Methods for Methanolysis of Sphingolipids and Direct Determination of Long-Chain Bases by Gas Chromatography

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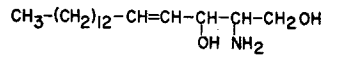
Abstract

A new, simple procedure is described for the direct gas chromatographic determination of sphingolipid bases as their trimethylsilyl derivatives. This method is used to study the nature of the mixture of bases produced by acidic methanolysis of ceramides, sphingomyelins, free sphingolipid bases, and *N*-acetyl derivatives of the latter. Cleavage of sphingolipids with anhydrous methanolic hydrogen chloride results in the production of considerable quantities of secondary products from sphingosine, such as the *O*-methyl ethers, which are not formed during methanolysis of free sphingosine. A modified reagent for methanolysis, containing methanol, water and hydrochloric acid, reduces the yield of these by-products to low levels. Preliminary purification of the sphingolipid bases, before gas chromatography, is achieved by chromatographic procedures. Evidence is presented for the occurrence of C_{16}-sphingosine in sphingomyelin from human blood plasma.

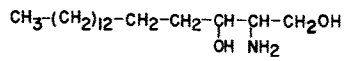
Introduction

AT THE PRESENT TIME, six long-chain bases are known to occur as constituents of various sphingolipids such as ceramide, sphingomyelin, cerebroside, more complex neutral glycolipids, and gangliosides. Their structures are shown below.

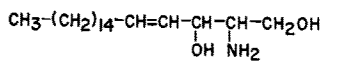
SPHINGOSINE



DIHYDROSPHINGOSINE



C_{20}-SPHINGOSINE



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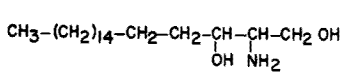
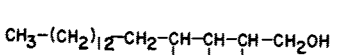
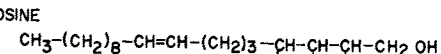


PHYTOSPHINGOSINE



DEHYDROPHYTOSPHINGOSINE



In addition, evidence has been found for the occurrence of a number of related bases whose structures have not yet been elucidated (11,19,27,29). Studies on the chemistry and metabolism of the sphingolipid bases have been hindered to some extent by a lack of adequate micromethods for isolation, purification, and qualitative and quantitative determinations of these compounds.

A great variety of conditions has been used for the cleavage of sphingolipids, including sulfuric or hydrochloric acid in methanol, ethanol or water, or sodium, potassium or barium hydroxide in one of the same solvents. The most commonly employed reagent, acidic methanol (6,11,13,15,28), generally gives good yields of total long-chain base but also leads to the formation of undesirable by-products of sphingosine such as 3-*O*-methylsphingosine (4), 5-*O*-methyl-Δ^3-sphingosine (33) and other products (10); as a result, low yields of sphingosine are always observed. Although these secondary products of acidic methanolysis are not formed under alkaline conditions (3,32), the latter reagents appear to be unsuitable for the isolation of free sphingosine in high yields from sphingomyelin (6).

Sphingolipid bases are extracted from hydrolysates with solvents such as diethyl ether (5), chloroform (17), ethyl acetate (15) or mixed solvents such as 2% isoamyl alcohol in heptane (1). The bases are further purified, usually in the form of a derivative, by fractional crystallization (5), countercurrent distribution (2) or column chromatography (12,20,35), and frequently more than one of these techniques is necessary to achieve complete purification.

Various colorimetric methods are available for the quantitative determination of total sphingolipid bases after hydrolysis (1,15,16,21–23); none of the procedures described to date is specific for a particular base or by-product.

Most promising are the recently described techniques for separating the bases by thin-layer chromatography (TLC) or gas chromatography. Several TLC systems have been reported for the separation of the bases (7,14,24), but none of them gives sufficient separation of the various stereoisomers of sphingosine and dihydrosphingosine for quantitative determinations of the isomers. Gas chromatography can be used for a rapid and precise qualitative and quantitative estimation of the bases, but requires a relatively time-consuming preliminary oxidation of the bases with periodate (29).

In this report we wish to describe a simplified procedure for the isolation and purification of sphingolipid bases, and a method for their direct determination by gas chromatography. The modified reagent described for acid-catalyzed methanolysis of the sphingolipids greatly reduces the yield of *O*-methyl sphingosine and other by-products.

