The Biochemistry of Long-Chain, Nonisoprenoid Hydrocarbons. I. Characterization of the Hydrocarbons of *Sarcina lutea* and the Isolation of Possible Intermediates of Biosynthesis*

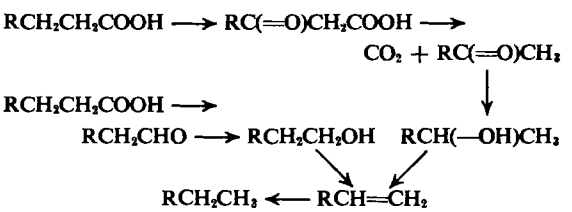
Phillip W. Albro† and John C. Dittmer

ABSTRACT: The hydrocarbons of an adequately identified strain of *Sarcina lutea* were characterized by a combination of thin-layer and gas–liquid partition chromatography, infrared spectroscopy, and oxidative cleavage of the double bonds. The major hydrocarbon fractions had 27, 28, and 29 carbons (18.4, 12.7, and 65.3% of the total, respectively) and the proportions did not vary greatly with the age of the culture. In 48-hr cultures, 90% of the hydrocarbons were monounsaturated and the percentage decreased as the cells continued into stationary phase. Only hydrocarbons with *cis* configuration of the double bond were detected. Chromatographic analysis of the hydrocarbons and the fatty acids produced by oxidation of the double bonds was used to establish the detailed structure of the three major fractions. A large percentage of these had branched methyl groups in either the *iso* or *anteiso* configuration on both terminae and the double bonds were near the center of the primary chain. This is consistent with biosynthesis by head-to-head condensation of two fatty acids. Also, because 36% of the fatty acids obtained by oxidation of the double bonds of the hydrocarbons were found to be a C-14 *anteiso* fatty acid which was not found in the lipid fatty acids, decarboxylation of one of the fatty acids during condensation probably occurs. In further support for this biosynthetic mechanism, long-chain ketones and secondary alcohols that would be expected as intermediates in such a pathway were isolated from the nonsaponifiable lipids of *S. lutea*.

Two major pathways for the biosynthesis of long-chain nonisoprenoid hydrocarbons have been proposed. The first was proposed for the synthesis of hydrocarbons in the wax of *Brassica oleracea* by Chibnall and coworkers (Channon and Chibnall, 1929; Chibnall and Piper, 1934; Sahai and Chibnall, 1932), and involved the condensation of two moles of fatty acid with decarboxylation of one of them as follows:

$$RCH_2COOH + R'CH_2COOH \longrightarrow CO_2 + RCH_2C(=O)CH_2R' \longrightarrow RCH_2CH(-OH)CH_2R \longrightarrow RCH_2CH{=}CHR' \longrightarrow RCH_2CH_2CH_2R'$$

In support of this mechanism, they demonstrated the presence of the ketone and secondary alcohol that would serve as the intermediates in the synthesis of the major hydrocarbon, nonacosane, from 2 moles of pentadecanoic acid. They were unable to find the alkene that would also be an intermediate or pentadecanoic acid itself. This mechanism or variations of it have been revived and modified to explain hydrocarbon synthesis in *Pyrethrum* cuticle wax (Wanless *et al.*, 1955) and *Nicotiana tabacum* (Kaneda, 1968). Kaneda (1967) earlier showed that free ketones and alcohols were probably not incorporated into hydrocarbons, and he has postulated enzyme-bound intermediates to explain evidence, primarily based on the incorporation of labeled octanoic acid into branched-chain hydrocarbons, that head-to-head condensation of fatty acids is involved. Kolattukudy (1966) has presented evidence against the condensation mechanism in *B. oleracea*, and he supports a mechanism (Kolattukudy, 1966, 1967) that has evolved from proposals by Chibnall and Piper (1934), Stone and ZoBell (1952), Wanless *et al.* (1955), and Stumpf (1965). In general, the mechanism involves the elongation of a fatty acid with acetate, α or β oxidation, decarboxylation, and reduction to the hydrocarbon as follows:



The long-range goal of this study is to determine whether either, both, or neither of these pathways is involved in the biosynthesis of hydrocarbons in *Sarcina lutea*. The hydrocarbons in *S. lutea* were first described by Albro and Huston (1964), and they found in cells

* From the Department of Biochemistry, St. Louis University Medical School, St. Louis, Missouri 63104. *Received August 26, 1968.* This work is part of a dissertation submitted by P. W. A. in partial fulfillment of the requirements for the degree of Doctor of Philosophy and was supported by Grant AM-09766 of the U. S. Public Health Service.

† Predoctoral Fellow, Institute of General Medical Sciences, U. S. Public Health Service. Present address: Chemical Section, National Environmental Health Science Center, P. O. Box 12233, Research Triangle Park, N. C. 27709.

TABLE I: Composition of M73b Medium.

Ingredient	g/l.	Ingredient	mg/l.
Vitamin-free Casamino Acids[a]	17.0	Uracil	3.0
Bacto-Peptone[a]	3.0	Xanthine	3.0
Glucose	3.0	Adenine sulfate	3.0
$CH_3COONa \cdot 3H_2O$	2.0	Guanine hydrochloride	3.0
KH_2PO_4	0.5	Riboflavine	0.5
K_2HPO_4	0.5	Calcium *d*-pantothenate	0.4
$MgSO_4 \cdot 7H_2O$	0.15	Nicotinic acid hydrochloride	0.4
NaCl	0.012	Thiamine hydrochloride	0.4
$FeSO_4 \cdot 4H_2O$	0.007	Pyridoxine hydrochloride	0.4
$MnSO_4 \cdot 4H_2O$	0.007	Pyridoxal hydrochloride	0.04
		p-Aminobenzoic acid	0.08
		Biotin	0.002
		Folic acid	0.002

