Structure of plasma sphingadienine

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ABSTRACT The dienic long-chain base (sphingadienine) of human plasma sphingomyelins has been identified as D-*erythro*-1,3-dihydroxy-2-amino-4-*trans*-14-*cis*-octadecadiene. A similar sphingosine was also detected in plasma sphingomyelins of rat, rabbit, and cat. The key reaction in the structural studies was partial reduction of sphingadienine with hydrazine to *cis*-14-sphingenine and 4-sphingenine.

SUPPLEMENTARY KEY WORDS catalytic hydrogenation · sphinganine · specific rotation · 3-deoxysphinganine formation · thin-layer chromatography · partial hydrazine reduction · *cis*-14-sphingenine · 4-sphingenine

Several groups (1–3) have studied the structure of sphingadienine, the second largest long-chain base component of human serum sphingomyelins. Evidence has been presented that this LCB (Fig. 1, IV) is an *erythro*-1,3-dihydroxy-2-amino compound of a straight C_{18} chain, with olefinic bonds at positions 4 and 14; one of these double bonds appears to be *trans*, the other *cis*. The present work concludes these structural studies and shows that the base belongs to the D-series and has the *trans* double bond at C-4 and the *cis* double bond at C-14. In other words, sphingadienine differs from the most common animal sphingolipid base, 4-sphingenine, only in having an extra *cis* double bond at C-14. Sphingadienine is shown to be a significant component not only in human plasma, but in animals as well.

MATERIALS AND METHODS

Isolation of Sphingadienine

Lipids were extracted from a 2 liter sample of pooled plasma (4). Mild alkaline methanolysis of the extract was carried out as described previously (4); this treatment splits off the acyloxy groups of glycerolipids. Chromatography of the methanolysate (9 g) on a column of 150 g of silicic acid (Unisil; Clarkson Chemical Co., Williamsport, Pa.), according to Rouser, Kritchevsky, and Yamamoto (5), gave 755 mg of pure sphingomyelin. This was converted to ceramides with phospholipase C of *Clostridium welchii* (EC 3.1.4.3) as described elsewhere (6). The ceramides (610 mg) were hydrolyzed with 1N KOH in methanol–water 9:1 as described by Carter, Rothfus, and Gigg (7). The mixture of LCB (210 mg) was isolated by silicic acid chromatography (8) and acetylated with acetic anhydride and pyridine (6). Triacetyl sphingadienine was finally isolated by subjecting the total LCB triacetates (262 mg) to argentation chromatography on thin-layer plates with chloroform–methanol 98:2 (see Fig. 3). The yields were 182 mg of the fast-moving monoene fraction and 42 mg of the slow-moving diene fraction; both appeared homogeneous on argentation TLC. The diene fraction proved to be triacetyl sphingadienine.

Reference LCB. Essentially pure 4-sphingenine was obtained from bovine brain sphingomyelin by techniques similar to those described above for sphingadienine. Its catalytic hydrogenation gave sphinganine. *Threo*-sphinganine was a synthetic sample obtained from Dr. E. F. Jenny, Basel, Switzerland. Diacetyl 3-deoxysphinganine, together with triacetyl sphinganine, was obtained by catalytic reduction of triacetyl 4-sphingenine.

Chromatographic Methods

Thin-layer chromatography (TLC) on Silica Gel G (E. Merck A.G., Darmstadt, Germany) was carried out by the standard ascending technique on 200 × 200 × 0.25 mm plates activated at 110–120°C for 30 min (9). The tanks were not lined with filter paper. For argenta-

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Some of the experiments presented are taken from the doctoral thesis of E. L. Hirvisalo (Helsinki, 1969. Annales Academiae Scientiarum Fennicae, Series A. II. Chemica 146).

