The Metabolism of Hydroxystearic Acids in Yeast*

R. J. LIGHT,† W. J. LENNARZ,‡ AND KONRAD BLOCH

From the James Bryant Conant Laboratory, Harvard University, Cambridge 38, Massachusetts

(Received for publication, January 31, 1962)

The enzymatic desaturation of palmitoyl coenzyme A to palmitoleoyl coenzyme A by yeast enzymes is known to require molecular oxygen and reduced triphosphopyridine nucleotide (1). Since these requirements are characteristic of oxygenase reactions (2), it has been proposed that the desaturation of palmitate to palmitoleate or of stearate to oleate proceeds by way of hydroxy acid intermediates. In apparent support of this hypothesis, we have observed that 9- or 10-hydroxystearic acids substitute fully for oleic acid as growth factors for the growth of yeast under strictly anaerobic conditions (3). Moreover, preliminary experiments seemed to suggest that yeast extracts can catalyze a conversion of hydroxystearic acids to oleic acid. However, this enzymatic transformation could not be reproduced in subsequent experiments, nor could a conversion of hydroxystearic acid to oleic acid be demonstrated in growing yeast cells. Although we are unable to offer an explanation for the positive results obtained earlier, it is now clear that the hydroxy acids or their coenzyme A esters do not as such have the properties of intermediates in the biosynthesis of oleic acid. Nevertheless, hydroxystearic acids can replace oleic acid as growth factors for anaerobic yeast. Since this biological activity is not due to a conversion of the hydroxy acids to olefinic acids, it became of interest to investigate the metabolism of hydroxystearic acids in anaerobic yeast and the fatty acid requirements of this system in more detail. The present report describes the growth-promoting effects of various fatty acids and fatty acid derivatives, and also the transformation of 9(aa and 10)-hydroxystearic acid to an acetoxy derivative by growing yeast and by extracts of these cells.

EXPERIMENTAL PROCEDURE

Materials

The detergent, Cytexum (trade name employed by Fisher Scientific Company), as well as the enzymes used to solubilize particulate fractions, was obtained from commercial sources. 12-Hydroxy-9-octadecenoic acid (ricinoleic acid) was purchased from K and K Laboratories. 9-trans-Octadecenoic acid (elaidic acid) was purchased from Fluka AG., Buchs, Switzerland.

β-Diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525-A) was supplied by Dr. C. W. French of Smith, Kline and French Laboratories. 3-Hydroxydecanoic acid, 10-hydroxy-

* Supported by grants-in-aid from the Life Insurance Medical Research Fund, the United States Public Health Service, the National Science Foundation, and the Eugene Higgins Trust Fund of Harvard University.

† National Science Foundation Postdoctoral Fellow.

‡ Postdoctoral Research Fellow of the National Heart Institute.

decanoic acid, and 10-hydroxy-2-decenoic acid were gifts of Dr. J. Law of this department. 2-Hydroxystearic acid and 2-hydroxytetradecanoic acid were gifts of Dr. A. J. Fulco (4). 1-Monoo-

olein was a gift of Dr. F. H. Mattson of The Procter and Gamble Company. 10-Hydroxystearic acid, isolated from natural sources, was a gift of Dr. A. T. James. 9(aa and 10)-Aminostearic acid was a gift of Dr. Richard Fulmer of General Mills, Inc. 11 cis-Octadecenoic acid (cis-vaccenic acid), free from oleic acid as shown by gas-liquid chromatography of the products obtained by KMN04-KIO4 oxidation,1 and 11,12 methylene stea-

ric acid (lactobacillic acid) were gifts of Professor Klaus Hofmann. Dl-10-Methyl stearic acid (tuberculostearic acid) was a gift of Professor S. Hünig.

9-Hydroxystearic acid and 12,13-di-H3-9-hydroxystearic acid were prepared by catalytic hydrogenation of the naturally occurring 9-hydroxy-12-octadecenoic acid. In several experiments, a mixture of 9-hydroxy- and 10-hydroxystearic acid (referred to as 9(aa and 10)-hydroxystearic acid) obtainable from oleic acid by way of the epoxide (5) was used. Contrary to earlier claims that this procedure affords 10-hydroxystearic acid (5), the reaction product has more recently been shown to be a mixture of the isomeric 9- and 10-hydroxystearic acids in approximately equal amounts (6). It was felt that, for the purposes of the present experiments, the use of this mixture of isomers was admissible since its metabolic behavior was indistinguishable from that of the individual 9- or 10-hydroxystearic acid isomers. 12-Keto-

stearic acid was obtained by chromic acid oxidation of 12-hy-

droxystearic acid (7). 9-Octadecenoic acid (stearolic acid) was synthesized from oleic acid (8), purified by silicic acid chromatography, and shown to be at least 99% pure by gas-liquid chromatography.

