Chain Elongation, 2-Hydroxylation, and Decarboxylation of Long Chain Fatty Acids by Yeast*

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SUMMARY

Chain elongation and 2-hydroxylation pathways, which are specific for very long chain fatty acids (>C22), have been found in the yeast, Candida utilis. The chain elongation system acts specifically on fatty acids of chain length C22 to C24; there is no detectable elongation of C20 and only trace activity with C18. The product of the elongation enzyme or enzymes is C26 when C20, C22, or C24 is substrate and is a mixture of C22 and C24 when the substrates are C21 and C23. The 2-hydroxylation enzyme converts C26 acid, formed in situ by chain elongation, to 2-hydroxyhexacosanoic acid. The enzyme seems quite specific for the C26 chain length, although there may be some activity for chain lengths C24, C25, and C27.

In addition to the chain elongation and 2-hydroxylation systems, a relatively nonspecific system for the oxidative decarboxylation of 2-hydroxy acids has been studied in C. utilis. All 2-hydroxy fatty acids tested (C18 through C26 containing an even number of carbons) were converted, in part, to the unsubstituted acid containing 1 carbon less than the substrate. Decarboxylation was most efficient with 2-OH-C26 and least with 2-OH-C24 acids. Another product arising from the decarboxylation reaction was the aldehyde containing 1 carbon less than the 2-hydroxy acid substrate. The aldehydes were detected in trace amounts with the shorter chain substrates but were major products from the decarboxylation of 2-OH-C24 and 2-OH-C26. Presumably, the aldehyde is an intermediate between the 2-hydroxy acid substrate and the unsubstituted fatty acid which results as the final product in the oxidative decarboxylation process.

Although 2-hydroxyhexacosanoic acid has long been recognized as a major constituent of yeast cerebrin (1–3), and more recently the presence of n-hexacosanoic and n-tetracosanoic acids in yeast cerebrin has been reported (4), the origins of these acids and their metabolism by yeast remained unknown. In a preliminary survey carried out in this laboratory, a number of strains of Candida utilis and Saccharomyces cerevisiae were grown aerobically on a lipid-free medium in the presence of acetate-1-14C or stearate-1-14C and examined for the presence of radioactive 2-hydroxy acids and for tetracosanoic and hexacosanoic acids. In no case was there incorporation of label into fatty acids of chain length longer than C26, nor could labeled 2-hydroxy acids be detected. When docosanoic acid randomly labeled with tritium was tested, however, it was found to give rise to both labeled 2-hydroxy acid and to labeled chain elongation products, and it was these observations which prompted the present study.

EXPERIMENTAL PROCEDURE

Materials—Fatty acids, randomly labeled with tritium, were prepared from the unlabeled acids by New England Nuclear by the tritium gas exposure technique of Wilzbach (5). The tritiated materials were then rigorously purified as the methyl esters and then as the free acids by silicic acid chromatography to remove relatively large amounts of decarboxylated and oxidized material arising during the tritium exposure treatment. The 2-hydroxy acids were prepared from the tritiated, unsubstituted analogues as previously described (6) and purified by silicic acid chromatography and recrystallization from petroleum ether. Tritium-labeled trans-2-hexacosenoic acid was prepared from hexacosanoic-14II acid via the 2-hydroxy derivative by the procedure of Myers (7). The trans-2-hexacosenoic-14II acid was purified as the methyl ester by silicic acid chromatography and recrystallization from petroleum ether. Small amounts of isomeric acids (i.e. cis- and trans-3-hexacosenoic-14II) were removed by several crystallizations of the free acid from petroleum ether at 60–70°. The final product had a melting range of 77.5–78.5° and was homogeneous with respect to both mass and radioactivity by silicic acid chromatography. Analysis by gas radiochromatography (9) on a diethylene glycol succinate polyester column indicated a radioactivity of 98%.
only impurity detectable by this method being 3-hexacosanoic acid.

