The Configuration of Sphingosine Synthesized in Rat Brain Homogenates*

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In naturally occurring sphingolipids of animal tissue, sphingo-
sine and dihydrosphingosine have been detected. Both com-
 pounded have the n-configuration at carbon 2 and the erythro
 configuration at carbon 3. The threo isomer of sphingosine,
 which has an inversion at carbon atom 3, has been noted in
 methanolic acid hydrolysates of cerebrosides (cf. (1)). This, how-
ever, must have been derived by inversion during hydrolysis,
since an inversion of N-acyl derivatives of 1,2-amino alcohols is
 known to occur during hydrolysis by acidic reagents (2). Car-
ter and Fujino prepared a pure cerebroside and removed the N-
 acyl substituent by alkaline hydrolysis. The resulting galacto-
sidosphingosine was catalytically reduced and hydrolyzed with
 acid, and erythro-dihydrosphingosine was isolated in high yield
 (3). Since no inversion should occur during this procedure, the
 existence of threo-sphingosine in the original cerebroside in any
 appreciable quantity is ruled out.

Sribney and Kennedy have reported that cytidine diphosphate
 choline and N-acyl threo-sphingosine are converted by an enzyme
 system derived from chicken liver to a sphingomyelin, and that
 N-acyl-erythro-sphingosine was relatively ineffective as an ac-
ceptor in this reaction (4). This suggests the possibility that
 sphingosine formed in tissues as a free intermediate might be of
 the threo structure, although its configuration in the complex
 sphingolipid is erythro.

We have tested this possibility and show in this paper that
 free sphingosine synthesized in rat brain homogenates is of the
 erythro configuration.

EXPERIMENTAL PROCEDURE

Materials—3-C14-serine, 0.76 mc per mmole, was purchased
 from Isotopes Specialties Company, Inc. Palmitoyl coenzyme A
 was prepared as previously described (5). erythro-Sphingosine
 and erythro-dihydrosphingosine were derived from natural
 sources. erythro-Sphingosine was isolated and purified as the
 triacetate derivative (m.p., 101–102°), and the free base was ob-
tained by hydrolysis for 8 hours with 0.5 n methanolic potassium
 hydroxide under reflux. erythro-Dihydrosphingosine was puri-
fied as the tribenzoyl derivative (m.p., 144–145°), which was
 then hydrolyzed for 15 hours with 0.5 n ethanolic potassium hy-
droxide under reflux. Samples of synthetic threo-sphingosine
 were generously furnished by Ciba Pharmaceutical Products,
 Inc., and the Upjohn Company. Although these were crystalline,
 the presence of varying quantities of contaminants in the differ-
ent samples was revealed by thin layer chromatography on silicic
 acid as described below. threo-Dihydrosphingosine was pre-
 pared by catalytic hydrogenation of pure threo-sphingosine with
 palladium in 95% ethanol. Each of the sphingosine bases was
 purified before use.

Methods—The thin layer silicic acid procedure of Stahl (6)
 was used for the separation of lipids. For the sphingosine bases,
 0.01 ml of a 0.1% chloroform solution of each compound was ap-
 plied to a silicic acid coated glass plate, and ascending chroma-
tography was allowed to proceed in a closed chamber for 1 hour
 at room temperature in the solvent system, chloroform-methanol
 (4:1, volume for volume), or in chloroform-acetone (2:1). The
 plate was dried and sprayed with 0.04% bromophenol blue,
 which revealed lipids as blue spots on a light blue background. Rf
 values of the four bases are given in Table I.

