Biosynthetic Studies on 8,9,13-Triacetoxydocosanoic Acid. *In Vivo* Incorporation of Fatty Acid Precursors*

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ABSTRACT: The *in vivo* incorporation of various fatty acid precursors into 8,9,13-triacetoxydocosanoic acid (TADA) by the yeast NRRL YB-2501 was studied. Distribution of radioactivity in the TADA was determined by deacylation with BF_3–methanol and periodate cleavage at the vicinal hydroxyl groups. [1-^{14}C]Acetate was most rapidly incorporated into TADA in 4-day-old cultures. The distribution of radioactivity in TADA was close to that predicted for a random incorporation, and this distribution did not vary over a range of acetate pulses from 2 min to 24 hr. [1-^{14}C]Palmitic, [1-^{14}C]stearic, [18-^{14}C]stearic, [1-^{14}C]eicosanoic, and 13-hydroxy[13-^{3}H]docosanoic acids were incorporated intact into TADA. [1,13-^{14}C]Docosanoic, 11-hydroxy[1,11-^{14}C]eicosanoic, and 11-hydroxy[11-^{3}H]eicosanoic acids were incorporated randomly into TADA, and hence must have been degraded prior to incorporation. The cellular nonhydroxy fatty acids of this organism consisted of myristic (4%), palmitic (18%), stearic (1%), oleic (20%), linoleic (54%), and linolenic (3%) acids as determined by gas–liquid chromatography of their methyl esters.

An ester isolated from BF_3–methanol treatment of the saponifiable fraction from yeast cells was identified tentatively as 7-(6′-carbomethoxyhexyl)-5-nonyl-6,8-dioxabicyclo[3.2.1]octane, the methyl ester of the cyclic ketal of 8,9-dihydroxy-13-oxodocosanoic acid. This dihydroxyoxo acid has previously been identified in the saponified extracellular lipids from the same organism (Vesonder, R. F., and Stodola, F. H. (1969), *Can. J. Chem. 47*, 1247).

An extracellular lipid formed by the yeast NRRL YB-2501 was identified as 8,9,13-triacetoxydocosanoic acid (TADA)[1] by Stodola *et al.* (1965). This yeast has since been shown to be closely related to *Torulopsis fujisanensis* (Soneda, 1959) which also produces TADA or a similar compound (L. J. Wickerham, 1967, personal communication). A second compound produced by NRRL YB-2501 was identified as 13-oxo-8,9-diacetoxydocosanoic acid (Stodola *et al.*, 1967; Vesonder and Stodola, 1969) although the principal structural work was carried out on the deacylated compound. Tulloch and Spencer isolated 8,9,13-trihydroxydocosanoic acid (THDA) from a strain of *Rhodotorula* (Stodola *et al.*, 1967), but in that yeast the hydroxyl groups were partially acetylated and partially esterified with long-chain fatty acids.

No function for these hydroxy acids in the organism producing them is readily apparent. They might tentatively be classified as secondary metabolites (Bentley and Campbell, 1968; Bu'lock, 1965) along with many fungal and plant products. TADA is produced in almost gram-per-liter quantities by NRRL YB-2501. Even if this compound has no function in the cell the system is an attractive one for studying the mechanism involved in both fatty acid chain elongation and hydroxy fatty acid biosynthesis. Biosynthetic studies might shed light on the function of these hydroxy acids if there is one. This paper reports *in vivo* studies on the incorporation of some fatty acid precursors into TADA.

Experimental Section

Fatty Acid Precursors. Sodium [1-^{14}C]acetate, [1-^{14}C]palmitic acid, [1-^{14}C]stearic acid, [18-^{14}C]stearic acid, and [^{3}H]-$NaBH_4$ were obtained from New England Nuclear (Boston, Mass.). [1-^{14}C]Eicosanoic acid was obtained from Mallinckrodt Nuclear (Orlando, Fla.). The radioactive palmitic, stearic, and eicosanoic acids were purified by chromatography on a silicic acid column (eluting solvent, 5% ether in hexane) and their radiochemical purity was checked on tlc (developing solvent, hexane–ether–acetic acid, 95:5:2, v/v) by autoradiography of the thin-layer chromatography plates. 11-Oxoeicosanoic acid and 13-oxodocosanoic acid were prepared by the acylation of 1-morpholinocyclodecene and 1-morpholinocyclododecene with decanoyl chloride (Hunter and Light, 1970; Hünig *et al.*, 1967). 11-Oxo[1,11-^{14}C]eicosanoic acid and 13-oxo[1,13-^{14}C]docosanoic acid were prepared by the same procedure utilizing [1-^{14}C]decanoyl chloride (Hunter and Light, 1970). [1,13-^{14}C]Docosanoic acid was prepared by the Wolff–Kishner reduction of 13-oxo[1,13-^{14}C]docosanoic acid (Hunter and Light, 1970). 11-Hydroxy[1,11-^{14}C]eicosanoic acid was prepared by the reduction of the corresponding oxo acid with $NaBH_4$ in methanol containing 0.1 N KOH, and was purified by chromatography on a silicic acid column (eluting solvent, 2% methanol in chloroform).

11-Hydroxy[11-^{3}H]eicosanoic acid was prepared by the re-

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[1] Abbreviations used are: TADA, 8,9,13-triacetoxydocosanoic acid. THDA, 8,9,13-trihydroxydocosanoic acid. Fatty acid designation: C_x, where x = number of carbon atoms in the fatty acid chain; y-HO-C_x, where y represents the position of substitution by a hydroxyl group.

TABLE I: Some Major Fragment Ions Found in the Mass Spectrum of Compound I.

