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Studies on Sphingolipids, I. Gas-liquid Chromatographic Analyses of Sphingosine Bases

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Introduction

Sphingolipids are most principal one of the lipids accumulated in various organs in certain groups of metabolic disorders called as lipidoses. A number of investigations have been made to elucidate the chemical nature of these lipids and the cause of their abnormal accumulations.

Fatty acid and sugar moieties in the sphingolipids have been well investigated but only a few studies on the composition of sphingosine bases have been reported. Recently, the excellent analytical methods of sphingosine bases have been presented in succession by the utilization of gas-liquid chromatography (GLC).

The principles of the three methods hitherto reported are as follows: 1) GLC of fatty acid methylesters obtained from the bases by either permanganate-periodate oxidation or ozone cleavage[1~3]; analysis of the cleavage products at the position of either double bond or C-C bond of ethylene glycol or its amino derivative, 2) GLC of long chain fatty aldehydes obtained from the bases by periodate oxidation[4~7]; analysis of the cleavage products at the position of C–C bond of ethylene glycol or its amino derivative, 3) GLC of trimethylsilylated compounds derived directly from bases[8~10].

In this paper, the three methods mentioned above were compared to one another, using authentic dihydrosphingosine (C–18) as standard as well as the bases in sphingolipids isolated from pig brain and from yeast powder, with the data indicating the occurrences of short chain homologs of both sphingosine and dihydrosphingosine.

Materials and Methods

1. *Materials*

N-acetyl dihydrosphingosine and triacetyl sphingosine were the generous gifts of Dr. H. E. Carter, University of Illinois. Aldehyde standards, that is, dodecanal, tetradecanal and octadecanal were the generous gifts of Dr. E. A. Moscatelli, University of Texas, Southwestern Medical Sichool.

Samples of cerebroside and sphingomyelin were isolated from pig brain as described in another paper[16].

Yeast powder (100 g) gifted from Sapporo Beer Co. was extracted for 15 minutes with 500 mℓ of boiling chloroform-methanol-water (10 : 10 : 1) and the residue after filtration was re-extracted for 3 hours with 500 mℓ of warm chloroform-methanol-water (10 : 20 : 3). The combined extracts were concentrated to dryness. The residue was dissolved in chloroform-methanol (2 : 1) and subjected to mild alkaline hydrolysis, by adding 1

N methanolic NaOH to make a final concentration of 0.5 N. After Folch's partition, the alkaline stable lipids were separated by silicic acid column chromatography. The column was first eluted with chloroform to remove neutral lipids and then with appropriate amount of methanol. 538 mg of polar lipids consisted mainly of sphingolipids were obtained from the methanol eluate.

2. *Preparation of Sphingosine Bases*

Each of sphingolipids was hydrolyzed with 1 N aqueous methanolic HCl in seal tube at 75°C for 18 hours (8.6 mℓ of conc. HCl and 9.4 mℓ of water to 100 mℓ with methanol, 2–3 mg lipid per mℓ). The reaction mixture was then cooled to room temperature and extracted with hexane to remove fatty acid esters. The methanol phase was concentrated to a small volume and adjusted to PH 12–13 by adding conc. KOH solution cautiously. An equal volume of water was then added and the bases were extracted from the alkaline aqueous solution with diethyl ether. After washing with water, the ether extract was evaporated to dryness and the crude sphingosine bases were purified by silicic acid chromatography. the column was first eluted with chloroform to remove fatty acid esters which would interfere the gas chromatographic assay of aldehydes. The bases were then eluted with chloroform-methanol (1 : 4) and the eluate was evaporated to dryness.

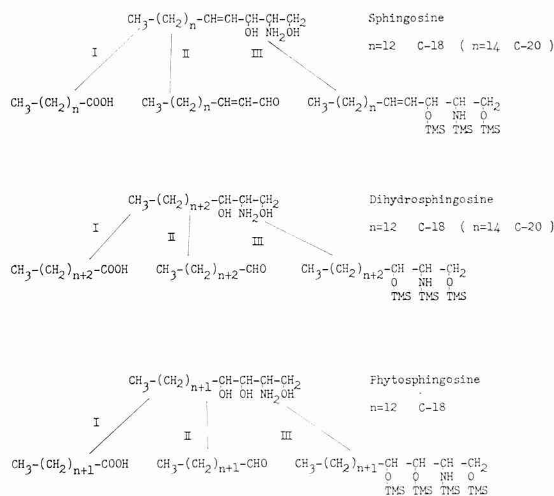
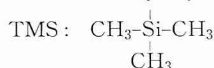


Fig. 1 Scheme of three methods for gas-liquid chromatographic analyses of sphingosine bases.

