The Biosynthesis of Lignoceric, Cerebronic, and Nervonic Acids

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The lipids of the mammalian brain and nervous system are characterized in part by a relatively high content of C24 acids, which occur as important fatty acid components of the sphingolipids.

The cerebrosides, in particular, contain large amounts of both the unsubstituted C24 fatty acids, lignoceric and nervonic, and their 2-hydroxy analogues, cerebronic and hydroxynervonic acids. Although detailed analyses of both rat brain (1) and human brain (2) cerebrosides fatty acids have recently been achieved and the quantitative pre-eminence of the C24 acids again demonstrated, little information is available concerning their biosynthesis. As early as 1940, Waelsch, Sperry, and Stoyanoff (3), using deuterium incorporation from body water as an indicator, showed that the brains of young rats contained significant amounts of newly synthesized fatty acids. Inasmuch as the brain fatty acids were isolated as a group, however, no conclusions could be drawn concerning the biosynthesis of the C24 acids. More recently, Klenk (4) has reported studies of the incorporation in vivo and in vitro of acetate-1-C14 into the brain lipids of rats. Although there was considerable radioactivity incorporated into both the saturated and unsaturated acids of the brain, cerebroside (the only C24 acid isolated) incorporated no measurable radioactivity. Thus, the mode of biosynthesis of the C24 acids remained obscure.

The experiments reported in this paper were designed with the hope of answering two questions about the C24 acids. First, is acetate the sole building block or is some non-acetate derived precursor also involved, particularly in the formation of the 2-hydroxy fatty acids? Second, what biosynthesis pathways exist among the C24 acids generally?

EXPERIMENTAL PROCEDURE

Materials—Tetracosanoic acid (m.p. 82-83°) and tricosanoic acid (m.p. 77-78°) were prepared from 1-bromodocosane by malonic ester synthesis and nitrile synthesis, respectively. 1-Bromodocosane (m.p. 44.0-44.9°) was prepared from 1-docosanol (5). 2 Hydroxydocosanoidie (m.p. 91.2-95.0°), 2 hydroxy docosanoic (m.p. 95.0-96.0°), and 2-hydroxytetracosanoic (m.p. 99.6-100.3°) acids were prepared from the corresponding unsubstituted analogues by α-bromination with phosphorous tribromide and bromine at 110° followed by hydrolysis. All other fatty acids used as carriers were obtained from commercial sources and purified by recrystallization from acetone and petroleum ether before use.

Treatment of Animals—Beginning from the 13th day after birth, 10 weaned, unsexed rats of the same litter were given daily intraperitoneal injections of sodium-1-C14-acetate. A total of 4 mc was administered over a 4-day period. The rats were killed by decapitation 31/2 hours after the last injection, and the brains were removed and frozen in Dry Ice until use.

Isolation of Crude Cerebroside Acids—After lyophilization, the dry brains (2.03 g) were twice extracted in a Waring Blender with 100 ml volumes of 2:1 chloroform-methanol. The extract was then filtered and the solvent removed under reduced pressure to yield 719 mg of total lipid. The brain lipids were then separated into five fractions by chromatography on a 105- x 44-mm (inner diameter) silicic acid column (Fig. 1). Crude sterol was eluted with ether and the remaining lipids were eluted with methanol-chloroform mixtures. The major components were identified by thin layer silicic acid chromatography (6) in conjunction with standards. The crude cerebroside fraction (191 mg), which actually contained phosphatidylethanolamine, phosphatidylserine, and several unidentified components in addition to cerebrosides, was subjected to methanolation (1), and the crude methyl esters thus obtained were separated into unsubstituted and 2-hydroxy esters by elution from a silicic acid column with 3% and 20% ether in pentane, respectively. These two fractions were then each separated into saturated and unsaturated esters by use of mercuric acetate as described by Kishimoto and Radin (1). The methyl esters were saponified and the unsaturated fatty acids were hydrogenated over 5% palladium on charcoal. A sample from each of the groups except the hydrogenated 2-hydroxy unsaturated acids was analyzed by gas chromatography (1) to determine the mole fraction of each component.

Isolation of Individual C24 Acids—It now remained to separate each group into its component fatty acids and to isolate each of the C24 acids in a high state of purity. The unsubstituted acids, after dilution with carrier, were readily separated by reversed phase chromatography (7). The two samples of tetracosanoic acid (representing lignoceric and hydrogenated nervonic acid) thus obtained were further diluted with inactive tetracosanoic acid and recrystallized to constant activity from petroleum ether.

Although the 2-hydroxy fatty acids, as such, could not be separated on the reverse phase column, it was found that the 2-acetoxy derivatives were readily separated by this technique. Thus, a sample of the saturated 2-hydroxy fatty acids (8 mg)
method of Dauben (11) and McFad and Howton (12) to heneicosanoic acid and two successive samples of benzoic acid, the carboxyl carbons of which represent carbon atoms 2 and 3 of the original cerebronic acid. Similarly, lignoceric acid was degraded by 3 carbons and tetracosanoic acid (representing hydrogenated nervonic acid) was degraded by one carbon. The sample of 2-hydroxy nervonic acid was lost before degradation results could be obtained.

