THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 237, No. 6, June 1962
Printed in U.S.A.

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(and 10)-hydroxystearic acid to an acetoxy derivative by growing yeast and by extracts of these cells.

EXPERIMENTAL PROCEDURE

Materials

The detergent, Cutscum (trade name employed by Fisher Scientific Company), as well as the enzymes used in attempts 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-Diethylaminoethyldiphenylpropylacetate hydrochloride (SKF 525-A) was supplied by Dr. C. W. French of Smith, Kline and French Laboratories. 3-Hydroxydecanoic acid, 10-hydroxydecanoic acid, and 10-hydroxy-2-decenoic acid were gifts of Dr. J. Law of this department. 2-Hydroxystearic acid and 2-hydroxytetracosanoic acid were gifts of Dr. A. J. Fulco (4). 1-Monoolein 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(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 $KMnO_4$-KIO_4 oxidation,[1] and 11,12-methylenestearic 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-H^3-9-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(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-hydroxy 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-Ketostearic acid was obtained by chromic acid oxidation of 12-hydroxystearic acid (7). 9-Octadecynoic 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-C^{14}-9(and 10)-acetoxystearate was synthesized by the acetylation of ethyl 1-C^{14}-9(and 10)-hydroxystearate. A solution of 1-C^{14}-hydroxy ester (78 mg, 2.03×10^4 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-C^{14}-9(and 10)-acetoxystearate with a specific activity of 1.83×10^4 d.p.m. per mg

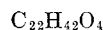
* Supported by grants-in-aid from the Life Insurance Medical Research Fund, the United States Public Health Service, the National Science Foundation, and the Eugene Higgins Trust Fund of Harvard University.

† National Science Foundation Postdoctoral Fellow.
‡ Postdoctoral Research Fellow of the National Heart Institute.

[1] Dr. Günter Scheuerbrandt, unpublished results.

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

(calculated, 1.80×10^4 d.p.m. per mg). The infrared spectrum in chloroform showed a strong ester band at 5.8 μ and a strong acetate band at 8.0 μ (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.



$C_{22}H_{42}O_4$

Calculated: C 71.30%, H 11.42%

Found: C 72.10%, H 10.86%

Methyl 1-C^{14}-9(and 10)-acetoxystearate and the free 1-C^{14}-9 (and 10)-acetoxystearic acid were synthesized by acetylation of methyl 1-C^{14}-9(and 10)-hydroxystearate and 1-C^{14}-9(and 10)-hydroxystearic acid, respectively. Methyl 1-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-C^{14}-acetyl-CoA was prepared from 1-C^{14}-acetic anhydride and CoA (11).

Methods

Yeast—Saccharomyces cerevisiae, strain LK 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 (0.25 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 Cutscum, or with trypsin, the procedures of Imai and Sato (13), of Levin, Levenberg, and Kaufman (14), and of Williams and Hager,[2] 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 $130,000 \times g$. 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 (50% 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 (15).

[2] L. P. Hager, personal communication.

Chromatography—The chloroform-methanol extracts were applied to silicic acid-Celite (1:2) columns for separation of neutral lipids from phospholipids (16) (Table I). Free fatty acids were separated from the neutral lipids by extraction with ethanolic ammonia (17). Silicic acid prepared and washed according to the method of Barron and Hanahan (18) was used for fractionation of the neutral lipids (Table II). For all other chromatographic procedures, the silicic acid was activated as described (1).

Gas-Liquid Chromatography—Free fatty acids were converted to their methyl esters, and the esters were analyzed without further treatment on a Research Specialties Company instrument. The column was packed with 5% diethyleneglycol succinate polyester on 60 to 80 mesh Chromosorb W (acid-washed). For counting, fractions were collected in cooled U-tubes as described (19). Specific activities were also determined as described (19), except that the areas under the peaks were determined by weighing paper cutouts from the charts. Methyl 1-C^{14}-9(and 10)-hydroxystearate of known specific activity was used as a standard.

