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Degradation of Sphingosine, Dihydrosphingosine, and Phytosphingosine in Rats*

Yechezkel Barenholz and Shimon Gatt

ABSTRACT: Tritium-labeled sphingosine, dihydrosphingosine, and phytosphingosine were administered by intravenous injection into rats. Two main changes were observed in the intact liver. The bases were converted in a biosynthetic route into ceramide (the *N*-acyl derivatives of the bases). In a degradative pathway they were cleaved to fatty acids which could be isolated from the liver triglycerides and lecithin. Using gas–liquid partition chromatography and determining the radioactivity of the effluents, the fatty acids, formed by cleavage of the respective bases, were identified. Hexadecanoic (palmitic) acid was the main product of the degradation of both sphingosine and dihydrosphingosine; pentadecanoic acid was the main product of phytosphingosine. The possible mechanisms leading to the formation of these fatty acids are discussed.

The three most abundant sphingosine bases are C_{18}-sphingosine (*trans*-D-erythro-1,3-dihydroxy-2-aminooctadec-4-ene; Figure 1A), C_{18}-dihydrosphingosine (D-erythro-1,3-dihydroxy-2-aminooctadecane; Figure 1B), and C_{18}-phytosphingosine (D-ribo-1,3,4-trihydroxy-2-aminooctadecane; Figure 1C). Sphingosine is the most abundant base of animal tissue sphingolipids, phytosphingosine is found mostly in plant sphingolipids, and dihydrosphingosine occurs in both tissues. These bases occur in nature in the phospho- and glycosphingolipids, which can be regarded as derivatives of ceramide, the N-acylated sphingosine base (reviewed in Hanahan and Brockerhoff, 1965). Enzymes which hydrolyze the sphingolipids of animal origin have been isolated (Gatt, 1963, 1966a,b, 1967; Barenholz and Gatt, 1966; Gatt and Rapport, 1965, 1966a,b; Frohwein and Gatt, 1966, 1967a,b, Leibovitz and Gatt, 1968; Heller and Shapiro, 1966; Brady *et al.*, 1965a,b, 1967; Kanfer *et al.*, 1966; Hajra *et al.*, 1966; Sandhoff and Jatzkewitz, 1967; Schneider and Kennedy, 1967). These enzymes can account for the complete, stepwise hydrolysis of the sphingolipids to a long-chain base, fatty acid, and the individual carbohydrate residues, or phosphorylchloline (summarized in Gatt, 1968). The metabolic fate of the sphingosine bases thus formed has been only slightly investigated. Kanfer and Gal (1966) have administered sphingosine to rats and have found that it was incorporated into ceramides and sphingomyelin. Kanfer and Richards (1967), using a similar procedure, have observed several, yet unidentified, degradation products of this base. Barenholz and Gatt (1967) have reported that tritium-labeled phytosphingosine, given by intravenous route, was converted, in the intact liver, into fatty acids, which can be completely oxidized to CO_2 and water. These fatty acids were isolated from the liver triglycerides and lecithin and identified by gas–liquid partition chromatography; most of the radioactivity was present in pentadecanoic acid. Roitman *et al.* (1967) have administered phytosphingosine and dihydrosphingosine, by intracerebral injection to rats. Similar to the results in liver, phytosphingosine was degraded in brain to pentadecanoic acid. Dihydrosphingosine, on the other hand, was degraded mostly to hexadecanoic (palmitic) acid.

This paper further extends these findings and provides evidence for the degradation of sphingosine, dihydrosphingosine, and phytosphingosine bases to fatty acids and most probably to, as yet unidentified, two- or three-carbon residues. The fatty acids can be incorporated into the liver lipids or can be completely oxidized to CO_2 and water by the fatty acid oxidation routes. These observations, together with previous data on the enzymatic hydrolysis of the sphingolipids, propose a metabolic pathway leading to the complete degradation of the sphingolipids of animal origin to CO_2 and water.