Experimental

Sphingolipids and Sphingolipid Bases

Synthetic *erythro*-dihydrosphingosine was obtained from D. Shapiro and was also synthesized in this laboratory by published methods (25,26). Synthetic *erythro-trans*-sphingosine, *threo-trans*-sphingosine, and *threo*-dihydrosphingosine were obtained from E. F. Jenny. *Erythro-trans*-sphingosine was also prepared from ceramide by the method of Tipton (32). Samples of triacetyl-sphingosine and *O*-methyl sphingosine were gifts of H. E. Carter.

N-Acetyl *erythro*-dihydrosphingosine (mp 122–127C) was prepared in quantitative yield by reacting the free base with an excess of acetic anhydride in methanol at room temp overnight. The product was

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collected by filtration following precipitation by the addition of water. Crystalline *N*-acetyl *erythro-trans*-sphingosine (mp 85–87C) was prepared in a similar manner. The *N*-acetyl derivatives were identified by melting points, IR spectra, elemental analyses, and TLC (Silica Gel G, chloroform:methanol 93:7) (8).

Crude sphingomyelin was prepared from beef brain by a hot ethanol extraction of the residue left after extraction of the tissue with acetone, cold ethanol, and petroleum ether. Preliminary purification was achieved by the method of Rapport and Lerner (20), and pure sphingomyelin was eluted from heat-activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.) with 20% methanol in chloroform containing 2.0% water. Sphingomyelin was also isolated from human plasma by a previously described method (28). Both samples of sphingomyelin gave a single spot on TLC.

Cerebroside was purified from commercially available preparations of crude sphingolipids (Armour spinal cord preparation and Wilson crude sphingomyelin) by repeated precipitations from hot glacial acetic acid, followed by chromatography on silicic acid and Florisil columns. A portion of this material was converted to ceramide (Ceramide C) by the procedure of Carter et al. (3).

Ceramides A and B were isolated from a commercial beef-lung preparation (Viobin Corp., Monticello, Ill.) by the method of Tipton (32).

Thin-layer chromatography of all of the above compounds is shown in Figure 5.

Methanolysis Procedures

Anhydrous Methanolic HCl. To a sample of 12.5 mg of beef-brain sphingomyelin, in a screw-capped (Teflon-lined) vial, was added 6 ml of freshly prepared 1.2N anhydrous methanolic HCl (gaseous HCl bubbled into reagent methanol to desired concn). The mixture was heated for 8 hr at 72C in a hot air oven. In a second experiment the methanolysis was carried out for 17 hr at 70C. Experiments with ceramides, free bases and their *N*-acetyl derivatives were carried out under the same conditions.

Aqueous Methanolic HCl. The reagent was prepared by diluting 8.55 ml of concd hydrochloric acid to 100 ml with methanol, giving 1N HCl. Ceramide B (4.0 mg) was reacted with 1 ml of reagent in a screw-capped tube for 18 hours at 70C.

Modified Aqueous Methanolic HCl. The modified reagent was prepared by diluting 8.6 ml of concentrated hydrochloric acid and 9.4 ml of water to 100 ml with reagent methanol, giving 1N HCl which was 10M with respect to water. Ceramide B, beef-brain sphingomyelin, and sphingomyelin from human plasma were reacted with this reagent (2 mg/ml) at 70C for 18 hr.

Isolation of the Free Bases

Following methanolysis, the reaction mixtures were cooled to room temp, evaporated to dryness under a stream of nitrogen, and the residues were dissolved in a small volume of methanol. This solution was applied to a small column containing 1 gm of CG-45 resin (Type 1, OH form, Rohm and Haas, Philadelphia, Pa.) in methanol. The resin was prepared for use by washing several times with methanol, decanting the fines each time, after which the resin was dried in vacuo. The column was eluted with 15 ml of methanol and the eluate was evaporated to dryness. The residue was dissolved in a small volume of chloroform and the solution was applied to a small column containing 1 gm of silicic acid (Unisil, 200–325 mesh). Fatty acids and methyl esters were eluted with 15 ml of anhydrous chloroform and the free sphingolipid bases with 15 ml of methanol.

