phosphorus-containing spots (molybdic acid spray) on TLC when the plate was developed with chloroform–methanol–water 65:25:4. Glycolipids became visible when the plates had been sprayed with orcinol–sulfuric acid and warmed. The fraction contained two cerebrosides (R_f 0.81 and 0.72) and a sulfatide (R_f 0.34). The same glycolipids were detected in the total polar lipid mixture by TLC.

Isolation of LCB

Approximately 60 mg of sphingomyelin was placed in a mixture of 4 ml of diethyl ether and 4 ml of 0.15 M Tris buffer (pH 7.3) containing 25 mg of phospholipase C. The mixture was stirred at 37°C for 3 hr. Ceramides were extracted from the reaction mixture with chloroform. The chloroform extract was washed with 10% aqueous sodium chloride and the solvent was removed under reduced pressure. Ceramides were then refluxed with 1.0 N KOH in methanol for 18 hr, and the LCB fraction from this hydrolysate was extracted into chloroform or ether. The extract was washed three times with 10% aqueous sodium chloride and then passed through anhydrous sodium sulfate. The solvent was removed under reduced pressure. LCB were purified by TLC in chloroform–methanol–water 65:25:4. In an

alternative isolation procedure, the chloroform or ether extract of the ceramide hydrolysate was applied to a 20 g Unisil column. Any fatty acids that were present in the extract were eluted with 100 ml of chloroform and the LCB were eluted with 200 ml of methanol. The homogeneity of the LCB fraction was judged by TLC in chloroform–methanol–water 65:25:4 or chloroform–methanol–ammonia 40:10:1 (20).

Preparation of 1,3-Dioxolanes

The LCB fraction was oxidized with 0.2 M aqueous sodium metaperiodate (1). Aldehydes were purified by elution from a 20 g Unisil column with 150 ml of chloroform and then converted to 1,3-dioxolanes (8). The 1,3-dioxolanes were purified by TLC in hexane–ether 90:10 (R_f 0.46).

Separation of Saturated and Unsaturated 1,3-Dioxolanes

Saturated and unsaturated dioxolane fractions were isolated by TLC. Silica Gel H plates were impregnated with $AgNO_3$ (21) and developed with hexane–ether 85:15. Bands were visible under UV radiation after the plates had been sprayed with 2′,7′-dichlorofluorescein. Bands were identified by comparing their R_f values with the R_f values of reference compounds synthesized from fatty aldehydes. The dioxolanes were extracted from TLC plates with chloroform–hexane 1:1.

Hydrogenation

The 1,3-dioxolanes were hydrogenated by the procedure of Rao, Ramachandran, and Cornwell (8) except that pyridine was not used in the hydrogenation procedure.

Cleavage

The unsaturated 1,3-dioxolanes were cleaved by a reductive ozonolysis procedure which employed DMS (9). Aldehydes were extracted into hexane, washed twice with small amounts of water, and converted to dioxolanes by the methods referred to above. Dioxolanes were purified by TLC on Silica Gel H. In an alternative procedure (see Fig. 1), dioxolanes in the diene fraction were cleaved by permanganate–periodate oxidation and the resulting dicarboxylic acids were converted to methyl esters (22).

In the reductive ozonolysis of intact sphingolipids (Fig. 1), the lipid was dissolved in 3 ml of chloroform–methanol 2:1 and this solution was added to 15 ml of pentane or methanol saturated with ozone. DMS was added, pentane or methanol was evaporated and aldehydes were extracted into hexane. The hexane solution was washed twice with small amounts of water. The aldehydes were then converted to dioxolanes and purified by TLC.

Gas–Liquid Chromatography

Dioxolanes and methyl esters were analyzed in Aerograph 200 and Aerograph 1200 chromatographs equipped with flame ionization detectors. 10-ft (3.3 m) stainless steel columns, 1/8 inch (3.2 mm) O.D., containing 20% ethylene glycol succinate polyester (EGS) on 80–100 mesh Gas-Chrom P, were used for separations on a polar phase. 5-ft (1.65 m) stainless steel columns, 1/8 inch (3.2 mm) O.D., containing 5% SE-30 (methylpolysiloxy gum) on 60–80 mesh Gas-Chrom W, were purchased (Varian Aerograph, Walnut Creek, Calif.) for chromatographic separations on a nonpolar phase. Peaks were identified by relative retention volumes ("R_{18} values"), calculated by dividing the retention volume of the peak by the retention volume of methyl stearate. Peak areas were measured by triangulation. Operating conditions are listed with the appropriate figures and tables.

