The Biochemistry of Long-Chain, Nonisoprenoid Hydrocarbons. II. The Incorporation of Acetate and the Aliphatic Chains of Isoleucine and Valine into Fatty Acids and Hydrocarbons by *Sarcina lutea in Vivo**

Phillip W. Albro† and John C. Dittmer

ABSTRACT: The *anteiso* aliphatic groups of fatty acids and hydrocarbons were shown to be derived from the five terminal carbons of isoleucine and the remaining carbons from acetate. Valine was selectively incorporated into *iso* fatty acids with an even number of carbons and probably contributes *iso* aliphatic groups in a manner analogous to that found for isoleucine and *anteiso* groups. Data based on the incorporation of isoleucine-U-^{14}C and acetate-2-^{14}C into the fatty acids and hydrocarbons of *Sarcina lutea* and the absence of incorporation of the methyl group of methionine-methyl ^{14}C- were consistent with biosynthesis of the hydrocarbons by head-to-head condensation of two molecules of fatty acid with decarboxylation of one of them and were completely or in part inconsistent with biosynthesis by a head-to-tail condensation of fatty acids or by elongation of fatty acids followed by decarboxylation. Neither *anteiso* nor *iso* fatty acids participated in the biosynthesis of the hydrocarbons by head-to-head condensation to a degree proportional to their concentration in the total lipids. This latter observation has been interpreted to indicate that either a specific pool of fatty acids, as perhaps determined by the fatty acid composition of a particular class of lipids, participates in the biosynthesis or the enzymes involved have specificity for certain fatty acids.

In the first paper of this series (Albro and Dittmer, 1969) we described a series of hydrocarbons isolated from *Sarcina lutea* that had from 22 to 30 carbons and of which 90% had one double bond near the center of the main aliphatic chain. Analyses of the fatty acids produced by oxidation of the double bonds along with additional information on the structure of the intact hydrocarbons and lipid fatty acids were consistent with a biosynthetic parthway involving the head-to-head condensation of two molecules of fatty acid with decarboxylation of one of them. Also, long-chain secondary alcohols and a series of long-chain ketones were isolated from the nonsaponifiable lipids of *S. lutea* that would be expected to serve as intermediates in a biosynthetic pathway based on the condensation of fatty acids. In this paper, evidence based on the *in vivo* incorporation of various low molecular weight compounds into the fatty acids and hydrocarbons is presented which strongly supports this general biosynthetic mechanism.

Experimental Procedures

General methods for the extraction of the lipids, the isolation of the hydrocarbons, gas–liquid partition chromatography, and thin-layer chromatography have been described in the first paper of this series, (Albro and Dittmer, 1969). All of the experiments described here were carried out with the strain of *S. lutea* designated as FD-533 in the previous paper. Cells were cultured in trypticase soy broth or a semidefined medium, M73b, as also described earlier.

The following labeled compounds with their sources and radiopurities were used: sodium acetate-1- and -2-^{14}C, Calbiochem, 99.9%; sodium acetate-2-^{14}C, Nuclear-Chicago, 98%; L-valine-U-^{14}C, New England Nuclear, 99.3%; L-isoleucine-U-^{14}C, Schwarz, 97.0–93.7%; and L-methionine-methyl-^{14}C, Calbiochem, 99%. Radiopurity was checked by chromatography on paper in a variety of solvent systems. An impurity that chromatographed with valine accumulated in the isoleucine over a 1-year storage period.

All radioactive samples were counted on a Beckman low-beta II geiger flow counter. Lipids were weighed on tared aluminum planchets and counted near "infinite thinness." Corrections for counting efficiency and self-absorption were determined with calibrated standards and absorption correction curves were prepared with appropriate standards. Fatty acids and hydrocarbons were counted on untreated planchets while fatty acid methyl esters were evaporated onto planchets in the presence of 0.05% ethanolic NaOH; 95% confidence limits were calculated using a table supplied with the instrument and adjusted for the reliability of the measurement of the sample weight (±0.01 mg). Labeled $BaCO_3$ collected in the Schmidt and Kuhn–Roth degradations described below were counted on micropore filter membranes or filter paper. The acetic acid produced by the Kuhn–Roth degradation was

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† Predoctorial Fellow, Institute of General Medical Sciences, U. S. Public Health Service. Present address: Chemical Section, National Environmental Health Science Center, Research Triangle Park, N. C. 27709.

plated as sodium acetate. With both the $BaCO_3$ and sodium acetate, corrections for self-absorption were made with curves prepared from standards.

