DEGRADATION OF SPHINGOSINE BASES BY CELL-FREE PREPARATIONS. α-HYDROXY PALMITIC ACID, AN INTERMEDIATE OF PHYTOSPHINGOSINE DEGRADATION

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Phytosphingosine (CH_3-$(CH_2)_{13}$-CHOH-CHOH-$CHNH_2$-CH_2OH) is the most abundant long-chain base of the plant sphingolipids, but has recently also been found in animal tissues (Karlsson, 1964; Michalec and Kolman, 1966; Karlsson and Martensson, 1968). Gatt and coworkers (Barenholz and Gatt, 1967, 1968; Roitman et al., 1967) have administered phytosphingosine, by intravenous or intracerebral route, to rats. This base was degraded by the animal and the main product, which consequently accumulated, was identified as pentadecanoic acid. When two other long-chain bases, dihydrosphingosine (CH_3-$(CH_2)_{14}$-CHOH-$CHNH_2$-CH_2OH) and sphingosine (CH_3-$(CH_2)_{12}$-CH=CH-CHOH-$CHNH_2$-CH_2OH) were similarly administered, the main degradation product was hexadecanoic (palmitic) acid (Roitman et al., 1967; Barenholz and Gatt, 1968; Stoffel and Sticht, 1967a, b). On this basis, Barenholz and Gatt (1968) suggested that the three bases might be degraded by two separate pathways. While phytosphingosine is cleaved between carbon atoms 3 and 4 to a C_{15} and C_3 units, the other two bases are split between carbon atoms 2 and 3 to a C_{16} and C_2 units. They also considered the possibility that phytosphingosine might, alternatively, be degraded in two-

stages. First, like dihydrosphingosine to a C_{16} and C_2 units; the C_{16} unit, most likely 2-hydroxy hexadecanoic acid, is then be further degraded to pentadecanoic acid and CO_2. The two-stage pathway could not be verified in studies with intact rats, as only little radioactivity was found in either the liver hydroxy fatty acids or in the non-hydroxy C_{16} fatty acids.

In the present investigation tritium labeled phytosphingosine was degraded to fatty acid using cell-free preparations of rat liver. The main radioactive component of the fatty acids was identified as 2-hydroxy hexadecanoic (α-hydroxy palmitic) acid.

METHODS

Tritium-labeled phytosphingosine (prepared according to Barenholz and Gatt, 1968) was suspended in 0.154 M KCl - 1 mM EDTA, pH 7.0 and incubated with cofactors (see "Results"), in a volume of 1 ml. The reaction was terminated by the addition of 4.8 ml of ethanol and 0.2 ml of 15 N KOH. After 30 min at 80°, the mixture was acidified with 0.6 ml of 10 N H_2SO_4 and shaken with 3 ml of water and 6 ml of heptane. The heptane layer was transferred to a second tube, washed once with 2 ml of pure solvents lower phase of Dole's solution (1956) and then treated with 4 ml of 0.1 N NaOH in 50% ethanol. 3 ml of the upper heptane layer, which contained the ceramide (N-acyl phytosphingosine), were transferred to a counting vial. The lower phase was washed twice with 2 ml of heptane and acidified with 1 ml of N H_2SO_4. The fatty acid was partitioned into 3 ml of heptane, this was transferred to a counting vial. 10 ml of scintillation fluid (5 g of 2,5-diphenyloxazole and 130 mg of 1,4-bis (2-(4-methyl-5-phenyloxazolyl) benzene per liter of toluene) was added and the vial was counted in a Tri Carb liquid scintillation spectrometer.

RESULTS

Tritium-labeled phytosphingosine, incubated with rat liver homogenates, in the presence of ATP, NAD and Mg ions underwent two main changes. It was degraded to fatty acids and acylated to ceramide (N-acyl phytosphingosine). These homogenates (1 pt of liver to 4-5 volumes of 0.154 M KCl - 1 mM EDTA, pH 7.0) were centrifuged for 10 min at 600 xg to remove debris and the supernatant was separated into the following subcellular fractions: Mitochondria (10 min at 12,000 xg), microsomes (1 hr at 100,000 xg) and the 100,000 xg supernatant. All three fractions converted the substrate to an N-acylated derivative (ceramide), but when tested individually, did not yield a radioactively-labeled fatty acid. Labeled fatty acid was obtained using mixtures of microsomes and supernatant, fortified with ATP, an ATP regenerating system, NAD, NADP, FAD or FMN and manganese ions.

Attempts were made made to extract and purify the components, responsible for the degradation of the base to fatty acid, in either the microsomes or supernatant. For this purpose the microsomes were lyophilized; the lyophilized powder retained its full activity for at least 2 months when stored in a dessicator at -20°. Trials were made to solubilize the enzymes by subjecting the lyophilized powder to sonic oscillation (3 min at 20,000 kc in a Branson sonifier), by incubating with M KCl or sodium taurocholate (20 mg/ml). There was only little loss of activity following these treatments, but the enzyme remained particle-bound. Treatment of the lyophilized powder with acetone or butanol resulted in loss of activity.

The 100,000 xg supernatant retained its activity for at least 2 months at -20°. It also retained its activity when dialyzed against 1 mM EDTA, pH 7.0, when treated with activated charcoal or filtered through a column of Biogel P-10. The preparations obtained after these treatments exhibited an improved linear proportionality of the activity with increasing protein concentrations. The super-

natant retained its activity when treated with a 10-15 fold oxcess of cold acetone, or with butanol (up to 0.07 ml per 1 ml of supernatant) but not when heated at 100°. Treatment of the supernatant with acetone, followed by ammonium sulfate fractionation and DEAE - cellulose chromatography (using a linear gradient of KCl in 1 mM EDTA, pH 7.0) resulted in a 10 fold increase of the specific activity.