Experimental Procedure

Substrates. BIOSYNTHETIC TRITIUM-LABELED PHYTOSPHINGOSINE. *Hansenula ciferri* (strain NRRL Y-1031 F-60-10, a generous gift of Dr. Wickerham) was grown according to Maister *et al.* (1962) in a medium contain-

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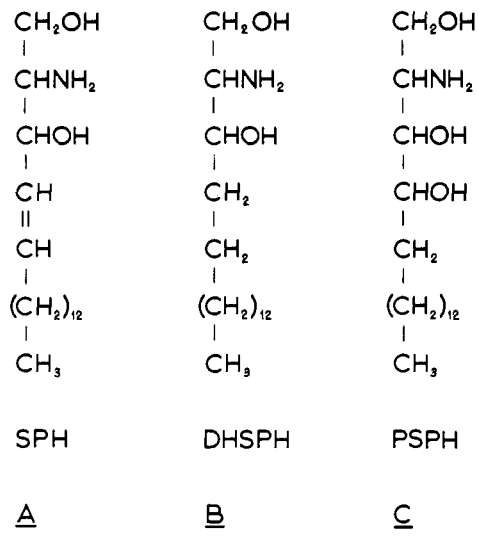


FIGURE 1: Structures of sphingosine (SPH), dihydrosphingosine (DHSPH), and phytosphingosine (PSPH).

ing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 5% glucose. Sodium [9,10-^{3}H]palmitate (280 μCi/μmole) was added to the growth medium at a concentration of 3.2×10^{-2} M. The yeast was grown in 2-l. erlenmeyers containing 600 ml of growth medium with continuous shaking (120 rpm) for 96 hr at 25° in a New Brunswick shaking incubator. The flasks were then placed for 72 hr, without shaking, at 4°. Under these conditions, most of the phytosphingosine is secreted into the medium as acetylated base. The contents of the flasks were centrifuged for 20 min at 5000 rpm. The sediment, which has the cells and about 95% of the acetylated bases, was extracted successively, four times each, with 2.5 ml of heptane/g of sediment. The extracts were combined, evaporated to dryness *in vacuo* at 45°, and the residue was hydrolyzed for 18 hr at 72° with 1 N HCl in 80% methanol (Gaver and Sweeley, 1965). The hydrolysate was shaken with petroleum ether (bp 30–60°) to remove the fatty acids, and the aqueous methanolic phase was adjusted to pH 12 with KOH. Chloroform and water were then added to a ratio of chloroform–methanol–aqueous KOH (8:4:3) (Folch *et al.*, 1957). After mixing, the phases were separated and the lower phase was evaporated to dryness. The quantity of bases thus obtained was about 300 μmoles/l. of growth medium. Pure, tritium-labeled phytosphingosine was isolated from the lower-phase residue by chromatography on a column (33 × 2.2 cm) containing 60 g of silica gel S (Riedel de Haen), using a continuous gradient of increasing concentrations of ammoniacal–methanol in chloroform, according to Barenholz and Gatt (1968). The quantity of the phytosphingosine was determined according to Lauter and Trams (1963). Aliquots were chromatographed on thin-layer silica gel plates (Sambasivarao and McCluer, 1963); the silica gel was scraped off and extracted, by heating, successively, once with 2.5 ml of chloroform–methanol (2:1) and three times with the same volumes of methanol; the phytosphingosine spot had over 97% of all radioactivity on the plate. The base was also oxidized with periodate and the pentadecanal thus obtained was identified by gas–liquid partition chromatography (Sweeley and Moscatelli, 1959). Over 95% of the radioactivity of the phytosphingosine was found in the pentadecanal. The specific activity of the base was 1.3×10^6 dpm.

TRITIUM-LABELED DIHYDROSPHINGOSINE. [4,5-^{3}H]*N*-Acetylsphingosine was prepared by catalytic hydrogenation of *N*-acetylsphingosine with tritium gas (Gatt, 1966a). The free, tritium-labeled dihydrosphingosine was obtained by hydrolysis of this compound in aqueous methanolic HCl (Gaver and Sweeley, 1965); it had an activity of 5×10^5 dpm/μmole. Its radiochemical purity was tested by chromatography on thin-layer silica gel plates in chloroform–methanol–2 N NH_4OH (77:23:2.3) (modification of Sambasivarao and McCluer, 1963). Ninety-five per cent of the total radioactivity on the plate migrated with the spot corresponding to the dihydrosphingosine base. The base was also oxidized with periodate (Sweeley and Moscatelli, 1959) and the product was subjected to gas–liquid partition chromatography; only one peak, that of hexadecanal, was obtained.