Methyl esters of the fatty acids were prepared by reaction with diazomethane in an ether solution containing a few drops of methanol. Ethyl esters were prepared by reaction with absolute ethanol and anhydrous hydrogen chloride at room temperature.

Ethyl 1-C14-9(aa and 10)-acetoxystearate was synthesized by the acetylation of ethyl 1-C14-9(aa and 10)-hydroxystearate. A solution of 1-C14-hydroxy ester (78 mg, 2.05 X 104 d.p.m. per mg) in pyridine (3 ml) and acetic anhydride (3 ml) was left at room temperature overnight. Ice was added, and the product was isolated by the usual extraction procedures. The crude product was purified by chromatography on 5 g of silicic acid, with gradient elution (mixing flask, 400 ml of Skellysolve B; reservoir, 400 ml of 10% ether in Skellysolve B). The radioactive peak (215 to 275 ml eluate) contained 68 mg of ethyl 1-C14-9(aa and 10)-acetoxystearate with a specific activity of 1.83 X 104 d.p.m. per mg.

1 Dr. Günter Scheuerbrandt, unpublished results.
(calculated, $1.80 \times 10^6$ d.p.m. per mg). The infrared spectrum in chloroform showed a strong ester band at 5.8 $\mu$ and a strong acetate band at 8.0 $\mu$ (9). According to gas-liquid chromatography, the acetoxy ester was more than 99% pure. However, the elemental analysis of the oily material was less satisfactory.

\[
C_{12}H_{16}O_4
\]

**Calculated:** C 71.30%, H 11.42%

**Found:** C 72.10%, H 10.86%

Methyl 1-$\text{C}^{14}$-9-(and 10)-acetoxystearate and the free 1-$\text{C}^{14}$-9 (and 10)-acetoxystearic acid were synthesized by acetylation of methyl 1-$\text{C}^{14}$-9-(and 10)-hydroxystearate and 1-$\text{C}^{14}$-9-(and 10)-hydroxystearic acid, respectively. Methyl 1-$\text{C}^{14}$-9-(and 10)-propionoxystearate was obtained by an analogous procedure with propionyl chloride and pyridine in ether.

Crude phytosphingosine was obtained from yeast lipids by the method of Sweeley and Moscatelli (10). 1-$\text{C}^{14}$-acetyl-CoA was prepared from 1-$\text{C}^{14}$-acetate anhydride and CoA (11).

**Methods**

**Yeast—**Saccharomyces cerevisiae, strain LA 2 G 12, was grown without aeration in flasks filled to the neck (microaerobically) in a medium containing 10% glucose, 0.67% Difco yeast nitrogen base, and 0.05 M succinate buffer, pH 5.0. After 1 to 4 days, the cells were collected by centrifugation and suspended in a solution containing 10% glucose and 0.05 M succinate buffer. For the anaerobic growth experiments (Table VII), aliquots of this suspension were used to inoculate 20 ml of the same growth medium. Ergosterol (0.55 mg) and fatty acids (0.33 mg) were added in ethanolic solution (0.1 ml). Anaerobic incubations were carried out in 50-ml flasks placed in a desiccator as previously described (12). In control flasks containing no additional fatty acid, growth was usually slight (Table VII), provided that the inoculum was small and that cells for inoculation were grown for only 1 day.

For the enzymatic experiments on fatty acid desaturation, crude extracts and particulate fractions were prepared as previously described (1). In attempts to solubilize the yeast particles with phospholipase, with Cutsenu, or with trypsin, the procedures of Imai and Sato (13), of Levin, Levenberg, and Kaufman (14), and of Williams and Hager, respectively, were followed.

For enzymatic acetylation experiments, crude extracts and particles were prepared in the same manner as for the desaturating system, except that the particulate fraction was obtained by centrifugation for 120 minutes at 130,000 X g. Also, the incubation with saliva was omitted. Extracts or particles frozen in buffer remained active for at least 2 months but gradually lost activity if thawed repeatedly.

