Intermediate Steps in the Incorporation of Fatty Acids into Long-Chain, Nonisoprenoid Hydrocarbons by Lysates of *Sarcina lutea**

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ABSTRACT: For reasons not yet understood, the acyl moiety of triglycerides, fatty acyl methyl esters, and wax esters are incorporated into monounsaturated hydrocarbons in lysates of *Sarcina lutea* only with decarboxylation. The double bond of the product is localized between those carbons that are derived from C-1 and C-2 of the fatty acid which is not decarboxylated upon incorporation into hydrocarbon. Long-chain ketones, secondary alcohols, and corynomycolic acid which have been postulated as intermediates in the conversion of fatty acids into hydrocarbons were either not incorporated to any significant extent or were not converted into the specific product expected.

In the consideration of alternative condensation mechanisms involving reduced derivatives of fatty acids, palmitaldehyde was found to serve as a precursor of hydrocarbon.

In previous papers of this series (Albro and Dittmer, 1969a–d), we have established that hydrocarbons are synthesized by *Sarcina lutea in vivo* from 2 moles of fatty acid with decarboxylation of one of the fatty acids (head-to-head condensation) as follows:

$$RCH_2COOH + R'CH_2COOH \rightarrow RCH{=}CHCH_2R' + CO_2$$

In cell-free preparations of *S. lutea*, the incorporation of fatty acids into hydrocarbons required ATP Mg^{2+}, coenzyme A, NADPH, and pyridoxal or better, pyridoxamine phosphate (Albro and Dittmer, 1969d). Data were reported consistent with the incorporation of acyl coenzyme A preferentially by a mode involving obligatory decarboxylation. The lack of incorporation of long-chain ketones into hydrocarbons *in vivo* led us to propose that one of the fatty acids may be reduced to the level of an aldehyde prior to the condensation (Albro and Dittmer, 1969c) and thus bypass the requirement for a condensation product at the oxidation level of a ketone. We present here further studies on the intermediary conversion of fatty acids into hydrocarbons *in vitro*.

Methods and Materials

Media and conditions for growing *S. lutea*, the methods for extraction of lipids from intact cells, and general chromatographic fractionation procedures for lipids are given in the first paper of this series (Albro and Dittmer, 1969a). The assay of radioactive fractions and the radiopurity and source of nonlipid precursors used in this study are given by Albro and Dittmer (1969b). Radiopurity and source of fatty acids-^{14}C used and the synthesis and characterization of heptacosanone-^{14}C are given by Albro and Dittmer (1969c). The preparation of cell-free lysates and assay of hydrocarbon biosynthesis has been previously described (Albro and Dittmer, 1969d). The cofactors and their concentrations used in the experiments reported here except as noted in the description of individual experiments were ATP, 1.7×10^{-4} M; coenzyme A, 9.0×10^{-6} M; Mg^{2+}, 5×10^{-5} M; pyridoxal or pyridoxamine phosphate (as indicated), 1.5×10^{-5} M; and thioglycollate, 3.8×10^{-6} M. Acrylate in a concentration of 1×10^{-4} M was used to inhibit β oxidation of fatty acids. Activity is reported in terms of the disintegrations per minute of labeled precursor incorporated into hydrocarbon per hour per gram dry weight of lysate per microcurie of precursor added to the lysate unless otherwise specified. All precursors were added to the lysate emulsified or complexed with bovine serum albumin.

Cetyl alcohol-1-^{14}C was obtained from Nuclear-Chicago and gave a single radioactive spot when chromatographed on silica gel thin-layer plates (hexane–diethyl ether–acetic acid, 80:20:1) after it had been purified by column chromatography on Florisil (Albro and Dittmer, 1968). Palmitate-9-10-*t* (Amersham-Searle,' Des Plains, Ill.) was freed of a labeled component which chromatographed with hydrocarbon by chromatography on silicic acid. The contaminant was eluted with hexane and the fatty acid recovered with 15% ether in hexane. Triplamitin labeled with ^{14}C in either C-1 or -16 of the acyl chain were synthesized from palmitoyl-1-^{14}C or -16-^{14}C chlorides (Borgström and Krabisch, 1963) as described by Mattson and Volpenheim (1962) and purified on Florisil as described by Carroll (1961). The product was found to be free of other labeled glycerides and free fatty acids when chromatographed on silica gel thin-layer plates developed with petroleum ether (bp 30–60°)–diethyl ether–acetic acid (88:22:1). Methyl esters of specifically ^{14}C-labeled

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palmitic acid were synthesized by refluxing the free acid in methanolic H_2SO_4 and were purified by chromatography on Florisil. The methyl esters were eluted from Florisil with 5% ether in hexane after first eluting the column with hexane.