Nominal *m/e*	Rel Intensity[a]	Formula[b]
382	9	$C_{23}H_{42}O_4$
351	12	$C_{22}H_{39}O_3$
228	42	$C_{13}H_{24}O_3$ $C_{12}H_{20}O_4$
227	56	$C_{13}H_{23}O_3$
211	18	$C_{13}H_{23}O_2$
210	100	$C_{14}H_{26}O$ $C_{13}H_{22}O_2$
195	18	$C_{12}H_{19}O_2$
185	24	$C_{10}H_{17}O_3$
178	49	$C_{12}H_{18}O$
155	52	$C_{10}H_{19}O$

[a] Obtained from low-resolution spectrum. [b] Obtained from high-resolution spectrum.

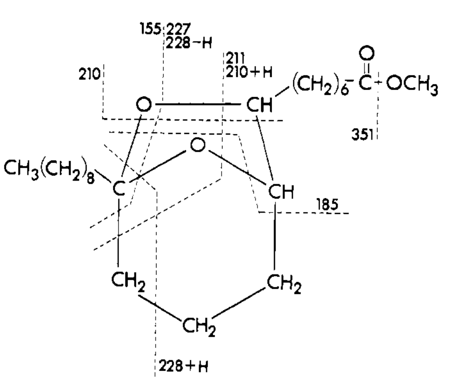
duction of 11-oxoeicosanoic acid with [^{3}H]NaBH$_4$. Oxo acid (9.6 mg) was reacted with 0.02 mequiv (0.9 mCi) of [^{3}H]-NaBH$_4$ in 0.5 ml of ethanol containing 0.05 ml of 4 N KOH. After 20 hr 4.0 mg of cold NaBH$_4$ was added to complete the reduction. After 2 hr 0.5 ml of acetone and 1 ml of 1 N HCl were added and the product was isolated by ether extraction. It was purified by silicic acid column chromatography (eluting solvent, 2% methanol in chloroform) and then recrystallized one time from absolute methanol. A small aliquot was diluted with carrier 11-hydroxyeicosanoic acid (mp 88–90°) and recrystallized once with no change in specific activity. This material (29 mg) was oxidized with 8 mg of CrO$_3$ in 1 ml of acetic acid at room temperature for 15 min. 11-Oxoeicosanoic acid was isolated and recrystallized five times from methanol. Specific activity of the recrystallized oxo acid was 1.6% of that of the 11-hydroxy[11-^{3}H]eicosanoic acid, showing that at least 98.4% of the tritium was located in position 11. In a similar set of reactions 13-hydroxy[13-^{3}H]docosanoic acid was produced by the reduction of 13-oxodocosanoic acid, except that the reduction was carried out on the methyl ester which was then hydrolyzed in 2% ethanolic KOH at 45° for 3 hr. 13-Hydroxydocosanoic acid had a melting point of 89–91° (lit. (Kuranova *et al.*, 1962) mp 86–87°). There was no decrease in specific activity upon addition of carrier and recrystallization four times from methanol. Reoxidation of a carrier diluted sample with CrO$_3$, followed by five recrystallizations from methanol showed that at least 99.5% of the tritium was located in position 13.

Culturing Methods. The yeast NRRL YB-2501 was obtained from Northern Regional Research Laboratory, Peoria, Ill. Cultures were maintained on malt agar slants (5% malt, 1% glucose, and 3% bactoagar). For a standard inoculum a portion of one slant was transferred to a 50-ml erlenmeyer flask containing 25 ml of the same growth medium. Cultures were grown on a rotary shaker at 25°. All manipulations were carried out using sterile techniques. The radioactive fatty acid precursors were added as solutions in 50 or 100 μl of ethanol to 4-day-old cultures and incubation was continued for 12 hr before isolation of TADA. Sodium [1-^{14}C]acetate was added at various times during growth and incubated for several periods of time (see Table II and Figure 3).

Isolation of Fatty Acids. After incubation of the cultures with radioactive precursors, the entire 25-ml culture was extracted four times with 15-ml portions of hexane. Evaporation of the hexane extracts usually yielded about 16 mg of residue from which about 10 mg of TADA could be isolated. The TADA was purified by preparative thin-layer chromatography (developing solvent, hexane–ether–acetic acid, 55 : 45 : 2, v/v) or by chromatography in a 2-g column of silicic acid (eluting solvent, 35% ether in hexane). Carrier TADA was isolated from larger cultures and purified by column chromatography. The structure of TADA was confirmed from the series of degradation steps carried out as described below.

The hexane-extracted lipid contained smaller amounts of an acid with an R_F slightly less than TADA on thin layer chromatography. This acid gave a yellow spot upon spraying with dinitrophenylhydrazine reagent. A small quantity of this material was collected from tailing chromatography fractions in the purification of carrier TADA. The compound was presumed to be 8,9-diacetoxy-13-oxodocosanoic acid on the basis of the following reactions, and on the basis of the recent identification by Vesonder and Stodola (1969) of 8,9-dihydroxy-13-oxodocosanoic acid in saponified extracts from the same organism. Treatment with BF$_3$–methanol reagent (14%, Applied Science Laboratories) followed by reduction with NaBH$_4$ produced two esters. One product was identified as methyl-THDA from the retention time of its trimethylsilyl ether derivative on gas–liquid chromatography, which was the same as that found for methyl-THDA prepared below.