Formation of the Trimethylsilyl Derivatives

The methanol eluate above was evaporated to complete dryness and mixed with sufficient silanes reagent to make a 1% solution. The silanes reagent, prepared according to the directions of Sweeley et al. (31), consisted of 1 ml of trimethylchlorosilane, 2 ml of hexamethyldisilazane and 10 ml of dry pyridine which had been predistilled over potassium hydroxide or barium oxide. The silanes were used as supplied by Applied Science Laboratories, State College, Pa.

Gas Chromatography

After thorough mixing and standing at room temp for several minutes, an aliquot of the reaction mixture was injected directly into an F & M Model 400 gas chromatograph, equipped with flame ionization detector. The U-shaped glass column (6 ft long and 1/8 in. I.D.) was packed with 2.5% SE-30 on 100/120 mesh acid-washed, silanized Gas Chrom S (34). The column was maintained at 180C and the injection port at approximately the same temp. A small quantity of added methyl stearate served as a reference compound for the chromatographic recordings. Areas were calculated from peak heights and widths at half height.

Thin Layer Chromatography

Preparative TLC plates (8 in. × 8 in.) were made from Silica Gel G (according to Stahl) using a commercial spreading device (Brinkman Instruments Inc., Great Neck, Long Island, N.Y.). Silica Gel G (30 gm) was weighed into a 250 ml glass-stoppered Erlenmeyer flask, 60 ml of 0.01M sodium carbonate was added, and the mixture was shaken vigorously for 15 to 30 sec to give a lump-free suspension. After spreading, the plates were left for about an hour at room temp and then heated at 125–130C for several hours or overnight before use.

The plates were developed, in a rectangular tank lined with Whatman No. 3 paper, with chloroform:methanol:water 100:42:6 as solvent. Spots were detected with iodine vapor, and permanent records were made using a Polaroid 800 camera with an Ednalite Duraklad coated 80C (clear flash) filter.

Results

Initial experiments on conditions for gas chromatography of the trimethylsilyl (TMS) derivatives were carried out with synthetic sphingolipid bases; comparisons were made of a variety of stationary liquid phases and operating parameters. Best results were obtained with a nonpolar SE-30 column, on which complete separation of the TMS derivatives of sphingosine and dihydrosphingosine was achieved. As shown in Figure 1, the dihydrosphingosine derivative (upper left) was eluted in about 30 min and its retention time relative to TMS sphingosine (upper right) was 1.14. The initial peak on each record, with retention time of about 10 min, is added methyl stearate. The minor peaks which occur along with the TMS sphingosine, from beef lung ceramide, are presumably due to other bases. After acetylation of the sphingosine with acetic anhydride in methanol, followed by reaction with the silanes reagent, the record shown at the bottom of Figure 1 was obtained. Although the TMS derivative of *N*-acetyl sphingosine can also be determined by this method, the retention time is more than twice that of the TMS derivative of

TABLE I

GLC Analysis of Long-Chain Bases from Beef Brain Sphingomyelin

	Rel. retention time[a]	Area percent	Area percent after acetylation
Unknown A	2.81±.05	26.1	13.1
3-*O*-Methyl sphingosine	3.08±.08	45.7	19.9
Sphingosine	3.68±.08	23.1	11.6
Dihydrosphingosine	4.19±.08	2.6	.....
N-Acetyl unknown A	6.84	.....	8.4
N-Acetyl 3-*O*-methyl sphingosine	8.00	.....	25.1
N-Acetyl sphingosine	8.38	.....	10.1

[a] Retention times are relative to methyl stearate.

the free base. The retention time of pure TMS *O*-methyl sphingosine was 0.83 relative to TMS sphingosine. *Erythro* and *threo* isomers of sphingosine and dihydrosphingosine could not be separated under these conditions.

To avoid selective enrichment of a particular base by isolations which employ extraction, an alternative method was developed for the purification of sphingolipid bases following acidic methanolysis. After evaporation residual HCl was removed by percolation of methanol solutions through columns of weak anion exchange resin. This step also served to liberate the bases as free amines, the necessary form for reaction with hexamethyldisilazane. Attempts to prepare volatile TMS derivatives from the amine hydrochlorides were not successful. Fatty acids and their methyl esters were completely separated from the bases by silicic acid chromatography. It was necessary to carry out this separation, because the retention times of methyl arachidate and methyl behenate on SE-30 were such that they would interfere with the determination of the TMS bases.