BIOSYNTHETIC TRITIUM-LABELED SPHINGOSINE. [9,10-^{3}H]Palmitic acid (0.05 μmole) (274 μCi/μmole) was neutralized with NaOH, suspended in 0.05 ml of saline, and injected intracerebrally into a 16-day-old male rat. After 24 hr, the animal was sacrificed and the brain lipids were extracted according to Folch *et al.* (1967). The extracts from eight rats, similarly injected, were pooled and hydrolyzed according to Gaver and Sweeley (1965). The hydrolysis mixture was shaken several times with petroleum ether to remove fatty acids and was adjusted to pH 12 with 15 N KOH. Chloroform and water were then added to give chloroform–methanol–aqueous NaOH (8:4:3). The phases were separated and the lower phase was evaporated to dryness. Sphingosine was isolated from this residue as follows (Barenholz and Gatt, 1968). The residue was dissolved in chloroform–methanol (9:1) and applied to a column (20 × 2.2 cm) of 20 g of silica gel (Merck, for column chromatography, 0.05–0.2 mm) which had been suspended in chloroform–methanol (9:1) and packed by gravity. The bases were eluted using a continuous exponential gradient of increasing concentrations of ammoniacal methanol in chloroform as follows. The lower mixing chamber contained 250 ml of chloroform–methanol–2 N NH_4OH (90:10:1); the upper reservoir had 250 ml of chloroform–methanol–2 N NH_4OH (50:50:5). Fractions (10 ml) were collected; the sphingosine which was eluted in fractions 14–20 had a radioactivity of 3.1×10^5 dpm/μmole. Its purity was determined by chromatography on thin-layer plates of silica gel (Sambasivarao and McCluer, 1963); the sphingosine spot contained 98% of the radioactivity on the plate.

Administration of Long-Chain Bases to Animals and Isolation of Liver Lipids. Chloroform–methanol solutions of the long-chain bases were evaporated to dryness. Saline was added gradually, and the mixture was heated for a few seconds in a boiling-water bath and stirred on a Vortex cyclomixer. The final concentration of the bases was 1 μmole/ml. Using this procedure, phytosphingosine and dihydrosphingosine yielded clear, sta-

ble dispersions. The sphingosine base could not be dispersed this way and a similar procedure was used for dispersion but in which mixtures of sphingosine and egg lecithin (1:10, w/w) were evaporated to dryness and suspended in saline.

Aliquots (0.5 ml) of the above suspensions were injected into the tail veins of rats weighing about 100 g each. At the desired times, the animals was sacrificed and the livers were excised and extracted with 19 volumes of a mixture of chloroform–methanol (2:1) (Folch *et al.*, 1957). After filtration, two-tenths volume of water was added, the phases were mixed, separated by centrifugation, and the upper phases were discarded. Each of the lower phases was evaporated to dryness, dissolved in petroleum ether (bp 60–80°), and applied to a "Unisil" silicic acid (Clarkson Chemical Co.) column (2 g of adsorbent/g of liver). The column was eluted with 25 ml of each of the following solvents: petroleum ether–benzene (2:1, 1:1, and 1:2), benzene (two fractions), benzene–chloroform (2:1, 1:1, and 1:2), chloroform (two fractions), chloroform–methanol (99:1, 98:2, 96:4, and 90:10), chloroform–methanol–2 N NH_4OH (90:10:1, 85:15:1.5, 80:20:2, 75:25:2.5, 60:40:4, and 40:60:6), and methanol–2 N NH_4OH (10:1). The triglycerides were eluted with benzene; ceramide, with chloroform–methanol (98:2); free, unreacted base, with chloroform–methanol–2 N NH_4OH (90:10:1 and 85:15:1.5), and lecithin with the same solvent mixture in a ratio of 75:25:2.5. The respective fractions were identified on thin-layer silica gel plates in the following solvent systems: neutral glycerides, in petroleum ether–ether–acetic acid (84:16:1) (Mangold, 1965) and in benzene–ether–95% ethanol–acetic acid (40:50:2:0.2) (Freeman and West, 1966); ceramide, in chloroform–methanol–acetic acid (94:2:4) (Gatt, 1966a); lecithin, in chloroform–methanol–2 N NH_4OH (75:25:4); and bases, in chloroform–methanol–2 N NH_4OH (77:23:2.3) (modification of Sambasivarao and McCluer, 1963).