Yeast Cultures—All cultures were obtained from the American Type Culture Collection (ATCC), with the exception of Fleischmann’s yeast (Standard Brands, Inc.), and were maintained on malt agar (Difeo Laboratories). Yeasts were grown in a defined medium (10) at pH 6 with shaking in a model C26 incubator shaker (New Brunswick Scientific Company). Fatty acid substrates were added to the medium in ethanol solution. Yeast cells were harvested and washed by centrifugation at 10,000 × g in the cold. The wet cells were then either directly saponified in 10% KOH in methanol-water (2:1) and the fatty acids isolated by either extraction of the acidified aqueous mixture or, when aldehydes were to be isolated, the cells were extracted with chloroform-methanol (2:1) (10 ml per g of packed cells), and the isolated lipids were transforensified by heating in a closed tube at 100° for 4 hours with 4% anhydrous HCl in methanol. Methyl esters, prepared either by the HCl-methanol treatment (which converted any aldehydes present to the dimethylacetales) or by the diazomethane treatment of the free fatty acid fraction, were separated on 1-g silica gel columns (Unisil, 100 to 200 mesh, 70 Hi-Flex gas chromatograph equipped with a Cary vibrating-reed electrometer (9). Liquid scintillation counting was performed with a Packard Tri-Carb liquid scintillation spectrometer (9) with an efficiency of 15 to 16% for tritium.

Results

Metabolism of Docosanoic-3H Acid by Yeast—In preliminary experiments, a number of strains of C. utilis and S. cerevisiae were grown in the presence of stearic-3H or docosanoic-3H acids to determine their ability to metabolically alter these substrates. There was no evidence for β oxidation of the substrates by any of the yeast strains. Stearic acid was converted in part to oleic acid, as expected (11), but in no case was it found to be chain elongated or to give rise to 2-hydroxy acids. On the other hand, docosanoic acid was not desaturated but was altered in other ways, and these results are summarized in Table I. In each case, the major chain elongation product was hexacosanoic acid. The 2-hydroxy acid fractions from two strains of C. utilis (12, 13) proved to be 2-hydroxyhexacosanoic (2-OH-C26) acid. The 2-hydroxy acids isolated from the various strains of S. cerevisiae were not further examined because of relatively low yields of labeled material. It should be noted that, in the cases examined, the C24 substrate was not itself hydroxylated, nor were significant amounts of 2-OH-C24 acid detected.

Effect of Growth Temperature on Chain Elongation and 2-Hydroxylation—C. utilis (ATCC 9226) was selected for further study, and in Table II the effects of temperature on chain elongation and the formation of 2-hydroxy-acids are presented. Again, in the two cases examined (Experiments 2 and 3), the 2-hydroxy acid fraction was the C26 homologue with only traces of other homologues. The effect of growth temperature on the metabolic alteration of docosanoic acid is apparent for both the chain elongation and the 2-hydroxylation reactions. There is essentially no alteration of substrate at 15° (Table II, Experiment 1), but at 25° (Experiment 2) a good yield of C26 acid is obtained, as well as a significant amount of the 2-hydroxy analogue. The amount of 2-OH-C26 more than doubles at 30° (Experiment 3) and doubles again at 35° (Experiment 4), while the total elongation products increase in a linear fashion. Although at all temperatures the major product is the C26 acid, significant amounts of tetrocosenoic acid are also formed. In addition, the odd carbon acids (C19, C21) begin to appear at 30°, and the two, in almost equal amounts, comprise 33% of the total products at 35°. Finally, the effects of a 5-fold decrease or increase in amount of substrate at 25° are shown in Experiments 2A and 2B, Table II. In both cases, the amount of 2-hydroxy acid formed remains relatively constant while the amount of C24 acid decreases sharply at the lower substrate level, but does not increase at the higher level.

