The Enzymatic Synthesis of Sphingomyelin*

MICHAEL SHIREN† AND EUGENE P. KENNEDY

From the Department of Biochemistry, University of Chicago, Chicago, Illinois

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The role of cytidine coenzymes in phospholipide biosynthesis is now well established (1, 2). Since sphingomyelin has a phosphorylcholine (PC) moiety similar to that found in lecithin, it has been proposed (3) that it is synthesized enzymatically in a reaction in which the phosphorylcholine moiety of CDP-choline is transferred to the free primary hydroxyl group of a ceramide (N-acylphosphoglycerine) to form sphingomyelin and CMP, as shown in Equation 1.

\[
\text{Ceramide} + \text{Cyt-P-P-choline} \rightarrow \text{sphingomyelin} + \text{CMP}
\]

This reaction is similar to the PC-glyceride transferase reaction (1).

It is the purpose of this report to describe some of the properties of an enzyme (PC-ceramide transferase) found in chicken liver and other tissues which catalyzes such a reaction. A preliminary account of some aspects of this work has been published (4).

EXPERIMENTAL

Materials and Methods

A particulate enzyme fraction was prepared from fresh frozen chicken liver (Swanson's brand) which was bought at the local market. The livers were thawed, minced, and homogenized in 4 volumes of ice-cold 0.25 M sucrose containing 0.001 M Versene (disodium salt of ethylene diaminetetraacetate, Dow Chemical Company) in an all glass Potter-Elvehjem type of homogenizer. Nuclei, debris, and whole cells were removed by centrifugation for 5 minutes at 500 \(\times\) g at 0\(^{\circ}\). A particulate fraction was obtained by centrifugation at 18,000 \(\times\) g for 30 minutes at 0\(^{\circ}\) with the use of a Servall refrigerated centrifuge. The pellet was resuspended and washed twice in sucrose-Versene solution. It was finally taken up in 0.02 M Tris buffer, pH 8.0, and 0.001 M Versene in a concentration of 20 to 40 mg. of protein per ml. and stored at \(-20^{\circ}\) for use as required. These preparations contained both mitochondria and microsomes. Since preliminary experiments indicated that both types of intracellular particles contained approximately the same enzymatic activity, further fractionation was not attempted.

Phosphorus determinations were done by the method of Fiske and SubbaRow (5). Ester values were obtained by the method described by Rapport and Alonzo (6). Determination of plasmalogen aldehyde was done by the method of Wittenberg et al. (7), and ninhydrin determinations, by the method of Cocking and Yemm (8). C, H, and N analyses were done by Micro-Tech Laboratories, Skokie, Illinois. CDP-choline labeled with \(^{3}H\) in 1,2-positions of choline was prepared as described previously (9).

Sphingomyelin from chicken liver was prepared in the following manner. A acetone powder was made from a large batch of frozen chicken livers (6 kg.). The acetone powder was extracted with ether for 48 hours with the use of a Soxhlet extractor to remove ether-soluble lipides. The residue was then extracted four times with 10 volumes of hot methanol. The methanolic extracts were combined, and enough methanolic KOH was added to make the extract 0.4 N. The mixture was then incubated at 37\(^{\circ}\) for 2 hours to remove alkali-labile phospholipides. After cooling, the KOH was neutralized with HCl and the methanol evaporated under vacuum to a small volume. The lipides were then extracted with chloroform, and the extract was thoroughly washed with water. The chloroform was removed and the lipides were dried in a vacuum, then chromatographed on alumina (Merck reagent grade) by means of the gradient elution technique described by Burch et al. (10). The lower mixing chamber contained 300 ml. of chloroform, and the upper reservoir contained a mixture of chloroform-methanol (1:1).