The reaction of sphingolipid bases with hexamethyldisilazane is very rapid when carried out in dry pyridine and in the presence of trimethylchlorosilane as catalyst. The TMS derivatives are presumed to be relatively unstable in the presence of moisture. Satisfactory results are obtained by direct injection of the reaction mixture itself. The white precipitate in the reaction mixture does not interfere and the silanes have the same retention time as pyridine.

An examination was made of the products of anhydrous methanolysis of sphingomyelin isolated from beef brain. As shown in Table I, the yield of sphingosine was only 23% of the total bases, as determined by gas chromatography. Almost 50% was accounted for by 3-*O*-methyl sphingosine, 26% by an unknown

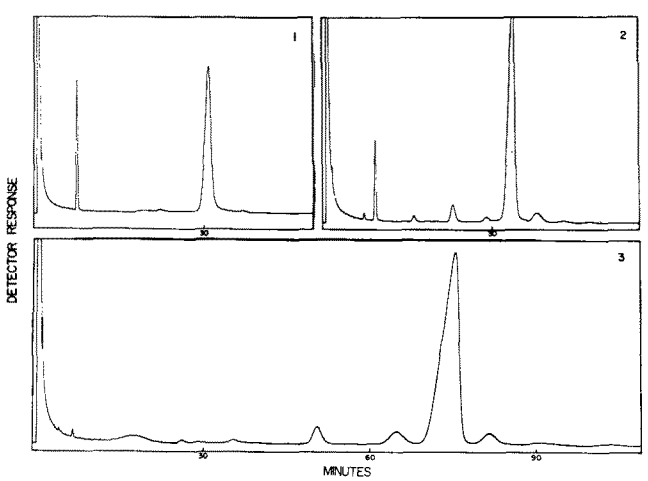

FIG. 1. GLC of the trimethylsilyl derivatives of synthetic and isolated sphingolipid bases: (1) synthetic *erythro*-dihydrosphingosine, (2) sphingosine prepared from ceramide by the method of Tipton (32), (3) *erythro-trans-N*-acetyl-sphingosine.

TABLE II

GLC Analyses of Long-Chain Bases from Ceramides Following Anhydrous and Aqueous Methanolysis

Component	Area percent				
	Anhydrous methanolysis			Aqueous methanolysis	
	Ceramide A	Ceramide C	Ceramide B	Ceramide B (5M H_2O)	Ceramide B (10M H_2O)
Trace unidentified[a]	2.6	1.2	3.0	1.7	.....
Trace unidentified[a]	4.2		5.7	5.7	5.4
Unknown A	19.1	17.3	14.4	5.1	.....
3-*O*-Methyl sphingosine	35.2	44.5	29.0	12.4	6.3
Sphingosine	27.6	27.1	40.6	69.8	82.4
Dihydrosphingosine	9.4	10.0	5.8	4.2	5.9

[a] The retention times of these trace constituents were 2.16 and 2.55, relative to methyl stearate.

base and 3% by dihydrosphingosine. Identifications of the products were based on comparisons of the various retention times with those of authentic standards, as well as by comparisons on TLC (Fig. 5, channel 18). After acetylation of the bases with acetic anhydride in methanol, which was not quantitative in this case, peaks corresponding to the TMS derivatives of *N*-acetyl sphingosine and 3-*O*-methyl-*N*-acetyl sphingosine were observed, along with an extra peak presumed to be the *N*-acetyl derivative of the unknown base.

To test whether the unknown base was a degradation product of a known sphingolipid base, free sphingosine, dihydrosphingosine, and their *N*-acetyl derivatives were subjected to anhydrous methanolysis. Sphingosine and dihydrosphingosine were stable under these conditions; no peaks were observed which corresponded to *O*-methyl ethers or the unknown. *N*-Acetyl dihydrosphingosine was converted quantitatively to the free base, with no concomitant production of any by-products. In contrast, however, methanolysis of *N*-acetyl sphingosine gave significant yields of 3-*O*-methyl sphingosine and a peak which corresponded to the unknown base. The remaining material could be accounted for as free sphingosine. *O*-Methyl derivatives and other products, therefore, appear to be formed from sphingosine, but only when it is in the *N*-acyl form.