The second product, compound I, could be formed just by BF$_3$–methanol treatment of the diacetoxyoxo acid and was identified as the cyclic ketal of methyl 8,9-dihydroxy-13-oxodocosanoic acid, 7-(6′-carbomethoxyhexyl)-5-nonyl-6,8-dioxabicyclo[3,2,1]octane on the basis of the following information: nuclear magnetic resonance spectra (2% in CDCl$_3$ δ 4.16 (m, 1), 3.97 (m, 1), 3.65 (s, 3, CH_3O), 2.31 (t, J = 7 Hz, CH_2COOR, superimposed on broad absorption, 2.2–2.6, total protons *ca.* 4), 1.50 (m), and 1.24 (m) integration not accurate but in approximately equal amounts, peaks due to CH_2 groups of the fatty acid chain (Chapman, 1965), and 0.89 ppm (broad t, 3, J = 6 Hz, CH_3CH$_2$); empirical formula from high-resolution mass spectrum, *m/e* 382.3091. *Anal.* Calcd for $C_{23}H_{42}O_4$: 382.3081. Table I shows some major ions found in the mass spectrum of I. Retention time of this compound on gas–liquid chromatography at 190° was 14.3 min, or 16 times the retention of methyl stearate. It was not altered by reduction with NaBH$_4$, by catalytic reduction with hydrogen, or by treatment with trimethylchlorosilane and hexamethyldisilazane in pyridine (Sweeley *et al.*, 1963). Alkaline hydrolysis followed by reesterification with diazomethane regenerated the same compound. The molecular formula indicates four oxygens and three rings or positions of unsaturation. Only two of the oxygens are associated with an ester group since only three methyl ester protons are observed in the nuclear magnetic resonance. The other oxygens are not present as ketones, aldehydes, alcohols, or a lactone as determined by the failure of the above chemical treatments to alter the compound. The two additional unsaturation positions are not reducible by hydrogen, and hence must be due to ring structures including the oxygen atoms. There are two low-field protons in the nmr which could be assigned to protons on carbons-1 and -7 of the bicyclic ring structure. These carbons are also attached to oxygen atoms. The mass spectrum of I is consistent with the proposed structure. Figure 1 shows possible cleavages which could produce some of the major ions in the spectrum. The fragmentation of the cyclic ketal ring structure is obviously quite complex, and no attempt has been made to propose mechanisms for these cleavages. Ions produced by cleavage α to the cyclic ketal ring are found at *m/e* 255 and 239, but they are of very low intensity. An ion at *m/e* 74, characteristic of fatty acid methyl esters, is also of low relative intensity (7% of base peak). Vesonder and Stodola (1969) reported that 8,9-dihydroxy-13-oxodocosanoic acid, isolated from the saponified lipid of this same organism, liquifies upon standing in a desiccator or upon refluxing in petroleum ether. They suggested that a closure between the keto and hydroxyl groups was taking place.



I

FIGURE 1: Proposed structure of I and some possible cleavages which could account for major ions in the mass spectrum.

For isolation of cellular fatty acids, cells were harvested by centrifugation and then saponified by refluxing in 50% methanol–5% KOH for 12 hr under nitrogen. The mixture was acidified and extracted with ether. The residue was heated for 5 min at 75° with 1 ml of BF_3–methanol to form the fatty acid methyl esters. Water was added and the methyl esters were extracted with hexane and analyzed by gas–liquid chromatography at 140°.

Chemical Degradation of TADA. TADA was degraded by deacetylation and oxidative cleavage at the vicinal hydroxyl groups. There was some variation in the details of the procedure and yields throughout the set of experiments. The most satisfactory general procedure is described here, with quantities quoted for a particular experiment.

Carrier TADA (about 200 mg) was added to purified TADA (about 10 mg) which had been isolated from the incorporation experiments. In some cases the diluted TADA was purified a second time by chromatography on a 50-g column of silicic acid (eluting solvent, 35% ether in hexane). The specific activity of the TADA was determined. The TADA was added to 3 ml of BF_3–methanol reagent, and the mixture was heated for 10 min in a 75° water bath. The mixture was extracted with 3 ml of hexane to remove unreacted TADA (about 6 mg) and 6 ml of water was added. The sample was extracted with chloroform until all the white solid was removed, and the chloroform extracts yielded 160 mg (91%) of methyl-THDA. The crude trihydroxy ester was purified by chromatography on a 20-g silicic acid column (eluting solvent, 2% methanol in chloroform). The eluted methyl-THDA was recrystallized from methanol to a constant specific activity. Five-times-recrystallized methyl-THDA had mp 135–137° (lit. (Stodola *et al.*, 1965) mp 134–135°). The trimethylsilyl ether, prepared according to the procedure of Sweeley *et al.* (1963), showed a single peak on gas–liquid chromatography at 190° with a retention time of 9.0 min, or 10.0 times the retention of methyl stearate.

To 121 mg (0.3 mmole) of methyl-THDA dissolved in 3 ml of warm dioxane was added 85 mg of crystalline H_5IO_6, and the mixture was warmed at 85° for 1 min. An equal volume of water was added, the mixture was extracted five times with hexane, and the hexane extracts were washed with water. Thin-layer chromatography of an aliquot showed two iodine-staining spots with R_F's of about 0.5 and 0.6 (developing solvent, hexane–ether, 75:25, v/v). After evaporating the hexane from this aldehyde mixture, it was dissolved in 3 ml of *tert*-butyl alcohol and 2 ml of 0.19 M $KMnO_4$ (0.38 mmole) was added slowly over a period of 6 hr. The reaction mixture was stirred at room temperature overnight, decolorized by the addition of a small amount of $NaHSO_3$, and extracted with hexane. The combined hexane extracts were washed with water and evaporated to yield 110 mg of a mixture of acids. The mixture was refluxed for 4 hr in 5 ml of benzene to lactonize 5-hydroxymyristic acid. The benzene was evaporated, 2 ml of 5% $NaHCO_3$ was added, and 5-hydroxymyristate lactone was extracted with hexane in a crude yield of 58 mg (85% based on methyl-THDA). The aqueous layer was acidified and extracted with ether to yield 39 mg (69%) crude monomethyl suberate, which was heated for 5 min at 75° in 1 ml of BF_3–methanol. Water was added and the mixture was extracted with hexane to yield 35 mg of crude dimethyl suberate.