Fatty acids were decarboxylated by Schmidt degradation with an apparatus similar to that described by Rabinowitz (1957). His procedure was used except no toluene was used in the CO_2 collection tubes. Kuhn–Roth degradation of the fatty acids and hydrocarbons was done by the procedure described by Ginger (1944). The evolved CO_2 was continuously flushed into a series of $Ba(OH)_2$ traps with a stream of nitrogen and the acetic acid derived from the methyl branch chains and terminal ethyl group was steam distilled into dilute NaOH.

Results

The Synthesis of Branched-Chain Fatty Acids from Acetate and Isoleucine or Valine and the Nonrandom Participation of Fatty Acids in Hydrocarbon Synthesis. INCORPORATION OF ISOLEUCINE AND ACETATE. The combined work of Lennarz (1961) with *Micrococcus lysodeikticus* and Kaneda (1963) with *Bacillus subtilis* has shown that the terminal branches of *anteiso* and *iso* fatty acids with an even number of carbons are derived from isoleucine, leucine, and valine, respectively. In the incorporation, the amino acids are deaminated and decarboxylated and the branched-chain five- or four-carbon fatty acid is then elongated by the addition of acetate to form fatty acids of different chain lengths. If this mechanism holds true for *S. lutea*, it would provide a means of labeling specific parts of the aliphatic chains of both fatty acids and hydrocarbons derived from the fatty acids. Comparison of the relative specific activities of these parts of the aliphatic chains in fatty acids and hydrocarbons should provide information on their possible metabolic interrelationship.

Accordingly, 10 ml of a concentrated suspension of *S. lutea* grown in M73b medium was added to 1 l. of fresh M73b medium and this culture was then divided into two equal portions. L-Isoleucine-U-^{14}C (5 μCi) was added to one portion and acetate-2-^{14}C (5 μCi) to the second portion. The specific activities of the two constituents in the media were initially 3780 dpm/μmole for the isoleucine and 1380 dpm/μmole for the acetate. The cells were harvested after 1 hr, and the total lipids and hydrocarbons were isolated, assayed for radioactivity, and analyzed by gas–liquid partition chromatography (Tables I and II). Control experiments showed that although isoleucine was extracted from the cells with the lipids it was not carried over into any of the purified lipid fractions. No radioactivity from similar controls with labeled acetate was found in any lipid extract or lipid fraction.

In addition to the distribution of the fatty acids in Table II, calculations based on the biosynthetic pathways outlined above for the contribution of carbons from acetate and isoleucine for each of these fatty acids are given. The number of carbons in the "average" fatty acid in the mixture would be the sum of the contributions of atoms from the various precursors or $10.1 + 4.22 + 0.70 = 15.02$. This average fatty acid would have 5.05 acetate units (10.11/2 carbons/acetate) and 0.84 *anteiso*-C-5 unit from isoleucine (4.22/5 carbons/isoleucine). The ratio of the specific activities of the fatty acids labeled with isoleucine should then be: (1380 dpm/μmole of acetate)(5.05)/(5/6 $\times$ 3750 dpm/μmole of isoleucine)(0.84) = 2.64. The ratio found was 4530/1767 = 2.56. The difference between the theoretical value and the experimental value is well within the accuracy of the techniques used. It would appear from this experiment that the branched-chain fatty acids with *anteiso* configurations were synthesized by the addition of acetate to 2-methylbutyrate derived from isoleucine. This was amply confirmed in an additional experiment described later in this paper.

If the *S. lutea* hydrocarbons are synthesized by random head-to-head condensation of fatty acids and each fatty acid participates in the process at a rate proportional to its relative concentration in the total fatty acid pool, the ratio of the specific activities of the hydrocarbons to that of the fatty acids should be the same whether acetate-2-^{14}C or isoleucine-U-^{14}C was used as a precursor. In this experiment, the ratios as calculated from the data in Table I differed by a factor of 1.21 with the isoleucine labeled fatty acids and hydrocarbons having a higher ratio. The fact that one fatty acid may be decarboxylated in the biosynthetic mechanism would not alter the ratio because neither isoleucine-U-^{14}C nor acetate-2-^{14}C, as will be shown subsequently, labels the carboxyl carbon. The results are consistent with nonrandom incorporation of the acids.