Approximately 200 gm. of alumina were treated with chloroform to give a slurry which was then poured into a column 30 cm. high and 3 cm. in diameter. The bed was thoroughly washed with chloroform before addition of the crude sphingomyelin. The sphingomyelin (10.5 gm.) dissolved in about 200 ml. of chloroform was then adsorbed on the column, which was washed with several portions of chloroform and then eluted. Three 9-ml fractions were collected per hour with the aid of a fraction collector. 9.3 gm. of lipide were obtained in tubes 25 to 45 inclusive. After chromatography, the lipide was further purified by recrystallization several times from ethyl acetate to which a little methanol was added. The sphingomyelin thus obtained contained negligible ester (6) and no detectable plasmalogen (7). The N:P was 1.96.

Analysis

C 66.82, H 11.77, N 3.10, P 3.50

Beef heart sphingomyelin was a gift of Sylvana Company. It was further purified by thorough washing with ether to remove any lecithines and by chromatography on an alumina column as described above. Analysis indicated a negligible amount of ester (6) and approximately 1 per cent of plasmalogen (7). The N:P was 1.94.
Ceramides were prepared from sphingomyelins with lecithinase D present in Clostridium perfringens Type A toxin (11) by a method based on the work of Macfarlane (12). The enzyme was a gift of Lederle Laboratories. To a flask were added the following: 10 mmoles of Tris buffer, pH 7.4, 2 mmoles of Ca++, 200 mg. of enzyme, and 200 mg. of sphingomyelin emulsified in water in a final volume of 200 ml. 100 ml. of ethyl ether were added and the reaction mixture was stirred at room temperature until the aqueous phase became clear. The reaction was usually complete in 2 to 4 hours. The extent of cleavage as determined by measurement of acid-soluble phosphorus (5) was 95 per cent. The addition of ether was found to stimulate the rate of cleavage of sphingomyelin in a manner similar to that found by Hanahan and Vercamer (11) in the cleavage of lecithin by lecithinase D. The ceramide which was in the ether phase was then purified by chromatography on a column of silicic acid-Celite, with gradient elution. The column, 15 cm. high and 1 cm. in diameter, contained 7 gm. of silicic acid-Celite (5:2). The silicic acid (Merek reagent grade) was activated by heating at 110° for 15 hours. The Celite was a product of Johns-Manville (No. 503). Fractions containing 5 ml. of solvent were collected with the aid of a fraction collector. The ceramide appeared in tubes 4 to 10, inclusive.

Since this paper will be concerned with several of the 8 possible isomers of sphingosine, the terms sphingosine or erythro-sphingosine will be applied to n-erythro-1,3-dihydroxy-2-amino-4-trans-octadecene which is the structure which has been assigned to the sphingosine isolated as a hydrolysis product of cerebrosides (13, 14). The term three-sphingosine will be applied to D-threo-1,3-dihydroxy-2-amino-4-trans-octadecene.

Sphingosine isomers were gifts of Dr. H. E. Carter, Dr. P. O’Connell, and the Upjohn Company, and Professor C. A. Grob and Ciba Pharmaceutical Products, Inc. Sphingosine was also prepared from ceramide and cerebroside as described below.

The N-acyl derivatives of the various sphingosine isomers were prepared by a method similar to that described by Carter et al. (13). The N-acetyl-, N-propionyl-, and N-succinyl-sphingosines were prepared with the use of the anhydrides of the respective acids. N-butyryl, N-octanoyl, N-lauryl, N-palmitoyl, N-oleyl, and N-linoleyl derivatives were prepared by use of the ayl chlorides of the respective acids. The preparation of N-acetysphingosine will be described in detail as a representative procedure.

To 35 mg. (100 μmoles) of sphingosine sulfate were added 0.5 ml. of 2.5 N NaOH and 2 ml. of ethyl ether. The mixture was shaken vigorously until all the sphingosine sulfate had been converted to the free base. An emulsion formed as the sphingosine dissolved in the ether phase. 40 mg. (400 μmoles) of acetic anhydride dissolved in anhydrous ethyl ether was added in several portions with vigorous shaking and cooling in an ice bath. The emulsion disappeared during the acetylation. The ether layer was removed and then washed with water until it was free from base. The ether was evaporated and the residue chromatographed on silicic acid with the use of gradient elution in the same manner as described for the purification of ceramides. Three fractions of 5 ml. each were collected per hour with a fraction collector. The N-acetylsphingosine appeared in tubes 7 and 8, a total of 26.4 mg. (75.5 μmoles) being obtained. A small amount (6 mg.) of unchanged sphingosine appeared in tubes 15 and 16.