To further identify the ceramide, it was subjected to mild alkaline hydrolysis with 0.4 N KOH in 90% methanol for 2 hr at 37°. The compound was not hydrolyzed and when shaken in the biphasic system of Dole (1956), it partitioned to the heptane phase. The ceramide was also hydrolyzed in aqueous methanolic HCl (Gaver and Sweeley, 1965) and the bases and fatty acids were isolated.

Isolation of Fatty Acids. The total liver extract or the separate lipid fractions were hydrolyzed for 45 min, at 72°, with 0.15 N KOH in 94% ethanol. (Sphingolipids are not hydrolyzed under these conditions; ceramide which was subjected to the same procedure underwent less than 5% hydrolysis.) The hydrolysis mixture was acidified, one volume of water was added, and the fatty acids were extracted into heptane. They were then separated into hydroxy and nonhydroxy fatty acids according to Preiss and Bloch (1964). The purity of the fractions obtained was tested by thin-layer chromatography (Mangold, 1965). The nonhydroxy fatty acids were dissolved in ether, methylated with distilled diazomethane in ether, and separated into saturated and unsaturated fatty acids (Goldfine and Bloch, 1961).

Gas–Liquid Partition Chromatography. The saturated methyl esters were chromatographed on a coiled preparative column (2.4 m long, 8-mm diameter) of ethyleneglycol succinate polyester on Gas Chrom P (70–80 mesh) (14%, w/w) in a Packard gas–liquid partition chromatograph, equipped with an ionization detector. The column temperature was 170° and the rate of flow of argon 60 ml/min. With the aid of a $^{50}/_{1}$ splitter, only 2% of the effluent was taken through the detector, while 98% was directed into a fraction collector and condensed onto glass wool in small glass vials. These were transferred into counting vials, 20 ml of scintillation fluid (3 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. of toluene) were added, and the radioactivity was determined in a liquid scintillation spectrometer.

Determination of Radioactivity. Chloroform–methanol solutions of radioactively labeled lipids were pipetted into glass counting vials. A chloroform–methanol solution (0.05–0.1 ml) of egg lecithin (20 mg/ml) was added and the solvents were evaporated under an infrared lamp. Toluene (1 ml) was added to the still-warm vials and after cooling to room temperature, 10 ml of scintillation fluid (3 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. of toluene) was added. The radioactivity was determined in a liquid scintillation spectrometer.

Results

Distribution of Radioactivity from Phytosphingosine in Rat Organs. Phytosphingosine (0.5 μmole/0.5 ml of saline) was administered intravenously into rats. The animals were sacrificed after 0.5 and 6 hr. Table I shows

TABLE I: Distribution of Radioactivity in Rat Organs after Intravenous Administration of [^{3}H]Phytosphingosine.[a]

Organ	Radioactivity (mμmole equiv)[b]	
	After 0.5 hr	After 6 hr
Liver	85.5	62.5
Blood	30.2	14.1
Lung	23.9	10.4
Spleen	11.5	3.0
Kidney	8.1	7.5
Brain	0	0

[a] ^{3}H-labeled phytosphingosine (500 mμmoles) (1.3 × 10^6 dpm/μmole) was suspended in 0.5 ml of saline and injected into the tail vein of each of two rats (120 g). After 0.5 and 6 hr, the animals were sacrificed and the organs were excised and extracted with 19 volumes of a mixture of chloroform–methanol (2:1) (Folch *et al.*, 1957). After filtration, two-tenths volume of water was added, the phases were separated, and aliquots of the lower phase were counted in a scintillation spectrometer. [b] These values were obtained by dividing the total radioactivity in the extract by the specific activity of the substrate.