Samples were counted in a Packard Tri-Carb scintillation counter. Ester groups were determined by the hydroxamate procedure of Stern and Shapiro (20). For paper chromatography, hydroxamates were prepared as in the analytical procedure, except that the addition of ferric chloride was omitted. After acidification, the hydroxamate solution was evaporated to dryness under a stream of nitrogen and desalted by adding 1 ml of ethanol and 9 ml of ether. The suspension was centrifuged, and the supernatant liquid was concentrated. The desalting procedure was repeated if necessary, the residue was applied to Whatman No. 1 paper strips, and the samples were chromatographed with saturated *n*-butanol-water (21). Spots were visualized by spraying with dilute ferric chloride solution and were eluted with ethanol. Acetylhydroxamate had an R_F of 0.52, whereas fatty acylhydroxamates moved with the solvent front (21).

RESULTS

Attempts to Demonstrate Enzymatic Conversion of Hydroxystearic Acid to Oleic Acid—Both 9-hydroxystearic acid and 9(and 10)-hydroxystearic acid, as well as their CoA esters (22), were incubated with crude homogenates or the particulate enzyme preparations which are known to catalyze the desaturation of palmitate and stearate or their CoA esters. After incubation and saponification, the fatty acids were assayed by chromatography on silicic acid. Nonpolar acids, *i.e.* oleic acid and unchanged stearate, were eluted with 10 to 15 ml of redistilled methylene chloride, and the hydroxystearic acid was eluted with a solution of 2% methanol in methylene chloride. The radioactivity found in the "oleic acid" fraction (2 to 5% of the total) after incubation with labeled hydroxy acid was never significantly greater than in control experiments with boiled enzyme. In contrast, when stearic acid was the substrate in the same crude extract, or when stearoyl-CoA was incubated with particulate enzyme, conversions to oleic acid ranging from 30 to 70% could be demonstrated by the mercuric acetate assay (12).

Further Studies on Stearate Desaturation—Attempts to clarify the mechanism of the desaturation process included the following experiments. Dinitrophenol, in concentrations of 10^{-5} to 10^{-3} M, does not inhibit the conversion of stearoyl-CoA to oleoyl-CoA. Interestingly, SKF 525-A, a drug known to inhibit many

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

oxygenase reactions involving TPNH and oxygen (23), has no inhibitory effect on desaturation. Glycerol 1-phosphate, ascorbate, and dimethylbenzimidazole 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, Cutscum (14), and a soluble acetanilide hydroxylase has been obtained by treatment with either lipase (24) or snake venom phospholipase (13). Cutscum 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-C^{14}-9(and 10)-hydroxystearic Acid or 1-C^{14}-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 C^{14}-hydroxystearate were therefore analyzed by silicic acid chromatography. The results are shown in Table I and may be compared with the distribution of C^{14} in the lipids from an analogous experiment with 1-C^{14}-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 C^{14} appears as free fatty acid. In the experiment with hydroxy acid, C^{14} 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 C^{14}-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-C^{14}-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.

Table I

Chromatography of lipid extracts of yeast

Cells were grown anaerobically in the presence of 1-C^{14}-9(and 10)-hydroxystearic acid or 1-C^{14}-oleic acid, and the lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (15).

Solvent	Chromatographic fraction[a]	Radioactivity incorporated	
		From C^{14}-hydroxystearate[b]	From C^{14}-oleate[b]
		%	%
CH_2Cl_2	I. Neutral lipids[c]	34	22
	II. Free fatty acids[c]	1	8
CH_3COCH_3	III. Polar acids	62[d]	2
CH_2Cl_2-CH_3OH (1:1)	IV. Phospholipids	3	31

[a] Fractionated on silicic acid-Celite columns (16).

[b] Total extracts contained 75% of the hydroxy acid and 32% of the oleic acid added to the growth medium. The numbers in the table are based on radioactivity recovered in the extracts.

[c] Separated into free fatty acids and neutral lipids by extraction with ethanolic ammonia (17).

[d] Shown to consist of more than 95% acidic material by extraction with ethanolic ammonia (17).