All the N-acyl derivatives were purified in the manner just described. Yields varied from 75 to 100 per cent. All the derivatives gave a negligible meter value (6) and a negative ninhydrin reaction (8) which indicates that O-acyl derivatives and sphingosine were not present.

Cerebrosides were prepared from beef spinal cord lipides as described by Carter et al. (13), and other preparations were a gift of Dr. Oscar Reiss.

RESULTS

The first experiments on the enzymatic synthesis of sphingomyelin were carried out by measuring the transfer of the phosphorylcholine moiety of CDP-choline to an alkali-stable lipide. The assay was carried out as follows: The enzymatic reaction, which was usually run in a volume of 1 ml., was stopped by the addition of 3 ml. of absolute methanol. The labeled lipides were extracted four times with 3-ml. portions of methanol at 55° for 5 minutes. To the combined extracts, in a glass-stoppered vessel, enough methanolic KOH was added to make the solution 0.4 N. This mixture was then incubated at 37° for 2 hours, thereby hydrolyzing alkali-labile phospholipides. After incubation, the tubes were cooled and the solution neutralized with acid. 5 ml. of chloroform were then added and the entire contents thoroughly mixed. 20 ml. of 2 M KCl were added to each vessel, which was then inverted about 100 times. The upper aqueous phase was then removed and discarded. The extraction with 2 M KCl was repeated three times to insure complete removal of water-soluble radioactive contaminants. Each tube was finally washed with 20 ml. of water. Aliquots of the chloroform phase were then plated in aluminum cups, dried, and counted in a gas flow counter under conditions of negligible self-absorption. Control experiments showed that labeled lecithin is completely destroyed under these conditions, whereas sphingomyelin is quite stable.

Various compounds which might possibly act as lipide acceptors for the phosphorylcholine moiety of CDP-choline were tested, with the formation of radioactive alkali-stable lipide as an index of activity. The results are shown in Table I. The addition of free sphingosine or of a ceramide, derived from chicken liver sphingomyelin by the enzymatic method already described, did not significantly stimulate the formation of radioactive sphingomyelin from labeled CDP-choline. However, a crude ceramide prepared by partial hydrolysis of cerebroside (phrenosine) with acetic-sulfuric acid by a procedure of Klenk (15) promoted an extensive synthesis into radioactive alkali-stable lipides. Further experiments revealed that free sphingosine treated with acetic-sulfuric acid or propionic-sulfuric acid under the same conditions also stimulated incorporation into an alkali-stable lipide. These enzymatically active ceramides were isolated by chromatography on silicic acid, by means of the procedures described above. The active ceramides had properties to be expected of N-acetyl or N-propionyl derivatives of sphingosine or some isomer of sphingosine. Thus, no significant loss of activity was noted when the active ceramide was treated with nitrous acid, period acid, or m-alkali, which indicates that a free amino group or ester linkages were either absent or not essential for activity. Since the active materials contained short chain fatty acids, a long chain fatty acid is not

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Identification of Radioactive Product as Sphingomyelin—In order to identify the product of the reaction, the following experiment was performed. To a flask were added 5 mmoles of Tris buffer, pH 7.4; 2 mmoles of cysteine; 1 mmole of MnCl₂; 80 μmoles of CDP-choline labeled in 1,2-position of choline (16,100 c.p.m. per μmole); 100 μg. of Tween-20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Company); 400 pmol of active ceramide (prepared by the treatment of sphingosine with propionic-sulfuric acid); and 25 ml. of chicken liver particles in a final volume of 100 ml. The mixture was extracted, treated with alkali, and taken up in chloroform in a manner identical to that used in the assay procedure. It was then purified by chromatography on silicic acid by means of the gradient elution technique. The mixing vessel contained 300 ml. of chloroform, and the upper reservoir, a mixture of chloroform-methanol (1:1). The column, 28 cm. high and 1.2 cm. in diameter, contained 14 gm. of silicic acid-Celite (5:2). Three fractions of 14 ml. each were collected per hour. The results of the chromatography are shown in Fig. 1. A single radioactive peak was obtained, containing 28.5 mg. of lipide and a total of 573,000 counts.