The crude dimethyl suberate was purified by chromatography on a 5-g column of silicic acid (eluting solvent, 13% ether in hexane) to yield 28 mg of dimethyl suberate. A 10-mg aliquot was further purified by preparative thin-layer chromatography (developing solvent, ether–hexane, 15:85, v/v). In all cases purification was followed by gas–liquid chromatography analysis of the chromatographic fractions, and the samples used for specific activity determination were judged pure by thin-layer chromatography and gas–liquid chromatography. In some cases the purification employed only column chromatography or only preparative thin-layer chromatography. The dimethyl suberate had a retention time of 10.2 min on gas–liquid chromatography at 120°, or 0.65 times the retention of methyl stearate. This was identical with the retention time of an authentic sample of dimethyl suberate, and provides confirming evidence that the TADA isolated in our experiments was identical with that isolated by Stodola *et al.* (1965).

The crude 5-hydroxymyristate lactone was also purified either by column chromatography on 5 g of silicic acid (eluting solvent, 15% ether in hexane), by preparative thin-layer chromatography (developing solvent, ether–hexane, 20:80, v/v) or both, and the purification was followed by gas–liquid chromatography. Samples used for specific activity determination were judged pure by thin-layer chromatography and gas–liquid chromatography. The lactone had a retention time on gas–liquid chromatography at 160° of 14.2 min or 4.0 times the retention of methyl stearate. This was identical with the

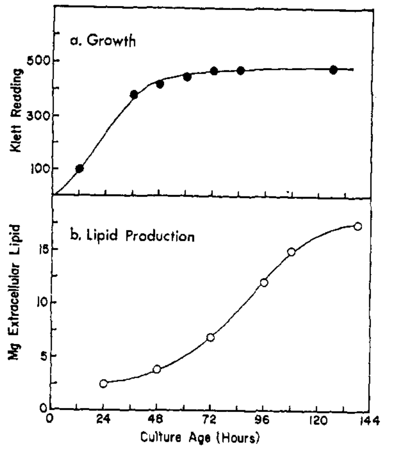


FIGURE 2: Growth of NRRL YB-2501 and the production of extracellular lipid. Flasks containing 25 ml of medium were inoculated and incubated at room temperature on a rotary shaker as described in the Experimental Section. (a) Samples were withdrawn from separate flasks at various times for the determination of turbidity using a Klett colorimeter with a 660-mμ filter. In the stationary phase a 25-ml culture contained about 0.2 g wet weight of cells, which corresponds to about 40 mg dry weight. (b) Separate cultures (both cells and culture medium) were extracted several times with hexane to obtain the extracellular lipid. Thin-layer chromatography analysis showed that about 90% of this lipid was accounted for as TADA.

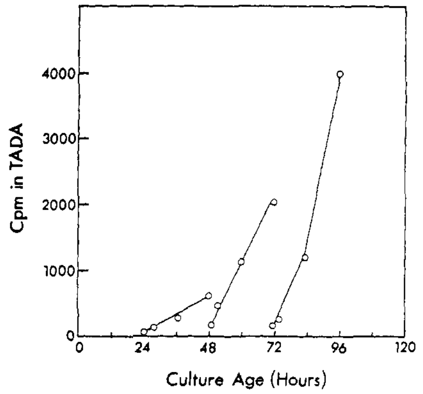


FIGURE 3: Incorporation of [1-^{14}C]acetate into TADA. Sodium [1-^{14}C]acetate (2 μCi) was added to groups of 25-ml cultures at 24-, 48-, and 72-hr growth. In each group of experiments a separate flask was extracted with hexane at 20 min, 1 hr, 12 hr, and 24 hr after the addition of [1-^{14}C]acetate. An aliquot of the extracted lipids (1% of the total) was purified by thin-layer chromatography, and the spot corresponding to TADA was scraped into a scintillator vial and assayed for radioactivity after addition of 5 ml of Cab-O-Sil and 15 ml of toluene scintillator. The total counts per minute incorporated into TADA per flask is therefore 100 times the counts per minute shown on the ordinate.

retention of an authentic sample of 5-hydroxymyristate lactone synthesized from 5-oxomyristic acid by NaBH$_4$ reduction and lactonization. 5-Oxomyristic acid was synthesized by the method of Robinson (1930).

In experiments with tritium-labeled hydroxy acid precursors, it was necessary to know how much tritium resided in position 5 of 5-hydroxymyristic lactone. The purified lactone (23 mg) was hydrolyzed in methanolic KOH (50% methanol and 5% KOH). The methanol was evaporated under a nitrogen stream and a few drops of 10% KOH and 0.5 ml of 0.19 M KMnO$_4$ were added. The reaction was left overnight at room temperature, an additional 0.1 ml of KMnO$_4$ was added. After 1 hr the mixture was acidified with 0.2 ml of 6 N HCl, decolorized with a small amount of NaHSO$_3$, and extracted with ether. Residue from the combined ether extracts was heated at 75° for 10 min in 1 ml of BF$_3$–methanol, and methyl 5-oxomyristate (10 mg) was isolated from unreacted lactone by preparative thin-layer chromatography (developing solvent, hexane–ether–acetic acid, 85:15:2, v/v).

In experiments with the 1,11-^{14}C and 1,13-^{14}C precursors, methyl-THDA was also decarboxylated by the Schmidt degradation. Purified methyl-THDA was hydrolyzed in methanolic KOH (50% methanol and 5% KOH) and aliquots of the THDA were decarboxylated using the procedure described in the accompanying paper (Hunter and Light, 1970).