[a] Difco, Detroit, Mich.

grown to late stationary phase that the hydrocarbons were primarily saturated and the major fractions had 27, 28, and 29 carbons. Subsequently, Tornabene *et al.* (1967a,b) reported that the hydrocarbons of *S. lutea* (ATCC 533) in early stationary phase were primarily monounsaturated and the major fractions had 25, 26, and 27 carbons. We have examined the hydrocarbons from a recently acquired culture of ATCC 533 and have confirmed their results in regard to the general distribution of the hydrocarbons; however, we have found by a variety of criteria that the culture obtained from the American Type Culture Collection is not *S. lutea*. We report here details of these findings, more detailed characterization of the hydrocarbons of an adequately identified strain of *S. lutea*, and some implication of the structure of the hydrocarbons on the biosynthetic pathway in this organism. Long-chain ketones and secondary alcohols that may be intermediates in biosynthesis have been identified.

Experimental Section

Bacterial Strains and Culture. This report is primarily concerned with an isoleucine-requiring strain of *S. lutea* obtained from the Department of the Army, Physical Defense Division, Fort Detrick, Frederick, Md., and listed in their collection by the designation FD-SCC-D-533. This is the same strain used by Albro and Huston (1964) and is believed to have originated from the American Type Culture Collection, strain ATCC 533. This strain will be referred to in this report as FD-533. A strain of *S. lutea* recently obtained from the American Type Culture Collection, ATCC-533, and presumably the same strain studied by Tornabene *et al.* (1967a,b) was also studied; this strain is designated as ATCC-533 in this report.

Cells were cultured in 1 l. of media in 2800-ml fernbach flasks on a rotary shaker driven at 200 rpm. Growth temperature varied from 23 to 25°. Each liter batch was started with 50 ml of a log-phase culture and the growth times given are from the time of inoculation to harvest. Cells were harvested by centrifugation at 4°. Two media were used: trypticase soy broth prepared as specified by the supplier (Baltimore Biological Laboratories, Baltimore, Md.) and a semidefined medium (Table I) for which the laboratory designation was M73b. The trypticase soy broth medium was found by extracting the original powder with chloroform–methanol and fractionating the extract as described below to have 0.62 mg of long-chain hydrocarbons/l. No lipid could be detected in extracts of freeze-dried M73b media.

Extraction of Lipids and Isolation of the Hydrocarbons. Because of difficulty in obtaining complete extraction of all of the lipid with neutral solvent systems, several alternative extraction procedures were used. In general, harvested cells were either extracted immediately or freeze dried for storage. Slow degradation of lipid occurred in moist cells even when stored at −20°. When neutral solvents were used, lyophilized cells were extracted as described by Huston and Albro (1964), and wet cells were extracted with chloroform–methanol (1:1 and 3:1) essentially as described for brain by Wells and Dittmer (1965). The equivalent of 20 times the packed volume of cells was used of each solvent and the residue was filtered with glass filter paper (Reeve Angel 934AH, chloroform washed) after each extraction. The combined extracts were partitioned with two-tenths volume of 1.19% aqueous KCl (Folch *et al.*, 1957).

When the cell residue from either extraction procedure was saponified and extracted as described by Huston and Albro (1964) or simply extracted with chloroform–methanol–12 N HCl (124:65:1) (Letters, 1966), additional lipid including hydrocarbon was obtained. No further lipid, fatty acids, or hydrocarbons could be detected in extracts of the residue left in either of the latter two procedures after refluxing with

TABLE II: Gas–Liquid Partition Chromatography Columns Used in This Study.[a]

Column	Liquid Phase	Support	Column Size (cm)	Temp	Carrier Gas and Flow Rate (cc/min)
1	3% SE-30 (methyl silicone)	80–100 mesh acid-washed silanized Chromsorb W	61 × 0.5	Gradient, 175–280° at 10°/min	Argon, 50
2	3% SE-52 (methyl phenyl silicone)	80–100 mesh Chromsorb W	305 × 0.3	Gradient, 175–270° at 5.6°/min	Helium, 82
3	5% cyclohexanedimethanol succinate	60–80 mesh Gas Chrom Z	248 × 0.4	220 or 230°	Argon, 60
4	3% cyclohexanedimethanol succinate	Same as column 1	248 × 0.4	160°	Argon, 60
5	3% W-98 (methyl vinyl silicone)	Same as column 1	184 × 0.4	Gradient, 190–275° at 7.5°/min	Argon, 60
6	3% W-98	Same as column 1	30 × 1.0 reduced to 80 × 0.4	Gradient, 150–270° at 10°/min	Argon, 85

[a] Liquid phases and supports were obtained from Applied Sciences Laboratories, State College, Pa. Columns 1, 5, and 6 were used for the fractionation of hydrocarbons, fatty acid methyl esters, and ketones with separation primarily on the basis of the number of carbons. Column 2 gave similar fractionations of hydrocarbons but with greater resolution. Columns 3 and 4 were used for the separation of fatty acid methyl esters and hydrocarbons and resolved compounds on the basis of the type of methyl side-chain branching as well as the number of carbons.

6 N HCl. When the harvested cells were extracted directly with acidified chloroform–methanol all of the lipid which was otherwise obtained by extracting in succession with neutral and acidified chloroform–methanol was extracted. The acidified chloroform–methanol extracts were routinely washed with 1.19% KCl solution as described above. A portion of the lipid not extracted with neutral chloroform–methanol was extracted with chloroform–methanol (2:1) if the cell residue was treated with lysozyme. The extracted cell residue was suspended in 50 volumes of 0.5% aqueous NaCl, 20 mg of crystalline lysozyme (Calbiochem) was added, and the suspension was stirred for 2 hr at 30° (Herbert, 1955). The mixture was then lyophilized and the lipids were extracted with 20 volumes of chloroform–methanol (2:1). The extract was partitioned with two-tenths volume of 1.19% KCl.