I. Permanganate-periodate oxidation
II. Periodate oxidation
III. Trimethylsilylation



3. *Permanganate-Periodate Oxidation*

The oxidation of sphingosine bases to fatty acids was accomplished according to the procedure of Green et al[2]. 1.2 mg of sphingosine bases were dissolved in 0.4 mℓ tert-butanol in screw capped tube and then 2 μmoles K_2CO_3 in 1.12 mℓ of solution, 10 mg $NaIO_4$ and 0.8 mg $KMnO_4$ in 0.54 mℓ of solution, and 0.34 mℓ of water were successively added. The tube was shaken at room temperature for 1 hour. After destruction of the reagents with $NaHSO_3$, the solution was acidified and extracted with ether to remove fatty acids. The acids were methylated by 1 mℓ of methanolic BF_3 reagent[12]. The fatty acid methyl esters thus obtained were analyzed by gas-liquid chromatography.

4. *Periodate Oxidation*

Sphingosine bases were converted to aldehydes by sodium periodate oxidation accord-

ing to the procedure of Sweely and Moscatelli[4]. 5 mg of sphingosine base preparation dissolved in methanol 1 mℓ and 0.2 mℓ of 0.2 M $NaIO_4$ solution were used. The reaction mixture was allowed to stand in the dark at room temperature for 45 minutes and then 2.4 mℓ of methylene chloride and 1.2 mℓ of water were added. After shaking with flash mixer, the mixture was centrifuged and the lower layer was then concentrated to dryness at low temperature. The aldehydes in dilute chloroform solution were stable for one week at −20°C but the analysis were usually carried out as soon as possible.

Catalytic hydrogenation: Adout 2–3 mg of aldehydes were dissolved in 8–12 mℓ of absolute ethanol in a tube and 4–6 mg of 5% palladium-charcoal catalyst were added. The tube was made to vacuo and then filled with hydrogen using three way cock and the mixture was subjected to rapid swirling for 20 minutes using a fiash mixer. The mixture was then applied on a cellulose column and aldehydes were eluted with chloroform. The eluate was evaporated to dryness and the residue then dissolved in chloroform for gas chromatography. 5% palladium-charcoal was prepared according to the procedure of Mozingo[11] and stored in dacicater besore use.

5. *Trimethylsilylation*

Dry sphingosine bases (ca. 1 mg) was mixed with 100 μℓ of silanes reagent. This silanes reagent, consisted of hexamethyl disilazane 2.6 mℓ, dry pyridine 2.0 mℓ and trimethylsilane 1.6 mℓ was prepared according to the method of Carter and Gaver[9]. Dry pyridine was obtained by the distillation over barium oxide before use.

6. *Gas-liquid Chromatography*

A Hitachi Parkin Elmer, Model F 6 gaschromatograph, equipped with a flame ionization detector was used for analyses.

Fatty acid esters and aldehydes were analyzed on a spiraled glass column, 1 m × 0.4 mm packed with 15% diethylene glycol succinate polyester on 100–120 mesh acid washed and silanized chromosorb W. The analyses were usually run at 155°C for aldehydes and at 170°C for fatty acid esters.

Trimethylsilyl derivatives of sphingosine bases were analyzed on a U-shapd stainless steel column, 2 m × 0.4 mm packed with 3.8% SE-30 on 80–100 mesh chromosorb W. The column was maintained at 210°C.

Results and Disccusion

1. *Isolation of Sphingosine Bases*

The recoveries of sphingosine bases in acid hydrolysis of pig brain sphingolipids are

Table 1 *Recovery of Sphingosine Bases in Acid Hydrolysis*

Sphingolipid	Amount for hydrolysis	Sphingosine bases		
		found	Recovery	Theoretical recovery
Cerebroside (Kerasine)	38.8 mg	12.0 mg	30.9%	36.2%
Cerebroside (Phrenosine)	45.2	12.6	27.2	35.7
Sphingomyelin	40.9	11.8	28.8	36.4

shown in Table 1. Sphingosine bases were isolated in high yield, 77.4–85.4% of theoretical values,

In the thin-layer chromatography developed with chloroform-methanol—2 N NH_4OH (40 : 10 : 1, v/v)[13], these sphingosine bases were separated into dihydrosphingosine, sphingosine, o-methyl sphingosine and upper spots than o-methyl sphingosine. The upper spots were not identified, but those were small quantaties and ninhydrin negative.