RESULTS

Isolation of LCB

When ceramide was prepared by the enzymatic hydrolysis of sphingomyelin with phospholipase C, the hydrolysis product showed only one spot on TLC in chloroform–methanol–water 65:25:4. The R_f value for this spot, 0.86, corresponded to the R_f value of a commercial ceramide sample. No unreacted sphingomyelin or other phosphorus-containing spot was detected. LCB was prepared by alkaline hydrolysis of the ceramide fraction. The product gave one spot (R_f 0.48) on TLC in chloroform–methanol–water 65:25:4 which corresponded to the LCB isolated from brain gangliosides (20). The product gave two spots on TLC in chloroform–methanol–ammonia 40:10:1 and these spots had the same R_f values as 4-sphingenine and sphinganine fractions isolated from brain gangliosides (20). No ceramide, 4*D*-hydroxysphinganine or *O*-methyl-4-sphingenine were detected on TLC with either developing solvent.

Separation of Saturated and Unsaturated 1,3-Dioxolanes

The saturated monoene and diene components of the LCB fraction yielded saturated, monoene, and diene aldehydes on periodate oxidation. These aldehydes were converted to their corresponding dioxolanes and the dioxolanes were separated by TLC on plates impregnated with $AgNO_3$ (Fig. 2). The R_f value of the saturated fraction, 0.69, was the same for dioxolanes prepared from LCB and dioxolanes prepared from tetradecanal. The R_f values for the monoene fractions were different for dioxolanes prepared from LCB where the double bond was in the α,β position (R_f 0.53) and dioxolanes prepared from 9-octadecenal (R_f 0.45). Similar differ-

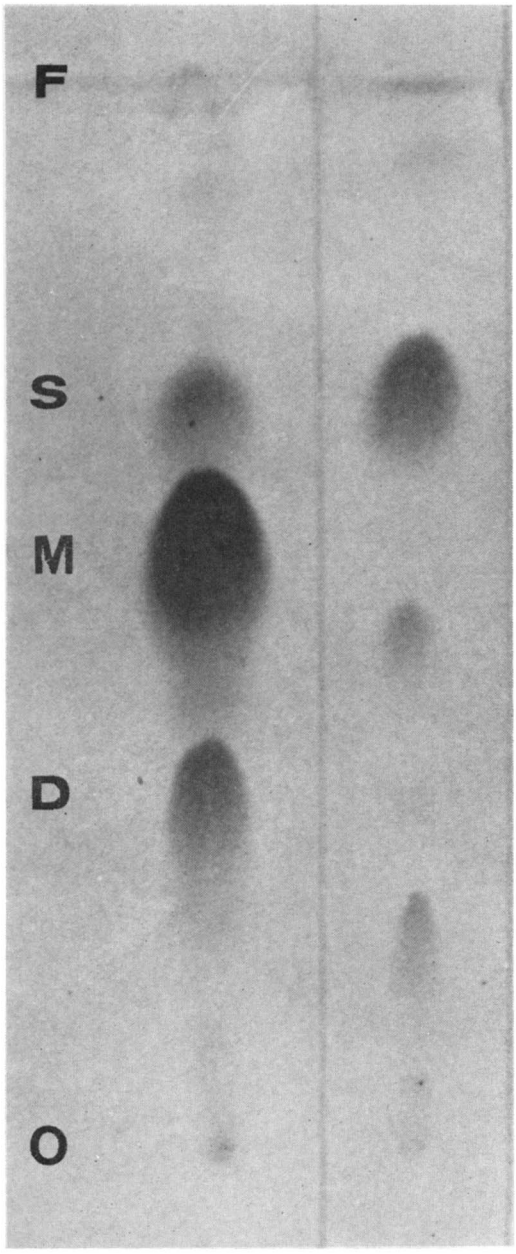

FIG. 2. Separation of 1,3-dioxolanes on $AgNO_3$-TLC: *F*, solvent front; *S*, saturated; *M*, monoene; *D*, diene; *O*, origin. Chromatogram was developed in hexane–ether 85:15 and charred with sulfuric acid. Right: dioxolanes from tetradecanal, 9-octadecenal, and 9,12-octadecadienal. Left: dioxolanes from LCB in which one double bond is α, β to the dioxolane group.

ences were seen in dioxolane R_f values for the diene fractions obtained from LCB (R_f 0.26) and 9,12-octadecadienal (R_f 0.16).

GLC Analysis of 1,3-Dioxolanes

The GLC analysis of 1,3-dioxolanes prepared from the LCB fraction of human aorta by periodate oxidation is shown in Fig. 3D. The R_{18} values for many dioxolane peaks did not correspond to the R_{18} values of dioxolanes