Palmitaldehyde was synthesized from palmitate-1-^{14}C by reduction of the acid chloride with $LiAlH_4$ (Rao *et al.*, 1967), purified as its 2,4-dinitrophenylhydrazone (Schwartz *et al.*, 1962) and converted into the dimethyl acetal (Mahadevan *et al.*, 1965). The free aldehyde was prepared by acid hydrolysis of the acetal (Farquhar, 1962), but only after it had been refluxed in 1 N NaOH in 80% methanol in order to remove methyl palmitate that apparently was formed during synthesis of the acetal. The final product was found to be free of alcohols, esters, and acids by thin-layer chromatography (Malins and Mangold, 1960; Neptune and Ide, 1963) and infrared spectroscopy (Rao *et al.*, 1967; Chapman, 1965). Its specific activity was 14.9 μCi/mmole. Alternatively, palmitaldehyde was prepared by oxidation of the palmitoyl-1-^{14}C mesylate with dimethyl sulfoxide (Mahadevan *et al.*, 1966; Bauman and Mangold, 1964). The product was purified by chromatography on silicic acid (Mahadevan *et al.*, 1966) and conversion into the bisulfite addition complex as follows: 8 ml of a saturated aqueous solution of sodium bisulfite to which a few crystals of hydroquinone had been added was mixed with a 2-ml solution in ethanol of 10 mg of the fraction obtained by silicic acid chromatography. After shaking the mixture for 2 min, the precipitate was collected by filtration and washed in succession with water and acetone. The free aldehyde was regenerated as needed by heating the sodium bisulfite adduct in 0.5 N HCl in 50% aqueous methanol under a nitrogen atmosphere and then extracting the aldehyde with *n*-hexane. Its specific activity was 120 μCi/mmole.

Tricosan-12-one (Pfaltz and Bauer, Flushing, N. Y.) was randomly tritiated by the Wilzbach procedure (Nuclear-Chicago). Exchangeable tritium was removed by alternately refluxing with methanol twice and 3 mM HCl in methanol once and drying *in vacuo*. The final product was crystallized from ethanol. Tricosanone-*t* used as a substrate was further purified by preparative chromatography on silica gel plates developed with hexane–benzene (40:60). Tricosanol-*t* was prepared by reduction of the ketone with sodium borohydride followed by column chromatography on Florisil. Preliminary elution with benzene removed unreacted ketone and the secondary alcohol was eluted with benzene–chloroform (1:1). This fraction was evaporated to dryness and suspended in 25 ml of 0.4% 2,4-dinitrophenylhydrazine in 90% ethanolic 2 N HCl. After 4 hr at room temperature, the lipid was extracted with petroleum ether, and the extract was freed of dinitrophenylhydrazones and unreacted reagent as described by Schwartz *et al.* (1962). The final product was found to be free of hydrocarbons, ketones, esters, and primary alcohols by chromatography on silica gel thin-layer plates developed with benzene. It had a specific activity of 8.0×10^8 dpm/mg. Tritium was assayed on samples dissolved in 1 ml of toluene mixed with 14 ml of toluene–Spectrafluor (Nuclear-Chicago) (100:4.2, v/v) with a Packard Model 3375 scintillation counter. A reference standard of hexadecane-1-*t* (Nuclear-Chicago) was used.