**Isolation of Fatty Acids**—1. By direct saponification: the cells were collected by centrifugation, washed twice with cold distilled water, and digested by heating with methanolic KOH (30% methanol containing 5% KOH) for 2 hours on the steam bath in a nitrogen stream. The fatty acids were isolated in the usual manner.

2. Total lipids were extracted from the washed cells with chloroform-methanol (2:1) by the method of Folch, Lees, and Sloane Stanley (13).

3 L. P. Hager, personal communication.
oxygenase reactions involving TPNH and oxygen (23), has no inhibitory effect on desaturation. Glycerol 1-phosphate, ascorbate, and dimethylenzimidazole cobamide neither stimulate nor inhibit the reaction.

In attempts to solubilize the particulate enzyme, procedures successful in various microsomal oxygenase systems were investigated. For example, the enzyme which catalyzes the hydroxylation of 3,4-dihydroxyphenylethylamine to norepinephrine has been solubilized by treatment with the surface-active agent, Cutsen (14), and a soluble acetanilide hydroxylase has been obtained by treatment with either lipase (24) or snake venom phospholipase (13). Cutsen only partially solubilized the yeast particles, and neither the supernatant fraction nor the remaining particles were enzymatically active. Treatment with phospholipase afforded partial dissolution, but the soluble fraction was inactive and the remaining particulate fraction was only slightly active in desaturating stearoyl-CoA. Brief trypsin digestion of the particles, followed by addition of soybean trypsin inhibitor, inactivated the particles. RNase treatment liberated nucleotides from the particles but did not solubilize them; the treated particles retained their desaturating activity. Sonication was also ineffective as a means for obtaining an active, soluble enzyme.

Fractionation of Lipids from Yeast Grown with 1-C14-9(and 10)-hydroxystearic Acid or 1-C14-oleic Acid—As previously reported, 9- or 10-hydroxystearic acid and also the mixture of the 9- and 10-hydroxy isomers satisfy the fatty acid requirement of anaerobic yeast and are as effective as oleate when tested at the same concentrations (3). Since the hydroxystearates exhibit this action without being converted to olefinic acids, the question arose whether these substances undergo any metabolic changes while serving as growth factors for anaerobic yeast. The lipids of anaerobic yeast grown in the presence of C14-hydroxystearate were therefore analyzed by silicic acid chromatography. The results are shown in Table I and may be compared with the distribution of C14 in the lipids from an analogous experiment with 1-C14-oleate. Oleic acid is incorporated to an equal extent into both the neutral lipid and phospholipid fractions of growing yeast, and only a small amount of the C14 appears as free fatty acid. In the experiment with hydroxy acid, C14 is found principally in the neutral lipid and polar fatty acid fractions (Table I). Further fractionation of the neutral lipids showed that oleic acid is incorporated into several lipid components, whereas radioactivity from C14-hydroxy acid is found almost exclusively in a single fraction (Fraction III, Table II). Of the oleic acid added to the growth medium, 32% was recovered in the lipid extracts of the cells, whereas the recovery of hydroxy acid was 75%.

The fractions obtained by chromatography of the chloroform-methanol extracts (see above) were saponified, and the fatty acids were isolated. Chromatography of the fatty acids on silicic acid demonstrated that less than 1% of the hydroxy acid had been converted to oleic or other nonpolar acids. Identical results were obtained when the whole cells were saponified directly and the fatty acids were examined by chromatography.

A similar analysis of cells grown in the presence of 1-C14-oleic acid revealed that less than 1% of the oleic acid had been converted into polar fatty acids such as hydroxystearic acid. Moreover, after separation of the methyl esters of the total fatty acids by the mercuric acetate method (12), less than 3% of the radioactivity was found in the fraction containing the saturated acids.

The reduction of oleate to stearate as well as the hydration of oleate to hydroxystearate by anaerobically growing yeast is therefore not detectable under the present experimental conditions.