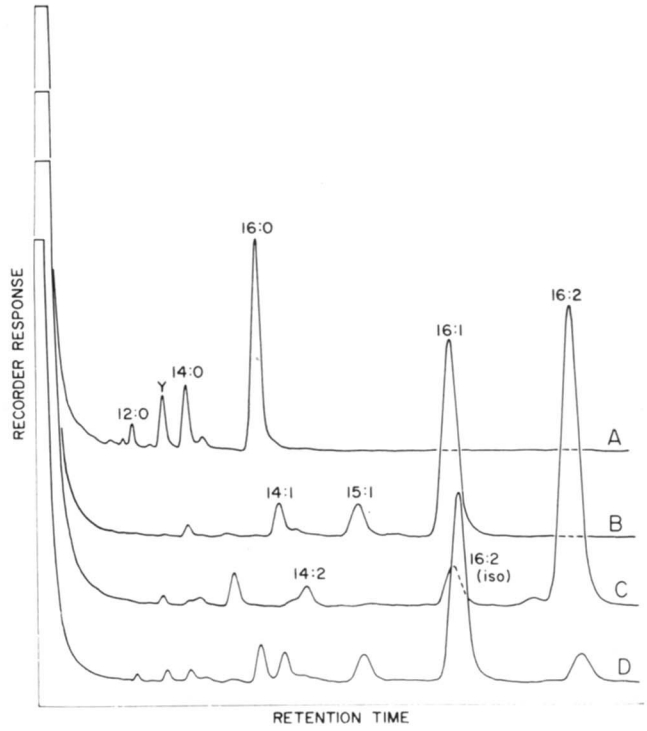

FIG. 3. GLC analysis of 1,3-dioxolanes prepared from the sphingomyelin LCB fraction of human aorta by periodate oxidation: *A*, saturated; *B*, monoene; *C*, diene; *D*, total mixture. Tracings were obtained from an EGS column in an Aerograph 200 equipped with a flame ionization detector. Operating temperatures were: injector 280°C, column 182°C, detector 280°C. Helium was the carrier gas and the flow rate was 30 ml/min.

prepared from known fatty aldehydes. Since reference compounds containing α,β-double bonds were not available, the GLC analysis of saturated and unsaturated dioxolane fractions and hydrogenation and reductive ozonolysis data were required for the identification of peaks. GLC tracings for saturated, monoene, and diene dioxolane fractions are shown in Fig. 3A, B, and C. The R_{18} values obtained from these GLC analyses are summarized in Table 1. These data and log R_{18} data were used for the tentative identification of 1,3-dioxolanes in the original mixture and these tentative peak assignments were then confirmed by catalytic hydrogenation. Thus 14:1 and 15:1 dioxolanes were converted to 14:0 and 15:0 dioxolanes, and 16:1 and 16:2 dioxolanes were converted to a 16:0 dioxolane, by catalytic hydrogenation (Fig. 4, Tables 1 and 2). A small peak in the hydrogenated mixture with a retention time greater than that of 16:0 dioxolane (Fig. 4) was not identified. The R_{18} for this peak did not correspond to a 17:0 or 18:0 dioxolane. No other peaks, except a 16:1br peak described below, were observed consistently or in measurable amounts during GLC after catalytic hydrogenation.

Reductive ozonolysis of α,β-double bonds in the monoene dioxolane fraction yielded saturated aldehydes, which were converted to dioxolanes. The major com-

TABLE 1 RELATIVE RETENTION TIMES FOR 1,3-DIOXOLANES OF STANDARD ALDEHYDES AND ALDEHYDES PREPARED FROM LONG-CHAIN BASES IN THE SPHINGOMYELIN OF HUMAN AORTA

	R_{18}† at 180–185°C					
			TLC with $AgNO_3$			
1,3-Di-oxolane*	Stan-dard	Total	Satu-rated	Monoene	Diene	Total Hydro-genated
12:0	0.6		0.6			
14:0	0.8	0.8	0.8			0.8
$Unknown_a$		0.9	0.9			
15:0	1.0					1.0
12:1		1.0		1.0		
16:0br						1.2
$Unknown_b$		1.3			1.3	
16:0	1.4	1.4	1.4			1.4
14:1		1.5		1.5		
$Unknown_c$					1.7	
15:1		2.0		2.0		
16:1br		2.3		2.3		
16:1		2.6		2.6		
$16:2_a$					2.6	
$16:2_b$		3.2			3.2	
$16:2_c$		3.4			3.4	

* The carbon number: double bond nomenclature refers to the parent aldehyde. Different unknown peaks and 16:2 isomers are indicated by subscripts.

† Relative retention time with respect to methyl stearate. The retention volume of methyl stearate at 182°C was 633 ml. Operating conditions are summarized in Fig. 3.

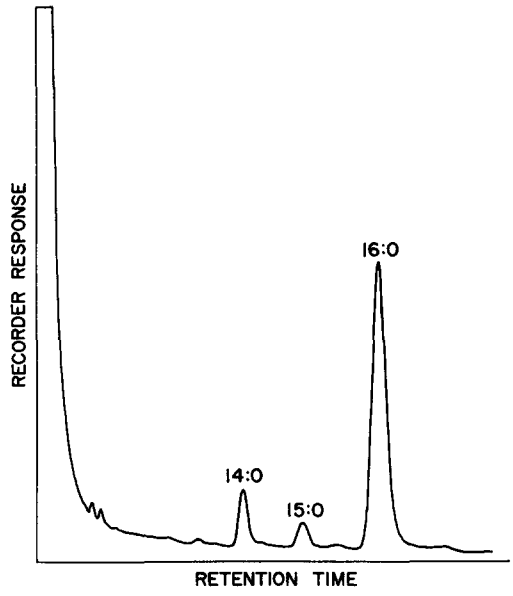

FIG. 4. GLC analysis of the total 1,3-dioxolane mixture (Fig. 3D) after catalytic hydrogenation. Operating conditions are summarized in Fig. 3.