RESULTS AND DISCUSSION

Table I presents the results of silicic acid chromatography of the brain lipids. It is of interest to compare the specific activity of the fatty acids obtained from silicic acid fractions F-4 and F-5 (brain phosphatide fatty acids) with that of the fatty acids obtained by saponification of the rat carcasses (Table I, Part A). It will be noted that the brain fatty acids are more than 18 times as active. Furthermore, crude brain sterol (mostly cholesterol)

![Fig. 1. Silicic acid chromatography of brain lipids. The eluting solvents were ether (E) and increasing percentages of methanol in chloroform (0%, 20%, etc.). Fraction F-3 (stippled area) contains the crude cerebrosides.

was diluted with 121 mg of an inactive mixture of 2-hydroxystearic, 2-hydroxy arachidic, 2-hydroxydocosanoic, and 2-hydroxytetraicosanoic acids. This mixture was refluxed for 2 hours with 2 ml of acetyl chloride, and the acetyl chloride was then distilled off. Distillation was repeated after the addition of 10 ml of acetone. Finally, 5 ml of 10:1 acetone-water were added, and the solution was allowed to stand for about 15 minutes. The solution was then pipetted into 50 ml of ether and dried over anhydrous magnesium sulfate. The yield of 2-acetoxy acids, after vacuum drying to remove traces of acetic acid, was 144 mg (theory, 145 mg). The mixture was chromatographed on a 40- x 1.2-mm (inner diameter) mineral oil-silicic acid column constituted in the usual way. The solvent system used and the separation obtained are illustrated in Fig. 2. After chromatography, acetone was removed from the collected fractions by reduced pressure distillation and the 2-acetoxy acids were saponified on the steam bath for 2 hours in 5% aqueous potassium hydroxide. The 2-hydroxy acids were extracted from the acidified solution with ether and chromatographed on silicic acid to remove mineral oil. Each acid was further diluted with inactive material and crystallized to constant activity from aqueous 1,4-dioxane and petroleum solutions. The over-all recovery of 2-hydroxy acids, before crystallization, was 95%. The hydrogenated unsaturated 2-hydroxy acids were isolated in the same manner and comparable yields were obtained.

**Stepwise Degradation of C44 Acids**—The carboxyl carbon of 2-hydroxytetraicosanoic (cerebronic) was removed by permanganate oxidation in acetic acid at 25°C (10) and collected as barium carbonate. The tricosanoic acid was degraded by the

1 It was noticed that 2-acetoxydocosanoic acid and 2-acetoxytetraicosanoic acid, prepared in this way, had melting points of 59° and 64°, respectively. However, if the derivatives were allowed to stand in the melting point tubes at several degrees above these melting points, solidification soon took place and new melting points (76.5° and 81.0°, respectively) were obtained. Indeed, the higher melting points were obtained if the lower melting forms were allowed to stand at room temperature for several weeks. Thus, the 2-acetoxy derivatives are polymorphic. These results probably explain the disagreement between Klenk and Clarenz (8) and Chibnall et al. (9) concerning the melting point of 2-acetoxytetraicosanoic acid.

*All samples were counted with a liquid scintillation counter as described previously (12).† Disintegrations per second.
has more than twice the specific activity of body nonsaponifiable materials (again, mostly cholesterol). These values give some idea of the relatively high rate of lipid synthesis and storage in the brains of 2-week-old rats. Table II shows the amounts and C14-activities of the four crude cerebroside acid fractions, whereas Table III reveals the analyses of these fractions as obtained by gas-liquid chromatography. It should be noted that, with the exception of small amounts of C15 and C17 acids and a trace of tricosanoic acid, acids containing an odd number of carbon atoms could not be detected. Apparently, in 16-day-old rats, the long chain odd-numbered acids have not been synthesized in more than trace amounts. Kishimoto and Radin (1) found small amounts of these acids in 22-day-old rats and they noticed that the relative concentrations of the acids greatly increased with age. It is possible that the odd-numbered acids represent degradation products of the even-numbered higher homologues, a possibility which is presently being investigated in this laboratory.

Table IV presents the results of reversed phase chromatography of the various groups of crude cerebroside acids. The large loss of total activity after recrystallization of the docosanoic acid fraction (Table IV, Part A) cannot at present be explained. Recrystallization of the mother liquors and interpeak fractions with synthetic tricosanoic acid showed that most of the extraneous activity was definitely not due to contamination with a highly active trace of this acid. Silicic acid chromatography also ruled out the possibility that the extraneous activity was associated with an aldehyde since the activity was eluted with the fatty acid fraction.

Table V gives data on the C14 acids isolated by reversed phase chromatography and crystallized to constant activity.

