Biosynthesis of Phytosphingosine by the Rat*

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Labeled C₂₃-phytosphingosine was detected in a glycolipid-rich fraction from rat intestine 4 and 24 h after injection of D-erythro[4,5-³H]C₂₃-dihydrosphingosine. The criteria for identification were: (a) co-migration on gas-liquid chromatography of the trimethylsilyl ethers of the free and N-acetylated base with authentic standard, (b) co-migration on thin layer chromatography of the dinitrophenyl derivative with authentic standard, (c) labeled pentadecanol detection by gas-liquid radiochromatography after subjecting intact sphingolipids or a mixture of free long chain bases to periodate oxidation followed by sodium borohydride reduction, and (d) obtainment of the same fragmentation pattern as with authentic standard when the trimethylsilyl ethers of the N-acetylated base were subjected to gas-liquid chromatography-mass spectrometry.

Smaller amounts of labeled C₂₃-phytosphingosine were also detected in ceramide- and sphingomyelin-rich fractions of intestine and in liver and kidney. Experiments with germ-free rats strongly suggest that the intestinal flora is not the (sole) site for the biosynthesis of C₂₃-phytosphingosine.

Phytosphingosines are long chain bases that differ from sphingosines in that they have an additional hydroxyl at C-4 and have no double bond between carbon 4 and carbon 5. They were first isolated by Carter and collaborators in plants (1), hence their name "phyto"-sphingosine. The configuration of the amino and hydroxyl groups was determined by Carter and Hendrickson (2) to be D-ribo-1,3,4-trihydroxy-2-aminoocotadecane. These materials and their unsaturated and branched analogues have since been reported to occur in yeasts (3), protozoa (4, 5), sea urchin (6), fish (7), amoeba (8), and mammals where they have been detected as part of sphingolipids in human (9), bovine (10, 11), rat (11, 12) and equine (13) kidney, human (11, 12) and dinitrophenyl derivatives of long chain bases were obtained by the procedure of Carter and Gaver (20). Preparation of borate-impregnated Silica Gel G plates and dinitrophenyl derivatives of long chain bases were obtained as described by Karlsson et al. (12).

Periodate oxidation of intact sphingolipids and free long chain bases followed by sodium borohydride reduction was performed as previously described (11). Me₃Si ethers of the free and N-acetylated long chain bases were obtained by the procedure of Carter and Gaver (20). Preparation of borate-impregnated Silica Gel G plates and dinitrophenyl derivatives of long chain bases were obtained as described by Karlsson et al. (12).

Materials and Methods
Preparation of D-erythro[4,5-³H]C₂₃-dihydrosphingosine - Catalytic reduction of D-C₂₃-sphingosine (8 mg; from bovine brain, purified from Sigma Chemical Co.) with tritium gas was performed by New England Nuclear. The reduction product was diluted with an equal quantity of D-erythro-C₂₃-dihydrosphingosine (synthetic; Sigma Chemical Co.) and purified by preparative thin layer chromatography (type 0 plates, New England Nuclear) in chloroform:methanol:2 N NH₄OH (100:25:2.5). Labeled dihydrosphingosine was eluted from the silica gel with methanol and the solvent was removed under reduced pressure. Radiochemical purity was greater than 97% as judged by thin layer chromatography (11). Gas-liquid radiochromatography of the Me₃Si ethers of the free or N-acetylated (30) long chain base indicated that at least 98% of the label was in erythro-C₂₃-dihydrosphingosine. The specific activity was 15.7 Ci/mmol.

Injection of Labeled C₂₃-Dihydrosphingosine into Rats - A suspension of radioactive dihydrosphingosine (3.64 mCi) in sterile saline (0.7%; 4; 3 ml) containing bovine serum albumin (5%), was sonicated for three 30-s periods with a Heat Systems ultrasonic oscillator at an output setting of 7. Eight male rats (NLR strain, Wistar origin; 5½ to 6 weeks old; average weight 121 g; National Laboratories, O'Fallon, Mo.) were each injected in the tail vein with 0.3 ml of the above solution. Animals were killed by exanguination, after light anesthesia with ether, 4 and 24 h after injection. Intestine, liver, and kidneys were removed, washed with cold saline, minced, and lyophilized.