Metabolism of 2-Hydroxydocosanoic-3H Acid—Since the production, at 30° and 35° of labeled fatty acids containing an odd number of carbon atoms, suggested the operation of a 2-hydroxy acid oxidative decarboxylation pathway analogous to that found in brain (9) and plants (12, 13), 2-hydroxydocosanoic-3H acid was tested as a substrate and found to be readily decarboxylated (Table III). In Experiments 2 and 3 (Table III), the major products are approximately equal amounts of C19 and C21 acids which presumably arose by chain elongation of the oxidative deacyylation product, C24 acid. In Experiment 1, however, a major product is C26 acid, a finding difficult to explain. In all

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Table I

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Yield of cells</th>
<th>Added 2-H recovered in fatty acids</th>
<th>2-Hydroxy acids</th>
<th>Chain elongation products</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida utilis</em> (ATCC 9950)</td>
<td>3.2</td>
<td>55.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>C. utilis</em> (ATCC 8206)</td>
<td>3.0</td>
<td>74.1</td>
<td>2.3</td>
<td>13</td>
</tr>
<tr>
<td><em>C. utilis</em> (ATCC 9226)</td>
<td>2.7</td>
<td>64.1</td>
<td>2.9</td>
<td>19</td>
</tr>
<tr>
<td>Fleischmann’s yeast</td>
<td>1.6</td>
<td>14.1</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae (ATCC 4098)</td>
<td>1.7</td>
<td>27.1</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> (ATCC 4134)</td>
<td>1.9</td>
<td>56.1</td>
<td>0.6</td>
<td>8.5</td>
</tr>
<tr>
<td><em>B. cerevisiae</em> (ATCC 4110)</td>
<td>2.4</td>
<td>27.1</td>
<td>0.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*As millimicromoles of docosanoic-3H acid, based on radioactivity.

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1 As determined by gas radiochromatography of the KMnO4 decarboxylation product.
Long Chain Fatty Acids of Yeast

TABLE II

Effect of growth temperature on metabolism of docosanoic-3H acid by C. utilis (ATCC 9226)

Yeasts were grown at the temperature indicated to stationary phase in 100 ml of medium containing 100 mpmoles (5 x 10⁶ cpm) of docosanoic-3H acid, and the fatty acids were obtained by saponification and analyzed as described. In Experiments 2 and 3, the 2-hydroxy acid fraction was subjected to permanganate decarboxylation, and the products were analyzed by gas radiochromatography.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation temperature</th>
<th>Yeast recovered in fatty acids</th>
<th>Unsubstituted 3H-acids formed, b by chain length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>C₄  C₆  C₈  C₁₀ C₁₂ C₁₄ C₁₆ C₁₈ C₂₀ C₂₂ C₂₄ C₂₆ C₄₆ total</td>
</tr>
<tr>
<td>1</td>
<td>15°</td>
<td>2.3</td>
<td>44 &lt;0.2</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>2.8</td>
<td>59 1.1</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>2.7</td>
<td>66 2.4</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>1.7</td>
<td>93 5.5</td>
</tr>
<tr>
<td>2A</td>
<td>23</td>
<td>2.9</td>
<td>63 0.9</td>
</tr>
<tr>
<td>2B</td>
<td>23</td>
<td>2.5</td>
<td>48 1.0-1.5</td>
</tr>
</tbody>
</table>

a In Experiment 2A, only 20 mpmoles of substrate were added; in Experiment 2B, 500 mpmoles of substrate were used.

b See Footnote a, Table I.