The hydrocarbons were isolated from extracts by a combination of silicic acid and Florisil chromatography. The lipids in hexane were loaded (20 mg/g) on a silicic acid (Mallinckrodt, 100 mesh) column made up in hexane (Fisher, certified) and the hydrocarbons were eluted with hexane (7 ml/g of silicic acid). This fraction was concentrated *in vacuo* and chromatographed with a loading of 10 mg/g on a Florisil (60–100 mesh, Varian Aerograph, Walnut Creek, Calif.) column made up in hexane. Seven per cent by weight of water was added to the Florisil and allowed to equilibrate for at least 1 week in a closed container before it was used. The hydrocarbons were eluted with hexane (7 ml/g of adsorbent).

Fractionation of the Hydrocarbons. GAS–LIQUID PARTITION CHROMATOGRAPHY. Several different columns were used for the various gas–liquid partition chromatography fractionations of hydrocarbons, fatty acid methyl esters, and long-chain ketones and pertinent data for these columns are given in Table II. Most fractionations were run on a Barber-Colman series 5000 apparatus equipped with dual argon ionization detectors. It was modified by the addition of a heated outlet port which terminated in a male leur fitting. Fractions were manually collected in Pasteur pipets packed with Florisil coated with 3% Carbowax 20M (Matheson Coleman and Bell). The pipet was fitted with a silicone septum and attached by way of a 13-gauge syringe needle to the gas chromatograph outlet. Collected fractions were eluted from the adsorbent with benzene–chloroform (1:1) (8 ml/g of Florosil–wax). Recoveries as determined by rechromatography or recovery of ^{14}C label were from as little as 50% for fatty acid methyl esters to 95% for C-22 to C-30 hydrocarbons.

Fractions resolved from fatty acid methyl ester mixtures were identified by comparison with branched-chain fatty acid standards obtained from Applied Sciences Laboratories, State College, Pa. Similar standards were not available for branched-chain hydrocarbons and identification of major fractions were made on the basis of the chromatographic behavior of normal saturated and unsaturated alkanes (LaChat Chemicals, Chicago), infrared analysis of isolated fractions, the behavior of monounsaturated hydrocarbons before and after hydrogenation, and the analysis of the fatty acids

obtained by oxidation of the double bond of the isolated fractions. By a combination of these techniques, it was found that the equivalent chain lengths as calculated from the relative retention times (Miwa, 1963) on the cyclohexanedimethanol succinate liquid phase (column 3, Table II) decreased from that for a normal alkane with the number of carbons in the primary aliphatic chain of the hydrocarbon by an increment of 0.65 and 0.75 for each *iso* and *anteiso* methyl branched chain, respectively, and decreased by an increment of 0.2 when there was a double bond near the center of the molecule. Comparison of the increase of the equivalent chain lengths after hydrogenation of heptacos-13-ene and tricos-11-ene (+0.20) with that of alk-1-enes (+0.50) and of 1,13-heptacosadiene (+0.70) indicated that the change due to unsaturation decreases to a minimum of 0.2 as the double bond is found closer to the center of the molecule. Essentially the same change in the equivalent chain lengths due to *iso* and *anteiso* branches was found with fatty acid methyl esters on this column.

THIN-LAYER CHROMATOGRAPHY. Class purity of the hydrocarbons and fractionation on the basis of the number of double bonds were as described by Albro and Huston (1964). In addition, *cis* and *trans* isomers of monounsaturated hydrocarbons were separated on 500-μ silica gel G (Merck, Darmstadt, Germany) plates that had been sprayed with 10% aqueous silver nitrate and air dried (Morris, 1962). Normal hexane was used as solvent and *cis*- and *trans*-heptacos-13-enes (Lachat Chemical Corp., Chicago) were used as reference standards. Quantitative data were obtained by reisolation of the compounds and gas–liquid partition chromatography with an internal standard (Hornstein *et al.*, 1960) or by reduction of dichromate (Amenta, 1964).

Permanganate–Periodate Oxidation. Since 90% of the hydrocarbons from 48-hr cultures of FD-533 were unsaturated, oxidation at the double bond to form acids characteristic of the aliphatic groups to either side of the bond was particularly useful in elucidating the structure of the parent hydrocarbon. Oxidation was done by a modification of the general permanganate–periodate oxidation procedure described by von Rudloff (1956). Hydrocarbon (5 mg) was dissolved in 4 ml of *t*-butyl alcohol to which was added and mixed 1 ml of 0.002 M Na_2CO_3, 2 ml of 0.01 N sodium periodate–0.0025 N $KMnO_4$, and 3 ml of water. The resulting emulsion was vigorously stirred for 3 hr at 25° and decolorized by the addition of sodium metabisulfite. NaOH (1 ml of 1 N) was added and the mixture was concentrated *in vacuo* to remove *t*-butyl alcohol. Water (5 ml) was then added and the mixture was partitioned twice with 10 ml of hexane to remove saturated and unreacted hydrocarbons. After acidifying the water phase, the fatty acids were extracted from it with two 10-ml aliquots of diethyl ether. The ether extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo*.

The efficiency of the oxidation was checked by determining the recovery of radioactivity in the fatty acid fraction after the oxidation of ^{14}C-labeled unsaturated hydrocarbons. Cells (FD-533) were grown up in the presence of acetate-2-^{14}C and the hydrocarbons were isolated and freed of saturated molecules by the formation of mercuric acetate adducts. After oxidation of the unsaturated hydrocarbons, 98% of the radioactivity was recovered in the fatty acid fraction.