Analytical Procedures. Assay of radioactivity was made with a Packard Tri-Carb Model 3114 liquid scintillation spectrometer using 15 ml of one of the following scintillator solutions. The toluene solution contained 4 g of 2,5-diphenyloxazole and 50 mg of 1,4[bis2(5-phenyloxazolyl)][benzene per l. of toluene. The dioxane solution contained 8 g of diphenyloxazole and 100 g of naphthalene per l. of dioxane. Counting efficiency for ^{14}C was 78.8% in the toluene solvent and 71.0% in the dioxane solvent. Counting efficiency for tritium was 34.4% in the toluene solvent and 24.8% in the dioxane solvent. Samples of CO$_2$ from the Schmidt degradation were collected in 0.5 ml of 1 M Hyamine hydroxide in methanol, added to the toluene scintillator solution, and counted at an efficiency of 69%. Specific activities were determined by one of two methods. In some cases the compound was weighed into a 10-ml volumetric flask, made up to volume with a solvent, and an aliquot was measured by volume into the scintillator counting vial. In other cases 1–2-mg aliquots of compound were placed on small tarred dishes of aluminum foil and weighed on a Cahn Electrobalance (Cahn Instrument Co., Downey, Calif.) to the nearest microgram. The samples were washed into scintillator vials with ether, the ether evaporated, and the counting solution added. In all cases samples were counted long enough to minimize the statistical error of counting. TADA was counted in the toluene solution and methyl-THDA in the dioxane solution. The methyl-THDA values were corrected to the toluene counting efficiency for comparison of specific activities. 5-Hydroxymyristate lactone and dimethyl suberate were counted in the toluene solution in all experiments except those involving hydroxy acid precursors, in which case the dioxane solution was used.

Gas–liquid chromatography was performed on an F & M Model 700 lab chromatograph with dual hydrogen flame detectors. Dual glass columns (4 ft × 0.25 in.) were packed with 10% diethylene glycol succinate on 100–120 mesh Gas Chrom Q (Applied Science Laboratories), and the instrument was operated at indicated temperatures with a helium gas flow of 60 cc/min. Thin-layer chromatograms were prepared with Adsorbosil-1 (Applied Science Laboratories) at a thickness of 275 μ for analytical work or 750 μ for preparative work. Silicic acid column chromatography was carried out on Mallinckrodt Silicar CC-4 (100–200 mesh). Low-resolution mass spectra were obtained on a Nuclide mass spectrometer operated at 70 eV. High-resolution spectra were obtained on a G.E.C.–A.E.I. MS-902 operated at 70 eV with a resolution of

TABLE II: Incorporation of [1-^{14}C]Acetate into TADA.[a]

			Sp Act. ($10^{-5} \times$ cpm/mmole)[b]					
Expt	Pulse	% Incorp[c]	TADA	MeTHDA	Lactone (L)	Dimethyl Suberate (S)	Sum (L + S)	Ratio[d] (S/L)
1	2 min[a]	8.0	26.5	18.2	10.9	6.8	17.7	0.625
2	10 min	0.96	1.2	0.77	0.49	0.27	0.76	0.55
3	20 min	2.5	1.48	1.40	0.73	0.52	1.25	0.71
4	1 hr	2.9	1.44	0.86				
5	12 hr	14.7	6.4	3.82	2.46	1.54	4.00	0.625
6	24 hr	31.6	33.2	23.5	14.7	7.1	21.8	0.482

[a] 2 μCi of sodium [1-^{14}C]acetate (57 μCi/μmole) in 1 ml of water was added to 25-ml shake cultures of yeast at 96-hr growth. In expt 1, 4 μCi of sodium [1-^{14}C]acetate was added. TADA was extracted at the times shown and purified and degraded as described in the Experimental Section. [b] Specific activities are given for TADA (plus carrier), for MeTHDA formed with BF_3–methanol, and for the cleavage products 5-hydroxymyristate lactone and dimethyl suberate. Values are averages of duplicates counted to at least 10,000 counts, and duplicates agreed within 10%. [c] Calculated from the total radioactivity in the TADA fraction after thin-layer chromatography purification before addition of carrier, and then normalized to 10 mg of TADA to take into account losses in the purification. [d] The S/L (head/tail) ratio calculated for random incorporation is 0.571.

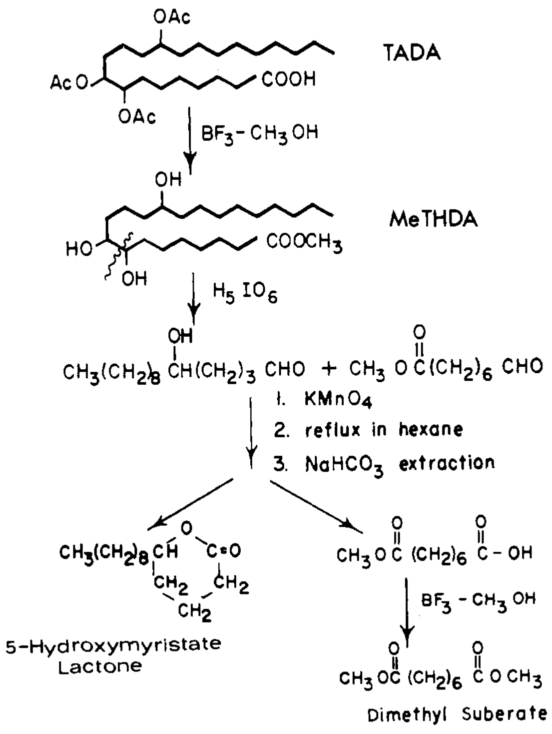
10,000. Perfluorokerosene was used as an internal standard. Nuclear magnetic resonance spectra were obtained on a 90-mHz Bruker nuclear magnetic resonance spectrometer.