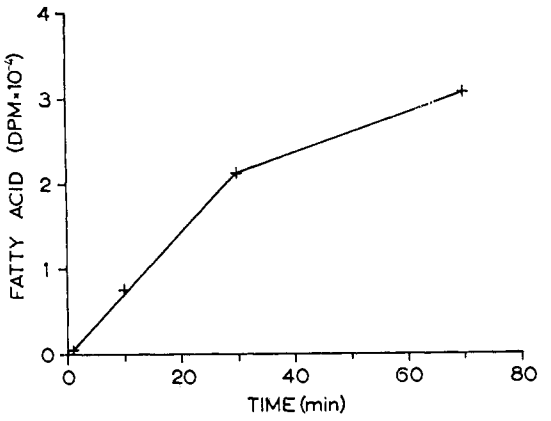


FIGURE 2: Dependence of the conversion of [³H]phytosphingosine into fatty acids upon the duration of the experiment. [³H]Phytosphingosine (0.25 μmole) (1.33 × 10⁶ dpm/μmole), suspended in 0.25 ml of saline, was administered into the tail veins of rats. At the specified times, the livers were excised and extracted. The extract was hydrolyzed in ethanolic KOH and the saponifiable fatty acids were isolated as described in the Experimental Procedure. To obtain a zero-time control, a rat was injected as above, and the liver was excised after 1 min and extracted immediately.

the distribution in blood and several organs; liver had the highest radioactivity and was used for further investigation.

Distribution of Radioactivity from Sphingosine Bases in Liver Lipids. Rats were injected with 0.5 μmole of each of the three bases (suspended in 0.5 ml of saline) and the livers were excised after 70 min. About 20–30% of the administered radioactivity was recovered in the liver extracts. The extracts were then chromatographed on "Unisil" silicic acid and the various lipid fractions were isolated as described in Experimental Procedure. Some variation in the distribution of the radioactivity was observed, depending upon the nature of the base used as substrate. The per cent radioactivity of the liver extracts present was in ceramide, 25–40%; in triglycerides, 15–25%; in lecithin, 17–21%; and recovered as the free base, about 25%. Only little radioactivity was present in either phosphatidylethanolamine or sphingomyelin.

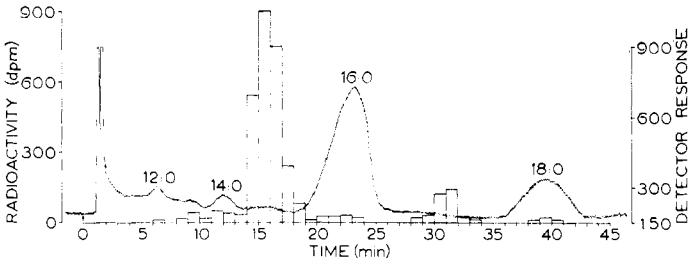


FIGURE 3: Fatty acid methyl esters (an aliquot having 2700 dpm) from animals injected with [³H]phytosphingosine. Distribution of radioactivity in the saturated fatty acid methyl esters. Rats were injected with suspensions (0.5 μmole/0.5 ml) of [³H]dihydrosphingosine or [³H]phytosphingosine, and the livers were excised after 1 hr and extracted. The extracts were saponified and the saturated fatty acids of the neutral and phosphoglycerides were prepared as described in Experimental Procedure. They were then methylated, subjected to gas–liquid partition chromatography, and the effluents were collected and counted in a scintillation spectrometer.

The above ceramide fractions were hydrolyzed in aqueous methanolic HCl (Gaver and Sweeley, 1965). With all three bases used as substrates, most of the radioactivity was present in the base portion of the ceramide and only little in the fatty acid. This suggests that the radioactive ceramide was formed by a condensation of the administered base with endogenous, nonradioactive fatty acids.

Incorporation of Phytosphingosine into Liver Glycerides. Phytosphingosine was injected into rats and the livers were excised after 2, 10, 30, 70, and 420 min. The livers were extracted with chloroform–methanol (2:1), the total lipid extract was hydrolyzed with 0.15 N ethanolic KOH, and the fatty acids were isolated as described in Experimental Procedure. This method releases the fatty acids of the neutral and phosphoglycerides, but not of the sphingolipids. Figure 2 shows the time course of incorporation of tritium-labeled phytosphingosine into these fatty acids. The radioactivity increased, in a linear fashion, for about 30 min, then leveled off. After 6 hr less than one-third of the radioactivity observed at 70 min after injection remained in the liver.