The results obtained after anhydrous methanolysis of ceramides A, B and C are given in Table II. Nearly identical results were obtained in each case. The combined yields of sphingosine and dihydrosphingosine

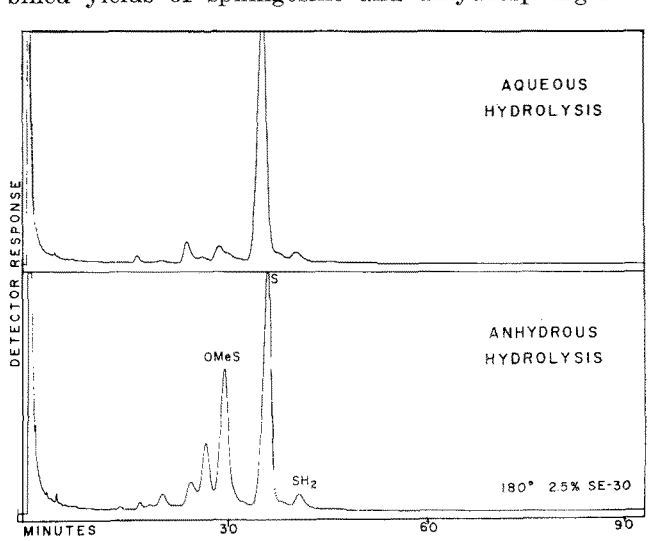

FIG. 2. Comparison of TMS derivatives of bases from ceramide B following aqueous (10M water) and anhydrous methanolysis. OMeS, 3-*O*-methyl sphingosine; S, sphingosine; SH_2, dihydrosphingosine.

TABLE III

Effect of Water on the Methanolysis Products of Sphingomyelin from Beef Brain

Component	Area percent	
	Anhydrous	Aqueous (10M water)
Unknown A	25.5	4.8
3-O-Methyl sphingosine	45.1	8.9
Sphingosine	23.4	76.6
Dihydrosphingosine	1.0	5.6
?	.....	2.9[a]

[a] The retention time of this unidentified constituent was 8.00 relative to methyl stearate.

were 37%–46%, whereas the yields of 3-*O*-methyl sphingosine were equal to or greater than those of sphingosine, and relatively high yields of the unknown base were found.

The reagent prepared from methanol and concentrated hydrochloric acid was approx 5M with respect to water. It had been reported previously that this reagent gave lower yields of *O*-methyl sphingosine than were found with anhydrous reagents (29). Comparisons of the course of methanolysis of ceramide B in aqueous and anhydrous methanolic HCl are shown in Table II. The relative yields of the unknown, 3-*O*-methyl sphingosine and several other unidentified components were reduced, with a corresponding increase in the relative yield of sphingosine. The production of by-products from *N*-acyl sphingosine is, therefore, lowered when water is present in the mixture during methanolysis.

Further reductions in the yields of by-products were observed with a special aqueous reagent consisting of 1N hydrochloric acid and 10M water in methanol. Comparisons of the products from sphingomyelin and ceramide B with this reagent and the anhydrous reagent are shown in Figures 2 and 3 and are tabulated in Tables II and III. None of the unknown base was found in the aqueous methanolic hydrolysates of ceramide B, and 3-*O*-methyl sphingosine was present in substantially lower amts, as compared with levels in anhydrous methanolysates. Similar results were obtained with sphingomyelin. The decreases in proportions of by-products can also be observed by TLC (Fig. 5). The dramatic effect of added water on the combined yield of sphingosine and dihydrosphingosine is apparent; from ceramide B these free bases accounted for 88% of total area, as compared with 46% under anhydrous conditions, and from sphingomyelin the yield increased from 24% to 82%.