Results

Characterization of the Bacteria and General Characteristics of the Fatty Acids and Hydrocarbons. Characteristics of the strain of *S. lutea*, FD-533, from the Fort Detrick collection which probably originated from the American Type Culture Collection strain 533 and a culture of this strain recently obtained from the ATCC are summarized in Table III. We have also indicated the characteristic where specified in the 7th edition of Bergey's Manual (Breed *et al.*, 1957) and summarized the data on the distribution of fatty acids and hydrocarbons as reported for ATCC-533 by Tornabene *et al.* (1967 a,b). It is our conclusion that these data show that the strain ATCC-533 which we have is not *S. lutea*, and with respect to fatty acid and hydrocarbon content, it more nearly resembles the organism studied by Tornabene *et al.* than does the authentic culture of *S. lutea*, FD-533.

Factors Affecting Yield and General Properties of Hydrocarbons. The hydrocarbons extracted from early stationary-phase cells with neutral chloroform–methanol represented only 20% of the total that could be extracted with acidified chloroform–methanol or extracted after saponification of the cells. An approximately additional 20% of the hydrocarbon could be extracted with neutral chloroform–methanol when the cell residue was treated with lysozyme. The remaining 60% was extracted with acidified solvent or after saponification. In general a larger proportion of the hydrocarbons was extracted with neutral chloroform–methanol from cells grown in the M73b media than from cells grown in trypticase soy broth. The proportions indicated above were for early stationary-phase cells and showed some variability with different batches of cells. The growth phase itself was found to alter the proportions and these differences are shown in Figure 1.

Usually the failure to extract lipids with neutral solvent is associated with polar lipids as with the polyphosphoinositides in brain (*e.g.*, Dittmer and Dawson, 1961) and phospholipids in yeast (Letters, 1966). In the latter case, the extent to which the cells are broken up is also a factor (Harrison and Trevelyan, 1963), and the effect of treatment with lysozyme suggests that this may also be a factor with *S. lutea*. *S. lutea* extracted with neutral solvent appeared intact under the microscope although the packets or octads of the cells were dissociated. Cells extracted with acidified chloroform–methanol appeared to have been shredded or broken up. Additional parameters appeared to be involved in this phenomena in *S. lutea*. When ^{14}C-labeled octadecane (Nuclear-Chicago) was added to log-phase cultures of FD-533, it was taken up from the media completely by the cells and was, even after 7 hr, completely recovered from the cells by extraction with neutral chloroform–methanol. This suggested that there may be at least two distinct pools of hydrocarbons in the cells that are distinguished by

TABLE III: Characterization of FD-533 and ATCC-533 Strains of Bacteria.

Characteristic	FD-533	ATCC-533	Published[a]
Agar colonies			
Size (mm)	4–5	<1	
Morphology	Raised, circular, and smooth	Crenulated	Raised, circular, entire
Color	Yellow	Yellow	Yellow
Tactility	Soft	Tough	Soft
Broth culture			
Optimum temperature (°C)	25	30	25
Cell morphology	Spheres in octads	Spheres, single and in chains	Spheres in packets
Cell diameter (μ)	1.3	0.9	1.0–1.5
Metabolism			
HS produced	+	−	+
Nitrate reduced	−	−	±
Indole produced	−	−	Slight
Litmus milk	Coagulated, alkaline	Coagulated, acid	Coagulated, alkaline
Isoleucine required	+	−	
Pigment released into medium	−	+	
	Total Lipid Fatty Acids[b]		
C_{11}–C_{12}	Trace	Trace	0.23
br-13	0.3	0.3	1.82
n-13	Trace	Trace	
br-14	1.6	0.4	4.14
n-14	0.2	0.9	6.45
br-15	94.1	89.5	70.09
n-15	0.7	Trace	
br-16	1.9	1.9	5.97
n-16	0.5	2.0	5.4
br-17	0.8	2.8	4.14
n-17	Trace	0.1	
br-18	0	1.6	
n-18	0	0.7	0.14
	Hydrocarbons[b]		
C_{23}	0.3	1.3	0.7
C_{24}	0.1	3.4	4.4
C_{25}	0.9	36.3	13.4
C_{26}	1.6	7.8	28.6
C_{27}	18.4	46.3	24.8
C_{28}	12.7	1.8	23.7
C_{29}	65.3	2.9	2.5
C_{30}	0.4		

[a] Published characteristics for colonies, cultured cells, and metabolism of *S. lutea* are taken from Bergey's Manual (Breed *et al.*, 1957). The published values for fatty acid composition of the lipids and hydrocarbon composition are taken from Tornabene *et al.* (1967) and are for strain 533 of *S. lutea* from the American Type Culture Collection. [b] Distribution given in terms of the per cent of the total area under a gas–liquid partition chromatography tracing. Fractionations were done on column 2 of Table II.

the ease with which they are extracted. The fact that the proportion of the hydrocarbon not extracted with neutral solvent decreased during log phase to a minimum in early stationary phase, and then increases again during stationary phase (Figure 1), was consistent with not only physically distinct pools (in terms of extractability) but perhaps even metabolically distinct pools. In this respect, it was surprising to find that the hydrocarbon extracted with neutral solvent with or without lysozyme treatment and the "bound" hydrocarbon that could be

TABLE IV: Gas–Liquid Partition Chromatography of FD-533 Hydrocarbons from Cells Grown on Trypticase Soy Broth and M73b Medium.[a]

Equivalent Chain Length[b]	% Distribution[c] Trypticase Soy Broth	M73b
<20	3.8	0.2
20.2		Trace[d]
22.5	0.3	0.1
23.0	0.2	0.1
23.5	Trace	Trace
24.0	0.3	0.2
24.5	0.6	0.4
25.3	1.4	0.9
25.5	Trace	Trace
26.0	10.3	24.0
26.5	8.1	0.9
27.0	0	0
27.3	12.4	14.5
27.5	0.3	0.4
28.4	65.3	57.8
29.4	0.2	0.2
30.2	0.2	0.1
>30.2	Trace	Trace

[a] The hydrocarbons were from cells grown for 48 hr and were separated on column 2 of Table II. [b] Equivalent chain length as calculated by Miwa (1963). [c] Calculated on the basis of the per cent of the total area under each peak on the gas–liquid partition chromatography tracing. [d] Less than 0.1%.

extracted only with acidified solvents when examined on SE-52 and XE-60 gas–liquid partition chromatography columns appeared to have the same quantitative distribution of components.