Table II

Chromatography of neutral lipid fractions from yeast

Chromatographic fraction[a]	Radioactivity incorporated	
	From C^{14}-hydroxystearate[b]	From C^{14}-oleate[b]
	%	%
Total neutral lipids[c]	34	22
I. Skellysolve B	0.02	3
II. 15% benzene in Skellysolve B	0.03	1.5
III. 5% ether in Skellysolve B	32.5	11
IV. 20% ether in Skellysolve B	1.5	3
V. 50 to 95% ether in Skellysolve B	0.1	3

[a] Fractionated on silicic acid according to the method of Barron and Hanahan (18), except that Skellysolve B was substituted for hexane.

[b] Numbers are based on radioactivity in total yeast extract.

[c] Fraction I, Table I.

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-C^{14}-9(and 10)-hydroxystearic acid (specific activity, 3.03×10^6 d.p.m. per mg). The cells were combined, harvested, and extracted as described. After preliminary fractionations of the chloroform-methanol extracts, the neutral lipid fraction containing the bulk of C^{14} (Fraction III, Table II) was purified further by chromatography on silicic acid (1 g) with gradient elution (mixing flask, 100 ml of Skellysolve B; reservoir, 200 ml of 10% ether in Skellysolve B). The radioactivity emerged as a single peak in the 50 to 75 ml fractions. It contained 0.75 mg of material with a

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

specific activity of 2.14×10^6 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 H^3-labeled 9(and 10)-hydroxystearic acid and also uniformly labeled C^{14}-glucose. The H^3-labeled hydroxy acid substrate was a mixture of unlabeled 9(and 10)-hydroxystearic acid combined with an essentially weightless amount of 12,13-di-H^3-9-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 H^3 and C^{14} (Table III). Although the C^{14} 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 C^{14}. From the C^{14} to H^3 ratio, it is estimated that the ester contains 4 to 6 carbon atoms more than the free hydroxy acid (Table III). Although this estimate is very approximate because of the low level of C^{14} activity, it appears certain that the unknown component(s) of the ester is a substance of relatively low molecular weight.

TABLE III

Incorporation of radioactivity from glucose into hydroxy acid ester

Cells were grown on uniformly labeled glucose-C^{14} (specific activity, 4.44×10^3 d.p.m. per mg) and 12,13-di-H^3-9-hydroxystearic acid diluted with unlabeled 9(and 10)-hydroxystearic acid (specific activity, 6.54×10^4 d.p.m. per mg).

Compound analyzed	C^{14}		H^3		Glucose carbon atoms per hydroxy acid (A/B)
	Activity[a]	A Glucose carbon[b]	Activity[a]	B Hydroxy acid	
	d.p.m.	*μatom*	*d.p.m.*	*μmole*	
Ester 1	15 ± 2	0.12	395 ± 10	0.020	6.0
Ester 2	16 ± 2	0.13	550 ± 10	0.028	4.6

[a] Peaks were collected from the gas-liquid chromatograph and counted for C^{14} and H^3.

[b] Calculated from specific activity of glucose added to the growth medium.

Approximate specific activities were determined for the hydroxy acid derivatives isolated from cells grown in the presence of 1-C^{14}-9(and 10)-hydroxystearic acid and H^3-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 diethyleneglycol succinate polyester column and on an Apiezon column. Methyl 9(and 10)-propionoxystearate was indistinguishable from the unknown ester on the diethyleneglycol 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.

TABLE IV

Retention times of hydroxy acid derivatives on gas-liquid chromatography

Compound	Relative retention times[a]	
	Diethyleneglycol succinate[b]	Apiezon L[c]
Ethyl 9(and 10)-hydroxystearate	8.7	
Methyl 9(and 10)-hydroxystearate	7.8	
Ethyl 9(and 10)-acetoxystearate	6.4	1.2
Methyl 9(and 10)-propionoxystearate	6.4	1.0
Methyl 9(and 10)-acetoxystearate	5.6	
Ester isolated from yeast	6.4	1.2

[a] Retention times are based on a value of 1.0 for methyl stearate. Values did not vary by more than 3% in duplicate determinations.

[b] Column operated at 195° with an argon pressure of 10 pounds.

[c] Column operated at 200° with an argon pressure of 30 pounds.

On the basis of hydroxamate determinations as performed initially, it appeared 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 at 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

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

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)-acetoxysteaıic acid.