TABLE I: The Incorporation of L-Isoleucine-U-^{14}C and Acetate-2^{14}C into the Fatty Acids and Hydrocarbons of *S. lutea*.

Precursor	Lipid Class	Total (dpm)	dpm/mg	dpm/μmole[a]	Ratio of Sp Act. (Hydrocarbons/Fatty Acids)
Isoleucine	Hydrocarbons	4,362	1,563	635	0.36
	Fatty acids	51,110	6,880	1,767	
Acetate	Hydrocarbons	9,908	3,310	1,348	0.30
	Fatty acids	186,860	17,631	4,530	

[a] Calculated using an average molecular weight for fatty acids of 242 and for hydrocarbons of 406; 95% confidence limits did not exceed $\pm$0.5 for any value in the table.

TABLE II: Fatty Acid Composition of Total Lipids and the Theoretical Contribution of Acetate, Isoleucine, and Other Intermediates in Their Biosynthesis.

Fatty Acid[a]	% of Total	Theoretical Number of Moles of Each Precursor in Each Fatty Acid			Theoretical Increment of Carbons from Each Precursor to Total Fatty Acids[c]		
		Acetate	Isoleucine	Other[b]	Acetate	Isoleucine	Other
i-13	0.1	4	0	1 (5)	0.008	0	0.005
a-13	0.2	4	1	0	0.016	0.002	0
i-14	1.6	5	0	1 (4)	0.160	0	0.064
n-14	0.2	7	0	0	0.028	0	0
i-15	10.6	5	0	1 (5)	1.060	0	0.530
a-15	83.5	5	1	0	8.350	4.175	0
n-15	0.7	6	0	1 (3)	0.084	0	0.021
i-16	1.9	6	0	1 (4)	0.225	0	0.076
n-16	0.5	8	0	0	0.080	0	0
a-17	0.8	6	1	0	0.096	0.040	0
Total number of increments of carbons					10.110	4.217	0.696

[a] Designated by the total number of carbons and the configuration of the aliphatic chain, *i.e.*, *i* = *iso*, *a* = *anteiso*, and *n* = normal. [b] Other precursors are valine, leucine, and propionate which would contribute four, five, or three carbons, respectively, as designated by the number in parentheses. [c] Calculated as the product of the proportion of the acid, the number of carbons contributed by the precursor, and the number of moles of the precursor in the fatty acid.

INCORPORATION OF VALINE. This experiment was designed to establish whether the *iso* branched-chain fatty acids were randomly incorporated into the hydrocarbons by head-to-head condensation with decarboxylation of one of the acids. M73b medium (1 l.) containing 20 μCi of L-valine-U-^{14}C was inoculated with a negligible mass of *S. lutea* and incubated for 19 hr. The C-27 hydrocarbon fraction was isolated by preparative gas–liquid partition chromatography and oxidized with periodate–permanganate. The specific activities of the *iso*-C-13 and -C-14 fatty acids produced by the oxidation and of the *iso*-C-13, -14, -16 and the *anteiso*- and normal C-15 fatty acids from the total lipids and the relative proportions of all the fatty acids of the lipids are given in Table III.

It should first be noted that the label from valine appeared to be preferentially incorporated into the *iso* fatty acids with an even number of carbons as would be expected if synthesis occurred by the mechanism described above. Because the labeled precursor was present throughout the incubation and the culture was started with a negligible mass of cells, the specific activity of any given fatty acid should be the same regardless of the class of lipids with which it was associated. If the hydrocarbon synthesis occurred by random condensation of fatty acids in proportion to their relative concentrations in the total cell lipids, it would be possible to predict the specific activities of the fatty acid residues derived by oxidative cleavage of the hydrocarbon. The possible combinations by which *iso* fatty acids would be incorporated into C-27 hydrocarbons through head-to-head condensations with decarboxylation of one of the fatty acids is given in Table IV. On this basis, the predicted specific activities of the *iso*-C-13 residue obtained by oxidation

TABLE III: Fatty Acid Composition of Total Lipids and the Specific Activities of the Fatty Acids of the Total Lipids and the *iso* Fatty Acids Obtained by Oxidation of the C-27 Hydrocarbons after Incubation of *S. lutea* with L-Valine-U-^{14}C.