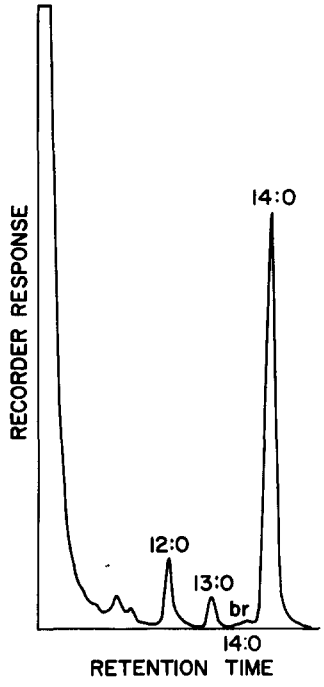

FIG. 5. GLC analysis of dioxolanes from the monoene 1,3-dioxolane fraction (Fig. 3B) after reductive ozonolysis. Operating conditions are summarized in Fig. 3.

ponents of this mixture had two fewer carbon atoms and with the exception of 13:0 were in the same relative concentrations (Fig. 5) as the dioxolanes in the monoene fraction (Fig. 3B). Unreacted ozonides from α,β unsaturated dioxolanes were not detected by TLC after reductive ozonolysis with DMS although some unreacted ozonide was detected after the reductive ozonolysis of 9-octadecenyl-1,3-dioxolane. The conversion of this ozonide to aldehydes was improved and ozonides were not detected when methanol was substituted for pentane in the ozonolysis reaction.

A 16:1br dioxolane with an α,β-double bond was tentatively identified as a minor component from the following data. The peak occurred between the 15:1 and 16:1 peaks in the total mixture and the monoene fraction (Table 1). A small peak with the same relative concentration was found between the 15:0 and 16:0 dioxolanes obtained after hydrogenation (Table 2 and Fig. 4). Finally, a small peak was found between the 13:0 and 15:0 dioxolanes obtained after reductive ozonolysis (Fig. 5).

The total dioxolane mixture contained three unknown minor components. One of these unknown peaks was found in the saturated fraction and the other two unknown peaks were found in the diene fraction (Table 1). Hydrogenation and reductive ozonolysis did not provide any additional data for the identification of these peaks. One peak in the diene fraction, $16:2_a$, had the same R_{18} value as the 16:1 dioxolane. Since the 16:1 dioxolane was the major component of the total mixture, this diene probably represented monoene contamination in the diene fraction rather than a 16:2 positional isomer. The $16:2_b$ and $16:2_c$ peaks probably do represent positional isomers. GLC peaks which corresponded to

TABLE 2 COMPOSITION OF MIXTURE OF LONG-CHAIN BASES IN THE SPHINGOMYELIN OF HUMAN AORTA*

Long-Chain Base	1,3-Dioxolane	Total†	TLC with $AgNO_3$			Total Hydrogenated‡
			Saturated	Monoene	Diene	
		area %		*area %*		*area %*
Tetradecasphinganine	12:0		4.4			
Hexadecasphinganine	14:0	0.6 ± 0.1	9.4			6.6, 5.4
$Unknown_a$		1.3 ± 0.4	12.1			
	15:0					4.5, 4.2
4-Tetradecasphingenine	12:1	0.8 ± 0.9		3.0		
	16:0br					0, 2.3
$Unknown_b$		0.8 ± 0.5			4.5	
Sphinganine	16:0	4.6 ± 1.6	74.1			88.8, 88.1
4-Hexadecasphingenine	14:1	5.4 ± 2.3		9.4		
$Unknown_c$					2.6	
4-Heptadecasphingenine [Sphingenine(iso)]	15:1 [16:1(iso)]§	8.8 ± 1.9 [4.3]§ [4.5]§		13.3		
4-Sphingenine (br)	16:1br	1.2 ± 0.6		0.9		
4-Sphingenine	16:1	63.2 ± 1.4		73.3		
$Sphingadienine_a$	$16:2_a$				10.3	
$Sphingadienine_b$	$16:2_b$	1.0 ± 1.0			2.7	
$Sphingadienine_c$	$16:2_c$	12.4 ± 2.3			79.9	

* Dioxolane nomenclature is described in Table 1. Operating conditions are summarized in Fig. 3.
† Mean ± SD for sphingomyelins from five different aortas. Lipids from two aortas were combined.
‡ Hydrogenation data from two dioxolane preparations.
§ See text for the tentative identification of sphingenine(iso) and the assumptions used in the quantification of this compound.

minor dioxolane components were not obtained when blank Silica Gel H plates and blank Silica Gel H plates impregnated with $AgNO_3$ were extracted with chloroform–methanol 2:1.

