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Studies on Sphingolipids, II. Sphingolipid Bases in Rat Brain, Liver and Kindney

Toyoaki AKINO

Department of Biochemistry (Section 1), Sapporo Medical College
(Chief: Prof. K. Ohno)

Introduction

By the utilization of gas-liquid chromatographic methods for analysis of sphingosine bases, various homologs other than common bases, that is, C_{18}-sphingosine and C_{18}-dihydrosphingosine, have been recently found in sphingolipids of mammalian tissues. For instance, phytosphingosine which had been believed to occur mainly in plant sphingolipids[1] has been found in lower species[2], in yeast[3] and in mammalian sphingolipids such as cerebroside of human[4,5], rat and beef kindney[6]. The occurrences of homologs different in chain length from common bases, such as C_{20}-sphingosine have been also reported[7~9].

The data on the sphingosine base composition of different sphingolipids in rat tissues such as brain, liver and kidney are presented in this paper, with some considerations on the distribution of sphingosine homologs in various types of sphingolipids as well as tissues.

Experimental

1. *Preparation of Sphingolipids (sphingomyelin, cerebroside, sulfatide and mucolipid)*

The preparation of sphingolipids was performed twice, using six and seven rats respectively.

Brain was extracted with 20 volumes of chloroform-methanol (2 : 1) and washed by the procedure of Folch et al[10]. Mucolipid fraction was obtained from the upper phase by lyophilization after dialysis. The lower phase was evaporated to dryness and the residure was dissolved in chloroform-methanol (2 : 1) and was hydrolyzed with 1 N methanolic NaOH at room temperature for 6 hours. After Folch's partition, the alkaline stable lipids were then fractionated by silicic acid column chromatography. Neutral lipids were first eluted with chloroform. The fractions eluted with chloroform-acetone (1 : 1) and chloroform-methanol (9 : 1) were further treated by DEAE-cellulose column chromatography to isolate cerebroside and sulfatide. The fractions eluted with higher concentration of methanol from silicic acid column, which contained much of sphingomyelin and small quantities of cerebroside and lysoplasmalogen, were treated by preparative thin-layer chromatography to isolate sphingomyelin in pure form.

The preparations of sphingolipids from liver and kinkey were performed as shown in scheme of Fig. 1. Liver and kidney were extracted in the same way as in brain. The lipid extract including mucolipid was concentrated and hydrolyzed with 1 N KOH (about 1 mℓ/4 g of fresh tissue weight) at room temperature for 24 hours according to the method of Schmidt[11]. The hydrolysate was neutralized to pH 6-7 by the dropweise addition of HCl, with vigorous stirring. The lipids were extracted from the hydrolysates with 10

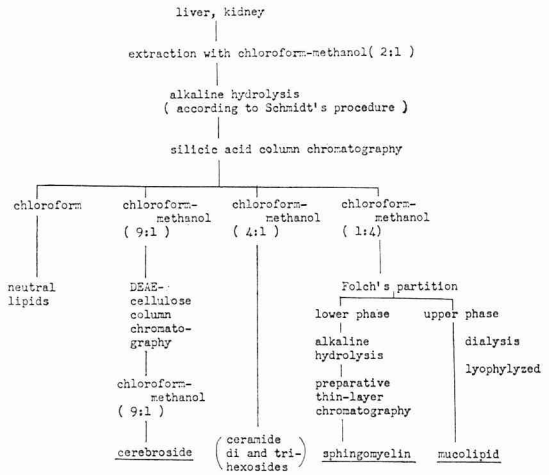

Fig. 1 Isolation of sphingolipids from rat liver and kidney.

volumes of chloroform-methanol (2 : 1). The extract was treated by silicic acid column chromatography. Cerebroside eluted with chloroform-methanol (9 : 1) from the column was further treated by DEAE-cellulose column chromatography. The fraction eluted with chloroform-methanol (1 : 4) from silicic acid column was partitioned by Folch's solvent. Mucolipid was isolated from the upper phase in the same manner as described above. The lower phase was subjected to mild alkaline hydrolysis using 1 N methanolic NaOH to remove a minute contamination of alkaline labile phospholipids. Lipid was extracted in usual manner from the hydrolysate and treated by preparative thin-layer chromatography to obtain sphingomyelin.