The purified material was identified as sphingomyelin by the following properties: (a) stability toward mild alkali, (b) N:P ratio of 2.08; (c) insolubility in ether and acetone; (d) infrared spectrum similar to that of authentic sphingomyelin;2 (e) cleavage by lecithinase D regenerating the active ceramide.

A less purified sample of the isolated lipide (estimated purity, 81 per cent) contained 5.05 per cent P and 4.77 per cent N, or 86 and 89 per cent, respectively, of the theory, calculated for sphingomyelin with propionic acid in amide linkage.

A control experiment was carried out with identical conditions of extraction and chromatography, except that boiled enzyme was used. Less than 0.5 μmole of phospholipide was isolated in the fractions corresponding to those containing the labeled sphingomyelin, indicating that the labeled lipide isolated above is the product of a net enzymatic synthesis.

Chemical Identification of Active Ceramide—Since the active ceramide prepared by the treatment of sphingosine with acetic-sulfuric acid appeared to have properties resembling those of N-acetylphosphosine, it was decided to prepare the latter compound by an unambiguous route for testing in this system. It was preferable to work with the N-acetyl rather than the N-propionyl derivatives, because much more information is available in the literature concerning the N-acetyl compounds.

A crude preparation of sphingosine sulfate was obtained by the hydrolysis of phrenosine with sulfuric acid in methanol by the method of Carter et al. (13). This material was freed of dihydro-sphingosine sulfate by recrystallization from methanol (13) but still consisted of a mixture of isomers of sphingosine. Crude N-acetylsphingosine was prepared from the crude sphingosine by treatment with acetic anhydride in ether and in the presence of alkali (13). This crude N-acetylsphingosine was enzymatically active in promoting the synthesis of sphingomyelin (Table II, No. 1).

Since N-acetylsphingosine is an amorphous material, it was purified by converting it to the crystalline triacetylsphingosine, which was then recrystallized (13). The crystalline triacetylsphingosine was then reconverted quantitatively to N-acetylsphingosine by mild treatment with alkali (13). It showed little activity in the enzymatic assay (Table II, No. 2). However, treatment of the purified N-acetylsphingosine with acetic-sulfuric acid converted it to "active ceramide" (Table II, No. 3).

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2 The authors are indebted to Dr. H. E. Carter for infrared analysis.
TABLE III
Configuration of active ceramide required for enzymatic synthesis of sphingomyelin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sphingomyelin synthesized</th>
<th>mpmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetyl-DL-erythro-trans-sphingosine*</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>N-acetyl-nL-three-trans-sphingosine*</td>
<td></td>
<td>105</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetyl-DL-erythro-trans-sphingosine†</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>N-acetyl-DL-three-trans-sphingosine†</td>
<td></td>
<td>83</td>
</tr>
<tr>
<td>N-acetyl-DL-erythro-cis-sphingosine†</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>N-acetyl-DL-three-cis-sphingosine†</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* The sphingosine samples from which these N-acetyl derivatives were prepared were gifts of Dr. Paul O'Connell and the Upjohn Company.
† The sphingosine samples from which these N-acetyl derivatives were prepared were gifts of Professor C. A. Grob and Ciba Pharmaceutical Products, Inc.