Fatty Acid	% Distribution	cpm/mg[a]
Total lipids		—
≤-12	0.25	
i-13	0.19	30.8 ± 6.0
a-13	0.65	—
n-13	0.05	
i-14	3.65	960.6 ± 2.8
n-14	14.98	—
i-15	8.89	—
a-15	54.67	0
n-15	0.80	0
i-16	5.03	695.0 ± 2.0
n-16	9.45	—
i + *a*-17	0.04	—
Oxidized C-27 hydrocarbons		
i-13		333 ± 29
i-14		248 ± 22

[a] The ranges shown are for 95% confidence limits. Fatty acids not assayed are designated with a dash.

of the hydrocarbon would be equal to the sum of the contribution of condensations D and E divided by the contributions of condensations A + B + C + D + E, times the specific activity of the original *iso*-C-14 fatty acid or (82.9/95.8)(960) = 828 cpm/mg. Likewise, the predicted specific activity of the *iso*-C-14 fatty acid

TABLE IV: Theoretical Condensation of Fatty Acids Which Would Yield C-27 Hydrocarbons.

Condensation	Precursor Acids	Sets of Acids Produced by Oxidation of the Hydrocarbons[a]		Rel Contribution of Condensation to Total C-27 Hydrocarbon Synthesis[b]
A	*i*-13 + *i*-15	*i*-13† + *i*-14†	*i*-12 + *i*-15	1.8
B	*i*-13 + *a*-15	*i*-13† + *a*-14	*i*-12 + *a*-15	10.9
C	*i*-13 + *n*-15	*i*-13† + *n*-14	*i*-12 + *n*-15	0.2
D	*i*-14* + *i*-14*	*i*-13* + *i*-14*	*i*-14* + *i*-13*	27.4
E	*i*-14* + *n*-14	*i*-14* + *n*-13	*i*-13* + *n*-14	55.5
F	*i*-15 + *a*-13	*i*-14† + *a*-13	*i*-15 + *a*-12	5.3
G	*i*-15 + *n*-13	*i*-14† + *n*-13	*i*-15 + *n*-12	0.9

[a] The C-27 hydrocarbon resulting from the condensation of the acids indicated was assumed to consist of equal amounts of two isomers, the two resulting from decarboxylation of either one or the other of the precursor acids. Those residues that would be labeled from valine are indicated by *. Those not labeled by valine but which would dilute the *iso*-C-13 and -14 pools are indicated by †. [b] Calculated as the product of the relative concentrations of the precursor fatty acids. The value for condensation "D" was doubled since it will yield two of each residue. The relative concentrations of the precursor fatty acids are given in Table III.

obtained by oxidation of the hydrocarbon would be the ratio of the sums of condensations D + E divided by A + F + G + D + E, times the specific activity of the original *iso*-C-14 fatty acid or (82.9/90.9)(960) = 878 cpm/mg.

Comparison of the predicted values with the data in Table III shows that the *iso* acids obtained by oxidation of the hydrocarbons had only about one-third the predicted specific activities and the deviations were considerably larger than the experimental error. It might be concluded from these results that condensations A, B, C, F, and G or certain ones of them were preferred over D and/or E and hence the fatty acids do not participate in a random manner or that the C-27 hydrocarbons were produced by a mechanism other than head-to-head condensation of the fatty acids.

Metabolic Relationship of the anteiso Aliphatic Groups of the C-29 Hydrocarbons. When the C-29, monounsaturated hydrocarbons were oxidized with periodate–permanganate it was found that the major fatty acids produced were *anteiso*-C-15 and -C-14 fatty acids (Albro and Dittmer, 1969). If the *anteiso*-C-14 acid, which was not found as a natural constituent of the lipids, was derived by decarboxylation in a head-to-head condensation of two molecules of *anteiso*-C-15 fatty acid, it should have the same specific activity as the *anteiso*-C-15 fatty acid also obtained by oxidation of the hydrocarbons derived from isoleucine-^{14}C.