TABLE III

Metabolism of 2-hydroxydocosanoic-3H acid by C. utilis (ATCC 9226)

Incubation and isolation and analysis of the fatty acids were carried out as previously described.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation temperature</th>
<th>2-OH-C₂₀-3H added</th>
<th>Yield of cells</th>
<th>Unsubstituted 3H-acids formed, b by chain length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mpmoles</td>
<td>%</td>
<td>C₄  C₆  C₈  C₁₀ C₁₂ C₁₄ C₁₆ C₁₈ C₂₀ C₂₂ C₂₄ C₂₆ C₄₆ total</td>
</tr>
<tr>
<td>1</td>
<td>23°</td>
<td>100</td>
<td>2.9</td>
<td>39 0.6 0.2 0.2 Trace 2.6 4.6 3.6 0.2</td>
</tr>
<tr>
<td>2</td>
<td>23°</td>
<td>400</td>
<td>2.5</td>
<td>25 30 2.2 0.7 Trace 12.0 1.1 13.6 2.7</td>
</tr>
<tr>
<td>3</td>
<td>35°</td>
<td>50</td>
<td>1.7</td>
<td>50 5 0.8 Trace Trace 1.8 0.6 1.8 0.2</td>
</tr>
</tbody>
</table>

a As millimicromoles of 2-hydroxydocosanoic-3H acid, based on radioactivity.
b Tentatively identified by retention time.

table IV

Metabolism of C₁₈- through C₂₄-unsubstituted 3H-acids by C. utilis (ATCC 9226)

All incubations were carried out at 30° in 100 ml of medium containing 50 mpmoles of the tritiated substrate. Total lipids were obtained by shaking each batch of centrifuged, packed cells for several hours with 50 ml of chloroform-methanol (2:1). The lipids were treated with HCl-methanol and the products were isolated as described.

<table>
<thead>
<tr>
<th>Chain length of -H-fatty acid added</th>
<th>mpmoles</th>
<th>Distribution of -H-fatty acids recovered, as percentage of total by chain length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C₁₀  C₁₂  C₁₄  C₁₆  C₁₈  C₂₀  C₂₂  C₂₄  C₂₆</td>
</tr>
<tr>
<td>18</td>
<td>2.1</td>
<td>&gt;99° 36 75 1 1 1 22</td>
</tr>
<tr>
<td>20</td>
<td>2.1</td>
<td>36    75 1 1 1 22</td>
</tr>
<tr>
<td>22</td>
<td>4.5</td>
<td>36    75 1 1 1 22</td>
</tr>
<tr>
<td>2₄</td>
<td>4.2</td>
<td>36    75 1 1 1 22</td>
</tr>
<tr>
<td>2₆</td>
<td>4.6</td>
<td>36    75 1 1 1 22</td>
</tr>
</tbody>
</table>

a In these experiments no analysis was carried out for 2-hydroxy acids because of the relatively poor recovery of activity.
b About 40% of the C₁₈ fraction was present as oleic and linoleic acids (by gas radiochromatography).
c Original (30°) sample lost. Results are from a run done at 35°. The C₁₄ and C₁₆ substrates were tested at 15°, 25°, and 35° as well as at 30°. At all temperatures, C₁₈ was recovered unchanged, while C₂₄ was recovered unchanged at 15° and 25°.

e In these experiments, a small peak corresponding to C₂₄ aldehyde was also detected, but no quantitative significance can be attached to the amount, since the saponification process leads to considerable destruction of aldehyde.

Metabolism of C₁₄- through C₂₄-Unsubstituted and 2-Hydroxy Acids—The results of growing C. utilis (ATCC 9226) in the presence of the C₁₄ through C₂₄ even carbon unsubstituted or 2-hydroxy acids are presented in Tables IV and V. In the experiments utilizing the unsubstituted acids (Table IV), the extraction of radioactive lipids was substantially incomplete while there was better, but still incomplete, extraction of the lipids in the experiments in which 2-hydroxy acid substrates were used. Therefore, direct comparison of results between the two tables would not necessarily be valid. A major point of interest in Table IV is the excellent conversion of the C₂₀ acid to C₂₄, as opposed to the complete lack of chain elongation of stearic acid.