Finally, in regard to other factors affecting the hydrocarbons, the growth curves for FD-533 grown in either M73b or trypticase soy broth media were essentially identical and the curve shown in Figure 1 for trypticase soy broth is typical. The hydrocarbons of 48-hr cultures in either media showed only small differences when separated by gas–liquid partition chromatography on SE-52 columns (Table IV). One observation that may be of some potential interest was that the pH of the media changed during growth with either media, and the change correlated with hydrocarbon content. The pH drops from 7.2 at start of incubation to 6.0 at 30 hr or the end of log phase and then rises to pH 8.0 by the 48th hr.

Characterization of the Hydrocarbons of FD-533. THIN-LAYER CHROMATOGRAPHY. In 48 hr, trypticase soy broth shake cultures, 9–10% by weight of the hydrocarbon, as determined by the distribution of the mercuric acetate adducts, were saturated. This compares with 89% saturated hydrocarbons in 84-hr cultures grown in the same media aerated with a sparger as reported by Albro and Huston (1964) or 22.6% saturated when grown in shake flasks. Approximately 1.3% of the hydrocarbons were polyunsaturated in log phase and this decreased to 0.2–0.4% in stationary phase with cells grown in either trypticase soy broth or M73b media in shake flasks. The monounsaturated hydrocarbons represented approximately 90% of the total in 48-hr trypticase soy broth cultures and when these were chromatographed on silver nitrate impregnated silica gel plates no *trans* isomers could be detected.

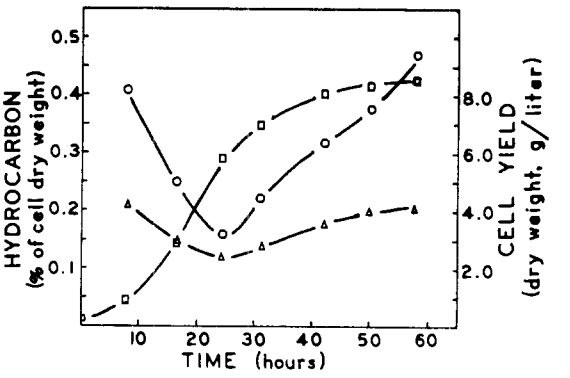


FIGURE 1: Changes in the content of hydrocarbon with growth in a trypticase soy broth culture of FD-533. Total hydrocarbon extracted with acidified chloroform–methanol (○), hydrocarbon extracted with neutral chloroform–methanol (△), and weight of dry cells per liter of culture medium (□).

INFRARED SPECTROSCOPY. A Perkin-Elmer Model 21 spectrophotometer was used to examine hydrocarbons spread as films on NaCl or AgCl plates or in CCl_4 solution. Spectra of the total hydrocarbon fraction or of the saturated and unsaturated hydrocarbons scanned separately showed no indication of the presence of *t*-butyl groups, three- to six-membered rings, phenyl groups, conjugated unsaturation, carbon-to-carbon triple bonds, vinyl groups, or allenes on the basis of the characteristic group frequencies given by Szymanski (1967) and Rao (1963). The saturated hydrocarbons, freed of unsaturated ones by two treatments with mercuric chloride followed by elution from Mallinckrodt silicic acid with *n*-hexane, gave spectra typical of long-chain branched alkanes (Szymanski, 1967; O'Conner, 1960). In particular, absorption at 7.25 and 7.35 μ (1379 and 1361 cm^{-1}), which was stronger at 7.25 μ, indicated the presence of both *iso* and *anteiso* branching. These assignments of absorption were confirmed by the presence of a peak at 12.9 μ (765 cm^{-1}) which indicated the presence of an isolated ethyl group (Wheeler, 1954), a band at 7.45 μ (1342 cm^{-1}) attributable to a carbon at a branch point, and the absence of bands at 13.5 and 8.8 μ (741 and 1150 cm^{-1}) which would have indicated the presence of *n*-propyl groups and quaternary carbons, respectively (Wheeler, 1954). Saturated hydrocarbons spread as thin films showed a doublet at 13.75, 13.95 μ (728, 717 cm^{-1}) due to orthorhombic packing of polymethylene chains of four or more sequential methylene groups (Chapman, 1965). This doublet was found only with solid-state spectra.