The C-29 hydrocarbons from *S. lutea* grown for 8 hr in the presence of L-isoleucine-U-^{14}C were isolated by preparative gas–liquid partition chromatography on a W-98 column and subjected to periodate–permanganate oxidation. The *anteiso*-C-15 and -C-14 acids were isolated by preparative gas–liquid partition chromatography of the methyl esters and their specific activities were found to be 880 ± 40 and 836 ± 40 dpm per μmole, respectively. These results were consistent with our original explanation of the origin of the *anteiso*-C-14 fatty acids as oxidation products of hydrocarbons. Actually, some of the C-29 hydrocarbons have *iso-anteiso'* and *anteiso-iso'* as well as *anteiso-anteiso'* configurations of the terminae of the aliphatic chains to either side of the double bond, and the fact that the specific activities agree suggests that the *anteiso* terminae of the three different isomers were derived from the same precursor pool.

Confirmation of the Biosynthetic Origin of the Different Portions of anteiso Fatty Acids and Hydrocarbon Aliphatic Chains from Acetate and Isoleucine. FATE OF ISOLEUCINE IN *anteiso* FATTY ACIDS. A 24-hr M73b culture of *S. lutea* was treated with 5 μCi of isoleucine-U-^{14}C and incubation was continued. Aliquots were harvested after 1, 3, 5, and 8 hr. The branched (*iso* + *anteiso*) C-15, *iso*-C-16, and *n*-C-16 fatty acids were isolated and assayed for radioactivity. Fractionation of the branched C-15 acids showed that 99.3 ± 1.5% of the radioactivity was in the *anteiso* acid.

The function R = dpm in br-C-15 fatty acid/dpm in br-C-15 + br-C-16 + *n*-C-16 fatty acid was used as an index of general randomization of the label and of β oxidation since the structure of these fatty acids preclude their direct interconversion. R varied from 0.07 to 0.92 when palmitic-^{14}C acid was used in preliminary experiments, and acetate-2-^{14}C gave R = 0.91 after 1 hr and 0.10 after 23 hr. A series of R values decreasing with time would be expected if fatty acids labeled from isoleucine-^{14}C were subject to β oxidation, and if the acetate-^{14}C coenzyme A released in this process was reincorporated into fatty acids.

The 1-, 3-, 5-, and 8-hr samples examined in this experiment all gave R = 0.984 ± 0.004. This high and constant R value was considered to indicate that (1) isoleucine-^{14}C did not directly label *iso*- or *n*-C-16 acids to any significant extent and that (2) the branched C-15 acid derived from isoleucine was not degraded to labeled fragments which could be reincorporated into *iso* or normal fatty acids.

COMPARISON OF THE DISTRIBUTION OF LABEL FROM ISOLEUCINE-^{14}C AND ACETATE-^{14}C IN FATTY ACIDS AND

HYDROCARBONS. The hydrocarbons and fatty acid methyl esters were isolated from cells grown in the presence of either L-isoleucine-U-^{14}C for 8 hr or a mixture of acetate-1-^{14}C and -2-^{14}C for 7 hr. Aliquots of these fractions were subjected to chromic acid oxidation (Kuhn–Roth degradation) as is illustrated with the major *anteiso* fatty acid

$$CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2(CH_2)_9COOH \rightarrow 2CH_3COOH + CO_2 + 10CO_2$$

The carbons which it is proposed were derived from isoleucine and acetate are shown in regular and italic type, respectively.

The per cent distribution of the recovered counts after Kuhn–Roth degradation of the total lipid fatty acids and of the hydrocarbons from cells incubated with labeled isoleucine and acetate are given in Table V. In addition, the fatty acid methyl esters were examined by thin-layer chromatography after conversion into the free acids by saponification. After chromatography on silica gel with water saturated with 1-butanol (Singh and Gershbein, 1966), the fractions were detected with Rhodamine 6G and the regions corresponding to the C-15 and C-16 and all the other fatty acids as established with standards were separately scraped from the plate. The fatty acids were eluted from the scrapings with ethanol and counted for radioactivity. The C-15 fatty acids contained 91% of the total radioactivity recovered from the plates whether isoleucine or acetate was the original source of label. With acetate-^{14}C, 4.3% of the label was in the C-16 fatty acid but no label from isoleucine could be detected in this fraction. The amount of labeled material applied to the plate in the latter case was sufficient to have permitted the detection of as little as 0.7% of the total acid. These findings were substantially in agreement with those reported above, that is, that isoleucine was incorporated only into fatty acids with an *anteiso* configuration.