Dihydrosphingosine as the predominant base and phytosphingosine were found in the thin-layer chromatography of sphingosine bases isolated from yeast powder sphingolipids.

2. *Permanganate-Periodate Oxidation*

Gas chromatograms of fatty acid esters obtained after permanganate-periodate oxidation are illustrated in Fig. 2. The percentages of methylesters, that is, their parent bases are shown in Table 2.

As seen in Fig. 2–A, small peaks due to shorter chain compounds than palmitic acid were detected in the sample obtained from authentic dihydrosphingosine. They are

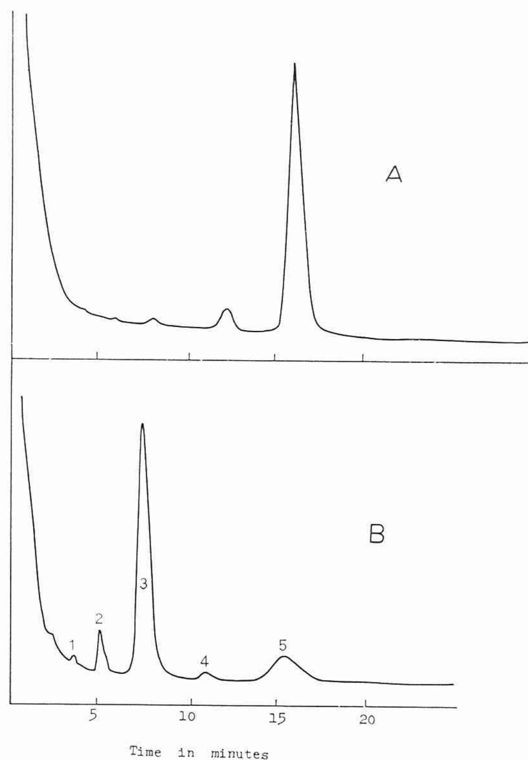


Fig. 2 Gas-liquid chromatographic analyses of fatty acids derived from $NaIO_4$–$KMnO_4$ oxidation of sphingosine bases of authentic dihydrosphingosine (A) and pig brain cerebroside (B).
Column: 15% diethylene glycol succinate polyester, 100–120 mesh acid washed and silanized chromosorb W. 1 m×0.4 mm spiraled glass column.
Carrier gas: N_2, 1.0 kg/cm^2. Temperature: 170°C.
Peak identification: 1. Lauric acid 2. n-Tridecanoic acid 3. Myristic acid 4. n-Pentadecanoic acid 5. Palmitic acid

Table 2 *Fatty Acids as Products of* $NaIO_4$–$KMnO_4$ *Oxidation of Sphingosine Bases of Authentic Dihydrosphingosine and Pig Brain Cerebroside*

Peak. No	Fatty acid (as methylester)	Probable parent base	% of Total	
			Authentic dihydrosphingosine	Pig brain cerebroside
1	Lauric acid	? (C_{16}-sphingosine)	0.2	0.6
2	n-Tridecanoic acid	? (C_{17}-sphingosine)	0.3	5.3
3	Myristic acid	C_{18}-sphingosine	1.3	78.0
4	n-Pentadecanoic acid	? (C_{17}-dihydrosphingosine)	5.4	1.7
5	Palmitic acid	C_{18}-dihydrosphingosine C_{20}-sphingosine	92.8	14.4

presumed to be overoxidation products, because no corresponding peaks were detected from the authentic base, using other two methods. The peaks corresponding with lauric (C–12), tridecanoic (C–13) and pentadecanoic (C–15) acids found in the case of pig brain cerebroside (Fig. 1–B) may be derived from sphingosine (C–16, C–17 and C–19), dihydrosphingosine (C–14, C–15 and C–17) or phytosphingosine (C–15, 16C– and C–18) and / or the overoxidation products produced from longer chain bases.

It is apparent that this method is less suitable for the determination of sphingosine base composition than the other two methods described below, but it is also useful for the datermination of double bond position and the identification of the individual sphingosine base.