2. *Thin-layer Chromatography*

Thin-layer plates of 0.5 mm thickness were prepared with Silica Gel G by the procedure of Stahl and activated for 1 hour at 110°C before use. The developing solvent mixtures used were chloroform-methanol-water (70 : 30 : 5, v/v) for separation of sphingolipids and chloroform-methanol–2 N NH_4OH (40 : 10 : 1, v/v) for separation of sphingosine bases[12]. The spots on the plate were detected by 50% H_2SO_4 spray followed by heating at 110°C for 15 minutes. Ninhydrin spray was also used for detecting sphingosine bases. The preparative thin-layer chromatography[13] was utilized for further purification of sphingomyelin, using the plates of 1 mm thick and 15–20 mg of lipids applied to each plate.

3. *Paper Chromatography of Sugar Components*

Each of sphingolipids was hydrolyzed with 1 N HCl. Hydrolysate was then passed through Dowex 1 OH form column and the eluate was lyophilyzed. The paper chromatography of sugar components was carried out on Toyo filter paper No. 51 A, the solvent being butanol-pyridine-water (6 : 4 : 3, v/v.). The spots on the paper were dected by silver nitrate reagent.

4. *Gas-liquid Chromatography*

Each of sphingolipids was hydrolyzed with 1 N aqueous methanolic HCl and the

sphingosine bases were isolated according to the procedure of Gaver and Sweely[14].

The periodate oxidation of sphingosine bases was carried out according to the method of Sweely and Moscatelli[15].

Gas-liquid chromatography of aldehydes was done using a Parkin Elmer Hitachi Model F 6 instrument with a flame ionization detector. The column was 45 m×0.5 mm i.d. packed with butanediol succinate (Hitachi Golay column BDS-45). Analyses were usually run at 155°C and nitrogen was used as the carrier gas.

Results and Discussion

1. *Isolation of Sphingolipids*

Thin-layer chromatogram of sphingolipids isolated from rat brain, liver and kidney is shown in Fig. 2 and the sphingolipid contents are listed in Table 1. The values listed are mean of two experiments and calculated from the weight of individual lipid isolated.

Each of sphingomyelin, cerebroside and mucolipid from rat brain, liver and kidney, and sulfatide from brain were semiquantitatively isolated in pure form on thin-layer chromatography as shown in Fig. 2 and Table 1.

Sugar components of each cerebroside and sulfatide were analyzed by paper chromatography. Galactose was only detected in brain cerebroside and sulfatide. However only glucose was found in liver cerebroside. Both glucose and galactose were found in approximately same proportion in kidney cerebroside.

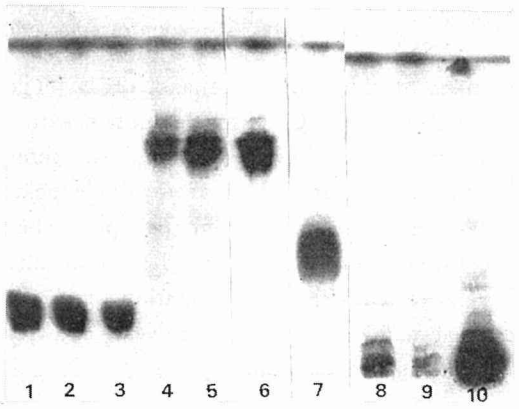

Fig. 2 Thin-layer chromatography of sphingolipids of rat brain, liver and kidney. Solvent system: chloroform/methanol/water (70 : 30 : 5, v/v) Detected by 50% H_2SO_4 spray followed by heating at 110°C for 15 min. Spot identification:

1. kidney sphingomyelin
2. liver sphingomyelin
3. brain sphingomyelin
4. kidney cerebroside
5. liver cerebroside
6. brain cerebroside
7. brain sulfatide
8. kidney mucolipid
9. liver mucolipid
10. brain mucolipid

2. *Sphingosine Base Composition of Eeach Sphingolipid*

Sphingosine bases of each sphingolipid were analyzed according to the periodate oxidation method, that is, by gas-liquid chromatography of fatty aldehydes.

Typical chromatogram obtained from brain sphingomyelin is shown in Fig. 3 and the sphingosine base composition of each sphingolipid in rat brain, liver and kidney is listed in Table 2.