The distribution of label shown in Table V shows that of the carbons incorporated into the fatty acids from isoleucine four out of five comprise the *anteiso* terminal group itself and one occurs most probably as the methylene carbon adjacent to the terminal group. This distribution is in complete accord with the labeling proposed in the reaction given above. If it also holds for labeling with acetate, none of the label from *anteiso* acids should have been recovered in acetate after degradation and all of it should be recovered as CO_2. The distribution observed was not quite that cleanly cut. Acetate would be expected to be incorporated into valine and leucine, which unlike isoleucine can be synthesized *de novo* by this strain of *S. lutea*. Also acetate would form the terminal ethyl group of straight-chain fatty acids and the terminal group would be recovered as acetate after Kuhn–Roth degradation. Under these circumstances the recovery of 5% of the label from the fatty acids as acetate after degradation was not too surprising nor inconsistent with the biosynthetic mechanism proposed.

TABLE V: Specificity of Labeling from Isoleucine and Acetate in Fatty Acids and Hydrocarbons.

Lipid Fraction	Labeled Precursor	% Distribution of Act. in Kuhn–Roth Degradation Products	
		CH_3COOH	CO_2
Total lipid fatty acids	Isoleucine	80.3 ± 0.8	19.7 ± 0.2
	Acetate	6.7 ± 3.0	93.3 ± 3.0
Hydrocarbons	Isoleucine	80.7 ± 1.0	19.3 ± 0.5
	Acetate	2.3 ± 1.0	97.7 ± 3.0

Derivation of the Carboxyl Carbon of Branched-Chain C-14 Fatty Acids Obtained by Oxidation of the Hydrocarbons. If the C-29 monounsaturated hydrocarbons were synthesized by head-to-head condensation of two fatty acids with decarboxylation of one of them, the C-15 fatty acids obtained by oxidation of the double bond could represent a carbon chain derived originally from a C-15 fatty acid incorporated intact or a C-16 fatty acid incorporated with decarboxylation. Similarly the C-14 fatty acid could have been derived from either a C-14 or a C-15 fatty acid. This assumes that the double bond marks the point of condensation which, as will be shown in a subsequent paper, is the case. Furthermore, if the C-15 fatty acids were synthesized by the addition of acetate to a preformed branched-chain acid derived from an amino acid as the experiments above indicate, then when these fatty acids are labeled with acetate-2-^{14}C, little or no activity should be in the carboxyl carbon. It follows that in the C-15 + C-15 → C-29 condensation, the C-14 fatty acid obtained by oxidation of the hydrocarbon should be labeled in the carboxyl carbon. The reverse would be true of the C-14 + C-16 → C-29 condensation, that is, the C-14 fatty acids would have no label in the carboxyl carbon whereas the C-15 fatty acid would be labeled in the carboxyl carbon. The over-all concept is illustrated for the C-15 + C-15 condensation in Scheme I.

SCHEME I

$$2CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2\overset{NH_2}{\overset{|}{C}}HCOOH \rightarrow 2CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2COOH + 10^{14}CH_3COOH$$

$$\rightarrow 2CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2(^{14}CH_2CH_2)_4{}^{14}CH_2COOH$$

$$\rightarrow CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2(^{14}CH_2CH_2)_4{}^{14}CH_2CH=\overset{CH_3}{\overset{|}{C}}{}^{14}CH(CH_2{}^{14}CH_2)_4CH_2\overset{CH_3}{\overset{|}{C}}HCH_2CH_3$$

$$\rightarrow CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2(^{14}CH_2CH_2)_4{}^{14}CH_2COOH + CH_3CH_2\overset{CH_3}{\overset{|}{C}}HCH_2(^{14}CH_2CH_2)^{14}COOH$$

In consideration of the composition of the fatty acids derived by oxidation of the hydrocarbons and the lipid

TABLE VI: Fate of the α-Carbon of Endogenous Fatty Acids in Hydrocarbon Synthesis.