Corynomycolic acid was isolated from *Corynebacterium rubrum*. (ATCC 14898) grown up at 37° in trypticase soy broth plus 0.25% (w/v) maltose for 55 hr. For ^{14}C-labeled corynomycolic acid, 50 μCi of palmitate-16-^{14}C was added to the medium. Lipids were extracted with chloroform–methanol and saponified by refluxing 2.5 hr with 2 N NaOH in 50% aqueous ethanol. The sodium salt of the mycolic acids were extracted into ether along with the nonsaponifiable lipids (Lacave *et al.*, 1967). An ether solution of the acetone-insoluble residue of the nonsaponifiable lipid was partitioned with aqueous 0.2 N H_2SO_4 and the free corynomycolic acid isolated from the ether phase by silicic acid chromatography (Etemadi *et al.*, 1965). The product gave an infrared spectrum typical of a hydroxy acid (*e. g.*, Chapman, 1965) and chromatographed as a single spot on silica gel thin-layer plates developed with hexane–ether–acetic acid (70:30:2). The compound had a higher R_F value after treatment with acetyl chloride or BF_3–methanol which was consistent with it being a hydroxy acid.

Results

Fate of Carbon Atoms 1 and 16 of Palmitate and Palmitate Derivatives on Incorporation into Hydrocarbons. Comparison of the incorporation of palmitate tagged with ^{14}C in either C-1 or C-16 distinguishes between the nondecarboxylative and decarboxylative mode of entry of palmitate into hydrocarbons. That is, with palmitate-16-^{14}C both modes of entry are assayed and with palmitate-1-^{14}C only the nondecarboxylative pathway is assayed. Data showing significant differences between free fatty acid and acid esters and the effect of using pyridoxal or pyridoxamine phosphate are given in Table I. When fatty acids were added to the system as triglyceride, methyl esters, or the thio ester of coenzyme A, the proportion incorporated with decarboxylation was increased to essentially 100%. Substitution of pyridoxamine phosphate for pyridoxal phosphate increased the proportion of the free acid incorporated by the decarboxylative pathway. Note that absolute activities cannot be for the most part compared in this table since different lysates were used in the different experiments. The otherwise irrelevant datum for the incorporation of tripalmitin with pyridoxamine phosphate is comparable with that for this precursor with pyridoxal phosphate and it is perhaps significant that no difference was observed. A similar observation with acyl-CoA has been interpreted to indicate that the pyridoxine derivative probably functions in the pathway by which fatty acids are incorporated without decarboxylation (Albro and Dittmer, 1969d).

Specificity of Double-Bond Formation in Monounsaturated Hydrocarbons. The carboxyl carbon of palmitate incorporated into monounsaturated hydrocarbons by *S. lutea in vivo* was located on the side of the double bond opposite that from the remainder of the palmitate molecule (Albro and Dittmer, 1969c). If the same specificity in the formation of the double bond occurs *in vitro*, methyl palmitate-16-^{14}C should be incorporated so that only normal C_{16} acids produced by oxidation of the monounsaturated hydrocarbons will be labeled. This follows from the fact that methyl palmitate, as shown above, is incorporated only with decarboxylation.

A cell-free preparation (100 ml) was incubated with methyl palmitate-16-^{14}C with basically the same conditions as described in Table I except 4×10^{-5} M acrylate was used. The fatty acids produced by oxidation of the hydrocarbons were methylated, fractionated by gas–liquid partition chromatography, and the collected fractions were assayed for radioactivity. Of the total ^{14}C recovered, 86% was in palmitate

TABLE I: Comparison of Incorporation of Palmitate-1-^{14}C and -16-^{14}C and Their Derivatives into Hydrocarbons.

Precursor[a]	Cofactor[b]	Act. -1-^{14}C	Act. -16-^{14}C	Rel Act.[c] -1-^{14}C/-16-^{14}C
Palmitic acid	Pyridoxal-P	261	358	0.73
	Pyridoxamine-P	139	593	0.23
Tripalmitin	Pyridoxal-P	244	3,796	0.06
	Pyridoxamine-P		3,780	
Methyl palmitate	Pyridoxal-P	11	361	0.03
Phospholipid[d]	Pyridoxal-P	522	2,255	0.23
Palmitoyl-CoA	Pyridoxal-P	40	16,275	0
	Pyridoxamine-P	5	21,750	0