Results

A typical growth curve for NRRL YB-2501 is shown in Figure 2a. Production of extracellular lipid, shown in Figure 2b, was most rapid after the growth had reached stationary phase. Thin-layer chromatography analysis showed that 90% of the hexane-extractable extracellular lipid was TADA, so that this curve essentially reflects TADA production. Four-day cultures were also shown to be more active than younger cultures at incorporating [1-^{14}C]acetate into TADA (Figure 3), which indicates that the TADA appearing in old cultures is newly synthesized and is not a degraded cellular constituent formed earlier in the growth period. For the following incorporation experiments all precursors were added to 4-day-old cultures.

Scheme I illustrates the chemical degradation used to determine the distribution of radioactivity in TADA. Radioactivity in the acetyl groups can be obtained by the difference in specific activities between TADA and methyl-THDA in most cases. In the case of hydroxy acid precursors, however, the precursor had chromatographic properties sufficiently similar to TADA that it was difficult to obtain TADA of good radiopurity. In those cases, the precursor was easily separated from methyl-THDA before further degradation. Periodate cleavage between carbons-8 and -9 of methyl-THDA, and subsequent oxidation and methylation, result in two fragments: dimethyl suberate, which represents the "head" end of the TADA molecule, and 5-hydroxymyristate lactone, which represents the "tail" end of TADA.

Table II shows that the length of time which cells were exposed to acetate had little effect on the distribution of ^{14}C between the head and tail portions of TADA. The values were close to the calculated random ratio of 0.571. This indicates that there is not a significant dilution of the radioactivity by a C_{16} or C_{18} fatty acid pool, which would result in a preponderance of radioactivity in the head end of the molecule. The acetyl pool furnishing the acetyl groups does seem to be of higher specific activity than that contributing to the carbon chain. This value can be obtained from the difference in specific activity between TADA and methyl-THDA, which represents the radioactivity in three acetyl groups. Comparison with the specific activity of methyl-THDA, which represents 11 acetyl groups, shows the acetyl groups to average twice the



SCHEME I

TABLE III: Incorporation of [^{14}C]Fatty Acids into TADA.[a]

			Sp Act. (10^{-5} × cpm/mmole)[b]				
Expt	Precursor	% Incorp[c]	TADA	MeTHDA	Lactone (L)	Dimethyl Suberate (S)	Ratio (S/L)
7	[1-^{14}C]Palmitate[d]	0.9	3.88	4.2	0.1	3.82	38.0
8	[1-^{14}C]Stearate[e]	1.4	7.65	7.35	0.14	6.1	43.5
9	[18-^{14}C]Stearate[f]	1.9	3.10	3.70	3.66	0.04	0.01
10	[1-^{14}C]Eicosanoate[g]	0.046	0.163	0.161	0.008	0.12	13.5

[a] Each [^{14}C]fatty acid was dissolved in a small volume of ethanol and 100 μl was added to 25-ml shake cultures of yeast at 96-hr growth. The TADA was extracted 12 hr later and purified and degraded as described in the Experimental Section. [b] Specific activities are given for TADA (plus carrier), for MeTHDA formed with BF_3–methanol, and for the cleavage products 5-hydroxymyristate lactone and dimethyl suberate. Values are averages of duplicates counted to at least 5000 counts, and duplicates agreed within 10%. [c] Calculated from the total radioactivity in the TADA fraction after thin-layer chromatography purification before addition of carrier, and then normalized to 10 mg of TADA to take into account losses in the purification. [d] 0.28 mg of specific activity 36.0 μCi/mg. [e] 0.24 mg of specific activity 33.1 μCi/mg. [f] 0.62 mg of specific activity 16.0 μCi/mg. [g] 2.1 mg of specific activity 6.4 μCi/mg.

TABLE IV: Incorporation of [^{3}H]Hydroxy Fatty Acids into TADA.[a]

			Sp Act. (10^{-3} × cpm/mmole)[b]						
Expt	Precursor	% Incorp[c]	TADA	MeTHDA	Lactone (L)	Dimethyl Suberate (S)	Sum (L + S)	5-OxoC_{14}	Ratio[d] (S/L)
11	[11-^{3}H]11-HO-C_{20}[e]	0.016	195	14.5	13.3	4.5	17.8	11.3	0.34
12	[13-^{3}H]13-HO-C_{22}[f]	0.006	68	5.84	6.2	0.1	6.3	0.0	0.016

[a] [^{3}H]Hydroxy fatty acids were prepared as described in the Experimental Section. Both were dissolved in a small volume of ethanol, and 100 μl was added to 25-ml shake cultures of yeast then at 96-hr growth. TADA was isolated 12 hr later, and was purified and degraded as described in the Experimental Section. [b] Specific activities are given for TADA (plus carrier), for MeTHDA formed with BF_3–methanol, for the cleavage products dimethyl suberate and 5-hydroxymyristate lactone, and for 5-oxo-C_{14} from oxidation of 5-hydroxymyristic acid. Values are averages of duplicates counted to at least 10,000 counts, and duplicates agreed within 5%. [c] Because of difficulty in purifying TADA from precursor, this value was calculated from the specific activity of purified methyl-THDA which in turn was corrected to total radioactivity in 10 mg of TADA prior to addition of carrier. [d] The ratio for tritium incorporation for [^{3}H]NADPH is calculated to be 0.545. [e] 11-Hydroxy[11-^{3}H]eicosanoic acid (1.3 mg) of specific activity 22.0 μCi/mg. [f] 13-Hydroxy[13-^{3}H]docosanoic acid (1.2 mg) of specific activity 15.7 μCi/mg.

specific activity of the rest of the chain in all but expt 3. If a pool of cold THDA in the cell were diluting the radioactivity incorporated into the carbon chain, one would expect that short pulses of [^{14}C]acetate would produce TADA with most of the radioactivity in the acetyl groups. Since the difference between the acetyl group and the carbon chain specific activities does not vary greatly in pulses from 2 min to 24 hr, the difference probably lies in precursor pools rather than in a trapping pool of THDA. The difference could, for example, represent the difference between acetyl and malonyl pools. One might expect a slightly lower head to tail ratio than observed if the acetyl starter group of the fatty acid chain also had a higher specific activity, however.