Identification of the Labeled Fatty Acids. Suspensions of each of the three bases were administered into rats. After 1 hr, the livers were excised and the lipids were extracted. Each extract was hydrolyzed and fatty acids were isolated and separated into several fractions as described in Experimental Procedures. Most of the radioactivity of the fatty acids was present in the normal, saturated acids (76% using sphingosine, 94% using dihydrosphingosine, and 85% using phytosphingosine), the rest was present in monoenoic acids. There was no radioactivity in the hydroxy or polyenoic fatty acid fractions.

The distribution of radioactivity among the normal, saturated fatty acid methyl esters is shown in Figures 3 and 4. In the experiments presented in these figures fatty acids, obtained after injection of phytosphingosine and dihydrosphingosine, were analyzed. A third similar run (not shown in the figures) was performed with the saturated fatty acids isolated after administration of [³H]sphingosine. Table II summarizes the results obtained with the three long-chain bases. There is a striking difference in the distribution of the radioactivity depending upon whether phytosphingosine or the two other bases were employed as precursors. With phytosphingosine, 81% of the radioactivity was located in pentadecanoic acid (C-15), 10% in heptadecanoic acid (C-17), and only less than 10% in the even-numbered fatty acids. With either dihydrosphingosine or sphingosine, 99% of the counts were in the even-numbered fatty acids, 80–90% in hexadecanoic, and the rest in tetra- and octadecanoic acids.

After administration of [³H]sphingosine, about 23% of the radioactivity of the fatty acids was present in monoenoic acids. Since sphingosine has a double bond between carbon atoms 4 and 5, the possibility was considered that hexadec-2-enoic acid might be formed as an intermediate. Unlabeled hexadec-2-enoic was added to the monoenoic acid methyl esters and these were sub-

TABLE II: Per Cent Distribution of Radioactivity in Saturated Fatty Acids.[a]

Precursor	Fatty Acid (%)				
	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}
[^{3}H]Phytosphingosine	2.8	81	4.2	10.2	1.8
[^{3}H]Dihydrosphingosine	2.6	<0.5	89.5	<0.5	7.8
[^{3}H]Sphingosine	7.3	<0.5	80.0	<0.5	12.8

[a] Conditions are the same as in Figures 3 and 4.

jected to gas–liquid partition chromatography. The effluents were collected as before and counted. Most of the radioactivity of this fraction was present in myristoleic, palmitoleic, and oleic acids. The effluent corresponding to the peak of hexadec-2-enoic acid was not radioactive.

Discussion

The purpose of this work was to establish whether the sphingosine bases are degraded in animal tissue and, if so, to identify the routes leading to the complete metabolic combustion of these bases to CO_2 and water. Both of these points were clarified by administration of the three most abundant bases, of eighteen carbon atoms, into rats (either intravenously as here described or intracerebrally; Roitman *et al.*, 1967) and following their metabolic fate. All three administered bases underwent two rapid changes, one synthetic, the other degradative. In the synthetic route, they were condensed with endogenous fatty acids to yield ceramide; in the degradative route, they were converted into fatty acids (or fatty acid derivatives) having two or three carbon atoms less than the parent base. The biosynthesis of ceramide can be brought about by one of two, formerly proposed, pathways. Either by a direct condensation with a free fatty acid, catalyzed by ceramidase (Gatt, 1963, 1966a), or by condensation with acyl coenzyme A (Sribney, 1966). The ceramide thus formed can be utilized for the formation of sphingomyelin (Sribney and Kennedy, 1958) and perhaps also of other sphingolipids. In the degradative route, the fatty acids formed from the bases will be oxidized by the fatty acid oxidation systems to CO_2 and water.

Of great interest was the type of fatty acid formed from each of the three bases. While phytosphingosine yielded pentadecanoic acid, both dihydrosphingosine and sphingosine were converted into hexadecanoic (palmitic) acid. The detailed mechanisms by which the bases are degraded to these fatty acid have not been established yet; any discussion of possible mechanisms is therefore hypothetical.

Scheme I proposes three possible mechanisms for the degradation of dihydrosphingosine. The pathways shown in Scheme Ia,b have hexadecanal as an intermediate; this aldehyde, if formed, can be oxidized to hexadecanoic acid by the liver aldehyde dehydrogenase. In the pathway shown in Scheme Ic, hexadecanoic acid is formed directly by a cleavage of the 3-ketodihydrosphingosine. There is no evidence to date for any of these mechanisms, and other possibilities (*i.e.*, phosphorylation of the primary hydroxyl group of the base prior to its cleavage), cannot be excluded.