[a] The amount of ^{14}C-labeled precursor added were as follows: Palmitic acid and methyl palmitate (0.5 μCi/μmole), 1.0 μCi; tripalmitin (2.3×10^{-2} Ci/mole), 0.25 μCi; phospholipid, 0.11 μCi; palmitoyl-CoA (5.6×10^{-2} Ci/mole), 0.1 μCi. [b] The cofactors used are given in the Methods except as indicated. [c] Calculated as the ratio of the specific activity of the isolated hydrocarbons. [d] Phospholipid isolated from *S. lutea* grown for 5 hr as a trypticase soy broth agar "lawn" with the appropriate ^{14}C-labeled palmitate. The fraction used was eluted from silicic acid with methanol (10 ml/g) after the column was first eluted with chloroform (20 ml/g) and chloroform–methanol (96:4, 3 ml/g). The latter solvent was found necessary to completely elute free fatty acids prior to elution of complex lipids.

and the remainder primarily in branched-chain C_{15} and C_{17} acids. Although the recovery in *n*-C_{16} acid is not as high as would be predicted for complete specificity, the relatively lower concentration of acrylate used to inhibit β oxidation could account for redistribution of part of the ^{14}C into branched-chain acids. In any case, the remainder of the label was not localized in normal C_{15} fatty acids as would otherwise be expected if the formation of the double bond was not specific.

The specificity was confirmed by another approach. With palmitate-16-^{14}C added to the cell-free preparation as the bovine serum albumin complex, the ratio of label in *n*-C_{15} to *n*-C_{15} + *n*-C_{16} fatty acids produced by oxidation of the monounsaturated hydrocarbons should be the same as the radio of ^{14}C incorporated from palmitate-1-^{14}C to that from palmitate-16-^{14}C. The experimental conditions were the same as given in Table I with pyridoxal phosphate. The ratio of label obtained was 0.69 which is in good agreement with the ratio of 0.73 reported in Table I.

Incorporation of Postulated Condensation Products. Ketones and secondary alcohols are logical intermediates in the formation of monounsaturated hydrocarbons by a head-to-head condensation of fatty acids. Or if the decarboxylation occurs in a step subsequent to the initial condensation, corynomycolic acid or analogous compounds may also conceivably serve as intermediates. Data on the incorporation of two ketones, a secondary alcohol, and corynomycolic acid are given in Table II. No significant incorporation was found except with heptacosanone; and with it, the distribution of ^{14}C between various hydrocarbons (Table III) indicates that the incorporation is not limited to a direct conversion of the ketone into hydrocarbon. The extent of labeling of hydrocarbons other than the expected C_{27} fraction suggests that some product of catabolism of the ketone may be a more immediate precursor than the intact molecule. With tricosanone, no significant amount of ^{3}H was detected in the fatty acid fraction of the total lipids after incubation. Consequently, it is unlikely

TABLE II: Incorporation of Heptacosanone, Tricosanone, Tricosanol, and Corynomycolic Acid into Hydrocarbon.

Expt	Precursor[a]	Cofactors[b]	Act.
1A	Palmitic acid-16-^{14}C	Complete	400
B	Heptacosan-14-one-14-^{14}C	Complete	1,580
C		Complete − acrylate	10,300
D		Complete − acrylate and pyridoxamine-P	990
2[c]	Palmitic acid-9,10-^{3}H	Complete	1,438
	Tricosan-12-one-*t*	Complete	59
		Complete − acrylate	54
3	Palmitic acid-16-^{14}C	Complete	3,130
	Tricosan-12-ol-*t*	Complete	0
		Complete − pyridoxal-P and coenzyme A	110
		Complete + arsenite (8×10^{-4} M)	0
4	Palmitic acid-16-^{14}C	Complete	1,440
	Corynomycolic acid-^{14}C	Complete	12[d]
		Complete − pyridoxamine	9