Identification of Metabolites of Hydroxy Acid—Yeast was grown anaerobically in 10 flasks with a total of 3.3 mg of 1-C14-9(and 10)-hydroxy acid and 1-C14-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Chromatographic fractiona</th>
<th>Radioactivity incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From C14-hydroxy acid b</td>
<td>From C14-oleate b</td>
</tr>
<tr>
<td>CH2Cl2</td>
<td>I. Neutral lipids e</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>II. Free fatty acids f</td>
<td>34</td>
</tr>
<tr>
<td>CH2COCH2</td>
<td>III. Polar acids g</td>
<td>1</td>
</tr>
<tr>
<td>CH2Cl2-CH2OH (1:1)</td>
<td>IV. Phospholipids h</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Total neutral lipids i</td>
<td>3</td>
</tr>
</tbody>
</table>

Identification of Metabolites of Hydroxy Acid—Yeast was grown anaerobically in 10 flasks with a total of 3.3 mg of 1-C14-9(and 10)-hydroxy acid and 1-C14-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15). The reduction of oleate to stearate as well as the hydration of oleate to hydroxystearate by anaerobically growing yeast is therefore not detectable under the present experimental conditions.

Identification of Metabolites of Hydroxy Acid—Yeast was grown anaerobically in 10 flasks with a total of 3.3 mg of 1-C14-9(and 10)-hydroxy acid and 1-C14-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15). The reduction of oleate to stearate as well as the hydration of oleate to hydroxystearate by anaerobically growing yeast is therefore not detectable under the present experimental conditions.

Identification of Metabolites of Hydroxy Acid—Yeast was grown anaerobically in 10 flasks with a total of 3.3 mg of 1-C14-9(and 10)-hydroxy acid and 1-C14-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15). The reduction of oleate to stearate as well as the hydration of oleate to hydroxystearate by anaerobically growing yeast is therefore not detectable under the present experimental conditions.

Identification of Metabolites of Hydroxy Acid—Yeast was grown anaerobically in 10 flasks with a total of 3.3 mg of 1-C14-9(and 10)-hydroxy acid and 1-C14-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15). The reduction of oleate to stearate as well as the hydration of oleate to hydroxystearate by anaerobically growing yeast is therefore not detectable under the present experimental conditions.
specific activity of $2.14 \times 10^5$ d.p.m. per mg, accounting for 26% of the total radioactivity recovered in the crude lipid extract. Analysis by gas-liquid chromatography revealed a single radioactive compound that had a retention time of 6.4 relative to methyl stearate and a minor impurity which contained no radioactivity. By comparison, methyl 9(and 10)-hydroxystearate had a retention time of 7.8 relative to methyl stearate.

Aliquots of this material were saponified in 0.05 N ethanolic sodium hydroxide for 1 to 2 hours at 37°. After acidification and extraction of fatty acid with ether, a test for glycerol by a periodate assay (25, 26) in the aqueous layer was negative. The ether layer was treated with diazomethane, and analysis of the ester by gas-liquid chromatography showed that the radioactivity was recovered as methyl 9(and 10)-hydroxystearate. The unknown compound is therefore a saponifiable derivative of hydroxystearate but not a glyceride.

For further identification of the unknown ester, yeast was grown in the presence of H²-labeled 9(and 10)-hydroxystearic acid and also uniformly labeled C¹⁴-glucose. The H²-labeled hydroxy acid substrate was a mixture of unlabeled 9(and 10)-hydroxystearic acid and also uniformly labeled C¹⁴-glucose. The H³-labeled hydroxystearic acid combined with an essentially weightless hydroxystearic acid combined with an essentially weightless amount of 12,13-di-H³-9-hydroxystearic acid. The hydroxy acid ester was isolated from the yeast in the manner already described, and the radioactive peak collected from the gas-liquid chromatograph was counted for H² and C¹⁴ (Table III). Although the C¹⁴ activity was low, the value was sufficiently above background to show that a small component of the ester molecule was derived from glucose. This component was removed on hydrolysis since the recovered free acid contained no C¹⁴. From the C¹⁴ to H² ratio, it is estimated that the ester contains 4 to 6 carbon atoms more than the free hydroxy acid (Table I). Although this estimate is very approximate because of the low level of C¹⁴ activity, it appears certain that the unknown component(s) of the ester is a substance of relatively low molecular weight.

Approximate specific activities were determined for the hydroxy acid derivatives isolated from cells grown in the presence of 1-C¹⁴-9(and 10)-hydroxystearic acid and H²-labeled hydroxystearic acid, respectively. The values, which ranged from 80 to 94% of the specific activity of the substrate added to the medium, are consistent with the double labeling experiment (Table III) by indicating that only a few carbon atoms are added to the hydroxy acid molecule during metabolism. They also show that the added hydroxy acid is not significantly diluted by material synthesized endogenously.

