THE UTILIZATION AND DEGRADATION OF PHYTOSPHINGOSINE BY RAT LIVER

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Received March 24, 1967

Phytosphingosine* (D-ribo-1,3,4 trihydroxy-2-amino octadecane) is the most abundant long chain base (LCB) of the plant sphingolipids (Hanahan and Brockerhoff, 1965) and has recently also been found in animal tissues (Michalec and Kolman, 1966; Karlsson, 1964). Greene et al. (1965) have shown that this compound is formed, biosynthetically, from palmitic acid and serine, similar to the formation of two other LCBs, sphingosine and dihydrosphingosine (Ansell and Hawthorne, 1964).

The interconversions and degradative pathways of the LCBs are not known to date. Brady and Koval (1958) and Brady et al. (1958) have shown that dihydrosphingosine is converted to sphingosine by a particulate preparation of rat brain. Weiss (1965) has suggested the possibility that sphingosine might be converted to phytosphingosine. Karlsson (1964) and Carter et al. (1966) have, however, discussed the alternate pathway, i.e., that phytosphingosine is an intermediate in the conversion of dihydrosphingosine to sphingosine.

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* The structures of the three most abundant sphingosine bases are as follows:

Sphingosine: $CH_3\,(CH_2)_{12}\,CH{=}CH\cdot CHOH\cdot CHNH_2\cdot CH_2OH$

Dihydrosphingosine: $CH_3(CH_2)_{12}\cdot CH_2\cdot CH_2\cdot CHOH\cdot CHNH_2\cdot CH_2OH$

Phytosphingosine: $CH_3\cdot(CH_2)_{12}\cdot CH_2\cdot CHOH\cdot CHOH\cdot CHNH_2\cdot CH_2OH$

We have considered the possibility that the formation of phytosphingosine might be the last stage in the interconversion of the three LCBs and that this compound is further degraded to simpler molecules. To test this, phytosphingosine was administered to rats and its metabolic fate followed. It underwent two main reactions in the intact liver: a) it was bound in amide linkage to fatty acids yielding free ceramide (N-acyl phytosphingosine), and b) it was degraded to a fatty acid of fifteen carbon atoms (pentadecanoic acid), which was isolated from lecithin and triglycerides after alkaline hydrolysis.

MATERIALS AND METHODS

^{3}H-labelled phytosphingosine was prepared by adding 9,10-di^{3}H-palmitic acid to the growth medium of the yeast Hansenulla ciferri which secretes acetylated LCBs (Maister et al., 1962; Greene et al., 1965). The acetylated bases were hydrolysed with aqueous-methanolic HCl (Gaver and Sweeley, 1965), and the free LCBs were separated by chromatography on columns of silica gel-S (Barenholz and Gatt, unpublished experiments). The tritium labelled phytosphingosine was identified on thin layer silica gel plates (Sambasivarao and McCluer, 1963). It was scraped off and extracted; it contained over 97% of all radioactivity on the plate. It was also oxidized with periodate and the pentadecanal obtained was identified by gas liquid chromatography (Sweeley and Moscatelli, 1959). Over 95% of the radioactivity of the phytosphingosine base was found in this pentadecanal.

Phytosphingosine, doubly labeled with ^{3}H and ^{14}C, was similarly prepared, except that both 9,10-di^{3}H-palmitic acid and 1-^{14}C palmitic acid were added to the growth medium. Since phytosphingosine is formed by condensation of palmitate and serine (Greene et al., 1965), the tritium should be present in positions 11,12 and the ^{14}C in position 3 of the base. Periodate exidation removes carbon atoms 1-3; the remaining pentadecanal should therefore have all the tritium and no ^{14}C. The

$^{14}C/^{3}H$ ratio in the phytosphingosine was 0.74. The pentadecanal had all the ^{3}H of the LCB and a $^{14}C/^{3}H$ ratio of 0.23. This indicates that about 30% of the ^{14}C resides in carbon atoms 1-15 of the phytosphingosine (possibly because of degradation of the 1-^{14}C palmitate and reincorporation of the ^{14}C).

For intravenous administration, the phytosphingosine base was dispersed in saline to give a concentration of 0.5 mM. 0.5ml of this suspension was injected into the tail veins of rats, the animals were sacrificed, the liver excised and the lipids extracted according to Folch et al. (1957). The lipids were separated on columns of Unisil (Clarkson Chemical Co.).

RESULTS

In a typical experiment, 250 mμmoles of ^{3}H-labelled phytosphingosine were injected into the tail vein of a 100 g rat. After 1 hour, 30% of the total radioactivity administered was found in the liver. The percent distribution of radioactivity among the liver lipids was as follows: ceramide - 46, triglycerides - 16, lecithin - 19, unchanged phytosphingosine - 19.