Extraction and Purification of Lipids - Lipids were extracted from the combined lyophilized intestines of four rats by the procedure previously described (11). The extract was washed according to Folch et al. (21) and the lower layer was taken to dryness under reduced pressure. Total lipids of animals 4 h after injection of labeled dihydrosphingosine contained 3.2 x 10⁶ dpm; 24 h after injection this fraction contained 4.6 x 10⁶ dpm. Total lipids were applied (in chloroform) to a silicic acid column (70 g; 30 x 2.8 cm) and a polar lipid-rich fraction was obtained (11). This fraction (2.8 x 10⁶ dpm from the 4-h rats and 2.0 x 10⁶ dpm from the 24-h rats) was dissolved in chloroform:methanol 2:1 (100 ml) and subjected to mild alkaline hydrolysis for 1 h at room temperature after the addition of an equal volume of 1 N methanolic KOH. Subsequent workup (11) provided a polar lipid fraction stable to mild alkali containing 2.1 x 10⁵ dpm from the 4-h rats and 1.0 x 10⁵ dpm from the 24-h rats. This latter sample was applied to a silicic acid column (5 g; 18 x 1.0 cm) and eluted with chloroform:methanol 97:3 (100 ml), chloroform:methanol:water 50:40:10 (100 ml). This latter fraction (1.9 x 10⁵ dpm) contained glycolipids and was used for analyses of most long chain bases.

Characterization of Long Chain Bases and Long Chain Alcohols - Free long chain bases were obtained by acid hydrolysis of sphingolipids in 1 to 2 ml of 1 N methanolic HCl (30 M H₂O) at 80° for 16 h as previously described (11). Me₃Si ether derivatives of free and N-acetylated long chain bases were obtained by the procedure of Carter and Gaver (20). Preparation of borate-impregnated Silica Gel G plates and dinitrophenyl derivatives of long chain bases were obtained as described by Karlsson et al. (12).

Periodate oxidation of intact sphingolipids and free long chain bases followed by sodium borohydride reduction was performed as previously described (11).

The abbreviations used are: Me₃Si, trimethylsilyl; DNP, dinitrophenyl.

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cording to Sweeley and Moscatelli (22), as modified by Carter and Hirscheberg (11). The resulting long chain alcohols were analyzed by gas-liquid radiochromatography. For this purpose, a Hewlett Packard 5711A chromatograph equipped with a gas effluent splitter was used. An SE-30 column (3% on Gas-chrom Q) (Applied Science Laboratories); 6 feet × 1/4 inches I.D.) was used for analyses of long chain bases. Injection port and detector were operated at 250°C. The column was operated at 200°C for 32 min followed by temperature increments of 1°C per min up to 210°C. Carrier gas was nitrogen (99.999% min). An ethylene glycol succinate column (15% on Gas-chrom P; 6 feet × 1/8 inches I.D.) at 160°C was used for analyses of long chain alcohols. Injection port and detector temperatures were 200°C. Nitrogen gas flow rate was 40 cc/min. Fatty acid methyl esters were analyzed on an ethylene glycol succinate column as described above, except that the column temperature was 180°C.

Fractions of the column effluent were collected with Pasteur pipettes for 1-min intervals. Radioactivity was eluted from the pipettes with toluene/Omnifluor (New England Nuclear). For liquid scintillation spectrometry, a Packard Tri-Carb model LS 3380 was used. Standard phytosphingosine was purchased from Aldrich Chemical Co.

Gas Chromatography-Mass Spectrometry—An LKB 9000 gas chromatograph-mass spectrometer was used for determining fragmentation pattern of the material that co-migrated with the Me₄Si ethers of N-acetyl-C₁₈-phytosphingosine. An OV-17 column (1%) at 230°C was used. The acceleration voltage was 2.5 kV and the ionizing potential 70 EV at 120 µA of emission. A Logos data system model DS-2000 WE was coupled to the mass spectrometer.

RESULTS

Previous studies had suggested C₁₈-dihydrosphingosine as a possible precursor of C₁₈-phytosphingosine in yeasts (23, 24); we therefore decided to investigate the existence of a similar pathway in rats, by injecting n-erythro[4,5-³H]C₁₈-dihydrosphingosine and determining the subsequent occurrence of labeled C₁₈-phytosphingosine in intestine, since this tissue has been reported to contain relatively high amounts of this base (10, 14, 15). Furthermore, preliminary studies had indicated to us that the presence of phytosphingosine carrier (in this case, of endogenous origin) would reduce breakdown of small amounts of labeled base and facilitate its isolation and characterization.

Preliminary Evidence for Occurrence of Labeled C₁₈-Phytosphingosine in Sphingolipids of Intestine—Preliminary evidence for the occurrence of labeled C₁₈-phytosphingosine in intestine was obtained by examining a fraction containing a crude mixture of sphingolipids (obtained after mild alkaline hydrolysis of polar lipids and subsequent purification on silicic acid chromatography). We reasoned that if labeled C₁₈-phytosphingosine occurred in such fraction, then according to the scheme of Fig. 1, one should be able to isolate and identify labeled pentadecanol as a reaction product upon periodate oxidation followed by sodium borohydride reduction. Fig. 2 shows a gas-liquid radiochromatogram of such a sample obtained 24 h after the injection of labeled C₁₈-dihydrosphingosine. The predominant mass peak and most of the radioactivity had the same mobility as pentadecanol. A similar mass profile was obtained from a sample isolated 4 h after injection of labeled C₁₈-dihydrosphingosine (Fig. 3). However, in this case, only 13% of the radioactivity had the same mobility as pentadecanol while the remainder co-chromatographed with hexadecanol. The absence of a hexadecanol mass peak suggests that this radioactivity was derived from free labeled C₁₈-dihydrosphingosine that had not (yet) been incorporated into lipids.