Each of aldehyde peaks was identified by semilogarithmic plots with authentic standard samples and by hydrogenation of aldehyde mixtures as described in the previous paper[16].

Sphingosine base compositions were calculated from the areas of each aldehyde peak. The value of C_{18}-sphingosine was obtained to add the areas of hexadecenal and

o-methylheptadecenal.

Sphingosine bases were mainly consisted of C_{18}-sphingosine (70–93%) and C_{18}-dihydrosphingosine (4–15%) in all sphingolipids of rat tissues examined. But some differences on the percentage of each component of sphingosine bases were observed between different sphingolipids and in lesser degree between a sort of sphingolipid in different tissues.

A considerable amount (23.2%) of C_{20}-sphingosine was found to occur in brain mucolipid and a minute amount (3.2%) in brain sphingomyelin. But brain cerebroside, sulfatide and other sphingolipids from other tissues were no detectable C_{20}-sphingosine. These results are very similar to the data on pig brain sphingolipids reported in another paper[17], although rat brain ceramide could not be separated in the present study. Sambasivarao and McCluer have reported that C_{20}-sphingosine is a specific component of brain ganglioside and does not occur in detectable amounts in the nonganglioside sphingolipids. Other studies[18~20], in which sphingosine bases from the nonganglioside sphingolipids obtained after washing with Folch's solvent were analyzed, have not demonstrated the occurrence of C_{20}-sphingosine. Most recently Klenk and Huang[21] have detected a small amount of C_{20}-sphingosine in human brain ceramide which contained stearic acids as the predominant acid. In the investigation on the change in sphingosine portion of the ganglioside in developing rat brain, Rosenberg and Stern[22] have reported that sphingosine portion of the brain ganglioside changes from almost exclusively C-18 at

Table 1 *Sphingolipid contents of rat brain, liver and kidney.*

Tissue	Sphingolipid	mg/g of wet tissue
Brain	Cerebroside	12.54
	Sulfatide	3.21
	Sphingomyelin	5.42
	Mucolipid	0.93
Liver	Cerebroside	0.69
	Sphingomyelin	1.76
	Mucolipid	0.12
Kidney	Cerebroside	1.36
	Sphingomyelin	2.67
	Mucolipid	0.52

a. Sugars detected in paper chromatography.
Brain cerebroside: galactose
sulfatide: galactose
Liver cerebroside: glucose
Kidney cerebroside: galactose, glucose
b. Average of two experiments.

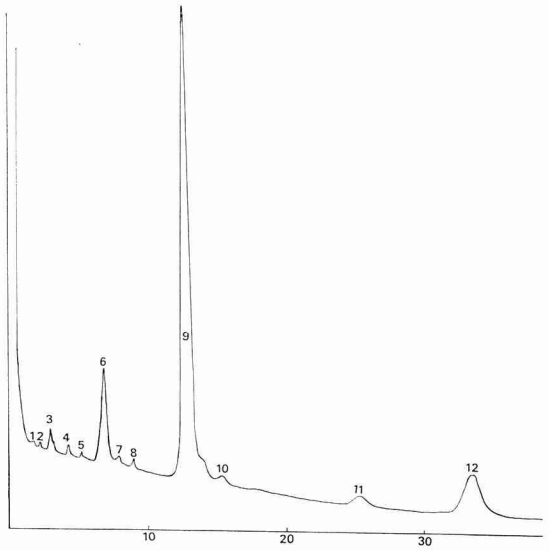

Fig. 3 Gas chromatogram of aldehydes derived from periodate oxidation of sphingosine bases of rat brain sphingomyelin.
Column: Hitachi golay column butanediol succinate, 45 m×0.5 mm.
Carrier gas: N_2 1.7 kg/cm².
Temperature: 155°C. Peak identification:

1. Dodecanal
2. Unidentified
3. Tetradecanal
4. Dodecenal
5. Pentadecanal
6. Hexadecanal
7. Tetradecenal
8. Unidentified
9. Hexadecenal
10. Unidentified
11. Octadecenal
12. O-methylheptadecenal