The unexpected finding (Table III) that the ceramide which is enzymatically converted to sphingomyelin must possess the three and not the erythro configuration made it of great interest to determine whether the sphingosine of sphingomyelin isolated from tissues is threo or erythro. Accordingly, ceramides prepared enzymatically from chicken liver and beef heart sphingomyelin were hydrolyzed to sphingosine with barium hydroxide under conditions not expected to cause isomerization (14). The sphingosine samples so obtained were converted to the N-acetyl derivatives in the usual way. The N-acetyl derivatives so obtained had little activity in the enzyme system (Table IV, Nos. 1 and 3) and therefore must be predominantly or entirely of erythro configuration. Treatment of these N-acetyl-sphingosines with acetic-sulfuric acid converted them to "active ceramide," i.e. N-acetyl-threo-sphingosine, in the expected manner. In a control experiment, a sample of N-acetyl-threo-sphingosine was carried through the entire barium hydroxide hydrolysis, hydrolyzed to the free base and, reacetylated without loss of activity.

Further experiments will be necessary to determine if the slight activity of the N-acetyl sphingosines derived from sphingomyelins (Table IV, Nos. 1 and 3) should be attributed to the
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TABLE V
Effect of other substrates as lipid acceptors for \( \text{P-choline} \)
Experimental conditions were identical to those in Table III.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spingomyelin synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N\text{-acetyl-dL-threo-dihydrosphingosine}^\ast )</td>
<td>1</td>
</tr>
<tr>
<td>( N\text{-acetylphytosphingosine}^\dagger )</td>
<td>1</td>
</tr>
<tr>
<td>( N\text{-acetyl-dL-threo-1,3-dihydroxy-2-amino-octadecyne}^\ddagger )</td>
<td>160</td>
</tr>
<tr>
<td>( N\text{octanoyl-dL-threo-1,3-dihydroxy-2-amino-octadecyne}^\ddagger )</td>
<td>208</td>
</tr>
</tbody>
</table>

* Gift of Dr. H. E. Carter.
† Gift of Dr. P. O'Connell and the Upjohn Company.

occurrence of a small proportion of threo-sphingomyelin, or a slight conversion of erythro- to threo-sphingosine during hydrolysis, or an incomplete specificity of the enzyme.

Determination of Threo or Erythro Configuration of Enzymatically Synthesized Sphingomyelin—Since \( N\text{-acetyl-dL-threo-trans-sphingosine} \) was required for the enzymatic synthesis of sphingomyelin, whereas the sphingomyelin which is isolated from tissues is largely or entirely erythro, the possibility was considered that conversion from threo to the erythro configuration took place during enzymatic synthesis of sphingomyelin itself. The enzymatically synthesized lipid was therefore isolated, purified as described, and then cleaved with lecithinase D to produce a ceramide. This ceramide had enzymatic activity which was equal to that of the original "active ceramide," and was thus of the threo configuration. Therefore, isomerization of the sphingosine moiety from threo to erythro does not take place during the enzymatic synthesis of sphingomyelin itself.

Amides of Dihydrosphingosine and Acetylenic Sphingosine—\( N\text{-acetyl-dL-threo-dihydrosphingosine} \) and \( N\text{-acetylphytosphingosine} \) were found not to be active as substrates in this reaction (Table V). \( N\text{-acetyl- and N-octanoyl-dL-threo-1,3-dihydroxy-2-amino-octadecyne} \) were found to be active acceptors for the phosphorylcholine moiety of \( \text{CDP-choline} \). This was unexpected since the enzyme is highly specific for the trans and not for the cis configuration at the double bond. However,

study of the molecular models of the acetylenic derivatives revealed that the carbon atoms adjacent to the triple bond are able to take a configuration resembling that of the trans isomer. The cis compound, on the other hand, is folded over, and is thus sterically different from the trans compound. In another experiment, not shown in the table, the acetylenic erythro derivative was tested and found inactive. Thus the specificity for threo isomers applies to acetylenic as well as ethylenic compounds.