Conversion of 1-C^{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^{14}-oleic acid (specific activity, 5.5×10^7 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)-acetox ystearate 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^{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). Acetoxystearic acid was eluted with methylene chloride (20 to 50 ml eluate), and radioactivity appearing in this fraction was taken as a measure of acetoxystearate synthesis. In some of these experiments, the product was identified by gas-liquid chromatography of its methyl ester (Table IV).

The acetylase 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 $\frac{1}{10}$ as active as the particulate fraction. Activity varied slightly with different preparations of particles, the incorporation of radioactivity into acetoxystearate 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^{-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-hydroxystearate and ricinoleic acid (12-hydroxy-9-octadecenoic acid) were appreciably active, whereas 10-hydroxydecanoic acid showed slight activity. 1-C^{14}-acetate was only approximately 3% as effective as 1-C^{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

Table V

Ester determinations by hydroxamate formation

Determinations were made on aliquots of the hydroxy acid ester isolated from yeast and on synthetic ethyl 1-C^{14}-9(and 10)-acetoxystearate with a slight modification of the method of Stern and Shapiro (20).

Ester and conditions	Hydorxy acid[a]	Ester[b]	Ester groups per molecule
	µmoles	*µeq*	
Isolated ester			
20 minutes at 22°	0.14	0.16	1.13
	0.18	0.18	1.00
	0.28	0.36	1.28
Synthetic ethyl 9(and 10)-acetoxystearate			
20 minutes at 22°	0.12	0.12	1.00
	0.29	0.31	1.07
	0.58	0.65	1.12
1 hour at 37°	0.29	0.51	1.76
	0.58	1.04	1.79
2 hours at 37°	0.29	0.54	1.86
	0.58	1.04	1.79

[a] Calculated from specific radioactivity.

[b] Based on standard curve obtained with either methyl 12-hydroxystearate or 1-monoolein, which gave the same values.

Table VI

Substrate specificity of yeast acetylase

The incubation mixtures contained 60 µmoles of phosphate buffer, pH 7.2; 1 µmole of substrate; 0.33 µmole of 1-C^{14}-acetyl-CoA, 220,000 d.p.m; and extract or particles obtained from 100 mg of wet yeast cells in a total volume of 1 ml. Incubation was for 2 hours at 37°. The values given are radioactivities in acetyl compounds relative to a value of 100 for 9(and 10)-hydroxystearate.

Substrate	Acetylation
None	2
9 (and 10)-hydroxystearate[a, b or c]	100
10-Hydroxystearate[a]	130
12-Hydroxystearate[a]	155
12-Hydroxy-9-octadecenoate[a]	95
10-Hydroxydecanoate[a]	8
10-Hydroxy-2-decenoate[a]	2
3-Hydroxydecanoate[a]	1
2-Hydroxystearate[b]	3
2-Hydroxytetracosanoate[b]	4
1-Monoolein[b]	4
Ergosterol[c]	5
Crude phytosphingosine[b]	2

[a] Added as the potassium salt.

[b] Added to particles as a solution in 0.1 ml of ethanol. The ethanol was removed under a stream of N_2, and the other components of the incubation, including a fresh portion of particles, were added to the resulting suspension.

[c] Added as a solution in acetone in the same manner as in footnote b.

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

TABLE VII

Anaerobic growth of yeast

Cells were grown for 4 or 5 days as described. All fatty acids were tested at a level of 0.33 mg per 20 ml of medium. Growth was measured after dilution of the cells with an equal volume of water and reading of the optical density in a Klett-Summerson colorimeter with a red filter. In earlier experiments (3), growth was measured by counting aliquots of cells in a hemacytometer counting chamber; the two methods gave comparable results.