Abbreviations: LCB, long-chain base(s); TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

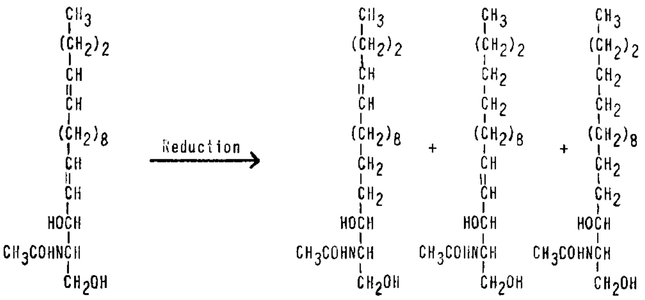

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FIG. 1. Structures of the three N-acetyl LCB isolated after partial reduction of N-acetyl sphingadienine (which in turn resulted from ester-bond deacetylation of triacetyl sphingadienine under reduction conditions).

tion TLC the adsorbent slurry was prepared in 10% $AgNO_3$ solution instead of in water. Staining of all analytical plates was carried out with ammonium hydrogen sulfate (10). In preparative TLC the plates were sprayed with water (11). The intact lipids were eluted from the adsorbent with chloroform–methanol–acetic acid 20:10:1; the eluates were washed with water to remove silver nitrate, acetic acid, and other impurities.

Gas–liquid chromatography (GLC) of fatty acid methyl esters (12), of $NaIO_4$-generated aldehydes (1), and of di-O-trimethylsilyl ethers of free and N-acetyl bases (13) was carried out as described in the cited papers. The dicarboxylic acid dimethyl esters were chromatographed on an EGSS-X column at 170°C.

Preparation of LCB Derivatives

Triacetyl LCB were prepared by treating free or N-acetyl bases with pyridine and acetic anhydride as described elsewhere (6). N-acetyl LCB were prepared by subjecting the triacetyl compounds to mild alkaline methanolysis (4), which cleaves off the ester-linked acetyl groups. Free bases were obtained from triacetyl and N-acetyl derivatives by strong alkaline methanolysis under conditions described by Carter, Rothfus, and Gigg (7). Trimethylsilyl ethers of free or N-acetyl bases were prepared by the silylation procedure of Carter and Gaver (13).

Oxidation Procedures

Free LCB were oxidized with periodate to aldehydes by the procedure of Sweeley and Moscatelli (1).

Rudloff oxidation of N-acetyl or triacetyl LCB to carboxylic acids was carried out by a modification of the procedure of Davidoff and Korn (14). Reagent solution A consisted of 51.2 mg of K_2CO_3, 60 ml of *tert* butanol, and 40 ml of water. Reagent solution B consisted of 16 mg of $KMnO_4$, 854 mg of $NaIO_4$, and 100 ml of water. The lipid (0.5–1 mg) was evaporated to dryness, dissolved in 0.5 ml of solution A, and 0.5 ml of solution B was added. The mixture was stirred at room temperature for 2 hr, decolorized with acidic sodium bisulfite (and occasionally with sodium thiosulfate), and extracted 2–3 times with 3-ml portions of ether. Free fatty acids were identified in the ether extract by TLC on prefabricated silica gel plates (E. Merck A.G., Darmstadt, Germany) in hexane–ether–acetic acid 50:50:1; the fatty acids were detected by staining according to Dudzinski (15). The ether extract was finally treated with diazomethane, and the fatty acid methyl esters were subjected to GLC. Dicarboxylic acids generated by Rudloff oxidation were identified as dimethyl esters after TLC on Silica Gel G in hexane–ether 9:1. Subsequently they, too, were identified by GLC.

Certain samples were also oxidized by a combination of the two procedures described above. The aldehydes resulting from the periodate oxidation were simply subjected to Rudloff oxidation; when a double bond was present in the aldehyde chain, a dicarboxylic acid was obtained. This was isolated by TLC as a dimethyl ester and subjected to GLC for identification.

Reduction Procedures

For catalytic hydrogenation the samples were dissolved in methanol, platinum oxide was added, and hydrogen was bubbled through the mixture for 1 hr at 20°C.