[a] Palmitate (1 μCi) was used in each experiment. The specific activity of palmitic-^{14}C acid used in expt 1 and 3 was 4.8 Ci/mole and in expt 4, 0.51 Ci/mole. Palmitic acid-9,10-*t* had a specific activity of 500 Ci/mole. The microcuries of other precursors added to lysates and their specific activities in curies per mole (given in parentheses) were as follows: Heptacosanone (2.6), 0.29; tricosanone (1.9×10^{3}), 25.0; tricosanol (1.9×10^{3}), 1.7; and corynomycolic acid (0.91), 0.45. [b] The complete system was with the cofactors and acrylate in the concentrations given in the Methods. In expt 1, 2, and 4 pyridoxamine were used and in expt 3, pyridoxal phosphate. [c] The lysate used in this experiment was prepared by a different method and had significantly different characteristics with regard to cofactor requirements than the lysates generally used in the experiments reported here. Details of its preparation and of as yet unfinished studies with it of certain aspects of the metabolic pathway will be published later. [d] The ketone fraction isolated by thin-layer chromatography had 176 dpm. The addition of 3 mg of unlabeled corynomycolic acid to lysates did not decrease the incorporation of labeled palmitate.

TABLE III: Distribution of ^{14}C from Hepthcosanone in Hydrocarbon Fractions.[a]

Expt	Carbon No.	^{14}C Recovd dpm	^{14}C Recovd %
1B	25	65	22.4
	26	72	24.9
	27	70	24.2
	28	82	28.3
	29–33	0	0
1C	21–23	334	19.7
	24–25	270	15.9
	26	304	17.9
	27–28	414	24.4
	29	369	21.8
1D	25	85	27.5
	26	74	24.0
	27	84	27.2
	28	65	21.1

[a] Samples of the hydrocarbons isolated from the corresponding experiments for which data are given in Table III were chromatographed on a preparative W-98 column (Albro and Dittmer, 1969a). The fractions indicated were collected and assayed for radioactivity. Overall recovery was 65–85% depending on whether the full range from C_{21} through C_{33} was collected.

TABLE IV: Incorporation of Reduction Products of Fatty Acids into Hydrocarbons.

Expt	Precursor[a]	Cofactor and Other Addenda[b]	Act.	Rel Act.[c]
1	Palmitate-1-^{14}C	Pyridoxal-P	345	100
	Cetyl alcohol-1-^{14}C	Pyridoxal-P	48	14
2	Palmitate-1-^{14}C	Pyridoxal-P	256	100
		Pyridoxamine-P[d]	220	86
	Palmitaldehyde-1-^{14}C	Pyridoxal-P	875	341
		Pyridoxamine-P[d]	223	87
		Pyridoxamine-P	520	203
3	Palmitate-16-^{14}C	Pyridoxal-P	461	100
	Palmitaldehyde-16-^{14}C	Pyridoxal-P	750	162
4	Palmitate-1-^{14}C		73	100
		Palmitaldehyde (20.8 μmoles)	44	62
		Cetyl alcohol (20.4 μmoles)	71	97
		Palmitic acid (19.5 μmoles)	52	71
	Palmitate-16-^{14}C		312	100
		Palmitaldehyde (20.8 μmoles)	231	74
		Cetyl alcohol (20.4 μmoles)	202	97
		Palmitic acid (19.5 μmoles)	221	71

[a] One microcurie of palmitate-^{14}C was used except in expt 2 in which 2 were used. The specific activity of palmitate-1-^{14}C added to lysates was 4.36 Ci/mole except in expt 2 in which it was 0.5 Ci/mole. The specific activity of the palmitate-16-^{14}C used was 4.8 Ci/mole. The amount of ^{14}C in microcuries and the specific activity of the other precursors in curies per mole (given in parentheses) were as follows: cetyl alcohol (6.19), 6.1; palmitaldehyde-1-^{14}C (0.12), 0.27; palmitaldehyde-16-^{14}C (1.5×10^{-2}), 0.03. [b] The cofactors used are given in the Methods with pyridoxal or pyridoxamine phosphate as indicated. In expt 4, pyridoxamine phosphate was used throughout. [c] Calculated on the basis of the specific activities of the hydrocarbons isolated and in each experiment calculated relative to the incorporation of the free acid. [d] The pyridoxamine phosphate was preincubated with the precursor for 5 min prior to the addition of the lysate and other cofactors.

that conversion into fatty acid can account for the findings with heptacosanone and the problem requires further study.