The enzyme system was prepared by homogenizing brain tissue
 from four 15- to 20-day-old rats with 16 ml of 0.25 m sucrose and
 0.001 M Versene (the disodium salt of ethylenediaminetetra-
 acetate) in a Potter-Elvehjem homogenizer. After incubation,
 the reaction was stopped with 1 ml of 15% trichloroacetic acid
 for the 2 ml of incubation mixture, and the precipitate obtained
 by centrifugation was extracted twice with 4 ml of chloroform-
methanol (2:1). The lipid extract was then washed by the method
 of Folch et al. (7), and the resulting chloroform layer was
 concentrated to approximately 0.3 ml under nitrogen. An ex-
 tract containing approximately 3 to 5 mg of total lipids was ap-
 plied in a band to the coated glass plate, developed, dried, and
 sprayed with dye. The colored bands were removed with a
 razor blade, and the powder was shaken several times with a
 small amount of methanol or acetone. Methanol eluted the dye
 also, so that treatment with a small amount of charcoal was
 necessary. Acetone removed sphingosine from the powder with-
 out elution of the dye, but a greater loss of sphingosine was noted
 with this solvent. Recoveries of radioactive sphingosine were
 not quantitative, as judged by rechromatography, but better
 recoveries were obtained when 2 or 3 mg of carrier erythro-
sphingosine were added to the washed chloroform extract before
 application to the plate. Some decomposition of the sphingo-
sine compounds was also observed because of exposure to air
 and manipulative procedures necessary for rechromatography.

RESULTS

Enzymatic Formation of Radioactive Sphingosine—An exper-
iment typical of many is shown in Table II. After incubation
 with palmitoyl coenzyme A, labeled serine, and other additions,
radioactivity was found to be present in the washed lipid extract. When the total lipids were chromatographed by the thin layer silicic acid procedure in chloroform-methanol, a band with RF between 0.17 and 0.25 was separated. This fraction was radioactive and is designated in Table II as the sphingosine fraction. It may be noted that addition of erythro-sphingosine (tube 4) or threo-sphingosine (tube 5) before incubation resulted in an approximately 3-fold increase in radioactivity in the total lipids and in the sphingosine fraction.

Preparation of Radioactive erythro-Tribenzoyldihydrosphingosine from Labeled Sphingosine Fraction—The radioactive sphingosine fraction isolated by chromatography may contain each of the four sphingosine bases, the two unsaturated and the two saturated compounds. It was first noted by Carter, Shapiro, and Harrison (8) that erythro-dihydrosphingosine forms a beautifully crystalline tribenzoyl derivative, whereas the saturated threo compound yields only an oil. We have repeated their experiments with the same result. This observation has made it possible to determine whether labeled erythrosephingosine or labeled threosedihydrosphingosine was derived from labeled tribenzoyldihydrosphingosine. The procedure used does not distinguish between the saturated and unsaturated sphingosines, however.

The radioactive sphingosine fractions from two experiments as described in Table I, tube 4, in which erythro-sphingosine was present during incubation, were combined. The total radioactivity was 818 c.p.m. To this mixture, 30 mg of pure erythro-sphingosine were added, followed by 1 ml of ethyl ether. The solution was treated with potassium hydroxide and benzoyl chloride as described for the preparation of N-benzylsphingosine (8), the crude oily product was hydrogenated with 10% palladium on charcoal, and the saturated N-benzyl compound was isolated. This was treated next with benzoyl chloride in pyridine to obtain crude erythro-tribenzoyldihydrosphingosine, which was recrystallized three times from 95% ethanol. The melting point of the crystalline product was 143-144° (reported, 144-145°), and the final yield was 3.0 mg. This material had a radioactivity of 42 c.p.m. If the small amount of erythro-sphingosine and erythro-dihydrosphingosine present before addition of 30 mg of carrier was neglected, the theoretical yield of crystalline derivative was expected to be 61.3 mg. The corrected radioactivity corresponding to theoretical yield is (61.3/3.0) x 42, or 588 c.p.m., which agrees well with the original activity of 818 c.p.m.