LCB Composition of Sphingomyelin From Human Aorta

The relative composition of the LCB moieties in the sphingomyelin of human aorta was obtained from the GLC analysis of the total dioxolane mixture before and after hydrogenation and the GLC analysis of the saturated, monoene, and diene fractions (Table 2). Aldehydes analyzed by GLC on an EGS column prior to their conversion to dioxolanes had the same composition as the total dioxolane mixture. The principal LCB in the saturated fraction was sphinganine. 4-Sphingenine and a sphingadienine were the principal LCB components of the monoene and diene fractions. The saturated fraction contained a small amount of tetradecasphinganine and the diene fraction contained a small amount of an unknown component, $unknown_c$; neither of these components was detected in the total mixture.

Composition data suggested that the 15:1 dioxolane peak contained both the expected 15:1 dioxolane with the double bond in the α,β position and a second component in which the double bond was not in the α,β position. The evidence for the identity of this dioxolane was obtained from hydrogenation and reductive ozonolysis experiments. Thus the 15:1 peak, 8.8% of the total mixture, was larger than the 15:0 peak, 4.3% of the hydrogenated mixture (Table 2) and it appeared that the 15:1 dioxolane peak contained an additional dioxolane which did not yield a 15:0 product on hydrogenation. The composition of the dioxolane mixture from the monoene fraction after reductive ozonolysis was: 12:0, 10.6%; 13:0, 5.9%; 14:0br, 1.3%; 14:0, 82.1% (data from Fig. 5). Since the relative concentration of the 15:1 dioxolane in the monoene fraction was 13.3% (Table 2), it appeared that the 15:1 dioxolane peak contained an additional dioxolane which did not yield a 13:0 dioxolane on reductive ozonolysis. These data would be explained by the presence of a 16:1 positional isomer which had the same retention volume as the 15:1 dioxolane with the double bond in the α,β position. Dioxolanes with α,β-double bonds which were prepared from LCB had larger retention volumes than dioxolanes prepared from fatty aldehydes (8) and it is possible that a 16:1 isomer and the 15:1 dioxolane from an LCB had the same retention volume. Furthermore, the sum of the 16:0 + 16:1 + 16:2 peaks in the original mixture, 81.7% (Table 2), was less than the 16:0 content of the hydrogenated mixture, 88.4% (Table 2), and these data also suggested that the 15:1 peak may have contained a 16:1 positional isomer which appeared as a part of the 16:0 component after hydrogenation. 4-Heptadecasphingenine was, therefore, estimated from the 15:0 content of the hydrogenated mixture. The unknown component, which may be a sphingenine isomer, was estimated as the difference between the concentration of the 15:1 peak in the original dioxolane mixture and the concentration of the 15:0 peak in the hydrogenated dioxolane mixture (Table 2).

Structure of Sphingadienine From Human Aorta Sphingomyelin

The 1,3-dioxolanes isolated in the diene fraction by TLC were cleaved by reductive ozonolysis and the dialdehyde products were converted to their α,ω-didioxolane derivatives. Reference C_6, C_8, and C_9 α,ω-didioxolanes were synthesized from 1,7-octadiene, 1,9-decadiene, and 9-octadecenal, respectively. Dioxolanes were chromatographed on SE-30 and EGS columns (Fig. 6) and the major peak was identified as a derivative of the C_{10} dialdehyde from log R_t data. Small amounts of C_9, C_8, and C_7 didioxolanes were also identified. The dibasic acids, which were obtained by periodate–permanganate oxidation, were analyzed as their methyl esters and the major peak had the same retention time as the methyl ester of a C_{10} dibasic acid on SE-30 and EGS columns (Fig. 7). These data showed that the two double bonds were separated by 10 carbon atoms and the data suggested strongly that the major diene LCB was 4,*x*14-sphingadienine since this LCB has been identified as the major diene LCB in human plasma (4). The minor C_9, C_8, and C_7 peaks probably included both overoxidation artifacts and sphingadienine isomers. It is interesting that periodate–permanganate oxidation yielded larger quantities of the C_9 and C_8 derivatives (Fig. 7) than did reductive ozonolysis (Fig. 6).

Reductive Ozonolysis of Intact Sphingolipids

The reductive ozonolysis of intact sphingolipids yielded long-chain aldehydes from their sphingenine moieties and short-chain aldehydes such as the 9:0 dioxolane from their unsaturated fatty acid moieties. GLC of the dioxolanes prepared from brain gangliosides is illustrated in Fig. 8. In contrast to cerebrosides, gangliosides contained a large amount of 4-eicosasphingenine which was identified as the 2-hexadecyl-1,3-dioxolane derivative. Direct reductive ozonolysis thus confirmed other studies on the LCB components of brain gangliosides and cerebrosides (20). No 4-eicosasphingenine was detected by the reductive ozonolysis of sphingomyelin, sulfatide, or cerebroside-sulfatide fractions from human aorta (Fig. 9). The 12:0 and 13:0 dioxolane peaks which had been detected after the reductive ozonolysis of the monoene dioxolane fraction (Fig. 5) were also detected after the reductive ozonolysis of intact sphingolipids.