3. *Periodate Oxidation*

Gas chromatograms of aldehydes obtained after periodate oxidation are shown in Fig. 3. The percentages of aldehydes, that is, their parent bases are listed in Table 3.

As seen in Fig. 4, semilogarithmic plots of retention time versus chain length were used to identify the aldehydes of saturated and unsaturated series of bases with aldehyde standards. The retention times for peak 1, 3, 5, 6 and 10 yield a straight line. The peak 1, 3, 6 and 10 were identified with authentic samples, that is, dodecanal, tetradecanal, hexadecanal and octadecanal respectively, showing that peak 5 may be pentadecanal. The retention times for peak 4, 7, 9 and 12 gave a straight line nearly parallel to that obtained for the saturated aldehydes. The peak 9 was identified with hexadecenal obtained from authentic triacethylsphingosine and the peak 13 with o-methylheptadecenal, obtained in high yield from the same sample by methanolysis in higher concentration of HCl. The spot 12 in Fig. 4 was identical with one of the peaks obtained from ganglioside-bases, known to a substantial amount of C_{20}-sphingosine. The retention time of the spot also shows that it might be octadecenal derived from C_{20}-sphingosine. Peak 12 in Fig. 3, obtained from hydrogenation, is corresponding with either o-methylheptadecanal or octadecenal, but it seems not to be o-methylheptadecanal since this peak does not appear in the analysis of authentic dihydrosphingosine treated by the same hydrolysis procedure. The corresponding peak 12 obtained from base prior to hydrogenation would be octadecenal derived from C_{20}-sphingosine.

The aldehyde peaks were clearly separated to one another and individual peak was well identified using authentic samples as well as by the semilogarithmic plot of retention

