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ORIGIN OF PALMITIC ACID CARBON IN PALMITATES FORMED FROM HEXADECANE-*1*-C^{14} AND TETRADECANE-*1*-C^{14} BY *MICROCOCCUS CERIFICANS*

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ABSTRACT

FINNERTY, W. R. (University of Iowa, Iowa City), AND R. E. KALLIO. Origin of palmitic acid carbon in palmitates formed from hexadecane-*1*-C^{14} and tetradecane-*1*-C^{14} by *Micrococcus cerificans*. J. Bacteriol. **87**:1261-1265. 1964.—Degradation of the palmitic acid moiety of cetyl palmitate and myristyl palmitate formed from hexadecane-*1*-C^{14} and tetradecane-*1*-C^{14} by *Micrococcus cerificans* was carried out. The patterns of C^{14} labeling in palmitic acid from cetyl palmitate showed that hexadecane is oxidized at the C_1 position, and cetyl alcohol and palmitic acid thus formed are directly esterified. Palmitic acid arising from tetradecane and esterified to tetradecanol appeared to have been synthesized by the addition of two carbon atoms to an existing 14-carbon atom skeleton. Considerable mixing of C^{14} occurred in the C_1 and C_2 positions of palmitic acid thus synthesized.

During studies (Stewart et al., 1959) on the mechanism of *n*-alkane oxidation by a species of *Micrococcus*, cetyl palmitate was isolated from cultures growing at the expense of *n*-hexadecane. Subsequently, Stewart and Kallio (1959) established that the acid moiety of esters produced from *n*-alkanes by this organism was predominantly palmitic acid regardless of the length of the alkane carbon skeleton serving as growth substrate. Yields of such waxes were not high but, because the alcohol moieties of the esters invariably had carbon skeletons identical to that of the alkane substrate used, these materials appeared to be useful tools for investigating microbial alkane oxidations. Interestingly, the structural integrity of olefins (including the double bond) is preserved in the alcohol moiety of esters produced by the organism from olefins (Stewart et al., 1960).

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We were interested primarily in the origin of palmitic acid carbon in the case of cetyl palmitate, as palmitate was a predominant but not invariable ester constituent in wax formation by *M. cerificans* from alkanes and olefins. For example, 17-octadecenyl margarate is a major ester formed from octadecene-1. We felt that data obtained from hexadecane-*1*-C^{14} might strengthen the "direct esterification" hypothesis (Stewart et al., 1959), at least in the case of *n*-hexadecane oxidation by this organism. Additionally, data obtained from tetradecane-*1*-C^{14} utilization might provide clues for more extensive study of palmitate carbon origins should the direct esterification hypothesis prove to be in error.

MATERIALS AND METHODS

Mineral media, characteristics of *M. cerificans*, and techniques of growing organisms on alkane substrates were previously described (Finnerty, Hawtrey, and Kallio, 1962). Hexadecane-*1*-C^{14} and tetradecane-*1*-C^{14} were obtained from Isotopes Specialties Co., Inc., Burbank, Calif., and were prepared by the hydrolysis of the Grignard compound of the appropriate alkyl bromide. The material contained no detectable alkyl bromide, less than 1% of double-length hydrocarbon, and no alcohol. Hexadecane-*1*-C^{14} was diluted 1:100 with American Petroleum Institute Standard *n*-hexadecane. Tetradecane-*1*-C^{14} was diluted 1:50 with American Petroleum Institute Standard *n*-tetradecane; the lower dilution was used to offset the lower ester yields obtained when tetradecane constituted the carbon source for the bacteria.

Growth experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml of freshly prepared media and augmented with 1% (v/v) of the appropriate alkane. Sterile air was scrubbed by passing through 20% NaOH solution followed by two distilled water washes, and was then drawn through the culture flask. Trial runs with non-

radioactive substrates were used to determine time to peak ester production under these conditions by periodically carrying out ester assays as outlined by Bauer and Hirsch (1949). Repeatedly recystallized methyl palmitate (homogeneous by gas liquid chromotography) was used to construct standard curves. Under the conditions used, peak ester production occurred 70 hr after 1 ml of a heavy cell suspension was introduced into a flask of basal medium amended with 1% (v/v) of the alkane. Radioactive alkanes were then employed as substrates under the same conditions. At the end of the incubation period, cells were recovered by centrifugation, washed three times with distilled water, and assayed for C^{14} activity. All washings were added to the original culture fluid. Synthetic cetyl palmitate (2 g) was added to the culture fluid, and total ester was recovered by exhaustive ether extraction. The product so obtained was recrystallized three times from ether. The same procedure was used for isolation of tetradecyl palmitate except that isotope dilution was carried out with 2 g of tetradecyl palmitate.