DISCUSSION

Several investigators have described the formation of ceramide by the enzymatic hydrolysis of sphingomyelin (2, 23) and the formation of LCB by the alkaline hydrolysis of ceramide (2). The advantages of these hydrolysis

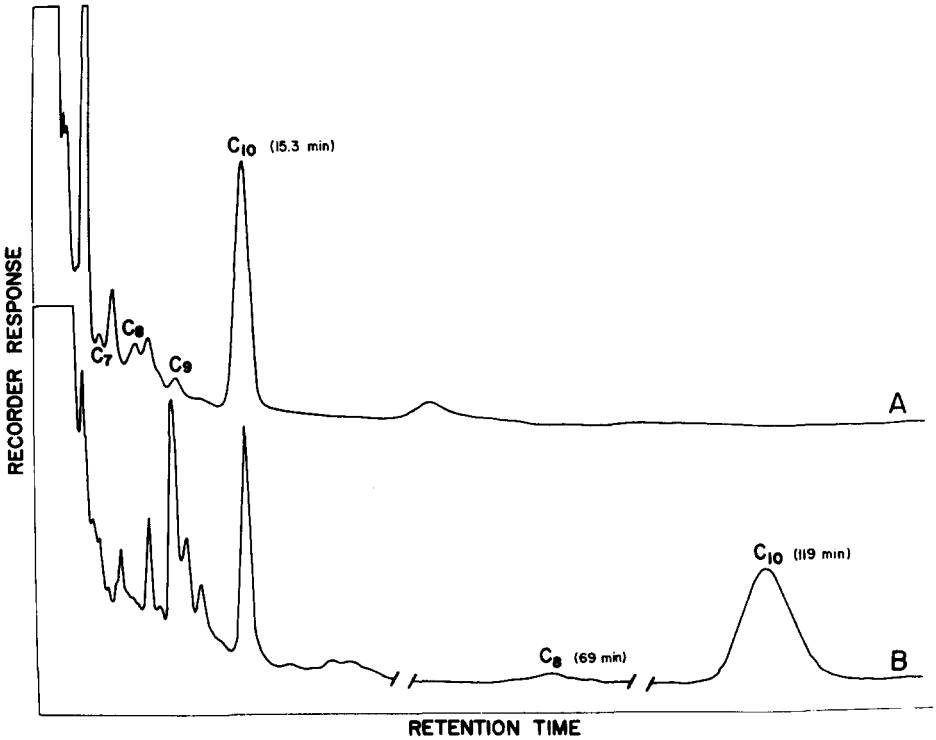

FIG. 6. GLC analysis of α,ω-didioxolanes prepared from the sphingadienine dioxolane fraction of sphingomyelin after reductive ozonolysis: *A*, SE-30 column at 172°C; *B*, EGS column at 174°C. Other operating conditions are summarized in Fig. 3.