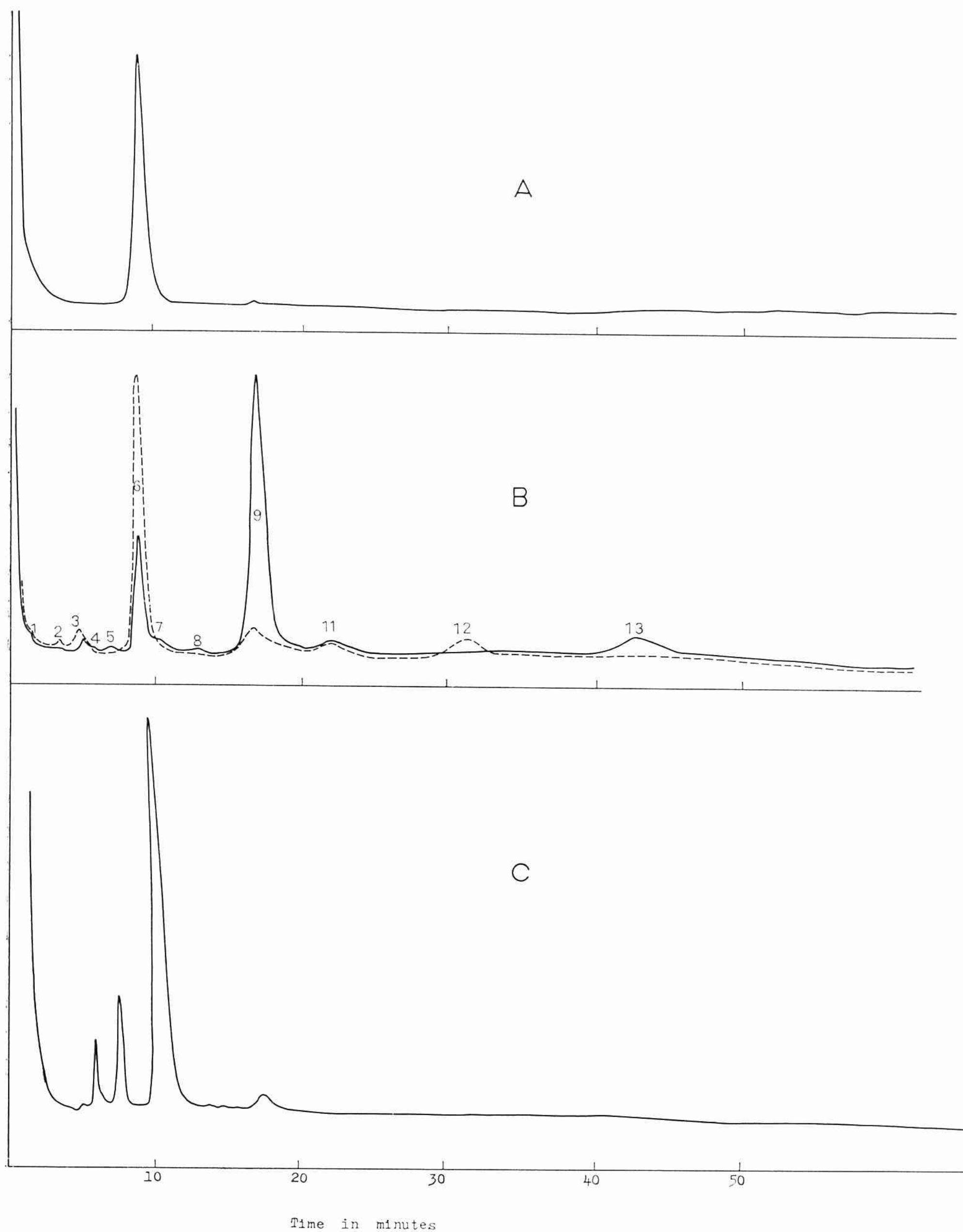


Fig. 3 Gas-liquid chromatographic analyses of aldehydes derived from periodate oxidation of sphingosine bases of authentic dihydrosphingosine (A), pig brain cerebroside (B) and yeast sphingolipids (C).

Column: 15% diethylene glycol succinate polyester, 100–120 mesh acid washed and silanized chromosorb W. 1 m×0.4 mm spiraled glass column.

Carrier gas: N_2, 1.2 kg/cm^2.

Temperature: 155°C

Peak identification is shown in Table 3.

Table 3 *Aldehydes as Products of Periodate Oxidation of Sphingosine Bases of Authentic dihydrosphingosine, Pig Brain Cerebroside and Yeast Sphingolipids*

Peak. No	Aldehyde	Probable parent base	% of Total		
			Authentic dihydro-sphingosine	Pig brain cerebroside	Yeast sphingolipids
1	Dodecanal	C_{14}-dihydrosphingosine	—	trace	trace
2	Unidentified	?	—	trace	trace
3	Tetradecanal	C_{16}-dihydrosphingosine	—	1.0	7.3
4	Dodecenal	C_{14}-sphingosine	—	trace	—
5	Pentadecanal	C_{18}-phytosphingosine	—	trace	11.6
6	Hexadecanal	C_{18}-dihydrosphingosine	99.8	16.1	76.2
7	Tetradecenal	C_{16}-sphingosine	—	trace	—
8	Unidentified	?	—	trace	—
9	Hexadecenal	C_{18}-sphingosine	trace	73.0	—
10	Octadecanal	C_{20}-dihydrosphingosine	—	—	5.8
11	Unidentified	?	—	1.8	—
12	O-methylheptadecanal	O-methyldihydro-shingosine	—	—	—
13	O-metylheptadecenal	O-methylsphingosine	—	7.2	—

* The peaks present at less than 0.5% are repoted as trace and no detectable peaks as—.

time. The data demonstrated that this method was very useful for the analysis of sphingosine base composition, except that uncertainty such as, C-15 saturated aldehyde could be derived from C_{17}-dihydrosphingosine or C_{18}-phytosphingosine, still remains to be solved by another method metioned below.

It should be noted that the over-oxidation products were also observed by this procedure if the higher concentration of sodium periodate than mentioned in the method-4 was used.

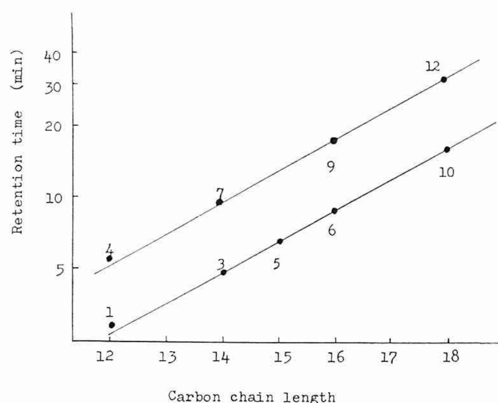


Fig. 4 Semilogarithmic plots of retention times of aldehyde peaks in Fig. 3.