Carbon-14 activity was counted in a model 314 X automatic Tricarb liquid scintillation spectrometer. Scintillation solvent was composed of 2,5-diphenyloxazole (PPO), 4 g; and 1,4-bis-2-(phenyloxazolyl)-benzene, 100 mg in 1,000 ml of toluene. Preparation of C^{14} standard for counting was by the method of Williams et al. (1956). Carboxyl-labeled benzoic acid (1 mg) was weighed and dissolved in toluene (1,000 ml) containing 100 mg of nonradioactive benzoic acid. Standardization samples were prepared for counting by adding 1 ml of the standard ϕ $C^{14}OOH$ solution to 5 ml of scintillation solvent. Samples were counted at approximately 60% efficiency. Experimental samples were monitored for radioactivity in 5 ml of the scintillation solvent. No significant quenching occurred, as indicated by addition of internal standard carboxyl-labeled benzoic acid to sample vials.

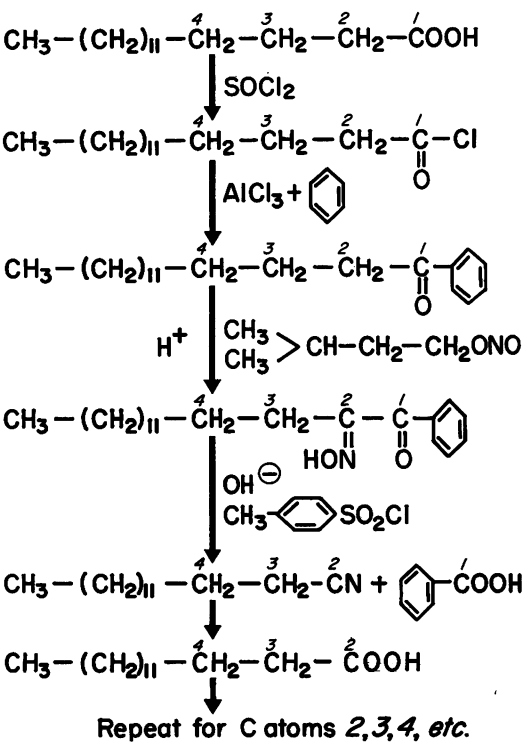

FIG. 1. *Flow sheet for degradation of palmitic acid.*

Isolated C^{14}-containing esters were saponified in aqueous KOH (10%) in methanol (1:4). Methanol was removed by distillation, and the residue was washed three times in distilled water. This residue was then brought to a rapid boil for 10 min in 3 N KOH. After cooling and washing, the residue was placed in a 50 C oven until dry; then the acid and alcohol moieties were separated by *n*-hexane extraction. Carbon atoms 1, 2, 3, and 4 were removed from palmitic acid as benzoic acid-*1*-C^{14} essentially as shown in Fig. 1 (Dauben, Hoerger, and Petersen, 1953). Fatty acids and alkyl-aryl ketones resulting from the degradation of palmitic acid were checked during the procedure by paper chromatography using methods based on those described by Mangold, Lamp, and Schlenk (1955) with 85% acetic acid as solvent. Chromatograms were visualized under ultraviolet light, which simplified location of spots; the long chain α-dextrin inclusion complexes appeared white against the dark background of α-dextrin-iodine covered paper. Table 1 lists R_F values obtained. Alkyl phenyl ketones were synthesized by use of Friedel-Crafts conditions (Dauben et al., 1953). Acid chlorides were made by refluxing $SOCl_2$ (2 ml) and the corresponding acid (1 g) at 50 C for 30 min. Excess $SOCl_2$ was removed under vacuum, the acid chloride was dissolved in 3.5 ml of dry benzene; then 730 mg of anhydrous aluminum chloride were added and the mixture was stirred for 16 hr at room temperature.

Table 2 lists the melting points of the alkyl-aryl ketones along with those of the 2,4-dinitrophenyl phenylhydrazone derivations. Infrared analysis

TABLE 1. *R_F values of intermediates in palmitic acid degradation procedure**

Compound	R_F value
n-Dodecanoic acid	0.73
n-Tridecanoic acid	0.68
n-Tetradecanoic acid	0.63
n-Pentadecanoic acid	0.58
n-Hexadecanoic acid	0.50
n-Heptadecanoic acid	0.43
n-Octadecanoic acid	0.38
n-Undecylphenyl ketone	0.89
n-Dodecylphenyl ketone	0.74
n-Tridecylphenyl ketone	0.61
n-Tetradecylphenyl ketone	0.45
n-Pentadecylphenyl ketone	0.28
n-Dodecanol-1	0.82
n-Tetradecanol-1	0.79
n-Hexadecanol-1	0.75
n-Octadecanol-1	0.70
Tetradecyl palmitate	0.81
Cetyl palmitate	0.65

* Chromatographic techniques used were those of Mangold, Lamp, and Schlenk (1955).