Characterization of Labeled C₁₈-Phytosphingosine in a Glycolipid-Rich Fraction of Rat Intestine—Several studies have shown that phytosphingosines occur in many instances as part of glycosphingolipids in mammals (10-15). A glycolipid-rich fraction was obtained by applying the crude sphingolipid mixture (used in the previous experiment) to a silicic acid column...
and eluting the column with chloroform, chloroform: methanol (97:3) and chloroform: methanol (60:40). Almost half of the radioactivity eluted in this latter fraction from a sample obtained 24 h after injection of labeled C₁₅-dihydrosphingosine co-migrated on thin layer chromatography with bovine brain cerebrosides. The remainder of the radioactivity had a mobility consistent with ceramide polyhexosides. No labeled free long chain bases could be detected. Periodate oxidation of this fraction followed by sodium borohydride reduction yielded pentadecanol as the only long chain alcohol detectable by gas-liquid chromatography and virtually all the radioactivity co-migrated with this peak (data not shown).

When this glycolipid-rich fraction was subjected to acid hydrolysis and the resulting free long chain bases were separated by gas-liquid chromatography (as the Me₃Si ethers), the profile shown in Fig. 4 was obtained. Two major mass peaks can be seen. The first to elute (33% of the total peak areas) had the same mobility as the Me₃Si ethers of C₁₅-sphingosine. The second peak (67% of the total peak areas) co-migrated with the Me₃Si ethers of C₁₅-phytosphingosine. This same figure shows that a substantial portion of the radioactivity (~33%) co-migrated with the Me₃Si ethers of C₁₅-phytosphingosine while the remainder co-migrated with the Me₃Si ethers of C₁₅-sphingosine and C₁₅-dihydrosphingosine.

A similar analysis of the sample obtained 4 h after injection of radiolabeled C₁₅-dihydrosphingosine showed that only 6% of the radioactivity co-migrated with the Me₃Si ethers of C₁₅-phytosphingosine. The remainder co-migrated with the Me₃Si ethers of C₁₅-dihydrosphingosine. Because of the relative small amount of labeled C₁₅-phytosphingosine in this sample, all further characterizations of the labeled C₁₅-phytosphingosine were done with the sample obtained 24 h after injection of labeled C₁₅-dihydrosphingosine.

To determine whether the mass peak which co-migrated with the Me₃Si ethers of C₁₅-phytosphingosine on Fig. 4 was homogeneous, the mixture of free long chain bases was converted to the N-acetyl derivative and subsequently subjected (as the Me₃Si ethers) to gas chromatography-mass spectrometry analyses. The mass fragmentation patterns on both sides of the gas chromatograph peak that had been tentatively identified as the Me₃Si ethers of N-acetyl-C₁₅-phytosphingosine were determined and found to be very similar, arguing against a mixture of N-acetyl-C₁₅-phytosphingosine with some other unknown compound. The following ions that have been reported to be characteristic for the Me₃Si ether derivative of N-acetyl-C₁₅-phytosphingosine were observed: m/e 239 (20% of base peak); m/e 560 (M-15), and m/e 491. The base peak was m/e 132 or m/e 73, in agreement with the fragmentation patterns previously described (18, 23, 25).

The free long chain bases were also converted to the dinitrophenyl derivatives. Upon separation by thin layer chromatography, the radioactivity profile shown in Fig. 5 was obtained. Approximately 30% of the radioactivity that co-migrated with the DNP derivatives of long chain bases had the same mobility as DNP C₁₅-phytosphingosine. The DNP derivatives of long chain bases of a glycolipid-rich fraction of free long chain bases of a glycolipid-rich fraction of intestine from rats 24 h after injection of D-erythro[4,5-³H]C₁₅-dihydrosphingosine. A, C₁₅-sphingosine; B, C₁₅-dihydrosphingosine; C, C₁₅-phytosphingosine.
per cent of the radioactivity co-migrated with pentadecanol, as to be expected if it was derived from C₁₄-phytosphingosine (Figs. 1 and 4). The remainder of the radioactivity co-migrated with hexadecanol and Δ²-hexadecanol, in the percentages to be expected if it was derived from C₁₆-dihydrosphingosine and C₁₈-sphingosine (Figs. 1 and 4).