4. *Trimethylsilylation*

Gas chromatograms of trimethylsilyl derivatives of sphingosine bases and semilogarithmic plots of retention times of the derivatives are illustrated in Fig. 5 and 6 respectively. The percentages of silylated bases are shown in Table 4.

As seen in Fig. 6, the retention times of trimethylsilyl derivatives were plotted on semilogarithmic graph and identified as follows: peak 2; C_{16}-sphingosine, peak 3; C_{16}-dihydrosphingosine, peak 4; C_{17}-sphingosine, peak 5; o-methylsphingosine, peak 6; C_{18}-sphingosine, peak 7; C_{18}-dihydrosphingosine, peak 8; phytosphingosine, and peak 9; C_{20}-dihydrosphingosine. The peak 1 is plotted on straight line of dihydrosphingosine series, suggesting that it might be C_{15}-dihydrosphingosine, although the occurrence of such base

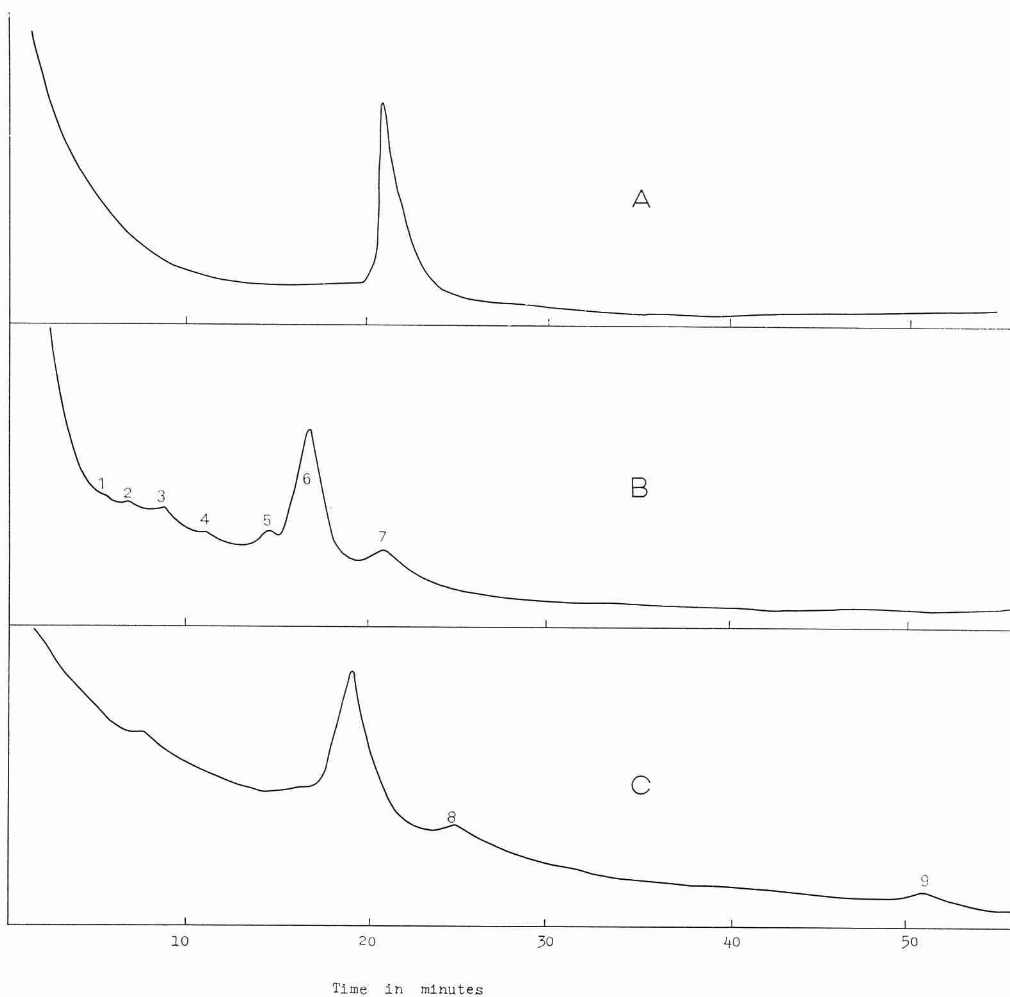


Fig. 5 Gas-liquid chromatographic analyses of trimethylsilylderivatives of sphingosine bases of authentic dihydrosphingosine (A), pig brain cerebroside (B) and yeast sphingolipids (C).