Addition	Colorimeter reading			
	I	II	III	IV
None	22	22	18	57
Oleic acid	210	199	162	195
9(and 10)-Hydroxystearic acid[a]	181	196	138	
10-Hydroxystearic acid		190		
11-*cis*-Octadecenoic acid				195
9-Octadecynoic acid				174
12-Hydroxystearic acid		111		
11,12-Methylenestearic acid		76		
12-Ketostearic acid		81		
Stearic acid		22		
10-Methyl stearic acid			24	
9,10-Epoxystearic acid		27		
9,10-Dihydroxystearic acid		0		
9-*trans*-Octadecenoic acid	5			
9 (and 10)-Aminostearic acid				43
Ethyl 9(and 10)-acetoxystearate				87
Ethyl 9(and 10)-hydroxystearate				160
9(and 10)-Acetoxystearic acid				158

[a] 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).

structural features, can support the anaerobic growth of yeast (Table VII). Structural modifications which confer this ability include a hydroxyl group at carbon atoms 9, 10, or 12; a *cis* double bond between C-9 and C-10 or between C-11 and C-12; and a triple bond between C-9 and C-10. The 12-keto and 11,12-methylene derivatives are less efficient growth factors, and the 10-methyl, 9,10-epoxy, 9,10-dihydroxy, 9(and 10)-amino, and the 9,10-*trans*-olefinic derivatives are completely inactive. Ethyl 1-C^{14}-9(and 10)-hydroxystearate and 1-C^{14}-9(and 10)-acetoxystearic acid both support growth almost as well as oleic acid or as the free hydroxy acids. These compounds were converted to ethyl 9(and 10)-acetoxystearate, the ester accounting for 38% and 48%, respectively, of the radioactivity in the crude lipid extracts. On the other hand, with synthetic ethyl 9(and 10)-acetoxystearate, growth is only slight.

DISCUSSION

From the requirement for TPNH and molecular oxygen in the desaturation reaction, it was reasonable to expect that monounsaturated 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-hydroxystearic 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(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(and 10)-acetoxystearate. It therefore seems possible that the acetoxy derivative, rather than the free hydroxy acid, is the active growth factor. This is supported by the finding that 9(and 10)-acetoxystearate is as such 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 C_{21} position of various steroids (30), as well as the occurrence of (+)-10-acetoxy-*cis*-7-hexadecen-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^{14}-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 ricinoleic 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(or 10)-hydroxystearate. Acetylase activity can be demonstrated in extracts from aerobic as well as anaerobic cells, and therefore it is

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

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 acetoxysterate 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^{14}-oleic acid, some 10% of the incorporated radioactivity is recovered in the form of ethyl oleate, showing that esterification with ethanol may occur rather nonspecifically 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 aeration, 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 (C_{12} and C_{14}) than in the lipids of aerobic cells. Thus anaerobic cells, lacking a supply of olefinic acid from either exogenous or endogenous 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 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.

TABLE VIII

Relative concentrations of fatty acids in aerobic and anaerobic yeast

Yeast cells were saponified, and the methyl esters of the fatty acids were separated by gas-liquid chromatography as described in the text (170° with an argon pressure of 10 pounds). Areas of peaks were estimated by the method described by Carroll (33). C′ refers to monounsaturated fatty acid.

Acid	Growth conditions	
	Aerobic	Anaerobic[a]
C_{12}	0.2	10.3
C_{14}	1.0	13.4
C_{16}	12.5	59.5
C_{18}	5.0	14.6
C'_{16}	37.0	0.2
C'_{18}	43.9	1.8

[a] Supplemented with 9(and 10)-hydroxystearic acid, which was removed by silicic 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%.

SUMMARY

1. The particulate enzyme system from yeast which catalyzes the desaturation of stearoyl coenzyme A (stearoyl-CoA) to oleoyl-CoA or of palmitoyl-CoA to palmitoleoyl-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.