The data from Table II provide appropriate controls for studying the incorporation of other precursors. If a potential precursor is first degraded to acetate before incorporation, then the head-to-tail ratio distribution of radioactivity in TADA should be close to the expected random value of 0.571 (or the actual average acetate value of 0.6). Table III shows the data for the incorporation of the normal fatty acids C_{16}, C_{18}, and C_{20}. In each case the extent of incorporation was low, but the compound was incorporated intact as shown by the distribution of radioactivity in TADA. As an additional control, both [1-^{14}C]stearate and [18-^{14}C]stearate were employed, and in each case the radioactivity was found in the appropriate cleavage product expected for intact incorporation. Furthermore, there was very little radioactivity in the acetyl groups.

Table IV shows results from the incorporation of two tritium-labeled hydroxy acid precursors. 13-Hydroxy[13-^{3}H]-docosanoic acid is incorporated intact since most of the radioactivity from this acid is found in the lactone-cleavage product. A further degradation of the lactone by oxidation to 5-oxo-

TABLE V: Incorporation of [1,*i*-^{14}C]Fatty Acids into TADA.[a]

			Sp Act. (10^{-5} × cpm/mmole)[b]					
Expt	Precursor	% Incorp[c]	TADA	MeTHDA	Lactone (L)	Dimethyl Suberate (S)	Sum (L + S)	Ratio[d] (S/L)
13	[1,11-^{14}C]-11-HO-C_{20}[e]	0.85	6.3	3.79	2.28	1.33	3.61	0.588
14	[1,13-^{14}C]-C_{22}[f]	0.17	1.53	1.10	0.67	0.42	1.09	0.625

[a] The [1,*i*-^{14}C]fatty acids were dissolved in a small volume of ethanol, and 100 μl was added to 25-ml shake cultures of yeast at 96-hr growth. TADA was extracted 12 hr later and was purified and degraded as described in the Experimental Section. [b] Specific activities are given for TADA (plus carrier), for MeTHDA formed with BF_3–methanol, and for the cleavage products 5-hydroxymyristate lactone and dimethyl suberate. Values are averages of duplicates, each counted to at least 10,000 counts, and duplicates agreed within 5%. [c] Because of the difficulty in purifying TADA from the hydroxy acid precursor, this value for expt 13 was calculated from the specific activity of purified methyl-THDA which in turn was corrected to total radioactivity in 10 mg of TADA prior to addition of carrier. [d] Ratio for intact incorporation should be 1.0. [1-^{14}C]Acetate gave an average ratio of 0.6 (Table II), while the calculated random ratio is 0.571. [e] 11-Hydroxy[11-^{14}C]eicosanoic acid (1.15 mg) of specific activity 3.35 μCi/mg. [f] [1,13-^{14}C]Docosanoic acid (1.3 mg) of specific activity 7.7 μCi/mg.

myristate showed that all of the radioactivity in the lactone was present in position 5. 11-Hydroxy[11-^{3}H]eicosanoic acid, on the other hand, appears to be incorporated randomly. No control values for random tritium incorporation were available. If one assumes that tritium would enter TADA by way of [^{3}H]NADPH, and that each of the odd carbons is equally labeled from [^{3}H]NADPH (Foster and Bloom, 1963), then the calculated random head to tail ratio would be 0.55. Since the observed ratio is lower than this, some intact incorporation cannot be ruled out. When the lactone from this experiment was oxidized to 5-oxomyristic acid, about 15% of the tritium was lost. The expected random value depends on whether tritium is furnished equally to both hydrogens on the odd carbon atoms, and should lie somewhere between 9% and 16%. Again this value does not rule out some intact incorporation.

It should be noted that the specific activity of the lactone in expt 12 is higher than that of the methyl-THDA, and the sum of lactone and dimethyl suberate specific activities in experiment 11 is higher than that of methyl-THDA. This can be explained in part at least if the permanganate oxidation of the aldehydes in the cleavage reaction produces some 5-oxomyristic acid. An isotope effect in that oxidation would cause an accumulation of tritium in the 5-hydroxymyristate lactone.

Table V shows the incorporation of two fatty acids which were labeled equally with ^{14}C in two positions. Both 11-hydroxy[1,11-^{14}C]eicosanoic acid and [1,13-^{14}C]docosanoic acid were incorporated randomly as judged from the head-to-tail ratio of about 0.6. In each case a ratio of 1.0 would be expected for intact incorporation. To confirm the random value, which is not as clear cut from this degradation as it would be for singly labeled fatty acids, the methyl-THDA from each experiment was hydrolyzed and decarboxylated by the Schmidt degradation. Table VI shows that the radioactivity in the carboxyl group is in accord with a random incorporation. This data confirms the tritium data for 11-hydroxyeicosanoic acid and also shows that the C_{22} acid does not serve as a direct precursor as do the C_{16}, C_{18}, and C_{20} acids.