Reduction with hydrazine was done as described by Privett and Nickell for fatty acids (16). 10-mg samples of triacetyl sphingadienine were dissolved in 1 ml of a 10% methanolic solution of hydrazine hydrate. After 3 hr at 37°C the reaction was stopped by addition of hydrochloric acid, and the lipids were separated from hydrophilic compounds by partition in chloroform–methanol–water 8:4:3.

Physical Measurements

IR spectra were recorded with a Perkin-Elmer Infracord type 457; the samples were in the form of a film on potassium bromide.

Specific rotations were determined with a Perkin-Elmer polarimeter type 141, using a thermostated tube 10 cm long and 0.9 ml in volume.

RESULTS AND DISCUSSION

Identification of Triacetyl Sphingadienine

Our sample migrated on regular Silica Gel G (without silver nitrate) with the same mobility as triacetyl 4-sphingenine, but on argentation plates it moved much slower than the 4-sphingenine derivative (Table 1). The same relationship was observed previously by Karlsson (2), who studied the dinitrophenyl derivatives.

TABLE 1 Analytical Properties of the Four C_{18}-LCB Isolated after Partial Reduction of Sphingadienine

Configuration of LCB		3-DS Formation during Catalytic Hydrogenation	GLC* of		TLC† of		Argentation TLC† of	
Double Bond at C-4	Double Bond at C-14		TMSi Derivatives on SE-30	HIO_4-Generated Aldehydes on EGSS-X	Free Bases on Silica Gel G	Triacetates on Silica Gel G	*N*-Acetyl Derivatives	Triacetates
+	+	+	0.87	2.56	1.20	1.05	1.00	1.00
−	+	−	0.99	1.28	1.00	1.00	1.14	1.00
+	−	+	0.87	1.95	1.20	1.05	1.41	1.50
−	−	−	1.00	1.00	1.00	1.00	1.50	1.50

3-DS, 3-deoxyspinganine.
* Relative retention times.
† Relative R_f-values.

The IR spectrum of triacetyl sphingadienine was very similar to that of triacetyl 4-sphingenine; in particular the peaks at 970 cm^{-1} were of similar intensity. This confirms the observations of Karlsson, who found that the *trans* double bond peaks of dinitrophenyl derivatives of sphingadinenine and 4-sphingenine were of equal intensity (2) and concluded that sphingadienine contains only one *trans* double bond.

Conversion of triacetyl sphingadienine into free base, and subsequently into the trimethylsilyl ether gave a sample which in GLC on SE-30 showed a major peak (98%) with the same retention time as the trimethylsilyl ether of 4-sphingenine (Table 1). Gaver and Sweeley (17) have also reported that these two bases are inseparable on SE-30. Catalytic hydrogenation of the free base gave a sample with the R_f of sphinganine on TLC (Fig. 4). Trimethylsilylation of the hydrogenated base gave a derivative with the same GLC behavior as di-*O*-trimethylsilyl sphinganine. Periodate oxidation of sphingadienine and GLC of the resulting aldehyde gave a peak with the same relative retention time (2.56 times that of hexadecanal) as reported by Sweeley and Moscatelli (1) on a similar polyester column (Table 1). Analogous treatment of the hydrogenated base gave hexadecanal. These observations confirm previous findings of Karlsson and Sweeley (1–3) and show that sphingadienine has 18 carbon atoms.

In marked contrast to the monoene LCB (3, 8) of plasma sphingomyelin, sphingadienine is not accompanied by large amounts of homologues. One lower homologue, however, was probably present in our sphingadienine. GLC of the trimethylsilylated sample revealed a minor peak (2%) which was tentatively identified as the trimethylsilyl ether of heptadecasphingadienine. The carbon number of the minor peak was 16.68 and that of the sphingadienine peak 17.60 (based on trimethylsilyl ethers of saturated *erythro* LCB). Polito, Akita, and Sweeley (3) have also detected small amounts of this base.

Rudloff oxidation of triacetyl sphingadienine gave pure sebacic acid (C_{10} dicarboxylic acid), which was identified by TLC and GLC. This confirms the result of Karlsson (2, 8) and Polito, Akita, and Sweeley (3) and shows that the distance between the two olefinic centers is 10 carbon atoms.