The data in Table V indicate that the 2-hydroxy acids of all chain lengths tested can be decarboxylated by the yeast, although 2-OH-C₂₀ was most readily metabolized, while the C₂₄ and C₂₆ analogues were relatively poor substrates. In all cases the n - 1 aldehydes (aldehydes containing 1 carbon less than the substrate 2-hydroxy acid, 2-OH-C₂₀) were detected and analyzed as the dimethyl acetals and in two experiments (with 2-OH-C₂₀ and 2-OH-C₂₄ as substrates) represent a major fraction of the total products. In general, the pattern of chain elongation of the n - 1 products was that expected from an analysis.
Yeasts were grown for 48 hours in 1 liter of medium containing 0.5 pmole of the tritiated 2-hydroxy acid. A temperature of 24-25°C was maintained throughout to minimize 2-hydroxylation of decarboxylation products. The packed yeast cells were extracted in a Waring Blendor for 2 min with chloroform-methanol (2:1), and the lipids were isolated and treated as described to yield separated fractions containing unsubstituted methylesters, 2-hydroxy methyl esters, and dimethylacetals, respectively.

Incubations were carried out at 30°C in 50 ml of medium containing the 14C-substrate. Each batch of yeast (2.3 g, wet weight) was extracted with chloroform-methanol (2:1) to obtain total lipids, and the residual fatty acids were isolated.* Less than 0.5% of the yeast fatty acids were extracted by at most, 65 to 70% of the yeast fatty acids were extracted by 10% KOH in methanol-water (2:1) at 100°C for 4 hours, and the residual fatty acids were isolated.

*a ND. = not detected. The limit of detection of C26 was about 0.2 mpmoles under the conditions used.

The increase in activity in the ester fractions (eluted with 3% methanol) was maintained throughout to minimize 2-hydroxylation of decarboxylation products. The packed yeast cells were extracted in a Waring Blendor for 2 min with chloroform-methanol (2:1), and the lipids were isolated and treated as described to yield separated fractions containing unsubstituted methylesters, 2-hydroxy methyl esters, and dimethylacetals, respectively.

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additional $^3$H activity could be obtained by this method from the yeast grown in the presence of C$_{18}$ acid. However, when the C$_{18}$ and 2-OH-C$_{18}$-grown cells were treated in the same way, 10.8% and 13.0% additional activity, respectively, could be obtained by saponification. Siliceic acid chromatography of a portion of the chloroform-methanol extract of the C$_{18}$-grown yeast cells indicated that more than 95% of the activity was present in the free fatty acid fraction. On the other hand, the failure of stearic acid to give rise to chain elongation products cannot be ascribed to lack of activation or metabolism since the acid is incorporated into yeast lipids and is desaturated to oleic and linoleic acids (Tables VI and VII, Experiments 1 through 3). Likewise, acetate is incorporated into C$_{15}$ fatty acids readily but is not incorporated into longer chain lengths (Tables VI and VII, Experiments 4 and 5).

**DISCUSSION**

The present experiments provide evidence for the existence, in yeast, of metabolic pathways for the alteration of very long chain fatty acids, which are similar to pathways known to operate in mammalian brain (6, 9) and in higher plants (12, 13). One apparently unique aspect of several of the yeast pathways, however, is their relatively narrow specificities. Thus, the chain elongation system of C. utilis is most active with C$_{20}$ and C$_{24}$ acids but is completely inactive with C$_{18}$ acid and shows only trace activity with C$_{16}$ (See Tables IV and V). The end product of this chain elongation system is the C$_{24}$ acid when the substrate is C$_{18}$, C$_{20}$, or C$_{24}$ and is a C$_{23}$ and C$_{22}$ mixture when the substrate is C$_{18}$ or C$_{20}$ (Table V). In addition to chain elongation, the yeasts are also able to carry out 2-hydroxylation with 2-OH-C$_{18}$ acid being, in the cases examined here, the only major product. The specificities of these two systems perhaps explain why the author was unable to detect the presence of either very long chain fatty acids (> C$_{18}$) or 2-hydroxy acids in yeast grown on defined, lipid-free media, despite the numerous reports that these fatty acids were found as components of cerebrin from C. utilis and other yeasts. It would seem that for C. utilis and S. cerevisiae, the elongation and 2-hydroxylation pathways operate only when the proper substrate is included in the medium, and that the typical composition of commercial cerebrin may be a reflection of dietary factors during growth.