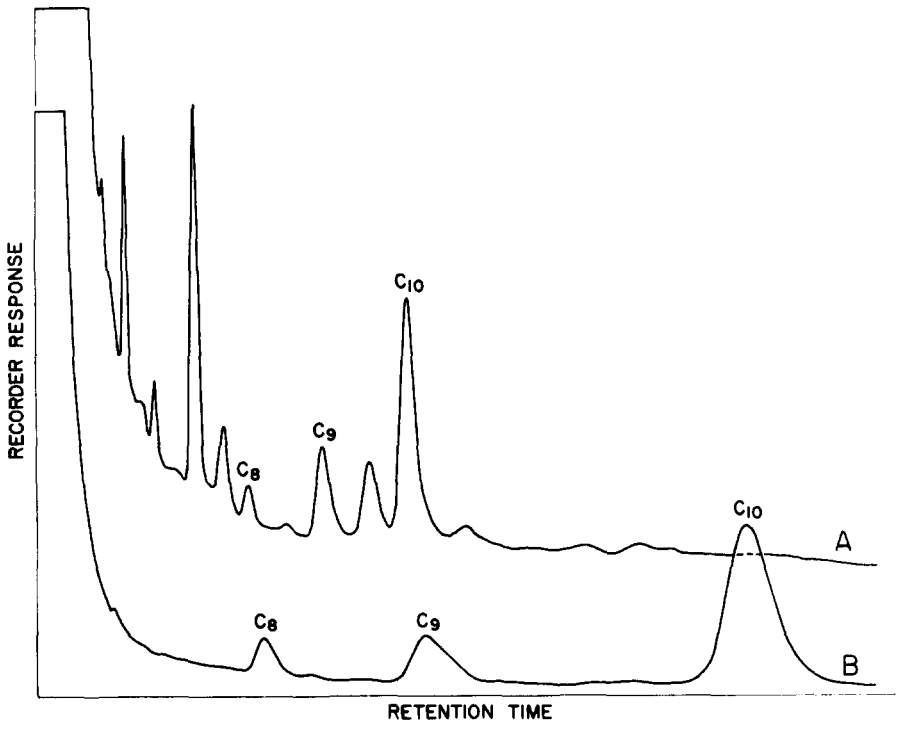

FIG. 7. GLC analysis of dibasic acid methyl esters prepared from the sphingadienine dioxolane fraction of sphingomyelin after periodate–permanganate oxidation: *A*, EGS column at 170°C; *B*, SE-30 column at 105°C. Other operating conditions are summarized in Fig. 3.

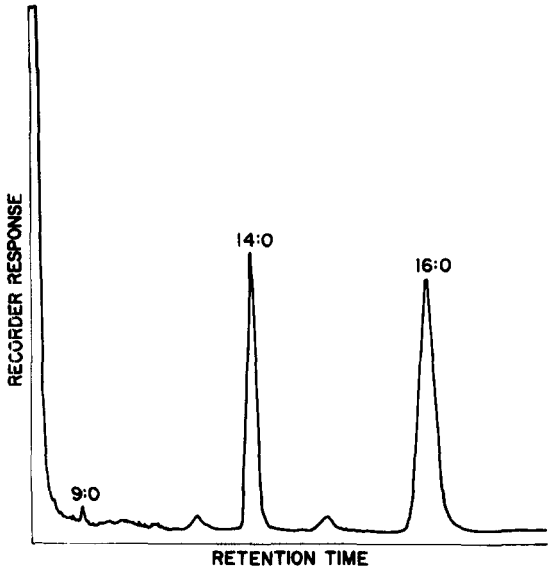

FIG. 8. GLC analysis of 1,3-dioxolanes prepared from purified brain gangliosides after ozonolysis–reduction. Operating conditions are summarized in Fig. 3.

procedures were confirmed in the present study. Both enzymatic and alkaline hydrolysis reactions were complete as judged by the absence of reactants on TLC. No side-products such as *O*-methyl-4-sphingenine were detected by either TLC or GLC. No 4*D*-hydroxysphinganine was detected in the LCB from aorta sphingomyelin by TLC (20).

Periodate oxidation of LCB is a well-established procedure (1). Aldehydes are readily identified by GLC (1, 2); however, aldehydes are unstable compounds and they tend to decompose and polymerize on standing (8). The 1,3-dioxolane derivatives of aldehydes were stable for long periods of time and these derivatives were well suited for the separation of saturated, monoene, and diene fractions on TLC plates impregnated with $AgNO_3$. Unsaturated LCB components yielded α,β-unsaturated aldehydes and the dioxolane derivatives of these compounds had somewhat higher R_f values than reference dioxolanes synthesized from fatty aldehydes in which the double bond was in the 9,10-position. Gunstone, Ismail, and Jie (24) found that the position of the double bond had the same effect on R_f values in a homologous series of methyl octadecenoates.

Dioxolanes were readily identified and quantified by GLC before and after catalytic hydrogenation. The position of the α,β-double bond was established by reductive ozonolysis and the GLC analysis of the aldehyde product as the dioxolane derivative. The R_{18} values for unsaturated dioxolanes prepared from LCB were much greater than the R_{18} values for unsaturated dioxolanes prepared from fatty aldehydes. For example, the 16:1 dioxolane from LCB had the same R_{18} value, 2.6, as the dioxolane from 9-octadecenal (8). Methyl octadecenoate with a *trans* double bond at the 2,3-position has an