With the information available from the above experiments, several simple hydroxy acid ester derivatives were synthesized, and their retention times on the gas-liquid chromatograph were compared with that of the unknown ester (Table IV). Of the derivatives tested, only ethyl 9(and 10)-acetoxystearate had the same retention time as the unknown ester both on a diethylenglycol succinate polymer column and on an Apiezon column. Methyl 9(and 10)-propionoxystearate was indistinguishable from the unknown ester on the diethylenglycol succinate column but had a different retention time on the Apiezon column. Ethyl 9(and 10)-acetoxystearate contains 4 extra carbon atoms in the molecule and has a calculated specific activity of 0.81 relative to hydroxystearic acid. All of the properties of the unknown ester are therefore consistent with the assigned structure.

On the basis of hydroxamate determinations as performed initially, it is apparent that the unknown ester contained only one ester group per molecule of hydroxy acid (Table V). This value, later found to be erroneous, was obtained when the ester was treated with hydroxylamine at an alkaline pH in a miscible ethanol-ether-water solvent system for 20 minutes at room temperature (20). Under these conditions, the analysis of synthetic ethyl 9(and 10)-acetoxystearate also gave values corresponding to only one ester group per molecule (Table V). However, when the reaction was carried out at 37° (in stoppered tubes) for 1 or 2 hours, the value of the number of ester groups per molecule was nearly doubled. This behavior can be explained by the fact that one of the groups reacting with hydroxylamine is the ester of a secondary alcohol which does not readily form a hydroxamate under the milder assay conditions.

An aliquot (0.15 µmole) of the ester isolated from yeast was treated with hydroxylamine for 2 hours at 37°, and the hydroxamates were chromatographed on paper. Two spots were visualized by spraying with ferric chloride solution, one with an $R_f$ of 0.52 and another at the solvent front. Since acetylhydroxamate has an $R_f$ of 0.52 under these conditions (21), this demonstrates the presence of an acetyl group in the ester.
and corroborates the structure of the ester as ethyl 9(and 10)-acetoxystearate.

The polar fatty acid fraction, separated from the total lipids by chromatography on silicic acid-Celite (Fraction III, Table I), was esterified with diazomethane and analyzed by gas-liquid chromatography. A single peak with a retention time of 5.5 relative to methyl stearate was observed, which identifies this acid as 9(and 10)-acetoxystearic acid.

Conversion of 1-C\textsuperscript{14}-oleic Acid to Ethyl Oleate by Yeast—To test whether the formation of ethyl esters as observed with hydroxystearate is a general phenomenon in anaerobic yeast, cells were grown in the presence of 0.3 mg of 1-C\textsuperscript{14}-oleic acid (specific activity, 5.5 \times 10\textsuperscript{7} d.p.m. per mg, and containing less than 0.05\% ethyl oleate). In this experiment, 29\% of the counts added to the medium were recovered in the crude chloroform-methanol extract; the neutral lipid fraction (Table I) contained 34\% of the total extractable counts. To aliquots of the neutral lipid fraction methyl oleate, ethyl oleate, and ethyl 9(and 10)-acetoxystearate were added as carriers. On gas-liquid chromatography, 10\% of the extractable counts were recovered in the ethyl oleate peak, 0.05\% in the methyl oleate peak, and 0.09\% in the ethyl 9(and 10)-acetoxystearate peak. The latter two values represent background from the column and are not considered significant. (Methyl oleate could have arisen by esterification or transesterification with methanol during extraction.)

Hydroxystearic Acid Acetylase—The presence of an acetylating enzyme responsible for the formation of the acetoxy derivative of 9(and 10)-hydroxystearic acid was tested as follows. Hughes' Press extracts of yeast cells were incubated with 9(and 10)-hydroxystearate and 1-C\textsuperscript{14}-acetyl-CoA, and the acidified incubation mixture was extracted with ethyl ether. The lipid-soluble material was assayed by chromatography on silicic acid (500 mg). Acetoxyacetic acid was eluted with methylene chloride (20 to 50 ml eluate), and radioactivity appearing in this fraction was taken as a measure of acetoxyacetic acid synthesis. In some of these experiments, the product was identified by gas-liquid chromatography of its methyl ester (Table IV).

The acetylating activity was sedimentable by centrifugation at 130,000 \times g. The washed particles were almost twice as active as the crude extract; the supernatant fraction was only 1/4 as active as the particulate fraction. Activity varied slightly with different preparations of particles, the incorporation of radioactivity into acetoxyacetic acid ranging between 5,000 d.p.m. and 15,000 d.p.m. under the conditions described (Table VI).