Sphingosine can be cleaved similar to any of the mechanisms proposed for the degradation of dihydrosphingosine. The products in this case will be a two-carbon fragment and hexadec-2-enoic acid. The latter, monoenoic acid will then be reduced to the saturated hexadecanoic acid. An alternate possibility might be a reduction of sphingosine to dihydrosphingosine, prior to its degradation. This dihydrosphingosine might then be cleaved by any of the mechanisms of Scheme I. In this connection, it is worth noting that after administration of [^{3}H]sphingosine to rats, there was no radioactivity in the effluent of the gas–liquid partition chromatogram corresponding to hexadec-2-enoic acid. Most of the label resulting from degradation of [^{3}H]sphingosine was present in the saturated fatty acids or in monoenoic acids, other than hexadec-2-enoic acid.

Phytosphingosine yielded pentadecanoic acid; this suggests a cleavage between carbon atoms 3 and 4 to a C_{15} and C_3 fragments. This could occur by any mechanism similar to those proposed in Scheme I for dihydrosphingosine (except that, dissimilar to the mechanism shown in Scheme IB, an aldol, rather than a ketol cleavage will take place). An alternative possibility might involve a two-stage cleavage as follows. In the first stage, the phytosphingosine base is cleaved to a C_2 unit and α-hydroxyhexadecanoic acid. This hydroxy acid might then be degraded by a fatty acid–α-oxidation system to pentadecanoic acid and CO_2. In this connection it is worth noting, that after administration of [^{3}H]phytosphingosine to rats, the fraction containing the liver hydroxy fatty acids was not

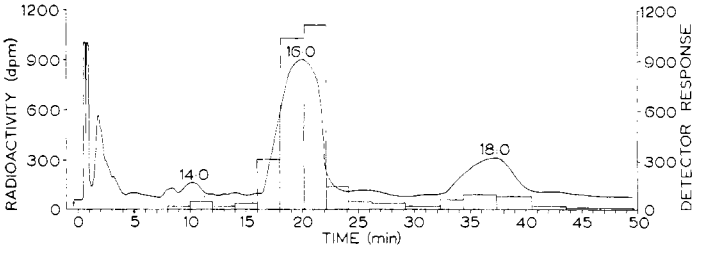
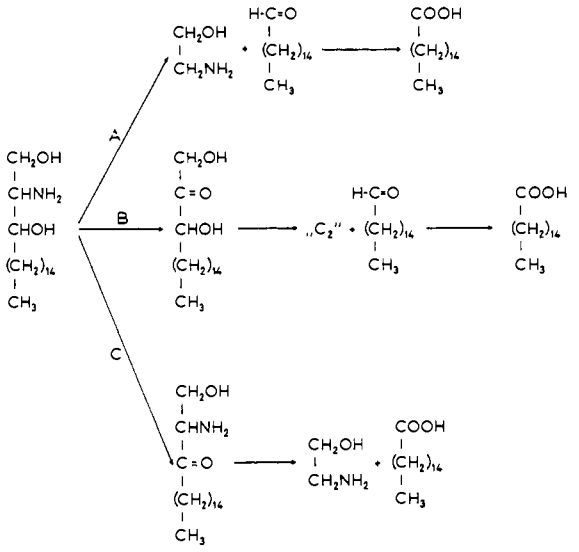


FIGURE 4: Fatty acid methyl esters (an aliquot having 5800 dpm) from animals injected with [^{3}H]dihydrosphingosine. See caption to Figure 3.

SCHEME I



radioactive; neither was there any appreciable radioactivity in the C_{16} fatty acids. These findings, therefore do not support the two-stage mechanism. In a former paper (Barenholz and Gatt, 1967), it was suggested that phytosphingosine might be an intermediate in the degradation of sphingosine and dihydrosphingosine. The fact that the latter two bases do not yield any labeled pentadecanoic acid (which should be formed if phytosphingosine is an intermediate) does not support this theory.