With tricosanol, the omission of cofactors that would cause a diminution of incorporation of fatty acids into hydrocarbons and hence promote incorporation of the alcohol (if it were an intermediate) did not enhance its incorporation significantly.

Incorporation of Reduction Products of Fatty Acids. While the lack of accessibility of the hypothetical intermediates described above to the site of synthesis or their failure to equilibrate with enzyme bound intermediates may explain the lack of evidence for direct conversion into hydrocarbons, it is also possible that such intermediates may be bypassed by the participation of reduction products of fatty acids in the condensation. Accordingly, primary alcohols and fatty aldehydes which are both found in the nonsaponifiable fraction of *S. lutea* (Albro and Dittmer, 1969a) were tested for the ability to serve as precursors of hydrocarbons (Table IV).

Cetyl alcohol was incorporated to a limited extent as compared with palmitate (expt 1), but it was found that 20% of the ^{14}C of the precursor was recovered in the lipid fatty acids after the 3-hr incubation. This conversion could account for the incorporation into hydrocarbon observed. An exact evaluation has to take into consideration the pool size of alcohols in the lysate and because of technical difficulties this has not been determined. Cetyl alcohol added to lysates did not significantly decrease the incorporation of ^{14}C from either palmitate-1-^{14}C or -16-^{14}C (expt 4).

Palmitaldehyde was incorporated to a greater extent than palmitate in every case when pyridoxal phosphate was used as a cofactor but its incorporation appeared to be inhibited when pyridoxamine phosphate was used (expt 2 and 3). The possibility of nonenzymatic interaction of pyridoxamine phosphate and aldehydes as an explanation of these data was supported by a marked decrease in incorporation when the palmitaldehyde was preincubated with pyridoxamine phosphate before adding the lysate and other cofactors (expt 2). It appeared that palmitaldehyde was a better precursor than palmitic acid but it was not possible to gain any insight into the relative role of the pyridoxine derivative in its

incorporation because of the anomaly described above. The swamping effect of unlabeled palmitaldehyde on the incorporation of ^{14}C-labeled palmitate (expt 4) into hydrocarbon was consistent with palmitaldehyde as an intermediate. It would appear relevant that the swamping effect was greater with palmitate-1-^{14}C than with palmitate-16-^{14}C since the former would only indicate incorporation by the nondecarboxylative pathway and aldehydes would most probably be incorporated without loss of C-1.

If aldehydes are intermediates in hydrocarbon biosynthesis, they should be synthesized *in vivo* from isoleucine which has been shown to be the source of the *anteiso*-branched methyl aliphatic group of both fatty acids and hydrocarbons. A 1-l. culture of *S. lutea* was grown for 17 hr and then incubated for another 8 hr with 10 μCi of isoleucine-U-^{14}C. The lipids of the harvested cells were extracted with chloroform–methanol–HCl. After washing and drying the lipids, they were refluxed for 1 hr with 10% HCl in anhydrous methanol. The methanolysis products were separated on Florisil into hydrocarbon and methyl ester plus dimethyl acetal fractions. The latter fraction was hydrolyzed with 2 N KOH in 50% ethanol and the dimethyl acetals extracted from the basic reaction mixture with petroleum ether. The aqueous phase was made acid with HCl and the fatty acids were extracted with diethyl ether. The dimethyl acetal fraction was treated overnight at room temperature with 2 N ethanolic HCl saturated with 2,4-dinitrobenzene. An equal volume of water was then added and the derivatives were crystallized at $-20°$. The monocarbonyldinitrophenylhydrazones were purified by elution from alumina with hexane–benzene (1:1) and the free carbonyl compounds were regenerated by acetone exchange (Demaecker and Martin, 1954). These were then chromatographed on silicic acid (eluted with 10% ether in hexane) and converted into the bisulfite derivative by shaking a 1 ml of 95% ethanol solution with 10 ml of saturated aqueous $NaHSO_3$. The precipitate was washed with water and acetone and the free aldehydes were regenerated by shaking with 0.5 N HCl and again converted into the 2,4-dinitrophenylhydrazones. These had a λ_{max} at 258 mμ; and using ϵ_{258} 22,500 (Schogt *et al.*, 1960), the yield of aldehydes was 0.63 μmole from 3 g dry weight of cells. This would be a minimum figure for cell content of fatty aldehydes since losses in the preparation were inevitable.