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

1. Bloomfield, D. K., and Bloch, K., *J. Biol. Chem.*, **235**, 337 (1960).
2. Mason, H. S., in F. F. Nord (Editor), *Advances in enzymology, Vol. 19*, Interscience Publishers, Inc., New York, 1957, p. 79.
3. Lennarz, W. J., and Bloch, K., *J. Biol. Chem.*, **235**, PC26 (1960).
4. Fulco, A. J., and Mead, J. F., *J. Biol. Chem.*, **236**, 2416 (1961).
5. Mack, C. H., and Bickford, W. G., *J. Org. Chem.*, **18**, 686 (1953).
6. Julietti, F. J., McGhie, J. F., Rao, B. L., and Ross, W. A., *Chem. and Ind. (London)*, 874 (1960).
7. Ross, J., Gebhart, A. I., and Gerecht, J. F., *J. Am. Chem. Soc.*, **71**, 282 (1949).
8. Khan, N. A., Deatherage, F. E., and Brown, J. B., *Org. Syntheses*, **37**, 77 (1957).
9. Bellamy, L. J., *The infrared spectra of complex molecules*, Ed. 2, John Wiley and Sons, Inc., New York, 1958, p. 178.
10. Sweeley, C. C., and Moscatelli, E. A., *J. Lipid Research*, **1**, 40 (1959).
11. Stadtman, E. R., in S. P. Colowick and N. O. Kaplan, (Editors), *Methods in enzymology, Vol. III*, Academic Press Inc., New York, 1957, p. 931.
12. Goldfine, H., and Bloch, K., *J. Biol. Chem.*, **236**, 2596 (1961).
13. Imai, Y., and Sato, R., *Biochim. et Biophys. Acta*, **42**, 164 (1960).
14. Levin, E. Y., Levenberg, B., and Kaufman, S., *J. Biol. Chem.*, **235**, 2080 (1960).
15. Folch, F., Lees, M., and Sloane Stanley, G. H., *J. Biol. Chem.*, **226**, 497 (1957).
16. Nelson, G. F., and Freeman, N. K., *J. Biol. Chem.*, **234**, 1375 (1959).

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

17. Weiss, S. B., Kennedy, E. P., and Kiyaso, J. Y., *J. Biol. Chem.*, **235,** 40 (1960).
18. Barron, E. J., and Hanahan, D. J., *J. Biol. Chem.*, **231,** 493 (1958).
19. Lennarz, W. J., Scheuerbrandt, G., and Bloch, K., *J. Biol. Chem.*, **237,** 664 (1962).
20. Stern, I., and Shapiro, B., *J. Clin. Pathol.*, **6,** 158 (1953).
21. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184,** 769 (1950).
22. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **204,** 329 (1953).
23. Cooper, J. R., Axelrod, J., and Brodie, B. B., *J. Pharmacol. Exptl. Therap.*, **112,** 55 (1954).
24. Krisch, K., and Staudinger, Hj., *Biochem. and Biophys. Research Communs.*, **4,** 118 (1961).
25. Burton, R. M., in S. P. Colowick and N. O. Kaplan, (Editors), *Methods in enzymology, Vol. III*, Academic Press, Inc. New York, 1957, p. 246.
26. Frisell, W. R., Meech, L. A., and Mackenzie, C. G., *J. Biol. Chem.*, **207,** 709 (1954).
27. James, A. T., Webb, J. P. W., and Kellock, T. D., *Biochem. J.*, **78,** 333 (1961).
28. Hebb, C. O., *Physiol. Revs.*, **37,** 196 (1957).
29. Zabin, I., Kepes, A., and Monod, J., *Biochem. and Biophys. Research Communs.*, **1,** 289 (1959).
30. Holmlund, C. E., Feldman, L. I., Rigler, N. E., Nielsen, B. E., and Evans, R. H., Jr., *J. Am. Chem. Soc.*, **83,** 2586 (1961).
31. Jacobson, M., Berozo, M., and Jones, W. A., *J. Am. Chem. Soc.*, **83,** 4819 (1961).
32. Stodola, F. H., and Wickerham, L. J., *J. Biol. Chem.*, **235,** 2584 (1960).
33. Carroll, K. K., *Nature (London)*, **191,** 377 (1961).
34. Christophersen, J., and Kaufmann, W., *Zentr. Bakteriol. Parasitenk., Abt. II*, **109,** 355 (1956).

Downloaded from http://www.jbc.org/ at Alma Mater Studiorum - Universita di Bologna on April 28, 2017

The Metabolism of Hydroxystearic Acids in Yeast

R. J. Light, W. J. Lennarz and Konrad Bloch

J. Biol. Chem. 1962, 237:1793-1800.

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

Downloaded from http://www.jbc.org/ at Alma Mater Studiorun - Universita di Bologna on April 28, 2017