* Corrected for dilution.
Lignoceric acid

The distribution of C$^{14}$ activity in cerebronic and lignoceric acids as determined by stepwise degradation of the molecules is revealed in Table VI. It is at once apparent that the C$^{14}$ activity in cerebronic and lignoceric acids is almost exclusively in the odd-numbered carbons of both acids. A further point of interest lies in the ratios of the C$^{14}$ activities of the narrow acid to the carbon atom 1 and to the carboxyl end of both molecules. It must be emphasized, however, that this bias of label towards the carboxyl group is relatively slight when compared with that of long chain (18 or more carbons) fatty acids synthesized by the nonnervous fatty acids of intermediate chain length. Some of the lignoceric acid is then converted to oleic acid with concomitant dilution of label and then chain shortening takes place to give n Vernon acid. Ex-

TABLE VI

<table>
<thead>
<tr>
<th>Sample</th>
<th>D.p.s./μmol × 10$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebronic acid</td>
<td></td>
</tr>
<tr>
<td>A. 2-Hydroxytetracosanoic</td>
<td>6.10</td>
</tr>
<tr>
<td>B. BaCOa (carbon atom 1)</td>
<td>0.026</td>
</tr>
<tr>
<td>C. Benzoic (carbon atom 2)</td>
<td>0.0298</td>
</tr>
<tr>
<td>D. Benzoic (carbon atom 3)</td>
<td>0.023</td>
</tr>
<tr>
<td>E. Heneicosanoic (carbon atoms 4-24)</td>
<td>4.83</td>
</tr>
<tr>
<td>F. Sum of degradation products</td>
<td>6.01</td>
</tr>
<tr>
<td>Ratio A:B</td>
<td>9.74</td>
</tr>
<tr>
<td>Ratio E:D</td>
<td>9.23</td>
</tr>
<tr>
<td>Lignoceric acid</td>
<td></td>
</tr>
<tr>
<td>A. Tetracosanoic</td>
<td>100.0</td>
</tr>
<tr>
<td>B. Benzoic (carbon atom 1)</td>
<td>10.3</td>
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<tr>
<td>C. Benzoic (carbon atom 2)</td>
<td>0.193</td>
</tr>
<tr>
<td>D. Benzoic (carbon atom 3)</td>
<td>9.16</td>
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<tr>
<td>E. Heneicosanoic (carbon atoms 4-24)</td>
<td>84.1</td>
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<tr>
<td>F. Sum of degradation products</td>
<td>103.8</td>
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<td>Ratio A:B</td>
<td>9.71</td>
</tr>
<tr>
<td>Ratio E:D</td>
<td>9.18</td>
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</table>

* All activities corrected for dilution of original acids with inactive material.
† Disintegrations per second.

TABLE VII

<table>
<thead>
<tr>
<th>Sample</th>
<th>D.p.s./μmol × 10$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tetracosanoic</td>
<td>18.8</td>
</tr>
<tr>
<td>B. Benzoic (carbon atom 1)</td>
<td>3.80</td>
</tr>
<tr>
<td>Tricosanoic (carbon atoms 2-24)</td>
<td>15.3</td>
</tr>
<tr>
<td>Ratio A:B</td>
<td>4.95</td>
</tr>
</tbody>
</table>

* Disintegrations per second.

Fig. 3 summarizes the biosynthetic pathways proposed for the C$^{14}$ acids of the brain. Acetate goes directly to lignoceric acid with little dilution along the way from fatty acids of intermediate chain length. Some of the lignoceric acid is then converted to cerebronic acid. Nervonic acid, on the other hand, does not arise directly from lignoceric acid. Rather, stearic acid is first converted to oleic acid with concomitant dilution of label and then chain shortening takes place to give n Vernon acid. Ex-
perperiments in vitro are currently in progress to study the details of these transformations.

**SUMMARY**

Two-week-old rats were given intraperitoneal injections of acetate-1-C\(^14\) over a 4-day period and killed 3½ hours after the last injection. The fatty acids obtained from the crude brain cerebrosides of these rats were then separated into four groups which included saturated unsubstituted, unsaturated unsubstituted, saturated 2-hydroxy, and unsaturated 2-hydroxy fatty acids. The individual unsubstituted acids were separated by reversed phase chromatography, and the C\(_{24}\) acids, lignoceric and nervonic, were isolated in a state of high purity. The 2-hydroxy acids were separated as the 2-acetoxo derivatives on the reversed phase column and the C\(_{24}\) acids, cerebronic and 2-hydroxynervonic, were obtained in the pure state. The C\(_{24}\) acids had high specific activities; the most highly active acid, lignoceric, had a specific activity 150 times greater than the average specific activity of the body fatty acids. Stepwise degradation of the C\(_{24}\) acids to determine the label distribution revealed that lignoceric acid is completely synthesized from acetate, with little dilution from acids of intermediate chain length during the chain elongation process. Cerebronic acid, in turn, is formed directly from lignoceric acid. Nervonic acid, on the other hand, is not derived from lignoceric acid by desaturation but rather appears to be formed by chain elongation of oleic acid.

**REFERENCES**

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