Mild alkaline methanolysis (4) converted triacetyl sphingadienine to the *N*-acetyl derivative. This was hydrogenated catalytically and subsequently silylated. GLC of the resulting *N*-acetyl-di-*O*-trimethylsilyl derivative on SE-30 (13) showed that it was identical with the corresponding derivative of sphinganine, and different from the synthetic *threo* sphinganine derivative. No *threo* isomer was detected in the natural sample. Karlsson (2) and Polito, Akita, and Sweeley (3) have previously suggested that plasma sphingadienine has *erythro* configuration. Our experimental evidence for this structure is worth recording since it concerns hydrogenated sphingadienine directly. Karlsson studied total LCB of plasma sphingomyelin and not pure sphingadienine; in addition he found traces of *threo* isomers. Polito, Akita, and Sweeley in turn draw their conclusion from GLC behavior of the native sphingadienine, and this implies that they could not make direct comparisons with synthetic models.

Specific Rotation of Triacetyl Tetrahydro-Sphingadienine

N-acetyl sphingadienine was hydrogenated catalytically and then converted to the fully acetylated form. The product appeared pure on TLC, and its specific rotation was determined:

$$[\alpha]_D^{22} = +6.3° \text{ (6.8 mg/ml in pyridine).}$$

The monoene LCB of plasma, mainly 4-sphingenine, were likewise converted to *N*-acetyl derivatives, hydrogenated, and acetylated. The product was pure according to TLC, and its specific rotation was:

$$[\alpha]_D^{22} = +6.3° \text{ (6.3 mg/ml in pyridine)}$$

$$[\alpha]_D^{22} = +16.9° \text{ (6.3 mg/ml in chloroform)}.$$

Carter, Norris, Glick, Phillips, and Harris (18) found that the specific rotation of triacetyl sphinganine prepared from natural lipids was:

$$[\alpha]_D^{28} = +18.1° \text{ (10 mg/ml in chloroform)}.$$

Thus it seems that both fractions of plasma LCB have the usual D-*erythro*-configuration at C-2 and C-3.

Hydrazine Reduction of Triacetyl Sphingadienine

Partial reduction of double bonds with hydrazine has been used to locate *cis* and *trans* bonds, if both are present in polyenoic fatty acids (16). This reaction seemed attractive also in the study of sphingadienine structure. If the reduction of sphingadienine proceeds nonselectively and the two double bonds are attacked at similar rates, the partially reduced sample should contain four products (shown in Fig. 1); these are fully saturated molecules (I), two monoenic LCB (II and III), and some intact starting material (IV).

TLC on Silica Gel G showed that the reaction products obtained from the triacetyl base were *N*-acetyl LCB; the alkaline conditions during the reduction had caused cleavage (possibly hydrazinolysis) of the ester-linked acetate groups. Argentation TLC of the reaction mixture (Fig. 2) showed that four components were indeed present. Comparison with reference samples on TLC showed that the fastest moving component (fraction I) was the fully saturated molecule; the second (fraction II) was the ordinary 4-sphingenine derivative; the third (fraction III) proved (see below) to be a novel LCB derivative which contains only a *cis* double bond at C-14; the slowest component (fraction IV) was sphingadienine that had survived the reduction.

Since the separations between I and II and between III and IV were not easily achieved in the argentation system, the following procedure was finally adopted for preparative fractionation of the reduction mixture. The sample was converted into fully acetylated form and the triacetyl LCB (25.4 mg) were separated by argentation TLC into two fractions (Fig. 3). The fast zone (9.7 mg) contained molecules I and II, and the slow zone (11.5 mg) consisted of III and IV. The two fractions were then hydrolyzed with alkali, and the free LCB were separated by TLC on ordinary Silica Gel G. Fig. 4 shows that both pairs were well resolved with chloroform–methanol–2 N ammonia 40:10:1 (19) as solvent; fractions II and IV moved faster than I and III in this system. We believe that the double bond at C-4 increases the mobility of the LCB by decreasing the basicity of the amino group at C-2.