Fatty Acid Fraction	% of Label in Carboxyl Carbon
Total lipid fatty acid	0.6 ± 0.1
C-14 fatty acid from oxidized C-29 hydrocarbon	70.0 ± 3.0
C-15 fatty acid from oxidized C-29 hydrocarbon	4.7 ± 0.6

fatty acids the C-15 + C-15 condensation probably greatly exceeds the C-14 + C-16 condensation.

Several lots of *S. lutea* grown in either TSB or M73b media for up to 48 hr with acetate-2-^{14}C were pooled and the fatty acid methyl esters and C-29 hydrocarbons were isolated as usual. The C-29 hydrocarbons were oxidized and the branched-chain C-14 and C-15 fatty acids were separately isolated by gas–liquid partition chromatography. Each fraction gave a single peak when rechromatographed on an analytical W-98 column. The esters of these acids and of the total lipid fatty acids were saponified and subjected to Schmidt degradation, and the carboxyl carbons were collected and assayed for radioactivity as $BaCO_3$. These data are given in Table VI.

As expected little or no label from C-2 of acetate was incorporated into the carboxyl carbon of the total lipid fatty acids. The distribution of the label in the acids derived from the hydrocarbons was consistent with head-to-head condensation. Since only 5% of the label in the C-15 fatty acid derived from the hydrocarbon was in the carboxyl carbon, the C-15 + C-15 condensation must have predominated. Had the C-14 + C-16 condensation predominated, a minimum of 16% of the label would have been found in the carboxyl carbon of the C-15 fatty acid. The high proportion of label in the carboxyl carbon of the C-14 acid relative to the rest of the molecule has consequences that are brought out in the Discussion.

The Absence of Incorporation of the Methyl Group of Methionine into the Hydrocarbons. The fact that most of the hydrocarbons of *S. lutea* have branched methyl groups either in *iso* or *anteiso* configuration at both ends was cited by us as evidence against biosynthesis by way of elongation of preformed fatty acid with acetate followed by decarboxylation and reduction. However, this is not a valid conclusion if, in fact, the methyl branch could be added after the aliphatic chain is formed. The derivation of the methyl branch of tuberculostearic acid (10-methyloctadecanoic acid) from methionine (Jaureguiberry *et al.*, 1965) is one such instance. Accordingly, the incorporation of ^{14}C into hydrocarbon from methionine labeled in the methyl group was checked. Because the methyl group of methionine can become the β-carbon of serine and eventually the methyl carbon of pyruvate, label could enter the hydrocarbon without the direct insertion of a methyl group. Branched-chain amino acids and acetic acid may be derived from pyruvate and would serve as logical intermediates in such an incorporation, so the incubation medium for this experiment was supplemented with both in order to suppress the *in vivo* synthesis of these known hydrocarbon precursors. As a control, the medium was also supplemented with alanine in order to make pyruvate available for metabolic pathways other than incorporation into alanine. The results of the experiment are given in Table VII. As can be seen, no incorporation of label in the absence of alanine could be detected and in the presence of alanine only an insignificant trace could be detected.

TABLE VII: Incorporation of Label from the Methyl Carbon of Methionine into *S. lutea* Hydrocarbons.

Expt[a]	Sp Act. of Hydrocarbons (dpm/mg)
1	0
2	237 ± 10

[a] A 250-ml, trypticase soy broth, 37-hr culture was augmented with 50 ml of fresh trypticase soy broth medium plus 100 mg each of L-valine, L-isoleucine, and L-leucine and 40 μCi (trace mass) of L-methionine-methyl-^{14}C. The culture was divided into two equal portions and one (expt 1) was incubated directly while the second (expt 2) was further augmented with 100 mg of L-alanine. Both cultures were incubated for 2 hr, the cells were harvested, and the hydrocarbons were isolated and asayed for activity.

The incubation conditions used in this experiment were comparable with those used in the experiment described previously with acetate-^{14}C and isoleucine-^{14}C since the M73b medium used in those experiments contained acetate and were richer in branched-chain amino acids than was the unaugmented trypticase soy broth medium. In those experiments, a conversion of 0.027% of the label supplied from isoleucine into hydrocarbon was obtained, and a corresponding value of 0.062%/hr with acetate was obtained. Since these values were from 30 to 80 times higher than the value obtained using methionine-^{14}C (with alanine, 0.0008%/hr), it seemed unlikely that "C-1" metabolism played a direct role in hydrocarbon synthesis in *S. lutea*.