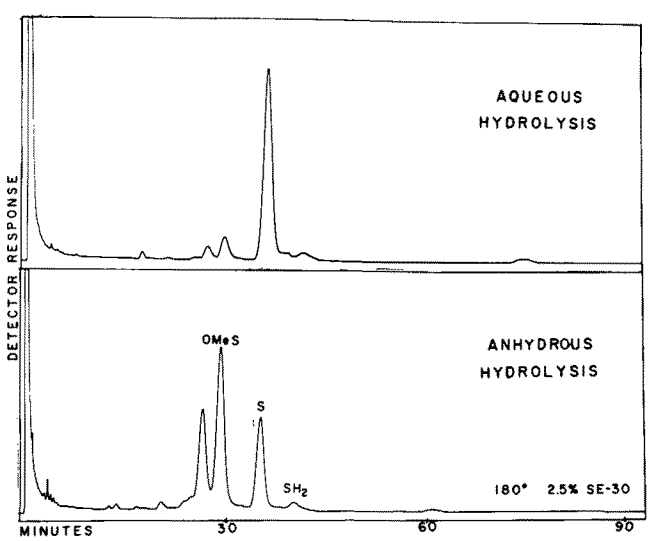

FIG. 3. Comparison of TMS derivatives of bases from beef brain sphingomyelin following aqueous (10M water) and anhydrous methanolysis. Identifications are the same as in Fig. 2.

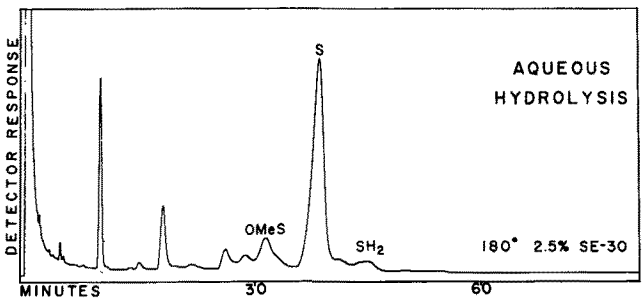

FIG. 4. GLC of TMS derivatives of bases from human plasma sphingomyelin following aqueous (10M water) methanolysis. Identifications are the same as in Fig. 2.

A sample of sphingomyelin from human plasma was hydrolyzed with the modified aqueous reagent described above. The TMS bases obtained are shown in Figure 4. The first peak after the solvent is methyl stearate. The major product was sphingosine, accompanied by a small proportion of dihydrosphingosine, as described previously (29). The reaction mixture contained small quantities of 3-*O*-methyl sphingosine, the unknown by-product base, and several unidentified substances. The most prominent unknown (8% of total area), with a retention time approximately twice that of methyl stearate, is believed to be the TMS derivative of C_{16}-sphingosine. This peak cannot be an artifact or by-product, since it is not present in mixtures obtained from other sphingolipids. Its retention time is almost exactly that which would be expected for C_{16}-sphingosine. Furthermore, the two small peaks just before that of the unknown have the same relative retention time and relative concns to the unknown as *O*-methyl sphingosine and the other by-product have to sphingosine.

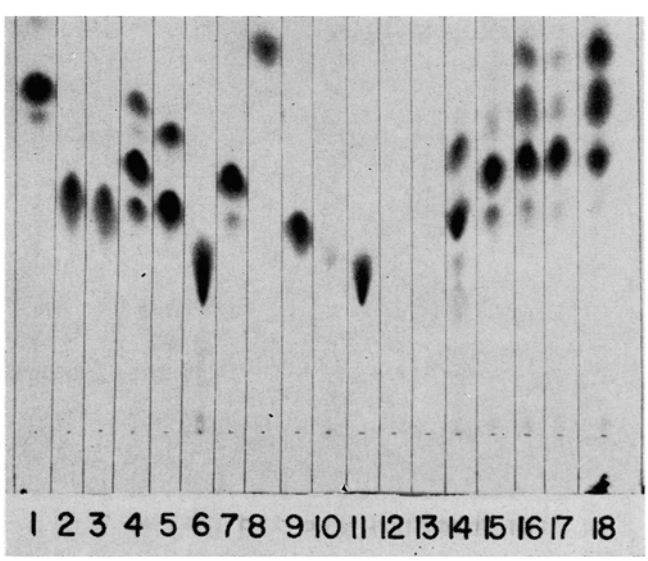

FIG. 5. TLC of sphingolipids and bases. *1* DL *erythro*-1,3-dihydroxy-2-amino-4-octadecyne, *2* DL *erythro*- dihydrosphingosine, *3* DL *threo*-dihydrosphingosine, *4* DL *erythro-trans*-sphingosine, *5* DL *threo-trans*-sphingosine, *6* sphingomyelin from Wilson's preparation, *7* sphingosine from ceramide B, *8* *erythro-trans-N*-acetyl-sphingosine, *9* synthetic *erythro*-dihydrosphingosine, *10* ceramide from cerebrosides by method of Carter et al. (3), *11* sphingomyelin from beef brain, *12* ceramide A from beef lung by method of Tipton (32), *13* ceramide B from beef lung by method of Tipton (32), *14* rabbit liver polar lipids, *15* bases from ceramide B after aqueous (10M water) methanolysis, *16* bases from ceramide B after anhydrous methanolysis, *17* bases from sphingomyelin after aqueous (10M water) methanolysis, *18* bases from sphingomyelin after anhydrous methanolysis.