The specific activities for the hydrocarbons, fatty acids, and aldehydes were found to be 850, 5552, and 6661 dpm per μmole, respectively. An average molecular weight of 406 for the hydrocarbons and 242 for the fatty acids was used for the calculation of specific activities.

Evidence for the Nature of Intermediates of Fatty Acids Incorporated with Decarboxylation. As indicated above, fatty aldehydes as intermediates in the conversion of fatty acids into hydrocarbons would most logically function in the nondecarboxylative mode of entry. Fatty acids or activated fatty acids would most probably serve in the decarboxylative mode. As shown above, the fatty acids of triglycerides, methyl esters, phospholipids, and coenzyme A thio esters are all preferentially incorporated *via* this latter mode. In addition it has been established that the fatty acid moiety of cetyl palmitate also serves as a precursor but those of dipalmitin are relatively poor (Table V).

Of the fatty acid esters investigated as precursors of hydrocarbons, triglycerides, and phosphoglycerides have been previously reported to occur in *S. lutea* (Huston and Albro, 1964; Huston *et al.*, 1965). The occurrence of wax esters would be consistent with the finding of primary alcohols in the nonsaponifiable fraction (Albro and Dittmer, 1969a) and their presence was tentatively confirmed by the detection of compounds in tetrahydrofuran extracts (Autilio and Norton, 1963) of *S. lutea* that had the R_F of cetyl palmitate on silica gel thin-layer plates developed with hexane–benzene (85:15). No methyl esters were detected under the same conditions. The participation of triglycerides as possible intermediates was investigated further *in vivo*. With media with a high acetate content, ^{14}C-labeled fatty acids added to the media were incorporated into hydrocarbon specifically without decarboxylation (Albro and Dittmer, 1969c). Should triglyceride be an obligatory intermediate in the decarboxylation pathway, the extent of incorporation of exogenous fatty acids into triglyceride *in vivo* with high acetate media should be decreased. When the triglycerides of *S. lutea* grown for 24 hr with 5 μCi of palmitate-16-^{14}C in 200 ml of trypticase soy broth or the same medium supplemented with 2 g/l. of sodium acetate were isolated by chromatography on Florisil (Carroll, 1961) and assayed for ^{14}C, no significant difference in the amount of ^{14}C could be detected.

If, as this experiment would indicate, triglycerides are not obligatory intermediates in hydrocarbon biosynthesis, the question then arises as to why they are apparently better precursors than free acids *in vitro*. One attractive explanation is that this reflects a metabolic pathway in *S. lutea* that serves primarily to cannibalize existing lipids for fatty acids to be used in the synthesis of hydrocarbons during stationary growth phase. Such cannibalization was not detected in preliminary studies in which the changes in hydrocarbon and acyl ester content of simple and complex lipid fractions were examined during stationary growth phase (Figure 1). An accumulation of fatty acids primarily in the phospholipid fraction occurs.

TABLE V: Wax Ester and Diglycerides as Hydrocarbon Precursors.

Expt	Precursor[a]	Cofactor	Act.
1	Palmitate-16-^{14}C	Pyridoxamine-P	370
	Cetyl palmitate-16-^{14}C	Pyridoxamine-P	2710
		Pyridoxal-P	2755
2	Tripalmitin-16-^{14}C	Pyridoxamine-P	1800
	Dipalmitin-16-^{14}C	Pyridoxamine-P	15

[a] The microcuries of ^{14}C-labeled precursor added and the specific activity in curies per mole (given in parentheses) were as follows: palmitate (4.8), 1.0; cetyl palmitate (2.9×10^{-2}) 0.22; tripalmitin (2.3×10^{-2}), 0.84; and dipalmitin (2.2×10^{-2}), 0.19.