Similarly, radioactive sphingosine fractions derived from incubation mixtures containing threo-sphingosine during incubation (Table I, tube 5) were converted to crystalline erythro-tribenzoyldihydrosphingosine. To such a mixture of fractions containing 832 c.p.m., 30.5 mg of carrier erythro-sphingosine were added, and 7.5 mg of derivative (m.p., 142-143°) were obtained. The total radioactivity was 96 c.p.m., corresponding to a theoretical yield of 797 c.p.m. This value also agrees well with the original quantity of 832 c.p.m.

The yields of crystalline erythro-tribenzoyldihydrosphingosine obtained in these experiments were low, in part because of the difficulty in handling small amounts of materials, and in part because of the presence of contaminants in the original mixture.

**Table I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chloroform-methanol (4:1)</th>
<th>Chloroform-acetone (2:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>erythro-Sphingosine</td>
<td>0.25</td>
<td>0.15</td>
</tr>
<tr>
<td>threo-Sphingosine</td>
<td>0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>erythro-Dihydrosphingosine</td>
<td>0.19</td>
<td>0.10</td>
</tr>
<tr>
<td>threo-Dihydrosphingosine</td>
<td>0.19</td>
<td>0.16</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Additions</th>
<th>Total lipids</th>
<th>Sphingosine fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None, zero time</td>
<td>0</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>430</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>Tween 20</td>
<td>630</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>Tween 20, erythro-sphingosine</td>
<td>1300</td>
<td>355</td>
</tr>
<tr>
<td>5</td>
<td>Tween 20, threo-sphingosine</td>
<td>1640</td>
<td>382</td>
</tr>
</tbody>
</table>

* Polyoxethylene sorbitan monolaurate.

For rechromatography of labeled sphingosine fraction—The radioactive sphingosine fraction isolated by chromatography may contain each of the four sphingosine bases, the two unsaturated and the two saturated compounds. It was first noted by Carter, Shapiro, and Harrison (8) that erythro-dihydrosphingosine forms a beautifully crystalline tribenzoyl derivative, whereas the saturated threo compound yields only an oil. We have repeated their experiments with the same result. This observation has made it possible to determine whether labeled erythro- or labeled threo-sphingosine was formed. The procedure used does not distinguish between the saturated and unsaturated sphingosines, however.

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The yields of crystalline erythro-tribenzoyldihydrosphingosine obtained in these experiments were low, in part because of the difficulty in handling small amounts of materials, and in part because of the presence of contaminants in the original mixture.

**Table III**

<table>
<thead>
<tr>
<th>Sphingosine Fraction</th>
<th>Radioactivity on rechromatography</th>
</tr>
</thead>
</table>
|                     | erythro-sphingosine | threo-sphingosine | erythro- and threo-
|                     | (c.p.m.)            | (c.p.m.)          | dihydrosphingosine |
| Derived from incubation mixtures containing erythro-sphingosine | 533 | 20 | 132 |
| Derived from incubation mixtures containing threo-sphingosine | 560 | 80 | 100 |

For rechromatography of labeled sphingosine fraction—The radioactive sphingosine fraction isolated by chromatography may contain each of the four sphingosine bases, the two unsaturated and the two saturated compounds. It was first noted by Carter, Shapiro, and Harrison (8) that erythro-dihydrosphingosine forms a beautifully crystalline tribenzoyl derivative, whereas the saturated threo compound yields only an oil. We have repeated their experiments with the same result. This observation has made it possible to determine whether labeled erythro- or labeled threo-sphingosine was formed. The procedure used does not distinguish between the saturated and unsaturated sphingosines, however.

The radioactive sphingosine fractions from two experiments as described in Table I, tube 4, in which erythro-sphingosine was present during incubation, were combined. The total radioactivity was 818 c.p.m. To this mixture, 30 mg of pure erythro-
Nevertheless, the excellent agreement between the observed and calculated values provides strong evidence for the conclusion that sphingosine or dihydrosphingosine (or both) of the erythro series, but not of the threo series, is formed in this system. This is the case when either erythro- or threo-dihydrosphingosine is present during incubation as a trapping agent.