Table 2 *Sphingolipid base composition of rat brain, liver and kidney.*

Sphingolipid	Tissue	C_{16} dihydro-sphingosine (%)	C_{18} dihydro-sphingosine (%)	C_{18} sphingosine (%)	C_{20} sphingosine (%)	C_{18} phyto-sphingosine (%)
Sphingomyelin	Brain	1.2	6.9	86.2	3.2	—
	Liver	0.7	5.4	93.3	—	—
	Kidney	1.4	6.1	88.2	—	—
Cerebroside	Brain	1.0	12.7	86.0	—	—
	Liver	1.2	14.8	74.4	—	7.5
	Kidney	1.5	10.2	76.1	—	3.5
Sulfatide	Brain	0.8	12.1	86.4	—	—
Mucolipid	Brain	2.7	3.8	70.1	23.2	—
	Liver	1.6	8.5	88.5	—	—
	Kidney	2.7	9.0	87.1	—	—

a. Peaks present at less than 0.5% are reported as—.
b. Kidney sphingomyelin and cerebroside contained substantial amounts of unidentified peaks, especially peak 10.
c. Average of two experiments.

birth to nearly equal quantities of C-18 and C-20 with myelination.

From the data obtained from pig and rat brain sphingolipids, it was concluded that C_{20}-sphingosine was localized not only in brain ganglioside but also in brain ceramide and sphingomyelin. This suggests that ceramide containing C_{20}-sphingosine may be a precursor of both ganglioside and sphingomyelin in brain.

There was a distinct tendency for more C_{18}-dihydrosphingosine to occur in cerebrosides than in sphingomyelins, which was found not only in brain but also in liver and kidney. The tendency has also been observed in the analysis of pig brain sphingolipids[17]. Taketomi and Kawamura[23] have reported that more C_{18}-dihydrosphingosine in cerebroside in developing rabbit brain was present than in adult one, from the analysis of sphingosine bases by trimethylsilylation method.

Mucolipids in liver and kidney contained no C_{20}-sphingosine and the sphingosine base patterns were similar to those of sphingomyelin in the tissues. Brain mucolipid contained stearic acid 98%, arachidic acid 4% as fatty acid component. However liver and kidney mucolipids contained stearic acid and palmitic acid as the predominant acids

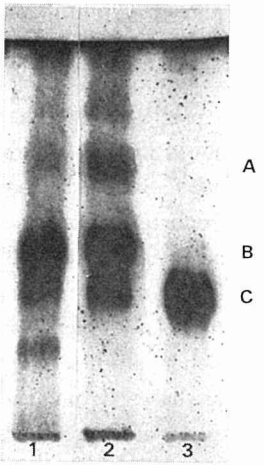

Fig. 4 Thin-layer chromatogram of sphingosine bases of rat 1. liver cerebroside, rat 2. brain cerebroside, and 3. authentic dihydrosphingosine.
Solvent system: chloroform/methanol/2 N NH_4OH (40 : 10 : 1, v/v)
Detected by 50% H_2SO_4 spray followed by heating at 110°C for 20 min. Spot identification:
A: o-methyl sphingosine
B: sphingosine
C: dihydrosphingosine

and the ratio of both acids was about 1 : 1.

3. *The Occurrence of Phytosphingosine in Rat Liver cerebroside*

Gas chromatograms of aldehydes obtained from sphingosine bases of rat liver and brain are shown in Fig. 5. Pentadecanal peak seen in the case of liver cerebroside was identified by semi-logarithmic plots with dodecanal, tetradecanal and hexadecanal to give a straight line and by the comparison with the analysis of yeast powder sphingolipids, known to contain a high percentage of phytosphingosine[15]. However, only a trace amount of the peak was found in brain cerebroside.

Thin-layer chromatogram of sphingosine bases of liver cerebroside brain cerebroside and authentic dihydrosphingosine is shown in Fig. 4 The lower spot than dihydrosphingosine was detected in liver cerebroside, which was ninhydrin positive. The spot is corresponding to phytosphingosine from Rf value. The occurrence of phytosphingosine in rat liver cerebroside was concluded from the data of thin-layer and gas-liquid chromatography.

Kidney cerebroside also contained 3.5% of phytosphingosine, but other sphingolipids contained only trace amounts of pentadecanal peak.