The yeast system for the decarboxylation of 2-hydroxy acids does not show the relatively narrow specificity of the chain elongation and 2-hydroxylation systems, but there is a decrease in activity with increasing chain length of the substrate (Table V). The accumulation of significant amounts of C$_{18}$ and C$_{24}$ aldehydes, when the 2-hydroxy-C$_{24}$ or C$_{24}$ acids are used as substrates, suggests that the rate of oxidation of the aldehyde to the acid also decreases with increasing chain length. Since, presumably, the C$_{18}$ - 1 aldehyde is an intermediate in the conversion of the 2-OH-C$_{18}$ acid to the C$_{18}$ - 1 acid, a low rate of aldehyde oxidation may well be a rate-limiting factor in the decarboxylation-chain elongation pathway at the C$_{24}$ through C$_{30}$ level.

Several questions, arising from the data, must await further experimentation before they can be answered with certainty. Why, for example, is C$_{18}$ acid, formed in situ from shorter chain length precursors, apparently readily hydroxylated, while C$_{24}$ acid, added to the medium, is not altered? The fact that added C$_{24}$ can be recovered chiefly as the free acid from cells grown in its presence, as well as the finding that none of the C$_{24}$ acid (as opposed to shorter chain substrates) is present in the cells in a form not extractable by chloroform-methanol, suggest that added C$_{24}$ acid is not easily activated to the CoA ester and hence not metabolized. On the other hand, C$_{24}$ acid resulting as an end product of the chain elongation system is presumably released as the CoA ester and this, or some active derivative, could be the actual precursor of the 2-hydroxy acid. This hypothesis is supported by the demonstration by Gatt (15) that tetrahydroxy acid is not readily metabolized by the rat, presumably because of lack of activation to the CoA ester; indeed, it can be recovered as the free acid from the liver of rats fed the acid 24 hours previously. Likewise, Kornberg and Pricer (16), with the use of a fatty acid activating system from guinea pig liver, reported a relatively constant rate of CoA ester formation for the saturated fatty acids of chain length C$_{18}$ through C$_{20}$ but a rate reduction of more than half for the C$_{24}$ acid and a very much lower rate of activation with C$_{26}$, the longest chain length tested. Although lack of activation seems the most likely reason for the failure of added C$_{24}$ to serve as a good substrate for 2-hydroxylation, the possibility also exists that some other C$_{24}$ derivative (i.e. a chain elongation intermediate) is the actual intermediate in hydroxylation, and it was this possibility which led in the testing of trans-2-hexacosenoic-$^3$H acid. Again, however, the results were negative, and it will be necessary to obtain a cell-free system in which the CoA esters can be tested directly before the true intermediate can be determined.

Another question concerns the origin of C$_{24}$ acid when yeast is grown in the presence of 2-hydroxydecosenoic acid at 23° (Table III, Experiment 1). Since the decarboxylation process would yield an odd carbon acid (i.e. C$_{21}$), chain elongation of the decarboxylation product cannot give rise directly to C$_{24}$, an even carbon acid. Thus, two decarboxylations must take place before an even carbon product can be obtained. One cannot, however, exclude the possibility of a dehydration-reduction of the 2-OH-C$_{21}$ acid to give C$_{22}$ followed by chain elongation to C$_{24}$. The author here accepts the preparation of 1-$^4$C-2-hydroxydecanoic acid since, with this type of labeling, the $^4$C would be retained in C$_{24}$ formed by a dehydration-reduction-chain elongation pathway but lost in the decarboxylation pathway.

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