TABLE 2. *Melting points of alkyl-aryl ketones and their 2,4-dinitrophenylhydrazone derivatives*

Compounds	Melting point	Melting point of 2,4-dinitrophenylhydrazone derivative
	C	*C*
n-Undecylphenyl ketone	45–46	86–88
n-Dodecylphenyl ketone	41–42	91–93
n-Tridecylphenyl ketone	54–55	95–96
n-Tetradecylphenyl ketone	50–51	98–99
n-Pentadecylphenyl ketone	59–60	101–102

of the ketones failed to reveal any functional groups except the carbonyl peak at 1,700 cm^{-1} and the phenyl peaks.

RESULTS

Basic data for radioactivities of cetyl palmitate, saponification products, C_1, C_2, C_3, and C_4 of palmitic acid from hexadecane-*1*-C^{14} are shown in Table 3 and in simplified form in Fig. 2. The symmetry of *n*-hexadecane makes it logical to assume that radioactivity of the C_5-C_{16} moiety is concentrated in the C_{16} position. If this assumption is granted, it would appear further that palmitic acid in this ester arises directly from a C_1 oxidation of *n*-hexadecane. Moreover, since cetyl alcohol has essentially the same activity as palmitic acid, it would seem that cetyl palmitate arises from a direct esterification of two oxidation products produced by C_1 oxidation of hexadecane, thus confirming an earlier postulate (Stewart et al., 1959). A confusing aspect of the study was an apparent loss of considerable radioactivity upon saponification of cetyl palmitate. The ester showed a specific activity of 14,017 counts per min, whereas the sum of specific activities of palmitic acid (878 counts per min) and cetyl alcohol (880 counts per min) equals 1,758 counts per min —a loss of 12,259 counts per min. We have no way to account for the loss of activity at present.

Contamination of the cetyl palmitate with radioactive hexadecane seems unlikely, because hexadecane does not have high enough volatility

TABLE 3. *C^{14} activities of cetyl palmitate, palmitic acid, and palmitic acid moieties derived from hexadecane-1-C^{14} by Micrococcus cerificans*

Compound	Counts per min per μmole*
Hexadecane	11,960
Cetyl palmitate	14,017
Cetyl alcohol	880
Palmitic acid	878
Pentadecanoic acid	665
Tetradecanoic acid	595
Tridecanoic acid	591
Dodecanoic acid	577
C_1 of palmitic acid (as benzoic acid)	451
C_2 of palmitic acid (as benzoic acid)	0
C_3 of palmitic acid (as benzoic acid)	<1
C_4 of palmitic acid (as benzoic acid)	<1

* Specific activity calculated on the basis of ester isolated after dilution with carrier cetyl palmitate. Dilution factor for the above data was 19.43.

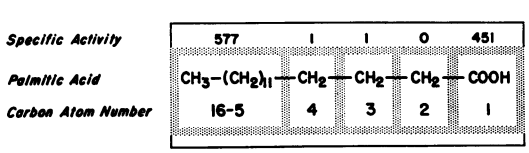

FIG. 2. *Specific activities of palmitic acid moieties from cetyl palmitate produced from n-hexadecane-1-C^{14} by Micrococcus cerificans.*

to escape the reflux condenser during the Soxhlet extraction of cetyl alcohol with hexane. Thus, any radioactive hexadecane contaminating the ester would have appeared in the hexane-cetyl alcohol solution and subsequently in the cetyl alcohol C^{14} measurements, and would have resulted in a disproportionate labeling between cetyl alcohol and palmitic acid. The results actually show almost equal labeling of these compounds, indicating negligible contamination of ester with hexadecane-C^{14}.

There may have been present (in the cetyl palmitate) a considerable amount of volatile material, perhaps esters of lower molecular weight, which would be lost during saponification procedures. Generally, chemical analysis of ester by hydroxamic acid assay yielded higher values than isotope-dilution techniques. In any case, there appears to be negligible labeling in the C_2, C_3, and C_4 positions of palmitic acid of cetyl palmitate.