Discussion

Gas chromatography of the sphingolipid bases as their trimethylsilyl derivatives provides a simple and direct method for qualitative and quantitative estimations of the bases. Compared with the method described earlier (29), in which the aldehydes liberated by periodate were separated by gas chromatography, this procedure has obvious advantages. Since analyses are made of the bases themselves, several lengthy steps can be eliminated from the method. Furthermore, the first two carbon atoms of the bases are not lost to analysis, as they were in the periodate method, so that the new method can be used for separations of isotope-labeled bases and determinations of their specific activities at the same time. On a semipreparative scale, analytical columns can be used to obtain pure TMS derivatives, from which the free sphingolipid bases can be recovered easily and quantitatively by mild hydrolysis (9). A disadvantage of the present method is that bases are not well separated according to differences in olefinic bonds. Selective liquid phases such as nitrile silicone (XE-60) are not much better than SE-30 in this respect, though no investigation has been made of separations on polyester columns.

Methanolysis of sphingolipids under anhydrous acidic conditions leads to the formation of 3-*O*-methyl sphingosine in yields of 50% or more, as illustrated by this study. This compound and a related unknown base are formed from any *N*-acylated sphingosine, but not from the free base or from *N*-acyl dihydrosphingosine. The unknown by-product may be the 5-*O*-methyl-Δ^3-sphingosine described recently by Weiss (33). The presence of *O*-methyl ethers complicates determinations of sphingolipid bases by TLC or gas chromatography and conditions were, therefore, sought which would completely eliminate their formation. Though we have not found such a reagent, the presence of water in the methanolysis mixture effectively inhibits the formation of *O*-methyl ethers to a large degree. Morrison and Smith (18) have described conditions for the quantitative liberation of sphingolipid bases and fatty acid methyl esters from sphingomyelin by methanolysis with boron trifluoride-methanol. We have not yet studied this condition with regard to the yield of *O*-methyl ethers; it may prove to be a superior reagent.

The presence of an unidentified sphingolipid base in sphingomyelin from human plasma presents an excellent example of the utilization of this method for structural work. The presence and concn of the compound have been demonstrated in this study. The base has been identified tentatively as C_{16}-sphingosine. Though it could probably never be separated from sphingosine by classical techniques, it will be possible to obtain small quantities of the pure base by preparative gas chromatography.

ACKNOWLEDGMENT

Investigation supported, in part, by grant A-4307, National Institute of Arthritis and Metabolic Diseases, USPHS.

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Individual Molecular Species of Different Phospholipid Classes. Part II. A Method of Analysis[1]

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Abstract

New analytical possibilities arise when glycerophosphatides are converted into diglyceride acetates or analogous compounds: In this less polar form the phospholipids can be subjected to the usual methods of triglyceride fractionation, including chromatography on silica gel mixed with silver nitrate. This opens a route to subfractionation of various glycerophosphatide classes, and makes analysis of the individual molecular species potentially possible in many cases. The same approach can also be applied to the analysis of sphingomyelins.

Two methods are suitable for the conversion of glycerophosphatides into "diglyceride acetates": 1) Acetolysis in a mixture of acetic anhydride and acetic acid, and 2) treatment with phospholipase C (E.C. 3.1.4.3.) followed by acetylation. Acetolysis was used successfully with phosphatidyl choline, phosphatidyl ethanolamine and corresponding alkoxy phosphatide (native cephalin B), phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol, phosphatidic acid and cardiolipin. Phospholipase C from *Clostridium welchii* was used for native choline and ethanol-

[1] Part I. Proposal for a Method of Analysis. ACTA Chem. Scand. *18*, 271 (1964).