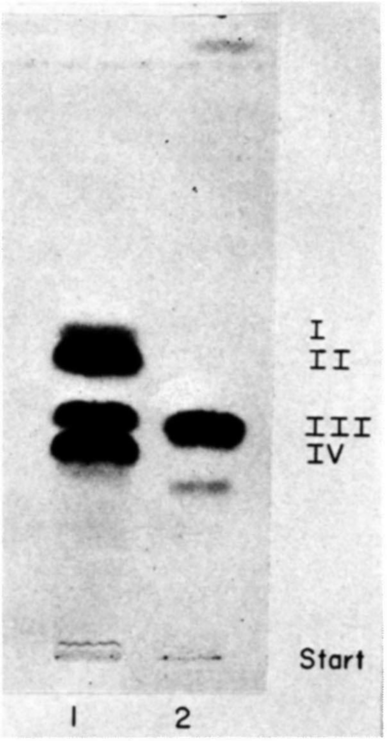

FIG. 2. Argentation TLC of *N*-acetyl LCB. 1, the reaction mixture obtained from partial reduction of *N*-acetyl sphingadienine with hydrazine; 2, *N*-acetyl *cis*-14-sphingenine isolated from the mixture. The plate was developed three times with chloroform–methanol 94:6.

FIG. 3. Argentation TLC of triacetyl LCB. 1, monoenic triacetyl LCB from plasma sphingomyelins; 2, triacetyl LCB derived from partial hydrazine reduction of sphingadienine triacetate. 3, triacetyl sphingadienine derived from plasma sphingomyelins. The plate was developed with chloroform–methanol 98:2.

The four fractions were acetylated and weighed: I, 2.1 mg; II, 4.6 mg; III, 3.9 mg; IV, 6.8 mg. The IR spectra were then recorded: fraction II revealed *trans* olefinic bonds (peak at 970 cm^{-1}), but fraction III did not (Fig. 5).

Fig. 4. TLC (on regular Silica Gel G) of free LCB. 1, reference sphinganine; 2, sphinganine derived from sphingadienine; 3, free LCB of the fast triacetate zone from argentation separation of the reduction mixture; 4, free LCB of the slow triacetate zone from argentation separation of the reduction mixture; 5, 4-sphingenine; 6, sphingadienine. The plate was developed with chloroform–methanol–2 N ammonia 40:10:1.

Rudloff oxidation of the triacetate of fraction II gave myristic acid, which was identified by TLC of the free acid and GLC of the methyl ester after preliminary purification on TLC (Fig. 6). This fixes the *trans* double bond at a distance of 14 C-atoms from the methyl end of the chain, i.e. at C-4. One of the double bonds in sphingadienine must be at C-4 also to explain the finding that catalytic hydrogenation of triacetyl sphingadienine yielded diacetyl 3-deoxysphinganine, which was identified by GLC (3) and TLC (Fig. 7). The reaction responsible for the formation of this compound (elimination of the acetoxy group at C-3) is typical of allylic systems and fixes one of the double bonds at C-4 (20).

Rudloff oxidation of the triacetate of fraction III did not yield any long- or medium-chain fatty acid. But periodate cleavage between C-2 and C-3 of the free base of fraction III followed by Rudloff oxidation of the double bond gave C_{12} dicarboxylic acid, which was identified as the dimethyl ester on TLC, isolated by preparative TLC, and finally identified also by GLC (Fig. 8). This fixes the *cis* double bond of fraction III, and presumably of sphingadienine, at a distance of 12 carbon atoms from C-2, i.e. at C-14. This finding fully confirms the oxidation results obtained with fraction II and with the original sphingadienine. Combination of the periodate cleavage and the Rudloff oxidation did not give C_{12} dicarboxylic acid from fraction II or from natural 4-sphingenine.

Table 1 shows some analytical properties which help to differentiate the four LCB found after partial reduction of sphingadienine. The regular patterns of these properties confirm our conclusions concerning structure.