Effects of Chain Length of Fatty Acid in Amide Linkage and Emulsifying Agents—When ceramides of varying chain length were tested for enzymatic conversion to sphingomyelin, the results were entirely dependent upon the presence or absence.
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FIG. 5. Effect of varying chain length and emulsifying agent. Experimental conditions were the same as shown in Table III. Each tube received 4 μmoles of N-acyl-n-threo-trans-sphingosine derivative in the presence and absence of Tween-20, as shown.

of emulsifying agents (Fig. 5). In the presence of emulsifying agent (Tween-20), the activity of the ceramide increases with chain length up to 8 carbon atoms but then abruptly declines. The lack of activity of derivatives of long chain, saturated fatty acids is related to the difficulty with which these compounds are emulsified. Thus N-palmitoyl-DL-threo-trans-sphingosine is completely inactive, but N-oleyl- and N-linoleyl-DL-threo-trans-sphingosine are much more easily emulsified and exhibit appreciable activity.

In the absence of emulsifying agent, activity falls with increasing chains length, and N-octanoyl-DL-threo-trans-sphingosine is quite inactive. The effect of Tween-20 on the activity of the N-octanoyl derivative was examined in greater detail in the experiment shown in Fig. 5. In the absence of Tween-20, there is no activity; optimal activity is observed at about 2 mg. per ml. of Tween-20, whereas higher concentrations are inhibitory.

Effect of Varying Substrate Concentration—An experiment in which the concentration of N-octanoyl-DL-threo-trans-sphingosine was varied, is shown in Fig. 7. Half the maximal rate was observed at a concentration of $7.5 \times 10^{-4}$ M. This result is only approximate since the substrate has to be dispersed in a surface active agent which inhibits the reaction as well as promoting the solubility of the substrate.

Occurrence of Enzyme—The enzyme (PC-ceramide transferase) has been found not only in chicken liver, but is present also in the liver, kidney, spleen, and brain of 10- to 20-day-old rats and in guinea pig and hog liver (Table VI). The enzyme in all the tissues investigated is specific for threo-sphingosine derivatives.

Metal Ion Activation—In the study of the effect of metal ion activation on enzymatic activity, Versene was omitted from the enzyme preparations. Manganese ions were found necessary for optimal transferase activity (Fig. 2). Magnesium ions are much less effective than manganese ions. Calcium ions inhibit the reaction. 0.002 M calcium reduces the PC-ceramide transferase activity to one-half in the presence of an optimal amount of manganese ions.

Effect of Temperature on PC-ceramide Transferase Activity—When the temperature was varied over a considerable range the enzyme exhibited maximal activity at 45°C under the conditions chosen (Fig. 3). Higher temperatures probably were of aid in increasing the solubility of the ceramide as well as increasing the reaction rate as anticipated.

TABLE VI
Occurrence of PC-ceramide transferase in various tissues

Experimental conditions were the same as shown in Table III. 0.25 ml. of enzyme was added to each tube as shown. Particulate fractions were prepared as described in the text. Other preparations were 20 per cent sucrose-homogenates.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Sphingomyelin synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken liver particles</td>
<td>210</td>
</tr>
<tr>
<td>Guinea pig liver particles</td>
<td>25</td>
</tr>
<tr>
<td>Hog liver particles</td>
<td>20</td>
</tr>
<tr>
<td>Rat brain homogenates</td>
<td>9</td>
</tr>
<tr>
<td>Rat kidney homogenate</td>
<td>11</td>
</tr>
<tr>
<td>Rat liver homogenate</td>
<td>31</td>
</tr>
<tr>
<td>Rat spleen homogenate</td>
<td>17</td>
</tr>
</tbody>
</table>
Effect of pH on Activity—The enzyme exhibits a sharp optimum between pH 7.5 and 8.0 (Fig. 4). Above and below these values the activity of the enzyme drops very rapidly.

Specificity for CDP-choline—The enzyme is highly specific for CDP-choline. Synthetically prepared UDP-choline, ADP-choline, and GDP choline (9) show no detectable activity.