Discussion

The synthesis of corynomycolic acid by condensation between C-1 and C-2 of two molecules of palmitic acid (Gastambide-Odier and Lederer, 1959, 1960) would by virtue of

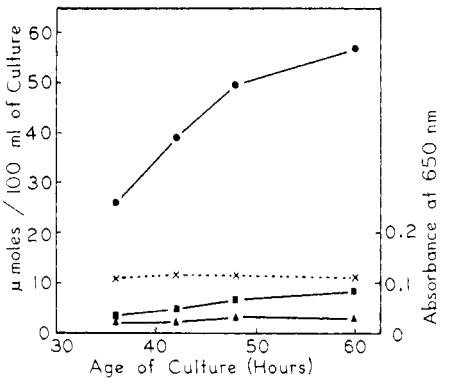

FIGURE 1: Changes in total hydrocarbon and fatty acid content of complex and simple lipids of stationary phase cultures of *S. lutea*. Trypticase soy broth cultures were grown for the time indicated. Total lipids were extracted from the harvested cells with chloroform–methanol–HCl and separated into hydrocarbon, simple lipid, and complex lipid fractions by chromatography on silicic acid columns eluted with hexane, chloroform, and methanol. The fatty acids of the complex (●) and simple (▲) lipids were assayed as their methyl esters (Dittmer and Wells, 1969). Hydrocarbons (■) were weighed and an average molecular weight of 406 was used to calculate the micromoles. The density of growth is given as the absorbance at 650 nm (×) of the equivalent of a 1:100 dilution of the culture in 33% glycerol.

analogy lend support to the intermediacy of long-chain ketones and secondary alcohols in a head-to-head condensation mechanism for the conversion of fatty acids into hydrocarbons. Indeed, Kaneda (1967) has proposed that compounds analogous to carboxylated ketones which might be intermediates in corynomycolic acid synthesis may serve as an intermediate in hydrocarbon biosynthesis in tobacco leaves. Such a mechanism would explain the lack of conversion of ketones themselves into hydrocarbons in plants (Kolattukudy, 1966) and *S. lutea* (Albro and Dittmer, 1969c) *in vivo*. However, failure of access of the ketone to the site of synthesis *in vivo* or the occurrence of enzyme-bound intermediates not in equilibrium with added compounds could also explain the results. Our failure to find significant incorporation *in vitro* of corynomycolic acid, tricosanol, and tricosanone into hydrocarbons or the specific conversion of heptacosanone into heptacosane strengthens the case against the condensation of two molecules of fatty acids or fatty acid derivatives on the oxidation level of fatty acids. Gastambide-Odier and Lederer (1960) proposed the participation of palmitaldehyde in one of three alternate pathways for corynomycolic acid synthesis, and in this way ketones would not be intermediates. The participation of ketones in hydrocarbon biosynthesis may be similarly excluded by analogy. The ready incorporation of palmitaldehyde into hydrocarbons under certain conditions provides direct evidence for such a condensation mechanism although the participation of compounds analogous to corynomycolic acid is not supported by our data. Since the product of such a condensation would be a secondary alcohol, the lack of incorporation of tricosanol is inconsistent with this mechanism. Data which lend support to yet another alternate pathway in which a vinyl ether participates in the condensation will be presented in the next paper of this series.

The participation of acyl-CoA or perhaps acyl-acyl carrier protein as the fatty acid derivative which eventually is decarboxylated is mechanistically consistent with the role of these compounds in other metabolic pathways. The preferential incorporation of acyl-CoA with decarboxylation was previously reported (Albro and Dittmer, 1969d). An explanation for the complementary findings reported here that other acyl esters are preferentially incorporated *via* the same route is elusive. The rationalization that a direct conversion of simple esters into thio esters would be advantageous to an organism that utilized the fatty acids of other lipids for hydrocarbon biosynthesis in stationary growth phase is not supported by the data given in Figure 1. The concept of hydrocarbons as secondary metabolites, as usually envisaged in plants, may very well not apply in bacteria. In any case, the relationship of hydrocarbons to other lipids and of the various lipids to other cell components during different phases of growth warrants closer study.

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