Presence of Sphingadienine in Different Plasma Samples

Sphingadienine was preparatively isolated and extensively characterized from three pooled plasma samples from 4–150 individuals. Furthermore, 10 individual plasma samples were analyzed quantitatively with GLC techniques. All sphingomyelin LCB contained 20–24% sphingadienine.

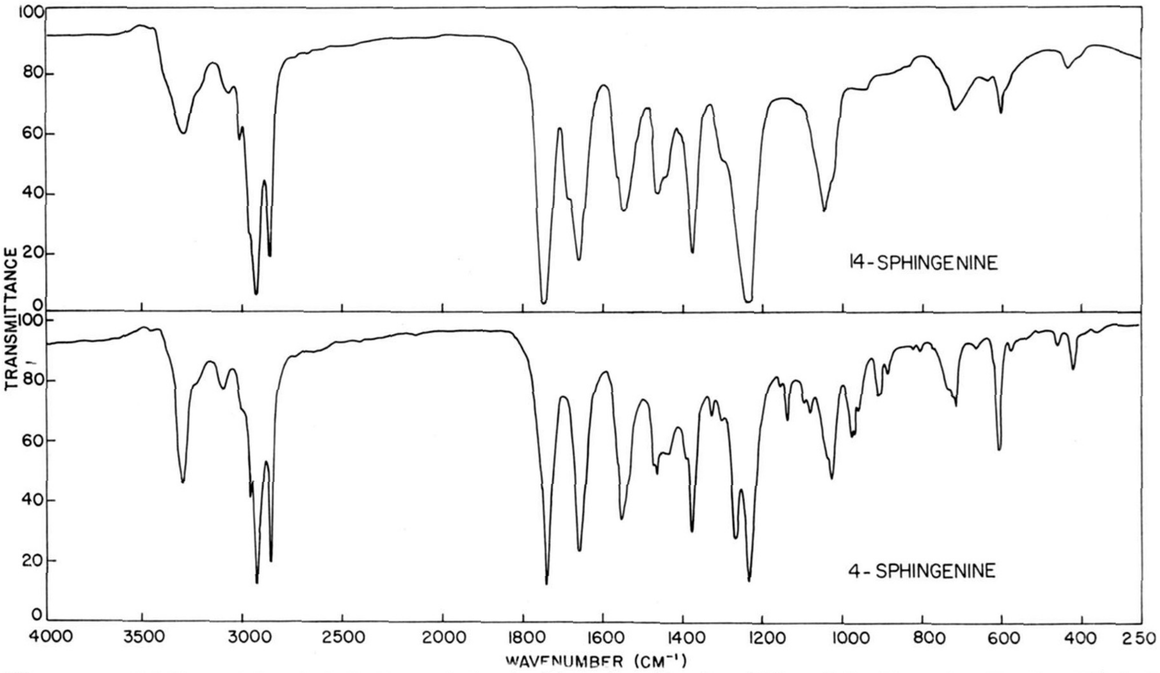

Fig. 5. IR spectra of fully acetylated derivatives of *cis*-14-sphingenine (fraction III) and 4-sphingenine (fraction II) derived from sphingadienine (film on KBr).

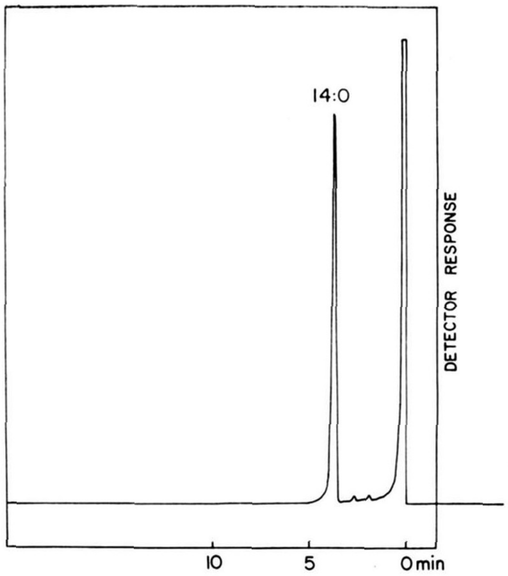

FIG. 6. GLC of fatty acid methyl esters derived by Rudloff oxidation of fraction II.