Column : 3.8% SE-30, 100-120 mesh acid washed and silanized chromosorb W. 2 m×0.4 mm stainless steel column.

Carrier gas : N_2, 1.0 kg/cm^2.

Temperature : 210°C.

Peak identification is shown in Table 4.

in animal tissue has never been reported.

Sphingoine base compositions determined by periodate oxidation and trimethysilylation methods were very similar to each other, as shown in Table 3 and 4, except that the somewhat lower value of C_{18}-dihydrosphingosine was found by periodate method in the case of yeast sphingolipids.

Both the trimethylsilylation method and the periodate method are useful for the determination of sphingosine base composition. The former due to the determination of

base itself has superiority for the qualitative identification of bases than the later due to the determination of the cleavage products of bases as seen in Fig. 5. But the later method is rather better than the former for the quantitative determination of base composition, because the aldehydes having simple structure are more clearly separable than the base derivatives with more complex structure, as seen in Fig. 3.

The lower homologs of sphingosine and dihydrosphingosine were found in pig brain cerebroside by trimethylsilylation method. This finding was partially confirmed by the data obtained by periodate method in which tetradecenal derived from C_{16}-sphingosine and tetradecanal derived from C_{16}-dihydrosphingosine were clearly demonstrated. But the existence of pentadecenal derived from C_{17}-sphingosine could not be detected by periodate method. The data by the permanganate-periodate method also suggest the occurrences of shorter chain homologs.

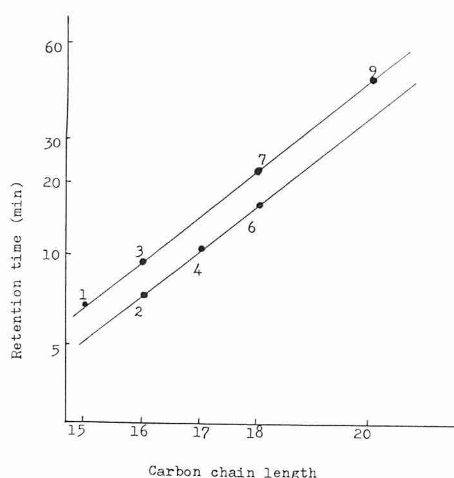


Fig. 6 Semilogarithmic plots of retention times of trimethylsilylderivatives of sphingosine bases in Fig. 5.

Table 4 *Identification of Trimethylsilylderivatives of Sphingosine Bases of Authentic Dihydrosphingosine, Pig Brain Cerebroside and Yeast Sphingolipids.*

Peak. No	Sphingosine base	% of Total		
		Authentic dihydrosphingosine	Pig brain cerebroside	Yeast sphingolipids
1	(C_{15}-dihydrosphingosine)	—	trace	—
2	C_{16}-sphingosine	—	trace	—
3	C_{16}-dihydrosphingosine	—	1.6	4.4
4	C_{17}-sphingosine	—	trace	—
5	O-methyl sphingosine	—	8.2	—
6	C_{18}-sphingosine	—	74.3	—
7	C_{18}-dihydrosphingosine	100	14.5	82.6
8	C_{18}-phytosphingosine	—	—	7.1
9	C_{20}-dihydrosphingosine	—	—	5.8

The possibility of naturally occuring lower homologs of sphingosine and dihydrosphingosine was suggested by various investigators[5,8,14,15] and reported that these homologs occurred at low or trace levels in mammalian tissues. Moscatelli and Mayes[5] have described that nonganglioside polar lipids in human white matter contained C_{16}-dihydrosphingosine at levels up to 2% and C_{14}-dihydrosphingosine in trace quantaties. Popovic[16] has reported the occurrence of C_{17}-sphingogine in addition to C_{16}-sphingosine in bovine heart sphingomyelin. The relative large amont (8%) of C_{16}-sphingosine in human plasma sphingomyelin was found by Gaver and Sweely[8] using trimethylsilylation method.

In the present study C_{16}-sphingosine and C_{16}-dihydrosphingosine occurred at low level in pig brain cerebroside. In yeast powder C_{18}-dihydrosphingosine was predominant and the occurrences of phytosphingosine and C_{20}-dihydrosphingosine were identified by both periodate and trimethylsilylation methods.