The nonoxygenated fatty acids of NRRL YB-2501 cells were analyzed by gas–liquid chromatography of their methyl esters and identified on the basis of retention time. The relative amounts were calculated from the peak areas and did not change over the first 4-days' growth: myristate (C_{14}), 4%; palmitate (C_{16}), 18%; stearate (C_{18}), 1%; oleate ($C_{18:1}$), 20%; linoleate ($C_{18:2}$), 54%; and linolenate ($C_{18:3}$), 3%. The total nonoxygenated fatty acids amounted to 2.5 mg/g wet wt of yeast, and hence about 12 mg/g dry wt, or 1.2%. By contrast the levels of TADA produced in the medium amounted to 75

TABLE VI: Decarboxylation of [^{14}C]THDA from Expt 13 and 14.[a]

			CO$_2$ Collected[c]		% Expected[d]	
Expt	Precursor	Amt[b] (cpm)	cpm	% of Total	Random Incorp	Intact Incorp
13	[1,11-^{14}C]-11-HO-C_{20}	1362	122	9.0	8.5	0
14	[1,13-^{14}C]C_{22}	347	26	7.5	8.5	47

[a] The [^{14}C]TADA isolated in expt 13 and 14 was treated with BF_3–methanol, and the methyl-[^{14}C]THDA produced was recrystallized to a constant specific activity. An aliquot was hydrolyzed in methanol and aqueous KOH to give [^{14}C]THDA, which was purified by column chromatography. Schmidt decarboxylations were performed as described in the accompanying paper (Hunter and Light, 1970). [b] An average of four samples, each containing about 1.3 mg. [c] An average of four samples, each counted for at least 12,000 counts. Values are corrected for the decreased counting efficiency caused by Hyamine quenching. [d] Expected values are corrected for an overall decarboxylation and collection efficiency of 94% which was determined using standard [1-^{14}C]fatty acids (Hunter and Light, 1970).

mg/g wet wt of cells at day 6 of growth (Figure 2). The fatty acid fractions isolated after saponification of the cells and then methylated with BF_3–methanol showed no evidence of the presence of methyl hydroxydocosanoate, which has a retention time of 34 min on gas–liquid chromatography at 180° (32.4 times the retention of methyl stearate). Nor were observable quantities of methyl eicosanoate or methyl docosanoate found. A peak on gas–liquid chromatography was observed, however, with a retention time of 14.4 min. This substance was purified and on the basis of the analytical data reported in the Experimental Section was identified as the cyclic ketal of methyl 8,9-dihydroxy-13-oxodocosanoate (I, Figure 1). The same compound was formed by treatment of an extracellular lipid tentatively identified as 8,9-diacetoxy-13-oxodocosanoic acid with BF_3–methanol. Whether the dihydroxyoxo acid or its cyclic ketal actually occurs as a cellular constituent, or whether it was derived from extracellular lipid that had not been washed from the cells remains uncertain.

Discussion

The incorporation data show that intermediate chain-length fatty acids can be activated and converted intact into the longer chain TADA. The extent of incorporation is low, which would indicate that free fatty acids themselves are not intermediates between acetate and TADA. The labeling pattern of TADA produced from [1-^{14}C]acetate also indicates there are no appreciable pools of intermediate chain-length fatty acid contributing to TADA synthesis, especially since this pattern is the same for short (2 min) and long (24 hr) pulses of acetate (see Table II).

The incorporation of 13-hydroxy[13-^3H]docosanoic acid into TADA suggests the sequence for introduction of the oxygen atoms. One might expect the next intermediates to be 13-hydroxy-8-docosenoic acid, and 8.9-epoxy-13-hydroxydocosanoic acid based upon biosynthetic mechanisms for other vicinal dihydroxy fatty acids (Knoche, 1968; Morris *et al.*, 1968; Schroepfer, 1966). Whether the intermediates are extracellular, compartmentalized within the cell, or present in only very low concentration in an activated form remains to be determined. The number of possible oxygenated intermediates is increased by the possibility of acetylation occurring at any of the intermediate stages. A careful examination of cellular and extracellular oxygenated acids both in young and old cultures may turn up some of the intermediates, although we have already indicated that the acetate-labeling data suggest they do not accumulate to any appreciable extent.

8,9-Diacetoxy-13-oxodocosanoic acid cannot be an obligatory intermediate on the way to TADA since tritium is retained in position 13 when 13-hydroxy[13-^3H]docosanoic acid is incorporated. Some tritium could be lost if one of the 13-hydroxylated intermediates is in equilibrium with a 13-oxo fatty acid side product. This might account for the low incorporation of 13-hydroxydocosanoic acid (0.006%) when compared to palmitate incorporation (0.9%). The per cent incorporation of 13-hydroxy[13-^3H]docosanoic acid, even though intact, was also slightly lower than the percent incorporation of 11-hydroxy[11-^3H]eicosanoic acid by a random pathway (Table IV). If the 13-HO-C_{22} acid were degraded as readily as 11-HO-C_{20} we would expect to see some random incorporation from 13-HO-C_{22} as well, yet from the specific activity of dimethyl suberate in expt 12 we can conclude that less than 4% of the TADA was formed by a random pathway. Consequently 13-HO-C_{22} must not be activated for β oxidation as readily as 11-HO-C_{20}, and perhaps activation is not required for further conversion into TADA. This observation may be helpful in designing cell-free experiments for this portion of the pathway.

The most puzzling observation in the current study concerns the chain length at which the first hydroxyl group is introduced. Neither docosanoic acid nor 11-hydroxyeicosanoic acid was incorporated intact into TADA. This may mean that at least one of these acids cannot be activated to the appropriate form for incorporation into the pathway. They both are activated and metabolized in some form, however, since radioactivity from these acids is converted randomly into TADA. Alternatively, the conversion of a C_{20} derivative into a 13-hydroxy-C_{22} derivative may involve a hydroxylation–chain elongation process that is either concerted or compartmentalized in such a way that the enzymes are not accessible to externally added substrates, even though these substrates are activated. A similar situation may exist for the formation of 2-hydroxyhexacosanoic acid in *Candida utilis* (Fulco, 1967). In that case externally added hexacosanoic acid was not incorporated into the hydroxy-C_{26} acid, while externally added docosanoic acid was. A better understanding of the pathway between eicosanoate and 13-hydroxydocosanoate will probably only come from studies in cell-free systems.

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