Rechromatography of Radioactive Sphingosine Fractions—To determine whether erythro-sphingosine or erythro-dihydrosphingosine was formed by the brain homogenate, the labeled sphingosine fraction was chromatographed again as shown in Fig. 1.

The results of the first part of this procedure are shown in Table III. Six sphingosine fractions, three of which were derived from incubation mixtures containing erythro-dihydrosphingosine during incubation (Table I, tube 4), and three from the threo experiment (Table I, tube 5), were each chromatographed again in chloroform-methanol. The band corresponding to erythro-sphingosine was separated carefully from that corresponding to the mixture of threo-sphingosine, erythro-, and threo-dihydrosphingosine and was eluted, dried, and counted. In every case, the erythro-sphingosine area was radioactive and contained at least 10 to 20% of the total activity in the recovered material. These results show that a portion of the total radioactivity was fixed in erythro-sphingosine.

The mixtures of threo-sphingosine and erythro- and threo-dihydrosphingosine from all six samples were combined, treated with a small amount of charcoal to remove traces of bromophenol blue, and then chromatographed in chloroform-acetone. The initial activity added was 371 c.p.m. threo-Sphingosine contained the negligible amount of 8 c.p.m., whereas the mixture of erythro- and threo-dihydrosphingosine contained 221 c.p.m. of radioactivity. Data of the preceding section show that all of the initial radioactivity in the mixture of sphingosine bases was present in the erythro compounds. It is clear, therefore, that all of the 221 c.p.m. must be in erythro-dihydrosphingosine.

**DISCUSSION**

The experiments presented here show that when palmitoyl coenzyme A and labeled serine are added to a brain homogenate, erythro-dihydrosphingosine and smaller amounts of erythro-sphingosine are produced. None or negligible amounts of threo-sphingosine or threo-dihydrosphingosine were formed. We have deliberately used a whole brain homogenate in order to insure the presence of all sphingosine-synthesizing systems, assuming that more than one exists in the brain. It is of interest to note that Brady and Koval have reported that a microsomal preparation of rat brain forms sphingosine and dihydrosphingosine of unspecified configuration, the latter in greater amount (9).

The finding of Sribney and Kennedy (4) that N-acetyl-erythro-sphingosine is converted to a sphingomyelin remains difficult to understand. Not only was N-acetyl-erythro-sphingosine ineffective, but N-acetyl-threo-dihydrosphingosine was also ineffective in forming sphingomyelin. In view of the lack of evidence for the natural occurrence of threo-sphingosine, the physiological importance of the reaction demonstrated by these investigators is in doubt. The explanation may be simply, as they have suggested, an "enzymatic artifact" due to the known high degree of insolubility of these compounds in aqueous systems, and a greater ease of emulsification of the threo derivative.

Finally, it should be mentioned that the isolation of free erythro-sphingosine and erythro-dihydrosphingosine does not prove that either or both of these compounds are intermediates in the biosynthesis of more complex sphingosine-containing lipids. It is conceivable that a true intermediate is a sphingosine-X compound, and that the labeled sphingosines isolated in these experiments arise by exchange or hydrolytic reactions. Observations which make this suggestion less likely, however, are (a) that addition of sphingosine to a brain homogenate decreases the incorporation of label from serine into N-acethylsphingosine (ceramide) (10) and (b) the reaction shown by Cleland and Kennedy, by which galactosidosphingosine (psychosine) is formed from uridine diphosphogalactose and erythro-sphingosine (11).

**SUMMARY**

The stereochemistry of sphingosine formed in rat brain homogenates to which palmitoyl coenzyme A and labeled serine were added was studied. Chromatography by the thin layer silica gel procedure and preparation of the crystalline erythro tribenzoyldihydrosphingosine derivative have yielded data which show that, under these conditions, erythro-dihydrosphingosine and, in lesser quantity, erythro-dihydrosphingosine were formed. The threo derivatives were not produced.

**REFERENCES**

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