A variety of hydroxy acids and alcohols was tested as substrates for the acetylase system. Because of the variation in activity between different enzyme preparations, the data given (Table VI) are recalculated for a value of 100 for 9(and 10)-hydroxystearate, which was the reference substrate in each experiment. Substrates were tested only at a concentration of 10\textsuperscript{-3} M, the saturating concentration for hydroxystearate. Radioactivity appearing in an appropriate chromatographic fraction of the lipid-soluble material was used as the measure of acetylation. Only 9,10- or 12-hydroxyacids and ricinoleic acid (12-hydroxy 9 octadecenoic acid) were appreciably active, whereas 10-hydroxydecanoic acid showed slight activity. 1-C\textsuperscript{14}-acetate was only approximately 3\% as effective as 1-C\textsuperscript{14}-acetyl-CoA as acetyl donor.

Effect of Various Fatty Acids on Anaerobic Growth of Yeast—Apart from oleic acid, several fatty acids, exhibiting a variety of properties, were added to the medium. Some of the results obtained are shown in Table VI.

### Table VI

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>9(10)-hydroxystearate\textsuperscript{a}, b</td>
<td>100</td>
</tr>
<tr>
<td>10-Hydroxystearate\textsuperscript{a}</td>
<td>130</td>
</tr>
<tr>
<td>12-Hydroxystearate\textsuperscript{a}</td>
<td>155</td>
</tr>
<tr>
<td>12-Hydroxy-9-octadecenoate\textsuperscript{a}</td>
<td>95</td>
</tr>
<tr>
<td>10-Hydroxydecanoate\textsuperscript{a}</td>
<td>8</td>
</tr>
<tr>
<td>10-Hydroxy-2-decanoate\textsuperscript{a}</td>
<td>2</td>
</tr>
<tr>
<td>3-Hydroxydecanoate\textsuperscript{a}</td>
<td>1</td>
</tr>
<tr>
<td>2-Hydroxystearate\textsuperscript{b}</td>
<td>3</td>
</tr>
<tr>
<td>2-Hydroxytetrasubstearate\textsuperscript{a}</td>
<td>4</td>
</tr>
<tr>
<td>Ergosterol\textsuperscript{c}</td>
<td>4</td>
</tr>
<tr>
<td>Crude phytochepinosin\textsuperscript{c}</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Added as the potassium salt.
\textsuperscript{b} Added to particles as a solution in 0.1 ml of ethanol. The ethanol was removed under a stream of N\textsubscript{2}, and the other components of the incubation, including a fresh portion of particles, were added to the resulting suspension.
\textsuperscript{c} Added as a solution in acetone in the same manner as in footnote b.
**Table VII**

**Anaerobic growth of yeast**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Colorimeter reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>22</td>
</tr>
<tr>
<td>9(9-and 10)-Hydroxystearic acid</td>
<td>22</td>
</tr>
<tr>
<td>10-Hydroxystearic acid</td>
<td>22</td>
</tr>
<tr>
<td>9-Octodecenoic acid</td>
<td>190</td>
</tr>
<tr>
<td>10-Octodecenoic acid</td>
<td>190</td>
</tr>
<tr>
<td>11,12-Methylene stearic acid</td>
<td>111</td>
</tr>
<tr>
<td>11,12-Methylenestearic acid</td>
<td>111</td>
</tr>
<tr>
<td>12-Hydroxystearic acid</td>
<td>76</td>
</tr>
<tr>
<td>12-Ketostearic acid</td>
<td>81</td>
</tr>
<tr>
<td>Searic acid</td>
<td>22</td>
</tr>
<tr>
<td>10-Methyl stearic acid</td>
<td>24</td>
</tr>
<tr>
<td>9,10-Epoxy stearic acid</td>
<td>27</td>
</tr>
<tr>
<td>9,10-Dihydroxystearic acid</td>
<td>0</td>
</tr>
<tr>
<td>9-trans- Octodecenoic acid</td>
<td>5</td>
</tr>
<tr>
<td>9 (and 10)-Aminostearic acid</td>
<td>43</td>
</tr>
<tr>
<td>Ethyl 9 (and 10)-acetoxystearate</td>
<td>87</td>
</tr>
<tr>
<td>Ethyl 9 (and 10)-hydroxy stearate</td>
<td>160</td>
</tr>
<tr>
<td>9 (and 10)-Acetoxy stearic acid</td>
<td>158</td>
</tr>
</tbody>
</table>