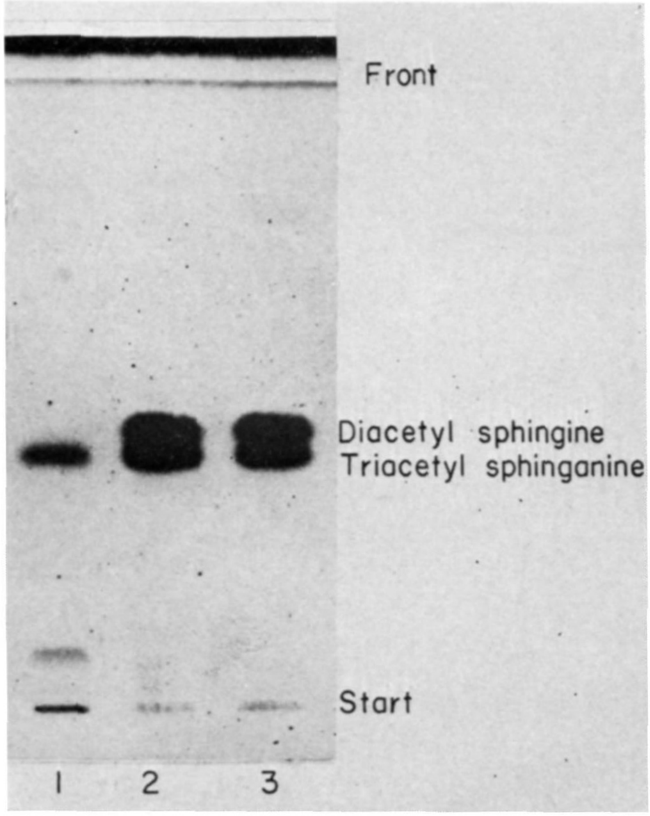

FIG. 7. TLC of diacetyl 3-deoxysphinganine (diacetyl sphingine) and triacetyl sphinganine. 1, triacetyl sphinganine; 2, reaction products of catalytic hydrogenation of monoenic LCB of plasma; 3, reaction products of catalytic hydrogenation of sphingadienine. The plate (Silica Gel G) was developed twice with chloroform–methanol 99:1.

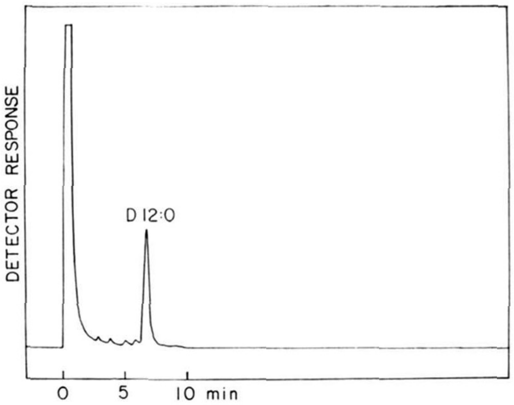

FIG. 8. GLC of dicarboxylic acid dimethyl esters derived by combined periodate cleavage and Rudloff oxidation of fraction III.

Sphingadienine in Plasma Sphingomyelins of Animals

LCB were isolated from serum sphingomyelins of rat, rabbit, and cat. In all these samples a sphingadienine-like compound was detected, and it was present in the same relative amounts as in human sphingomyelins. The dienic base was identified by argentation TLC of triacetyl LCB and by GLC of periodate generated aldehydes. This finding may stimulate in vitro studies on the biosynthesis of sphingadienine with liver homogenates.

In sources other than plasma, sphingadienine has been detected only in small amounts in kidney sphingolipids (21–22) and possibly in yeasts (3). It remains to be seen how widely distributed it is in natural lipids.

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