Subcellular preparations of rat liver can convert the sphingosine bases into fatty acids. Experiments are in progress in an attempt to isolate and characterize the enzymatic systems responsible for this conversion.

While this manuscript was in preparation, Stoffel and Sticht (1967a,b) published experiments, similar to ours, using labeled sphingosine and dihydrosphingosine. Similar to the results reported here, they also observed the conversion of these two long-chain bases into palmitic acid in the liver. In our experiments the animals were sacrificed after 1 hr, while Stoffel and Sticht terminated the experiments after 6–8 hr. This variation in the duration of the experiments resulted in two differences. While the radioactivity of the ceramide in our experiments resided mostly in the long-chain-base portion, 40% of radioactivity of the ceramide isolated by Stoffel and Sticht was found in the fatty acid fraction. Also, in our experiments, the phosphatidylethanolamine and sphingomyelin had very little radioactivity, while in their experiments these compounds were considerably labeled. In the experiments by Roitman *et al.* (1967), where the long-chain bases were injected intracerebrally and the animals sacrificed after 24 hr, considerable radioactivity was found in the sphingomyelins and gangliosides.

Added in Proof

In experiments conducted after submitting this paper for publication, it was found that a mixture of microsomes and dialyzed 100,000*g* supernatant degraded phytosphingosine to fatty acids. Only about 10% of these fatty acids was present as pentadecanoic, while about 65% was identified as α-hydroxypalmitic acid. This suggests that α-hydroxypalmitic acid is an intermediate in the conversion of phytosphingosine into pentadecanoic acid. In *in vivo* experiments, the formation of this hydroxy acid has not been observed, most likely because of rapid oxidative decarboxylation.

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Gas Chromatography and Mass Spectrometry of Sphingolipid Bases. Characterization of Sphinga-4,14-dienine from Plasma Sphingomyelin*

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ABSTRACT: A partially purified sample of sphinga-4,14-dienine was obtained by chromatographic procedures from acid-catalyzed methanolysates of human plasma sphingomyelin. The structure of this sphingolipid base was deduced from mass spectrometric data before and after osmium tetroxide oxidation, and by mass spectrometric identification of sebacic acid after permanganate–periodate oxidation of the base. Hexadecasphing-4-enine and heptadecasphing-4-enine were also identified conclusively in sphingomyelin from the mass spectra of *N*-acetyl-*O*-trimethylsilyl derivatives. Gas chromatography of the *N*-acetyl-*O*-trimethylsilyl derivatives on selective liquid phases separates sphingadienines from sphingenines and relative retention data are given for routine gas chromatographic identification of a variety of sphingolipid bases.

Sphingomyelin from human plasma contains several long-chain bases in addition to sphingosine (sphing-4-enine)[1] and sphinganine. They were originally detected by gas–liquid partition chromatography of an aldehyde fraction obtained by periodate oxidation of the bases (Sweeley and Moscatelli, 1959). On the basis of relative retention behavior before and after catalytic hydrogenation, one of the aldehydes was assumed to be derived from a doubly unsaturated base, a sphingadienine, closely related to sphingosine. Later, this new base was partially purified by silicic acid chromatography of a mixture of DNP derivatives and permanganate oxidation led Karlsson (1964) to conclude that it was a mixture of sphinga-4,14-dienine and sphinga-4,12-dienine.

Several lines of evidence have indicated the presence of lower homologs of sphingosine in sphingomyelin from human plasma and other sources. Hexadecasphing-4-enine and heptadecasphing-4-enine have been detected by gas–liquid partition chromatography of aldehydes from periodate oxidation (Sweeley and Moscatelli, 1959), gas–liquid partition chromatography of fatty acids from various types of oxidations at the olefinic center (Proštenik and Majhofer-Oreščanin, 1960; Karlsson, 1964; Popović, 1966), and gas–liquid partition chromatography of the intact bases as trimethylsilyl derivatives (Gaver and Sweeley, 1965; Karlsson, 1965).

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[1] The nomenclature for sphingolipid bases is based on recent recommendations of the Commission on Biochemical Nomenclature of IUPAC and IUB (see The Nomenclature of Lipids, *J. Am. Oil Chemists' Soc. 44*, 548A (1967)). Where new names challenge the imagination at first sight, they are used together with common names for the sake of clarity.