Identification of the radioactive LCB in ceramide

Liver ceramide, isolated after intravenous administration of ^{3}H-labelled phytosphingosine, was hydrolysed with aqueous methanolic HCl (Gaver and Sweeley, 1965) and the LCBs were isolated. All the radioactivity of the ceramide was present in the LCB fraction. Aliquots of the LCB fraction were chromatographed on thin layer silica gel plates (Sambasivarao and McCluer, 1963). The spots corresponding to the various LCBs were scraped off, extracted and counted. The distribution of radioactivity was identical with that of the substrate phytosphingosine, similarly treated with methanolic HCl and chromatographed on the same silica gel plates.

A second portion of these LCBs was oxidized with periodate (Sweeley and Moscatelli, 1959) and the resulting aldehydes were chromatographed on ethylene glycol

succinate polyester columns, in a Packard Tri Carb gas liquid chromatogram; all the radioactivity resided in the phytosphingosine base.

Identification of labelled fatty acid

^{3}H-labelled phytosphingosine was administered to the tail vein of rats. After 1 hour the livers were excised and the lipids isolated. The triglycerides and lecithin were hydrolysed, at 72°, with 0.15N KOH in 94% ethanol. The fatty acids were separated into hydroxy and non-hydroxy acids (Preiss and Bloch, 1964; Mangold, 1965); all the radioactivity resided in the non-hydroxy fatty acids. These non-hydroxy fatty acids were then methylated with distilled diazomethane in ether and separated into saturated and unsaturated fatty acids (Goldfine and bloch, 1961; Mangold, 1965). Over 85% of the radioactivity resided in the saturated fatty acids. This observation was also verified by hydrolysis of the lecithin with phospholipase-A of Crotalus adamanteus. The lysolecithin, isolated after such hydrolysis, had 85% of the radioactivity of the lecithin. It is well established that lysolecithin prepared in this manner has mostly saturated fatty acids, in the α'-position (Van Deenen and DeHaas, 1964).

The saturated methyl esters were chromatographed on a preparative column of ethylene glycol succinate polyester, the effluent fatty acids were collected on glass wool and the radioactivity determined in a liquid scintillation spectrometer. 95-100% of the radioactivity applied was recovered. Of this, 81% was present in pentadecanoic and 11% in heptadecanoic acid. This indicates that the phytosphingosine was split between carbon atoms 3 and 4 yielding a residue of 15 carbon atoms, which was converted to pentadecanoic acid and subsequently introduced into lecithin and glycerides.

In a separate experiment the ^{14}C and ^{3}H, doubly-labelled, phytosphingosine was used. The ^{14}C/^{3}H ratio of the phytosphingosine portion of liver ceramide was 0.8 and of the fatty acid moiety of the triglycerides or lecithin - 0.27. These ratios are

very similar to the corresponding values of the substrate phytosphingosine and the pentadecanal obtained from it by periodate oxidation. These results further support the assumption of a degradation which splits the molecule of phytosphingosine between carbon atoms three and four, yielding a residue of fifteen carbon atoms.

DISCUSSION

In the experiments here described, phytosphingosine was converted to pentadecanoic acid by rat liver. Since this acid can be further split to acetate and propionate, this might represent a pathway for the complete degradation of phytosphingosine to CO_2 and water. In similar experiments, phytosphingosine, administered intracerebrally, was also degraded to fatty acids, which were esterified to glycerol. On the other hand, the radioactivity of sphingosine, administered intracerebrally (Kanfer and Gal, 1966), was found only in the LCBs of ceramide and sphingomyelin; conversion to compounds other than LCBs has not been reported. The above results, together with the observation that dihydrosphingosine could be converted to sphingosine (Brady and Koval, 1958; Brady et al., 1958) tempt to suggest the following pathway for the metabolism of the LCBs: Palmitate + serine $\longrightarrow$ dihydrosphingosine $\longrightarrow$ sphingosine $\longrightarrow$ phytosphingosine $\longrightarrow$ pentadecanoic acid $\longrightarrow$ $CO_2 + H_2O$. One step in this scheme, the conversion of sphingosine to phytosphingosine, has not been demonstrated to date. It should be pointed out, that the data here reported, do not exclude, other, alternate, pathways.

The steps leading from phytosphingosine to pentadecanoic acid have not been identified yet. A possible pathway might involve a cleavage, of the aldol type, between carbon atoms 3 and 4. This would result in the formation of a three-carbon unit and pentadecanal, which could be oxidized to pentadecanoic acid by aldehyde dehydrogenase. The nature of the intermediates in the convertion of phytosphingosine to pentadecanoic acid is currently under investigation.

Acknowledgment: This investigation was supported in part by a U.S. Public Health Research Grant (NB02967). We wish to thank Dr. Wickerham for a culture of Hansenulla ciferri and Dr. Carter for a sample of phytosphingosine.

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