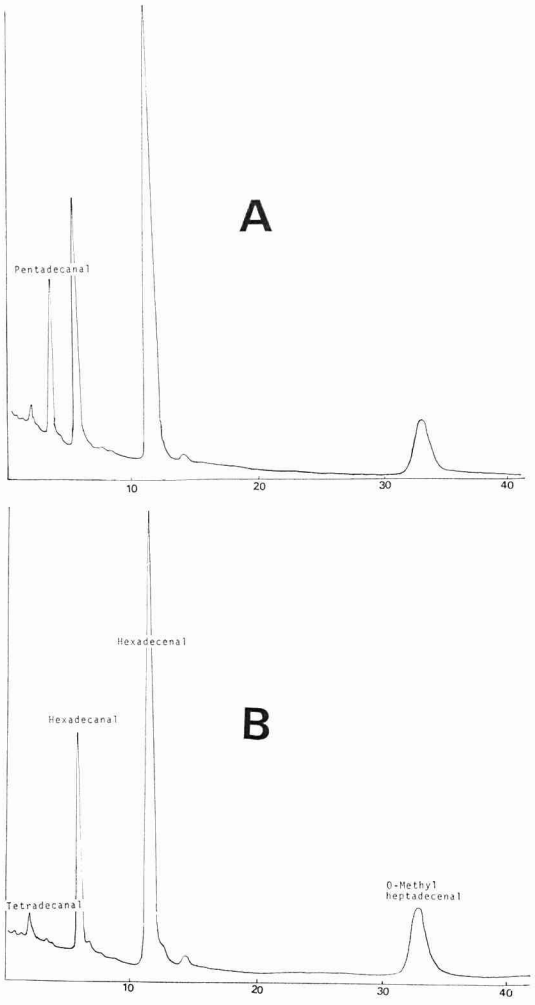

Fig. 5 Gas chromatograms of aldehydes obtained from sphingosine bases of rat liver (A) and brain (B) cerebroside.

Phytosphingosine, which has been known to constitute the major long chain bases in plants[1], lower species[2] and yeast[3], have also been detected in mammalian tissues. Recently Karlsson and Mårtensson have[5] reported the presence of phytosphingosine in human kidney glycolipids and noted remarkable differences in phytosphingosine content between glycolipids. Namely, there was a decreasing phytosphingosine content with increasing carbohydrate chain and a parallel relationship between the phytosphingosine and hydroxy fatty acid contents. Quantitative data have been presented by Carter and Hirshberg[6], who concluded that 25% of the base occur in rat and beef kidney cerebrosides.

The occurrence of phytosphingosine in other tissues than kidney has also been reported by Okabe and Schmidt[24] in intestinal mucosa of several species. But there is no report

on the occurrence of phytosphingosine in liver.

Summary

The sphingolipids, such as cerebroside, sphingomyelin and mucolipid were isolated from rat brain, liver and kidney. The isolation of sphingosine bases from each sphingolipid was performed according to the method of Gaver and Sweely. The analysis of sphingosine bases was carried out according to the procedure of Sweely and Moscatelli, that is, by gas-liquid chromatography of long chain aldehydes obtained from sphingosine bases by periodate oxidation, and the results are summarized as follows.

1. Sphingosine bases are mainly consisted of C_{18}-sphingosine (70–93%) and C_{18}-dihydrosphingosine (4–15%) in all sphingolipids of rat tissues examined. But some quantitative differences in sphingosine base composition were observed between different sphingolipids and in lesser degree between a sort of sphingolipids in different tissues.

2. Brain mucolipid contained a substantial amount (23%) of C_{20}-sphingosine and brain sphingomyelin also contained a small amount (3%), but no C_{20}-sphingosine was detected in the sphingosine bases of brain cerebroside, brain sulfatide and all sphingolipids from other tissues.

3. There was a distinct tendency for more C_{18}-dihydrosphingosine to occur in cerebroside than in sphingomyelin, which was seen not only in brain but also in other tissues.

4. Liver cerebroside contained 7.5% of phytosphingosine in addition to C_{18}-sphingosine and C_{18}-dihydrosphingosine. Lesser amount of phytosphingosine was found in kidney cerebroside. The occurrence of phytosphingosine was further confirmed by thin-layer chromatography.

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