Identification of the Reaction Products. The incubation mixture, in a volume of 1 ml, contained 80 mμmoles of tritium-labeled phytosphingosine (1300 dpm/mμmole), suspended in 0.2 ml of 0.154 M KCl-1 mM EDTA, pH 7.0; 5 μmoles of ATP; 10 μmoles of phosphoenol pyruvate; 8 μg of pyruvic kinase (Sigma); 0.5 μmoles each of NAD, NADP and FAD or FMN; 40 μmoles of nicotinamide; 20 mg of lyophilized microsomes; 0.3 ml of dialyzed supernatant and as the last addition, 7.5 μmoles of manganese chloride. 24 such incubation mixtures were kept for 1 hr at 37°, pooled and fatty acid and ceramide were isolated as described in Methods.

The ceramide fraction was adsorbed onto a 10 g silicic acid (Mallinckrodt) column and the ceramide (850,000 dpm) was eluted with 1-2% methanol in chloroform. Chromatography on thin layer plates of silica gel (in chloroform-methanol-acetic acid 94:2:4 and chloroform-methanol-conc. NH_4OH 97:3:0.3; Barenholz et al., 1966) showed that practically all the radioactivity migrated with the spot corresponding to N-acyl phytosphingosine. It was hydrolyzed in aqueous methanolic HCl (Gaver and Sweeley, 1965), the long-chain bases were extracted and chromatographed on thin layer plates of silica gel in chloroform-methanol-2N NH_4OH, 77:23:2.3 (modification of Sambasivarao and McCluer, 1963). The spots corresponding to phytosphingosine, dihydrosphingosine and sphingosine were scraped off and transferred to counting vials. The silica gel was suspended in 2 ml of Triton X-100-absolute ethanol, 1:1,

10 ml scintillation fluid were added and the vials were counted. 90% of the radioactivity was present in the spot corresponding to phytosphingosine.

The fatty acid fraction (80,000 dpm) was separated into hydroxy and non-hydroxy fatty acids on a column of Unisil-silicic acid (Clarkson, Williamsport, Pa.) according to Preiss and Bloch (1964). The individual fractions were further characterized on thin layer silica gel plates in hexane-ethyl acetate-acetic acid, 70:30:1 (Okui et al., 1963). The hydroxy fatty acids had 75% of the total radioactivity of the fatty acid fraction. The hydroxy and non-hydroxy fatty acids were then methylated with methanolic-HCl and with diazomethane in ether. The hydroxy fatty acids methyl esters were further acetylated with the perchloric acid-ethyl-acetate-acetic anhydride mixture of Fritz and Schenk (1959), to yield the α-acetoxy derivatives of the methyl esters. The hydroxy fatty acids were also oxidized with powdered $KMnO_4$ (Fulco and Mead, 1959; Hajra and Radin, 1963) to the normal fatty acids having one carbon atoms less than the parent compounds.

The hydroxy fatty acids were identified by liquid partition chromatography as follows:

a. The methyl esters were chromatographed (at 153°) on a 4 ft column of 14% ethylene glycol succinate on Gas Chrom P, 70-80 mesh (Applied Science).

b. The methyl esters were also chromatographed (at 193°) on a 6 ft column of 3.8% SE-30 on Chromosorb AW-DMCS, 100-120 mesh (Applied Science).

c. The α-acetoxy methyl esters were chromatographed (at 153°) on a 4 ft column of 14% ethylene glycol succinate on Gas Chrom P.

With all three methods, the column effluents were collected on glass wool in small vials. These were transferred to 20 ml of scintillation fluid and counted. In each case, over 80% of the radioactivity migrated with the peak corresponding to the appropriate derivatives of 2-hydroxy hexadecanoic acid.

d. The permanganate oxidation - product of the hydroxy fatty acid was methylated with diazomethane and chromatographed (at 174°) on a 15 ft column of 14% ethylene glycol succinate on Gas Chrom P. Most of the radioactivity of the non-hydroxy fatty acids thus obtained, migrated with pentadecanoic acid.

These results identify the hydroxy fatty acid, formed by degradation of phytosphingosine by rat liver microsomes and dialyzed supernatant, as mainly 2-hydroxy hexadecanoic (α-hydroxy palmitic) acid.

The non-hydroxy fatty acid methyl esters (25% of the total radioactivity of the fatty acid fraction) were chromatographed on a 15 ft column of 14% ethylene glycol succinate on Gas Chrom P. The column effluents were collected (using a 50:1 splitter) and counted as above. Over 40% of the radioactivity migrated with pentadecanoic acid. The rest of the radioactivity was distributed in several other fatty acids.

The distribution of radioactivity in the fatty acid fraction, using a mixture of microsomes and supernatant is therefore as follows: About 60-70% in 2-hydroxy hexadecanoic acid, 10% in pentadecanoic acid and 20% in other fatty acids. When phytosphingosine was similarly incubated with rat liver homogenate less debris, the hydroxy acids had only 35% of the radioactivity of the fatty acid fraction. This agrees with the assumption that phytosphingosine is first degraded to 2-hydroxy hexadecanoic acid, which is then further oxidized to CO_2 and pentadecanoic acid. In vivo, the intermediate hydroxy fatty acid did not accumulate; in the homogenate minus debris 35% of the fatty acids formed were hydroxy acids; in the microsomes plus dialyzed supernatant this value increased to 75% (most likely because of lack of enzymes or of necessary cofactors of the liver α-oxidation system (Stokke, 1968).

The conversion of phytosphingosine to α-hydroxy palmitic acid is conceivably a two-stage reaction, i.e. a cleavage of the bond between carbon atoms 2 and 3, and an oxidation of the group at carbon atom 3. The nature of these reactions is under investigation.

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