Occurrence of Labeled C₁₄-Phytosphingosine in Other Tissues and Lipid Fractions — Labeled C₁₄-phytosphingosine was also detected in ceramide-rich and sphingomyelin-rich fractions of intestine and in the corresponding ones of kidney and liver. However, in all these samples, the radioactivity was less than 8% of that in other long chain bases (C₁₈-sphingosine + C₁₆-dihydrosphingosine).

Experiments with Germ-free Rats — We had previously postulated (11) that the intestinal flora did not play a significant role in the biosynthesis of phytosphingosine. This hypothesis was tested at the University of Notre Dame by using germ-free rats from their colony.

Male rats (1 year old, 250 g average weight) were injected with sterile [4,5-³H]C₁₄-dihydrosphingosine (0.55 mCi in 5% propylene glycol in saline). Animals were killed 24 h after injection. Upon periodate oxidation (followed by sodium borohydride reduction) of a crude sphingolipid fraction of rat intestine, 60% of the radioactivity in long chain alcohols was in pentadecanol, strongly suggesting that C₁₄-phytosphingosine was not (solely) derived from the intestinal flora.

DISCUSSION

Assmann and Stoffel (26) observed in a previous study that radiolabeled C₁₄-phytosphingosine was incorporated into sphingolipids by the rat when injected intravenously or administered orally. These experiments led them to conclude that phytosphingosines in rats were of dietary origin. Our results with normal and germ-free animals clearly show that the rat can synthesize phytosphingosines and strongly suggest that the diet or the intestinal flora (or both) are not the sole source of phytosphingosines in mammals.

Several studies have suggested that C₁₄-phytosphingosine originates by hydroxylation of C₁₆-dihydrosphingosine (23, 24, 27). Although, in the present study, labeled C₁₄-phytosphingosine may be directly derived from C₁₆-dihydrosphingosine, it is also possible that labeled C₁₆-dihydrosphingosine was first broken down to 1,2,3-³H-palmitic acid and that subsequently this material was incorporated into phytosphingosine by some other mechanism. Furthermore, since the amount of label that was actually isolated and characterized as C₁₄-phytosphingosine was less than 0.1% of the radioactivity injected, we cannot rule out the possibility that the precursor of C₁₄-phytosphingosine was a small quantity of an unknown contaminant. The possibility that a small amount of labeled C₁₄-phytosphingosine (as a contaminant of the C₁₆-dihydrosphingosine) was actually injected into rats and subsequently concentrated by different tissues is highly unlikely, as preparative thin layer chromatography (used in the purification) clearly separates C₁₄-dihydrosphingosine from C₁₄-phytosphingosine; in addition, this latter base cannot be purified by this procedure because it breaks down during elution from the silica gel.

We have found that periodate oxidation of intact sphingolipids followed by sodium borohydride reduction is a rather sensitive method to detect C₁₄-phytosphingosine (in the form of pentadecanol). This procedure has enabled us to detect a mass peak for pentadecanol in only those fractions where subsequently C₁₄-phytosphingosine was detected as the Me₃Si ethers of the free base. The possibility that the labeled penta-

canol was not solely derived from C₁₄-phytosphingosine but also from α-hydroxypalmitate of high specific activity (bound to the amino group of the long chain bases) was also investigated. Essentially no radioactivity was detected in this fatty acid.

C₁₄-phytosphingosine (in small amounts) has been found to be rather unstable to chemical treatments in which other long chain bases such as C₁₈-sphingosine and C₁₆-dihydrosphingosine are relatively stable. For example, as previously mentioned, we have been unable to purify C₁₄-phytosphingosine by preparative thin layer chromatography. We have also detected breakdown of C₁₄-phytosphingosine during acid hydrolysis of small samples of sphingolipids under conditions where C₁₈-sphingosine and C₁₆-dihydrosphingosine are rather stable. Variations of the percentage of radiolabeled C₁₄-phytosphingosine (as compared to other long chain bases) in a given tissue of rats injected with the same amounts of radiolabeled C₁₄-dihydrosphingosine have also been observed. Some of this variability can be attributed to individual differences in metabolism; however, this problem is further complicated by the previously mentioned instability of small amounts of C₁₄-phytosphingosine isolated during such studies. One therefore should keep in mind that the 33% of radioactivity in the long chain bases that was characterized as C₁₄-phytosphingosine of a glycolipid-rich fraction of intestine from animals 24 h after injection of labeled C₁₄-dihydrosphingosine represents an average from four rats.

The occurrence of phytosphingosine mass in some sphingolipids but not others, even within the same tissue, raises some intriguing questions as to how this base is incorporated into sphingolipids and its relation to other long chain bases. We are currently attempting to gain further insights into some of these questions.

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