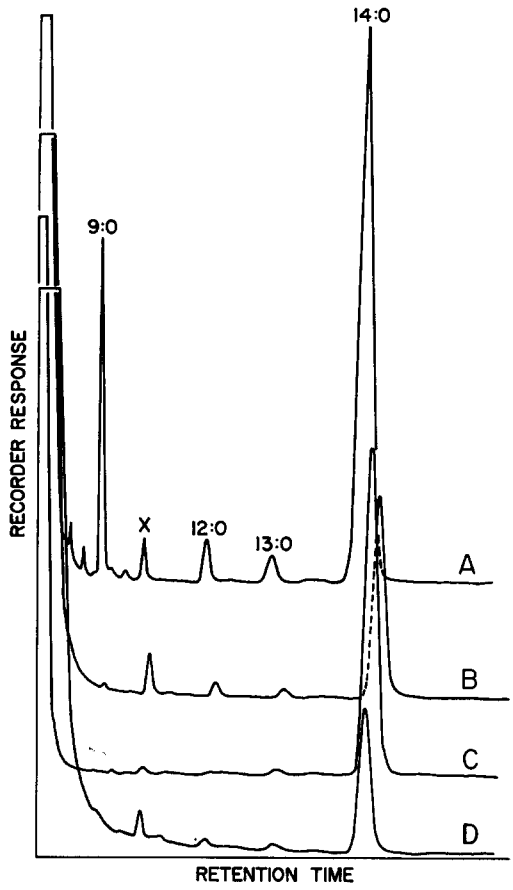

FIG. 9. GLC analysis of 1,3-dioxolanes prepared from intact aorta sphingolipids and an LCB fraction after ozonolysis reduction: *A*, sphingomyelin; *B*, sulfatides; *C*, LCB from sphingomyelin; *D*, cerebroside–sulfatide fraction. Operating conditions are summarized in Fig. 3.

anomalous carbon number which is much higher than the corresponding *cis* compound (24). The R_{18} data for unsaturated dioxolanes from LCB may be explained by the presence of a *trans* double bond in the α,β-position.

The location of double bonds in sphingadienine was indicated both by reductive ozonolysis with GLC analysis of the α,ω-didioxolane derivatives and by permanganate–periodate oxidation with GLC analysis of the methyl esters of the dibasic acid derivatives. The reductive ozonolysis procedure had no advantage over the permanganate–periodate oxidation procedure described by Chang and Sweeley (22) except that smaller amounts of overoxidation artifacts may have been formed (Figs. 6 and 7).

The major LCB components of sphingolipids are homologues of 4-sphingenines and these components are converted to aldehydes by the reductive ozonolysis of intact sphingolipids. These saturated aldehydes were readily identified and quantified as dioxolanes. The presence of 4-sphingenine and 4-eicosasphingenine in brain gangliosides was demonstrated by this procedure. Reductive ozonolysis of intact aorta sphingolipids showed that neither the cerebroside–sulfatide fraction nor the sulfatide fraction contained identifiable amounts of 4-eicosasphingenine.

The relative composition of the LCB from human aorta sphingomyelin was similar but not identical to the relative composition of LCB from human plasma sphingomyelin. Karlsson (2) found more 4-hexadecasphingenine and less sphinganine in human plasma. Aorta sphingomyelin contained no heptadecasphinganine or 4*D*-hydroxysphinganine since these LCB components would have yielded a 15:0 aldehyde by periodate oxidation. A small amount of heptadecasphinganine has been identified in plasma (2). Trace amounts of tetradecasphinganine and 4-tetradecasphingenine were found in aorta sphingomyelin and these LCB have not been reported in human plasma sphingomyelin. Aorta sphingomyelin also contained trace amounts of an unknown sphinganine and two unknown sphingadienines which have not been reported in human plasma.

The double bonds in the major sphingadienine from aorta were separated by 10 carbon atoms and it is very likely that this sphingadienine was identical with the major sphingadienine, 4,*x*14-sphingadienine, in human plasma (2, 4). Small amounts of a second isomer, sphingadienine$_b$, were identified by 16:2$_b$ dioxolane peaks in both the total mixture and the diene fraction separated on TLC. This isomer may correspond to the 4,*x*12-sphingadienine tentatively identified in human plasma sphingomyelin by Karlsson (25) or the 4,*x*13-sphingadienine found in bovine heart sphingomyelin by Popovič (7). Polito et al. (4) have suggested that the C_9 and C_8 cleavage products they obtained from human plasma sphingomyelin were overoxidation artifacts. The concentration of the 16:2$_b$ dioxolane was too low for us to distinguish between overoxidation and the presence of sphingadienine isomers. The third diene isomer that was separated as a dioxolane derivative on TLC, 16:2$_a$, had the same R_{18} as the major 16:1 dioxolane and this LCB derivative may merely represent the incomplete separation of monoene and diene dioxolane fractions.

Dioxolanes from the LCB fraction of human aorta sphingomyelin contained a small peak which was tentatively identified as the 16:1br derivative of a branched-chain 4-sphingenine. This LCB component may correspond to the branched-chain 4-sphingenine obtained from bovine kidney sphingomyelin by Carter and Hirschberg (26). Hydrogenation and reductive ozonolysis data suggested that the 15:1 dioxolane peak contained a second component, which did not yield the 15:0 dioxolane after hydrogenation or the 13:0 dioxolane after reductive ozonolysis. These data would be explained by the presence of an isomer of sphingenine in which the double

bond was not at the 4,5-position. Additional studies are required before the presence of this suggested isomer is established.

We are indebted to Doctor R. H. McCluer for suggestions and comments and to Doctor D. W. Buntine for the collection of aorta tissue.

This study was supported in part by PHS Research Grants HE-11897 and GM-09506, a grant from the United Health Foundations, Inc., and a postdoctoral fellowship (Doctor Panganamala) from the Central Ohio Heart Association.

Manuscript received 27 December 1968; accepted 14 April 1969.

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