In the case of the palmitic acid from myristyl palmitate derived from tetradecane-*1-*C^{14}, the results do not appear to be clear-cut. Table 4 and Fig. 3 show the pertinent data. If, again, the assumption is made that C^{14} activity of the moiety C_5-C_{16} is concentrated in the C_{16} position, it seems evident that palmitic acid was synthesized by the addition of 2 carbon atoms to a 14-carbon atom

TABLE 4. *C^{14} activities of tetradecyl palmitate, palmitic acid, and palmitic acid moieties derived from tetradecane-1-C^{14} by Micrococcus cerificans*

Compound	Counts per min per μmole*
Tetradecane	26,317
Tetradecyl palmitate	31,170
Tetradecanol	338
Palmitic acid	2,956
Pentadecanoic acid	1,211
Tetradecanoic acid	347
Tridecanoic acid	241
Dodecanoic acid	151
C_1 of palmitic acid (as benzoic acid)	1,729
C_2 of palmitic acid (as benzoic acid)	739
C_3 of palmitic acid (as benzoic acid)	90
C_4 of palmitic acid (as benzoic acid)	0

* Specific activity calculated on the basis of ester isolated after dilution with carrier tetradecyl palmitate. Dilution factor for the above data was 37.36.

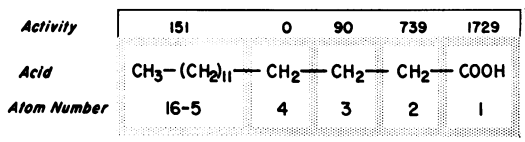

FIG. 3. *Specific activities of palmitic acid moieties from cetyl palmitate produced from tetradecane-1-C^{14} by Micrococcus cerificans.*

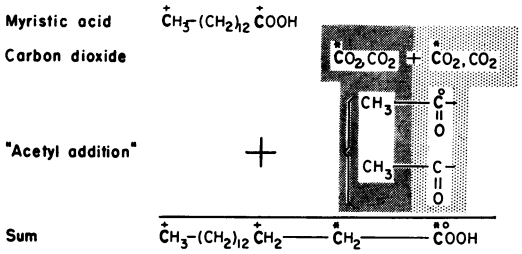

FIG. 4. *Speculative mechanism for C^{14} incorporation mechanism involving both acetyl and CO_2 additions to myristyl carbon skeleton.*

chain, probably myristic acid. The fact that the activity of myristyl alcohol equals that of the palmitic acid moiety C_3-C_{16} supports this notion. The nature of the 2-carbon addition to the 14-carbon atom skeleton presents a puzzle. First, there appears to be very considerable mixing of label in the C_1 and C_2 positions. Even more puzzling is the high order of activity in C_1 and C_2 compared with C^{14} activity of the remainder of the molecule. The appearance of such unexpected and unusual labeling prompted a complete repetition of the C^{14} protocol with essentially the same results.

Bacterial fatty acid oxidations appear to follow classical β oxidation pathways (Webley and Dekock, 1952; Webley, Duff, and Farmer, 1956), and in certain cases bacterial fatty acid syntheses may follow reversal of the oxidative pathway (Lynen, 1961). One would, therefore, expect classical labeling in the 2-carbon fragment added to the "myristic acid" skeleton. Conceivably, mixing may occur if the acetyl fragments derived from the fatty acid, which constitutes the sole carbon source, were cycled through some metabolic mechanism such as the tricarboxylic acid cycle in which there are several symmetrical components, but this would not explain the relatively high order of activity in (now) carbon atoms 1 and 2 of palmitic acid. If fatty acids are synthesized by *M. cerificans* by means of the

malonyl-coenzyme A (CoA) system first uncovered by Wakil (1958), the labeling pattern should remain essentially that of acids synthesized from acetyl-CoA moieties (Lynen, 1961).

One other possibility should be mentioned, if only to be discarded. Stumpf (1956) and Martin and Stumpf (1959) demonstrated a fatty acid peroxidase which carries out an α oxidation of long-chain, saturated fatty acids, eventuating in CO_2 and an aldehyde one carbon atom shorter than the substrate acid. Indirect evidence for the occurrence of α oxidation in *M. cerificans* has been provided by Stevenson, Finnerty, and Kallio (1962) in the demonstration of heptadecyl palmitate formation from *n*-heptadecane by this organism. If, as seems to be the case, α oxidation is limited to long-chain fatty acids, CO_2 arising early may be derived, in large measure, from this process and would, therefore, have a much higher specific activity than CO_2 arising later by way of conventional β-oxidation mechanisms. Thus, molecules of CO_2 arising from α oxidation of myristic acid would have specific activities equal to half the specific activity of the tetradecane-*1*-C^{14} substrate. If some mechanism for C_1 additions were operative in addition to or in conjunction with conventional "acetyl addition" fatty acid syntheses, Fig. 4 would represent the radioactivity pattern.

This speculative mechanism would account for the apparent "mixing" of activity in what have become C_1 and C_2 of palmitic acid in myristyl palmitate.

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