*In an earlier experiment in which growth was measured by counting of the cells, 9-hydroxystearic acid was as active as 9 (and 10)-hydroxystearic acid in supporting growth (3).*

Discussion

From the requirement for TPNH and molecular oxygen in the desaturation reaction, it was reasonable to expect that mono-unsaturated fatty acids arise from their saturated counterparts by way of oxygenated intermediates. It has not been possible, however, to demonstrate the conversion of 9- or 10-hydroxy stearic acid (or their CoA esters) to olefinic products, and hence the formation of free hydroxy intermediates seems exceedingly unlikely. A 10-hydroxystearic acid has been isolated from fecal lipids by James, Webb, and Kellock (27). The acid appears to be produced oxidatively from stearate, but it is not yet clear whether this transformation is direct. As to the possibility of an intermediary formation of fatty acid hydroperoxides, we have not obtained any evidence for or against this possibility. Another alternative mechanism which is attractive but difficult to test is a direct abstraction of hydrogen from the central carbon atoms of the fatty acid chain, oxygen serving as the electron acceptor without entering into covalent linkage with carbon.

Our results clearly demonstrate that hydroxystearic acids can serve as growth factors for anaerobic yeast without being converted to oleic acid. It is therefore interesting that, apart from the hydroxy acids, several other derivatives of stearic acid can substitute for oleic acid as growth factors (Table VII). Compounds which are active contain either a hydroxyl group, a cis double bond, a triple bond, or a cyclopropane ring in the central region of the fatty acid molecule. Although the precise location of these groups does not seem critical, one common feature is their electron-rich nature. On the other hand, the amino, epoxy, dihydroxy, and trans-olefinic derivatives, which are also electron-rich, do not support growth.

When 9(9-and 10)-hydroxystearic acid serves as a growth factor for anaerobic yeast, it is transformed in high yield into both 9 (and 10)-acetoxystearate and ethyl 9(9-and 10)-acetoxystearate. It therefore seems possible that the acetoxyl derivative, rather than the free hydroxy acid, is the active growth factor. This is supported by the finding that 9(9-and 10)-acetoxystearate is as effective in supporting anaerobic yeast growth. Whether the other active fatty acids listed in Table VII also undergo enzymatic transformations has not yet been determined. In the absence of such information, it is not profitable to define the structural features responsible for growth-promoting activity of a fatty acid molecule.

Acetylations of hydroxyl groups in biological systems are relatively rare, and the examples which are known, e.g. the acetylations of choline (28), of isopropyl β-D-thiogalactoside (29), and of the C24 position of various steroids (30), as well as the occurrence of (++)-10-acetyl-α-tetradecan-1-ol in the gypsy moth (31), give no clues as to the role which acetoxy fatty acids might play in supporting the anaerobic growth of yeast. In fact, there are no indications that acetoxystearate or related compounds are normal metabolic products. When yeast was grown anaerobically in the presence of 1-C[4]-oleic acid, there was no evidence for the formation of more polar fatty acids, but more experiments are necessary to rule out the possibility that acetoxystearate, or its ethyl ester, is a minor product in the metabolism of oleate.

The formation of acetoxystearic acid involving acetyl-CoA as the acetylating agent can be demonstrated in a cell-free particulate enzyme system. Other compounds which were tested as possible substrates for the yeast acetylase but gave negative results were long chain α-hydroxy fatty acids, several hydroxydecanoic acids, ergosterol, 1-monoolein, and phytosphingosine, which occurs as a tetraacetyl derivative in yeast (32). Only 9-, 10-, or 12-hydroxystearate and monocarboxylic acid were acetylated to an appreciable extent. These results, although not proving that the enzyme is specific for hydroxyl derivatives of long chain fatty acids, do suggest a relatively close structural resemblance between the natural substrate, if one exists, and 9(9-and 10)-hydroxystearate. Acetylase activity can be demonstrated in extracts from aerobic as well as anaerobic cells, and therefore it is
unlikely that the enzyme is synthesized by induction in response to anaerobic growth conditions.