Discussion

Tornabene and Oró (1967) have reported the results of a study of the incorporation of ^{14}C-labeled acetate, isoleucine, and leucine into the fatty acids and hydrocarbons of a micrococcus reported by them to be *S. lutea* but, for reasons which are given in the first paper of this series, was probably incorrectly identified. Nevertheless, their findings are pertinent to the work reported here because this micrococcus was found to incorporate isoleucine primarily into both fatty acids and hydrocarbons with *anteiso* aliphatic groups.

Leucine and acetate, on the other hand, were incorporated with few exceptions in similar patterns into all of the different fatty acids and hydrocarbons as resolved by gas–liquid partition chromatography. Their findings with respect to isoleucine are in accord with those reported by Lennarz (1961) for *M. lysodeikticus* and by Kaneda (1963) for *B. subtilis* as are our findings reported here. We have further shown, as did Lennarz and Kaneda, that only five carbons of the isoleucine were incorporated into the aliphatic chain of fatty acids, and we find that this is also true for the *anteiso* aliphatic groups of the hydrocarbons. Kaneda (1967) and Kolattukudy (1968b) have reported the specificity of isoleucine and valine in the labeling of *anteiso* and *iso* branched-chain hydrocarbons in tobacco.

Neither *iso* nor *anteiso* fatty acids were found to participate in the biosynthesis of hydrocarbons to a degree proportional to their concentration in the total lipid fatty acids. In this respect, it is of interest to recall the observation (Albro and Dittmer, 1969) that in the micrococcus ATCC-533 condensations involving *iso*-C-13 fatty acids are particularly favored in hydrocarbon biosynthesis. It appears that there is either a specific fatty acid pool, perhaps as represented by the fatty acids of a particular class of lipids, that participates in hydrocarbon biosynthesis or that the enzymes involved in the synthesis show specificity for certain fatty acids. It has been reported that the different lipids of *S. lutea* have different fatty acid compositions (Huston and Albro, 1964).

There is an anomaly in the data presented in Table VI which requires further consideration. On the basis of the biosynthetic pathway proposed for the branched chain C-15 fatty acids, only 20% of the label would be expected in C-2 if the label from acetate-2-^{14}C was evenly distributed throughout the portion of the molecule derived from acetate. Also, only 20% of the label should be in the carboxyl carbon of the C-14 fatty acid derived from it. In fact, 70% of the label of the C-14 fatty acids was found in the carboxyl carbon. The apparent descrepancy probably arises from the fact that the label derived from acetate was not evenly distributed along the chain. A similar unequal distribution was observed by Fulco and Mead (1959) in the 5,8,11-eicosatrienoic acid labeled from acetate-^{14}C *in vivo* by fat-deficient rats. They found 66% of the radioactivity in C-1 and -2 of this acid which had been synthesized by elongation and desaturation of oleate. Dilution of each elongation product by unlabeled material in the cell prior to each subsequent addition of C-2 units would account for the distribution in *S. lutea*.

Detailed studies of the distribution of C-2 of acetate in the lipid fatty acids and in the aliphatic groups to either side of the double bond of monounsaturated hydrocarbons and of the incorporation of the methyl carbon of methionine into hydrocarbon gave results fully consistent with a mechanism by which the hydrocarbons are synthesized by head-to-head condensation of 2 moles of fatty acid with decarboxylation of one of them. The data obtained were fully or in part inconsistent with either the synthesis of the hydrocarbons by head-to-tail condensation of fatty acids or by elongation of preformed fatty acids followed by decarboxylation and reduction. In the latter case, the biosynthetic pathway appears to differ markedly from that reported by Kolattukudy for *Brassica olercea* (1966, 1967), *Senecio odoris* (1968a), and *Pisum sativum* and *Spinacia olercea* (1968c). His findings support an elongation–decarboxylation mechanism. On the other hand, Kaneda (1968) finds that at least in part the biosynthesis of hydrocarbons in tobacco occurs by head-to-head condensation. In the next paper of this series in which data on the incorporation of intact fatty acids into hydrocarbons of *S. lutea* will be included, evidence will be presented that suggests the need for reevaluation of some of the data supporting the elongation–decarboxylation mechanism in plants.

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