The unsaturated hydrocarbons, isolated by regeneration from their mercuric acetate adducts and by thin-layer

TABLE V: Comparison of the Distribution of FD-533 Hydrocarbons on SE-30 and Cyclohexanedimethanol Succinate Gas–Liquid Partition Chromatography Columns.[a]

Reduced Carbon No.[b]	% Distribution						
	SE-30	Cyclohexanedimethanol Succinate					
	0.0	0.1	0.2	0.3	0.4	0.5	0.7
<20	2.3						
21	Trace						
22	0.5	0.2		0.3			
23	0.2	Trace		Trace		Trace	Trace
24	1.5	0.8			0.8		
25	1.5	0.1		1.3		0.2	
26	18.4	9.8	2.0		5.9		
27	12.9	1.0	11.3			0.6	0.4
28	65.3	4.0	11.3		48.8		Trace
29	0.5	0.4			0.2		
30	0.2	Trace			Trace		

[a] The conditions for gas–liquid partition chromatography are given in Table II, columns 1 and 3, respectively. The hydrocarbons were from cells grown in trypticase soy broth for 48 hr. [b] The whole number portion of the equivalent chain length. The fractional portion is indicated in the series of columns to the right.

chromatography on silver nitrate impregnated silica gel plates, gave spectra nearly identical with those from the saturated hydrocarbons. In addition, small bands at 3.27–3.30, 6.03–6.08, and 14.4 μ (3030–3660, 1645–1659, and 695 cm^{-1}) indicated the presence of isolated *cis* double bonds (Szymanski, 1967). Since this fraction was a liquid at room temperature, the 13.75, 13.95 μ doublet observed with the saturated hydrocarbons was replaced by a broad band at 13.9 μ (720 cm^{-1}).

The infrared spectra were consistent with the hydrocarbons being primarily a mixture of saturated and unsaturated, long-chain aliphatic compounds with *iso* and *anteiso* branch chains and *cis* double bonds. The possibility of the occurrence of ethyl branches cannot be ruled out.

GAS–LIQUID PARTITION CHROMATOGRAPHY AND MOLECULAR SIEVE FRACTIONATION. Gas–liquid partition chromatography on SE-30 (column 1 of Table II) separated the hydrocarbons primarily on the basis of the number of carbons in the main aliphatic chain and methyl branch chains increased the equivalent chain length by 0.5 unit. Since, as is shown on the basis of the oxidation studies described below, most of the hydrocarbons have branched chains at both ends, the equivalent chain lengths of the major fractions are whole numbers and are one unit larger than the number of carbons in the main aliphatic chain. Fractionation on cyclohexanedimethanol succinate (column 3 of Table II) separated the hydrocarbons further on the basis of the types of branched chains present as described in detail in the Methods section. This is clearly seen from the data on the equivalent chain lengths and distribution of fractions given in Table V.

When the hydrocarbons were hydrogenated for 16 hr in *n*-heptane with PtO_2 as catalyst and chromatographed on cyclohexanedimethanol succinate, all of the components comprising more than 0.2% of the total showed an increase in the equivalent chain lengths of 0.2 unit. This was consistent with the thin-layer chromatography and infrared data which indicated that the hydrocarbons were composed predominantly of monoenes.

Molecular sieve treatment of the hydrogenated total hydrocarbons on Type 5A Sieve (Union Carbide) by the procedures of Mold *et al.* (1963) and O'Connor and Norris (1960) showed that 95–97% by weight of the hydrocarbon were excluded and presumably had branched chains or ring structures. Ring structures were, as indicated above, found to be absent by infrared spectroscopy.

These data gave only an indication of the complexity of the hydrocarbon mixture and additional information was obtained by more detailed examination by thin-layer chromatography and gas–liquid partition chromatography of the three major fractions, those with 27, 28, and 29 carbons, and by oxidation of the double bonds of the hydrocarbons.

Characterization of the C-27, C-28, C-29 Hydrocarbons and Permanganate–Periodate Oxidation Studies. Monoenes make up more than 90% by weight of the total hydrocarbon fraction from 48-hr trypticase soy broth cultures and the data in Tables IV and V show that approximately 97% of the hydrocarbons have 27, 28, or 29 carbons (equivalent chain lengths 26, 27, or 28). Each of these three "families" of hydrocarbons were separately collected by preparative gas–liquid partition chromatography (column 6 of Table II). Rechromatography on cyclohexanedimethanol succinate (column 3 of Table II) showed that each fraction was homogeneous with respect to the total carbon numbers.

TABLE VI: Distribution of Total Lipid FattyAcids and Fatty Acids from Oxidation of Hydrocarbons.[a]

Fatty Acid[b]	% Distribution[c]				
	Total Lipid	Total Hydrocarbon	C_{27} Hydrocarbon	C_{28} Hydrocarbon	C_{29} Hydrocarbon
br-10	Trace	Trace	0.1	Trace	Trace
n-10	Trace	Trace	0.1	Trace	Trace
i-11	Trace	Trace	0.1	Trace	Trace
a-11	Trace	Trace	0.7	Trace	Trace
n-11	Trace	Trace	0.1	Trace	Trace
i-12	Trace	1.0	5.3	2.5	Trace
a-12	0	0.3	15.7	4.8	Trace
n-12	Trace	Trace	0.5	5.1	Trace
i-13	0.1	2.7	26.4	10.7	0.1
a-13	0.2	1.2	6.4	5.6	Trace
n-13	Trace	0.5	0.4	3.8	Trace
i-14	1.6	7.8	14.5	17.0	7.1
a-14	0	36.0	5.6	3.4	43.3
n-14	0.2	1.5	7.6	3.3	0.2
i-15	35.9	7.1	1.3	5.5	7.1
a-15	58.2	35.5	9.3	13.2	40.2
n-15	0.7	0.6	3.4	1.3	0.1
i-16	1.9	0.7	1.0	4.6	0.4
a-16	0	0.4	Trace	9.8	0.1
n-16	0.2	3.9	Trace	5.6	1.2
i-17	0.1	0.8	0.2	1.0	0.3
a-17	0.7	Trace	0.3	2.8	Trace
n-17	Trace	Trace	1.1	Trace	0.2
>17	0	0	0	0	0

[a] Lipids from 48-hr trypticase soy broth culture of FD-533. Fatty acids analyzed on column 4 of Table II. [b] *br* = branched, *i* = *iso*, *a* = *anteiso*, *n* = *normal*. The number given is the number of carbon atoms in the fatty acid. [c] Calculated as the per cent of the total area under each peak on the gas–liquid partition chromatography tracing.