DISCUSSION

Evidence is presented here for the first time concerning the mechanism of the enzymatic synthesis of sphingomyelin, which has been found to be analogous to the synthesis of lecithin (2), where the phosphorylcholine moiety of CDP-choline is transferred to the free hydroxy group of a \( \alpha, \beta \)-diglyceride. The PC-ceramide transferase reaction is unlike the PC-glyceride transferase reaction in that manganese ions are required instead of magnesium ions for optimal activity. The PC-glyceride transferase reaction is completely inhibited by 0.001 M calcium ions, whereas the PC-ceramide transferase reaction is inhibited to the extent of only 50 per cent by 0.002 M calcium ions. The addition of a surface active agent promotes the solubility of the ceramides in a manner similar to that found in the PC-glyceride transferase reaction where the \( \alpha, \beta \)-glyceride must be emulsified to promote the synthesis of lecithin.

Carter et al. (13) obtained only the erythro isomer of sphingosine from the cerebroside phrenosine, and in excellent yields, by a process which would not be expected to lead to isomerization. The results shown in Table IV indicate that the sphingosine moiety of sphingomyelin isolated from tissues is predominantly or entirely in the erythro configuration. It is not possible to decide as yet whether the slight activity of the \( N \)-acetyl derivatives of sphingosine derived from sphingomyelin is the result of a small proportion of threo-sphingomyelin in the original material. If, in fact, sphingolipids in tissues are a mixture of a large proportion of erythro and a small proportion of threo, it is possible that the minor component would be removed during the process of purification.

There are at least two possible explanations for the unexpected specificity of the phosphorylcholine-ceramide transferase for threo-ceramides. First, this specificity may in a sense be an artifact, resulting from the experimental conditions under which it is necessary to test the enzyme in vitro, whereas threo-ceramides are the actual substrates in vivo. According to this theory, the threo-ceramides may simply be more soluble or readily emulsified in vitro and thus penetrate more readily to the enzyme surface. The importance of emulsification is shown by the experiments described in Figs. 5 and 6. This theory requires two further assumptions: that the enzyme in fact has no specificity for the threo- or erythro-ceramides, and that erythro-ceramides can somehow penetrate to the enzyme surface in vivo. This explanation is also rendered somewhat less likely by the finding that with acetylenic as well as with ethylenic derivatives the enzyme displays a specificity for three derivatives.

The second theory is that the enzyme acts on threo-ceramides in vivo as well as in vitro and that the newly synthesized sphingomyelin is of the threo configuration, but is later converted to erythro-sphingomyelin, as is shown in Equations 3 and 4.

\[
\text{Threo-ceramide} + \text{Cyt-P-P-choline} \rightleftharpoons \text{three-sphingomyelin} + \text{CMP} \\
\text{Threo-sphingomyelin} \rightarrow \text{erythro-sphingomyelin}
\]

It should be made clear that no evidence for reaction (4) has as yet been obtained, but if such a reaction occurs, it would undoubtedly be of great physiological interest. Niemann-Pick's disease, for example, is characterized by an accumulation of sphingomyelin in the liver and spleen, and it is possible that the inability to carry out a transformation such as shown in Equation 4 might lead to such a pathological condition.

The present study, as well as the work of Zabin (20), points to the importance of ceramides as intermediates in the biosynthesis of sphingolipids. It is possible that ceramides may also be intermediates in the biosynthesis of cerebrosides, by transfer of galactose from uridine diphosphate galactose. The ceramides themselves may arise from a reaction between sphingosine and a long chain fatty acid thiolester of coenzyme A.

SUMMARY

1. The enzymatic synthesis of sphingomyelin has been found to occur by the transfer of the phosphorylcholine moiety of cytidine diphosphate choline to the free primary hydroxyl group of a ceramide.
2. The enzyme (PC-ceramide transferase) catalyzing this reaction is highly specific both for cytidine diphosphate choline and ceramide. The sphingosine of active ceramides must have the trans configuration of the double bond, and the hydroxyl group on carbon 3 must have the three relationship to the amino group on carbon 2. Ceramides of dihydrosphingosine are inactive, but derivatives of sphingosine containing a triple bond rather than a double bond at carbon 4 are active, if the hydroxyl group on carbon 3 is threo.
3. The enzyme is widely distributed in various animal tissues and is particularly active in chicken liver.
4. Other properties of the enzyme are described.
5. Evidence as to the structure of naturally occurring sphingomyelin is presented.

REFERENCES