IN VIVO CONVERSION OF ERYTHRO AND THREO DL-SPHINGOSINE-^{3}H TO CERAMIDE AND SPHINGOMYELIN

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The details of the metabolic pathways leading to the biosynthesis of the complex sphingolipids are still obscure. In vivo experiments from several laboratories employing simple carbohydrates have indicated that such materials are readily incorporated into complex sphingoglycolipids (Moser and Karnovsky, 1959; Radin et al., 1957; Burton et al., 1963; Suzuki and Korey, 1963).

In an earlier attempt to elucidate the manner in which sphingoglycolipids are formed, glucose-1-^{14}C cerebroside was administered to rats. The results indicated that this compound was converted only to ceramide and no radioactivity could be detected in the more highly glycosylated sphingolipids (Kanfer, 1965). This observations has subsequently been corroborated (Kopaczyk and Radin, 1965). As an extension of this earlier work, the present study describes the results of administering tritium labeled erythro and threo DL-sphingosine intercerebrally to rats.

Materials.

Tritium labeled erythro and threo DL-sphingosine were chemically synthesized by a suitable adaptation (to be described in detail elsewhere) of the procedure of Grob and Gadient (1957). In this procedure, the acetyleinic

erythro or threo 1,3-dihydroxy-2-amino-octadec-4-yne compounds were specifically reduced with $LiAl^3H_4$ in order to introduce the tritium at carbons 4 and 5 of the sphingosines. The melting point for the erythro DL-sphingosine was 84°C and threo DL-sphingosine was 97-98.5°C.

Water clear solutions of these substances were prepared by sonicating the suspension obtained by adding 0.5 ml of 0.1 M acetate buffer, pH 5.0 to 10 mgm of the sphingosine base. Aliquots ranging from 20 to 50 microliters were injected intercerebrally into 9 day old rats. In the experiments described, one half of the litter received the labeled threo sphingosine and the remaining animals the labeled erythro sphingosine. After 48 hours, they were sacrificed by decapitation, the brains removed and pooled. The brains from the animals which received erythro DL-sphingosine and those which received threo DL-sphingosine were kept separate and worked up simultaneously. The extraction procedure was that previously employed (Kanfer, 1965).

Ceramide was prepared from purified galactocerebroside by the method of Carter et al. (1961). Only one major component was detected on thin-layer chromatography. Impurities were removed from commercial samples of sphingomyelin by conventional procedures, and only one spot was evident on thin-layer chromatography of the final product.

Silica gel G purchased from Brinkmann was used to prepare thin-layer plates which were heated at 100°C for 2 hours prior to use. Silicic acid from BioRad Company was heated at 100°C for 3 days prior to use for column separation of the lipids.

Results.

Under these experimental conditions, 60-75% of the administered radioactivity was recovered in the chloroform phase after alkaline saponification. Gross separation of the labeled materials was effected by preparative thin-layer chromatography using the chloroform-methanol-2N NH_4OH (40:10:1) solvent system of Sambasivarao and McCluer (1963). Three radioactive fractions were obtained from both the threo and erythro plates. That is, radioactivity was

found in the sphingosine area as well as in areas which migrated more rapidly and more slowly than sphingosine. Since sphingosine was found to distribute into several different fractions on silicic acid columns, preparative TLC was employed as the most effective procedure to separate sphingosine from other sphingolipids. Approximately 85-95% of the radioactivity was recovered from the TLC plates by repeated extraction of the gel with boiling methanol. The extracts were concentrated to dryness and stored in vacuo. This dried material was then applied to a silicic acid column containing from 100 to 200 times the weight of the lipid sample. Fractionation was achieved by stepwise elution employing 10 bed volumes of chloroform and then chloroform-methanol mixtures in the ratio of 50:1, 30:1, 9:1, 4:1, 2:1, 1:1, and finally methanol.

Identification of Ceramide: The column fractionation of the labeled material which migrated more rapidly than sphingosine on the TLC revealed that most of the radioactivity was contained in the first 2 eluting solvents. Thin-layer chromatography indicated that most of the radioactivity co-chromatogramed with ceramide. Only one spot corresponding to ceramide could be visualized after charring a parallel strip. Identical observations were obtained with the material derived from the animals receiving the erythro sphingosine-^{3}H or from the animals receiving the threo sphingosine-^{3}H.

In order to characterize the product further, carrier dilution experiments were undertaken. To separate aliquots from the erythro sphingosine-^{3}H and threo sphingosine-^{3}H columns, 40 mgm of ceramide were added. The solvents were evaporated and the residue dissolved in methanol. The solutions were placed in the cold and crystallization occurred. The material was dried, weighed, dissolved in methanol and aliquots removed for radioactivity determination. This sequence was repeated until constant specific activity was obtained. These results are presented in Table I.

From this data, it is apparent that ceramide is produced in the animals which received threo sphingosine-^{3}H and also in those which were administered erythro sphingosine-^{3}H.

Table I

Ceramide Carrier Dilution Data

	Derived from animals receiving	
	Erythro-DL Sphingosine-^{3}H	Threo-DL Sphingosine-^{3}H
	cpm/mg	cpm/mg
First crystallization	286	366
First recrystallization	176	246
Second recrystallization	159	249
Third recrystallization	163	—

Identification of Sphingomyelin: The bulk of the radioactivity derived from the band of material which migrated less rapidly than sphingosine on the original thin-layer plate was obtained from silicic acid columns in the fractions eluted with 4:1 and 2:1 mixtures of chloroform-methanol. Thin-layer chromatography revealed that most of the radioactivity cochromatogramed with sphingomyelin standards. The samples from the erythro sphingosine and threo sphingosine animals behaved identically.

Carrier dilution experiments were also performed with these samples. To aliquots from the erythro sphingosine-^{3}H column and the threo sphingosine-^{3}H column 50 mg of sphingomyelin was added. The solutions were evaporated to dryness and the residue dissolved in hot ethyl acetate. Recrystallization to constant specific activity was carried out as in the ceramide experiments. The results are presented in Table II.

Table II

Sphingomyelin Carrier Dilution Data

	Derived from animals receiving	
	Erythro-DL Sphingosine-^{3}H	Threo-DL Sphingosine-^{3}H
	cpm/mg	cpm/mg
First crystallization	98	793
First recrystallization	91	670
Second recrystallization	101	665

It is apparent that sphingomyelin is produced in animals which received either erythro DL-sphingosine-^{3}H or threo sphingosine-^{3}H.

Discussion.

No evidence was obtained in these experiments for the conversion of these sphingosines-^{3}H into sphingoglycolipids. However, it is evident that the animals were able to convert both erythro DL-sphingosine-^{3}H and threo DL-sphingosine-^{3}H into ceramide and sphingomyelin.

Sribney and Kennedy (1958) found that N-acyl derivatives of threo sphingosine were far superior precursors for sphingomyelin production in vitro than were the erythro derivatives. From our results it appears that both bases are efficient precursors of sphingomyelin in vivo.

It is not possible to draw any conclusions, from the data presented in this communication, with respect to precursor-product relationships. However, in another study it has been demonstrated that intercerebrally administered N-stearoyl-1-^{14}C sphingosine was not incorporated into sphingomyelin (Kanfer and Brady, 1966). Therefore, it appears that the recently described acylation of sphingosinephosphorylcholine may be the physiological pathway responsible for sphingomyelin biosynthesis (Brady et al., 1965).

Experiments are currently in progress to determine the configuration of the sphingosine base in the biosynthesized ceramide and sphingomyelin.

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