Samples of the total hydrocarbon fraction, total monoenes isolated by thin-layer chromatography, and the C-27, C-28, and C-29 fractions were subjected to permanganate–periodate oxidation. The fatty acids were converted into methyl esters as described by Metcalfe and Schmidtz (1961) and analyzed by gas–liquid partition chromatography on 3% cyclohexanedimethanol succinate (column 4 of Table II). The total hydrocarbons and total monounsaturated hydrocarbons gave essentially identical results and the average distribution of the fatty acids derived from them are given in Table VI under "total hydrocarbons." The fatty acids produced by saponification of the total extractable lipid from the same culture from which this sample of hydrocarbons was obtained were also analyzed and the data are included in Table VI. If one assumes a random distribution of the various aliphatic groups as associated with each other in the monounsaturated hydrocarbons, the data in Table VI are consistent with over 200 different individual components in the hydrocarbons. Obviously complete isomeric composition cannot be determined on this basis but several general observations can be made and these have been in part summarized in Table VII.

The C-29 hydrocarbons were resolved into three fractions on cyclohexanedimethanol succinate (Table V) for which the equivalent chain lengths indicated structures corresponding to *iso*, *iso'*, *iso*, *anteiso'*, and *anteiso*, *anteiso'* configurations of the two aliphatic terminae. The fatty acids produced on oxidative cleavage of the double bonds are almost exclusively branched *iso*- and *anteiso*-C-14 and C-15 acids. Also 98% of these hydrocarbons have the double bond at C-13. The acids found could be combined in only four different ways to give C-29 hydrocarbons meeting these specifications. From this it is possible to calculate that the C-29 monounsaturated hydrocarbons consisted of the components indicated in Table VIII.

Three other observations based on the data in Table VI were of particular significance to the possible biosynthetic pathways for these hydrocarbons. Only 6.5% of the fatty acids from oxidation of the total hydrocarbons have straight chains, no fatty acids of greater length than the longest fatty acid present in the saponifiable lipids were produced by oxidation of the monounsaturated hydrocarbons, and *anteiso*, even carbon-numbered fatty acids, which were not found in the saponifiable lipids, were found in the hydrocarbon oxidation prod-

TABLE VII: General Characteristics of C-27, C-28, and C-29 Hydrocarbons of FD-533.

Configuration and Double-Bond Position	Per Cent		
	C-27	C-28	C-29
Distribution of the Structure of the Aliphatic Terminae			
iso	48.8	37.8	15.0
antesio	38.1	32.0	83.7
n	13.2	19.1	1.3
Distribution with Respect to the Position of the Double Bond in the Primary Hydrocarbon Chain			
Δ^9	0.6		
Δ^{10}	1.8		
Δ^{11}	28.0	25.0	
Δ^{12}	55.0	40.0	
Δ^{13}		24.0	98.0
Other	14.0	11.0	2.0

TABLE IX: Fatty Acids Obtained by Periodate–Permanganate Oxidation of ATCC-533 Hydrocarbons.

Fatty Acid	% Distribution
br-11	0.9
n-11	Trace
br-12	28.1
n-12	Trace
br-13 (*iso*)	38.0
n-13	0.5
br-14 (*iso* + *anteiso*)	31.3
n-14	Trace
br-15	0.4
n-15	Trace
br-16 (*anteiso*)	0.3
n-16	0.5
Others	Traces

ucts. The significance of these observations will be pointed out in the Discussion.

Fatty Acids Produced by Oxidation of ATCC-533 Hydrocarbons. The data for the distribution of the fatty acids obtained by oxidation of the hydrocarbons of a 48-hr trypticase soy broth culture of ATCC-533 are given in Table IX. The most striking difference between this *Micrococcus* and *S. lutea* is in the much larger proportions of branched-chain C-13 and C-12 fatty acids. Since most of the fatty acids obtained by oxidation have branched chains, it must be concluded that most of the hydrocarbons have both terminae branched.

Isolation of Possible Intermediates in the Biosynthesis of S. lutea Hydrocarbons. In 48-hr cultures of FD-533, most of the hydrocarbons were monounsaturated and the position of the double bond was consistent with a biosynthetic pathway involving the head-to-head condensation of fatty acids. If this is the pathway, then both long-chain ketones and secondary alcohols may be intermediates. Accordingly, FD-533 lipid extracts were saponified either by refluxing in 20 volumes of 1 N KOH in water–ethanol (3:1) or by heating for 3 hr in a closed tube at 100° with 20 volumes of 2 N KOH in 50% aqueous ethanol. The nonsaponifiable lipids were extracted with petroleum ether (bp 30–60°) and the ether extract was washed in succession with 1 M Na_2CO_3, water, and saturated NaCl, dried over anhydrous Na_2SO_4, filtered through glass wool, and concentrated *in vacuo*. This lipid was passed through a column of Florisil in ten volumes of ethyl acetate–acetone (9:1) to remove any contaminating traces of fatty acids or acidic pigments.

TABLE VIII: Composition of the C-29 Hydrocarbons.

Component	%
2,26-Dimethylheptacos-13-ene	6.1
2,25-Dimethylheptacos-13-ene	8.7
3,26-Dimethylheptacos-13-ene	8.7
3,25-Dimethylheptacos-13-ene	74.0
Other isomers	2.3

Two-dimensional thin-layer chromatography on silica gel G plates developed in benzene–chloroform (9:1) (first direction) and petroleum ether–diethyl ether–acetic acid (90:10:1) (second direction) were run. Spots were visualized by spraying first with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl followed by 0.005% Rhodamine 6G and finally with H_2SO_4–HNO_3–H_2O (5:3:3) for charring at 180°. A tracing of a typical chromatogram is shown in Figure 2. The lipid classes detected were tentatively identified by means of the *in situ* functional group analyses described by Purdy and Truter (1963) and by cochromatography with standards. On this basis spots corresponding to hydrocarbons, vitamin K's, aliphatic ketones, aldehydes, primary and secondary alcohols, and at least two hydroxylated pigments were identified.