Anaerobic yeast grown in the presence of hydroxystearate contains the acetoxy derivative both in the form of free acid and as ethyl ester. It is not clear whether the esterification with ethanol has any metabolic or physiological significance. As a growth factor, ethyl acetoxystearate is much poorer than either hydroxystearate, acetoxystearate, or ethyl hydroxystearate, which suggests that the yeast cell may have a limited permeability for the relatively nonpolar acetoxy ethyl ester. As to the sequence of steps in the formation of ethyl acetoxystearate, it appears from the experiments with the cell-free system that the substrate for the acetylase is the free hydroxy acid and that esterification with ethanol takes place subsequently. Before esterification, acetoxystearate is presumably converted to the CoA derivative, which can react with ethanol but apparently not with glycerol, glycerides, or sterol. When anaerobic yeast is grown in the presence of 1-C'-oleic acid, some 10% of the incorporated radioactivity is recovered in the form of ethyl oleate, showing that esterification with ethanol may occur rather non-specifically with various fatty acids.

As seen from the data in Table VIII, the fatty acid composition of yeast lipids is strikingly dependent on the environmental atmosphere during growth. In cells grown with aerobiosis, and without fatty acid supplement, palmitoleic and oleic acids comprise 60 to 80% of the total fatty acids, whereas in strictly anaerobic cells, grown in the presence of hydroxystearate, the content of unsaturated fatty acids is negligible. Although essentially all of the fatty acids synthesized by anaerobic cells are saturated, a very much greater proportion of these acids is of shorter chain length (C16 and C18) than in the lipids of aerobic cells. Thus anaerobic cells, lacking a supply of olefinic acid from either endogenous or exogenous sources, appear to respond to this deficiency at least in part by producing increased amounts of shorter chain saturated fatty acids. Other changes in the physical environment for growing yeast, such as lowered temperature, have also been observed to lead to an increased content of lauric and myristic acids in the yeast lipids (34). However, it is clear from our results that the enhanced synthesis of shorter chain fatty acids in response to oxygen deprivation does not relieve the requirement for an exogenous fatty acid derivative. At any rate, it is remarkable that an organism can be raised under conditions leading to the virtual absence of olefinic fatty acids in the cellular lipids. As far as it is known, monounsaturated fatty acids are universal cell constituents under natural conditions. Yet the fact that they are replaceable by compounds such as acetoxystearate suggests that the double bonds per se are not an essential structural feature for biological activity, at least for anaerobic forms of life.

SUMMARY

1. The particulate enzyme system from yeast which catalyzes the desaturation of stearyl coenzyme A (stearyl-CoA) to oleoyl-CoA or of palmitoyl-CoA to palmityl-CoA does not convert 9- or 10-hydroxystearate or their CoA esters to unsaturated acids. Attempts to solubilize the desaturating enzyme were unsuccessful.

2. 9(and 10)-Hydroxystearic acid, when substituting for oleic acid as a growth factor for anaerobic yeast, is converted to ethyl 9(and 10)-acetoxystearate and to 9(and 10)-acetoxystearic acid.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Aerobic</th>
<th>Anaerobic*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16</td>
<td>0.2</td>
<td>10.3</td>
</tr>
<tr>
<td>C18</td>
<td>1.0</td>
<td>13.4</td>
</tr>
<tr>
<td>C18</td>
<td>12.5</td>
<td>50.5</td>
</tr>
<tr>
<td>C18</td>
<td>5.0</td>
<td>14.6</td>
</tr>
<tr>
<td>C16</td>
<td>37.0</td>
<td>0.2</td>
</tr>
<tr>
<td>C18</td>
<td>43.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Supplemented with 9(and 10)-hydroxystearic acid, which was removed by silice acid chromatography before the other fatty acids were analyzed. The content of hydroxystearate in the total fatty acid fraction is estimated to be 10 to 20%.

3. The acetylation of hydroxystearic acids with acetyl-CoA as the acetyl donor is catalyzed by a particulate enzyme system from yeast. The only substrates acetylated are 9-, 10-, and 12-hydroxystearic acids.

4. Various fatty acids have been tested as growth factors for anaerobic yeast.

5. The fatty acid compositions of the lipids of aerobic and anaerobic yeast have been compared. Anaerobic yeast contains relatively high concentrations of lauric and myristic acids and only insignificant amounts of unsaturated fatty acids.

REFERENCES

The Metabolism of Hydroxystearic Acids in Yeast
R. J. Light, W. J. Lennarz and Konrad Bloch


Access the most updated version of this article at
http://www.jbc.org/content/237/6/1793.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/237/6/1793.citation.full.html#ref-list-1