Summary

Three different methods ; permanganate-periodate oxidation, periodate oxidation and trimethylsilylation, for gas-liquid chromatography of sphingosine bases were compared to one another, using authentic dihydrosphingosine as well as bases of cerebroside isolated from pig brain and sphingolipids from yeast powder, with the data on the occurrences of shorter chain homologs of sphingosine bases.

The trimethylsilylation method was most suitable for the qualitative determination and identification of individual sphingosine base. This method was also available for the quantitative determination of sphingosine base composition.

The periodate method was most suitable for the quantitative determination of sphingosine base composition, except that some uncertainties such as C-15 saturated aldehyde could be derived from either C_{17}-dihydrosphingosine or C_{18}-phytosphingosine, remain to be solved by other methods such as Trimethylsilylation method or thin-layer chromatography etc.

The permanganate-periodate method was less suitable for the determination of sphingosine base composition for higher appearance of some oxidation products as well as uncertainties due to same acids could be derived from different parent bases by this method than by periodate method. But it is still useful for the exact determination of the double bond position and helps the identification of the bases with other methods.

C_{16}-sphingosine, C_{16}-dihydrosphingosine and probably C_{17}-sphingosine occured at low level in pig brain cerebroside.

C_{18}-dihydrosphingosine was predominant base in yeast sphingolipids but the occurrences of C_{18}-phytosphingosine and C_{20}-dihydrosphingosine were also confirmed.

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References

1) Klenk, E. und Facllard, H.: Über sphingosine. Z. Physiol. Chem. **299**, 48-53 (1955).
2) Green, M. L., Kaneshiro, T. and Law, J. H.: Studies on the production of sphingolipid bases by the yeast, Hansenula ciferri. Biochim. Biophys. Acta **98**, 582-588 (1965).
3) James, A. T. and Webb,J.: The determination of the stucture of unsaturated fatty acids on a micro scale with gas-liquid chromatogram. Biochem. J. **66**, 515-520 (1957).
4) Sweely, C. C. and Moscatelli, E. A.: Qualitative microanalysis and estimation of sphingolipid bases. J. Lipid Res. **1**, 40-47 (1959).
5) Moscatelli, E. A. and Mayes, J. R.: Sphingosine bases of normal human white matter. Biochemistry **4**, 1386-1390 (1965).
6) Sambasivarao, K. and McCluer, R. H.: Lipid components of gangliosides. J. Lipid Res. **5**, 103-108 (1964).
7) Sweely, C. C.: A gas chromatographic meth od for sphingosine assay. Biochim. Biophys. Acta **36**, 268-271 (1959).
8) Gaver, R. C. and Sweely, C. C.: Methods for methanolysis of sphingolipids and direct determination of long-chain bases by gas chromatography. J. Am. Oil Chemist's Soc

42, 294–298 (1965).

9) Carter, H. E. and Gaver, R. C.: Improved reagent for trimethylsilylation of sphingolipid bases. J. Lipid Res. **8**, 391–395 (1967).

10) Karlson, K. A.: Studies on sphingosine 10, Use of trimethylsilyl ethers for the gas chromatography and massspectrometry of sphingosines. Acta Chem. Scand. **18**, 2425–2427 (1965).

11) Mozingo, R.: Palladium catalysts. Organic synthesis John Wiley and Sons, Inc, New York Chapman and Hall, Ltd, London **26**, 77–82 (1946).

12) Morrison, W. R. and Smith, L. L.: Preparations of fatty acid methylesters and dimethylacetals from lipids with boron fluoride-methanol. J. Lipid Res. **5**, 600–608 (1964).

13) Sambasivarao, K. and McCluer, R. H.: Thin-layer chromatographic separation of sphingosine and related bases. J. Lipid Res. **4**, 106–108 (1963).

14) Karlsson, K. A.: Studies on sphingosines 6, C_{16}-and C_{17}-sphingosines hitherto unknown sphingosines. Acta Chem. Scand. **18**, 2395–2396 (1964).

15) Popovic, M.: Sphingosine and its lower homologues from bovine heart sphingomyelin fraction. Biochim. Biophys. Acta **125**, 178–182 (1966).

16) Akino, T.: Sphingosine base and fatty acid compositions of pig brain sphingolipids. Tohoku J. Exp. Med. **98**, 245–253 (1969).