Our attention at this time was concentrated on establishing the identity of the ketones and secondary alcohols with the possibility of subsequently establishing whether they were hydrocarbon intermediates. The ketones were isolated in pure form after reaction with 2,4-dinitrophenylhydrazine as described by Cheronis (1954). The monocarbonyl derivatives were isolated with the column procedure of Schwartz *et al.* (1963) and the ketone derivatives were separated from these with Magnesia 2665 as described by Schwartz *et al.* (1962) or by first regenerating the free carbonyl compounds (Demaecker and Martin, 1954) followed by removal of the aldehydes with bisulfite (Cheronis, 1954). The dinitrophenylhydrazine derivatives of these ketones had a λ_{max} at 361 mμ in 95% ethanol and a peak at 525 mμ which was stable in 1% ethanolic NaOH (Jones *et al.*, 1958), as did the derivative of authentic 12-tricosanone. Infrared spectra of CCl_4 solutions of the free ketones showed the expected (Rao, 1963; Kawada *et al.*, 1966) bands at 7.10 μ (CH_2 adjacent to C=O) and 7.31 and 8.90 μ (aliphatic chain with more than two carbons). Methyl ketones

TABLE X: Distribution of Ketones and Hydrocarbons from a 48-Hr Trypticase Soy Broth Culture of FD-533.

No. of Carbons	Ketones RRT[a]	Ketones % Distribution	Hydrocarbons RRT[a]	Hydrocarbons % Distribution
<20	≤0.48	7.6	≤0.28	3.1
20	0.60	2.4	0.36	0.4
21	0.78	1.6	0.47	0.1
22	1.04	2.3	0.64	0.3
23–24	1.31	1.8	0.81	0.2
25	1.70	7.8	1.05	0.9
26	2.11	3.8	1.32	1.4
27	2.76	18.3	1.74	18.4
28	3.42	10.5	2.16	12.4
29	4.47	34.6	2.85	65.3
30	5.51	6.7	3.50	0.2
31	6.96	2.8	4.10	0.2

[a] Relative retention times calculated with *n*-tetracosane = 1 at 275°. Fractionations were carried out on column 1 of Table II.

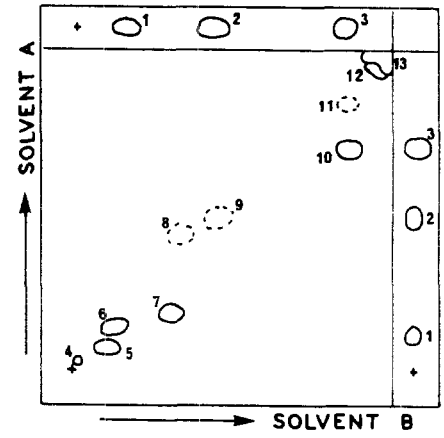


FIGURE 2: Thin-layer chromatogram of FD-533 nonsaponifiable lipids developed first with solvent A, benzene–chloroform (9:1), and second with solvent B, petroleum ether–diethyl ether–acetic acid (90:10:1). Standards run above the solvent fronts were: (1) behenyl alcohol, (2) tricosan-12-ol, and (3) tricosan-12-one. Comparison of the *S. lutea* fraction with these standards and the chromatographic behavior of the compounds on other plates after treatment with the reagents described by Purdy and Truter (1963) led to the following identifications: (4 and 5) yellow, hydroxylated pigments, (6) primary alcohols, (7) unidentified, (8) secondary alcohols, (9) aldehydes, (10) ketones, (11) unidentified, (12) unidentified fluorescent compound, and (13) hydrocarbons.

were not detected (absence of bands at 7.39 and 8.60 μ). Gas–liquid partition chromatography of the ketones on SE-30 (column 1 of Table II) showed the distribution of components given in Table X. Data for the distribution of the hydrocarbons from the same preparation are included for comparison. The separation factor for the corresponding ketone and hydrocarbon was 1.60. Under the conditions used, a C=O/CH_2 was 1.55 and a CH_2CH_2/CH=CH was 1.03. The data were therefore in agreement with a series of aliphatic saturated ketones of the general structure R(C=O)R′ with both R and R′ having two or more carbons. The similarity of distribution between the ketones and hydrocarbons suggests close metabolic interrelationship.

Primary and secondary aliphatic alcohols were isolated by preparative thin-layer chromatography on 500-μ silica gel G plates developed in benzene–chloroform (7:3). Each fraction was scrapped from the plate, eluted with chloroform–ethanol (1:1), and purified by column chromatography on Florisil. The column was made up in benzene and the alcohols were placed on the column in the same solvent. After a preliminary wash with 15 ml of benzene/g of Florisil, secondary alcohols were eluted with 15% ether in hexane (8 ml/g) and primary alcohols with diethyl ether–hexane (1:1) (8 ml/g). Both alcohol fractions, as well as their acetates (Pietrzyk and Belisle, 1966), gave single spots on thin-layer chromatography in benzene–ethyl acetate (8:1) and chromatographed the same as authentic primary (behenyl and oleyl) and secondary (12-tricosanol) alcohols and their acetates. The secondary alcohols showed the expected infrared absorption bands at 2.95, 7.85, and 8.8 μ while the primary alcohols absorbed at 2.95, 7.80, and 9.4 μ when scanned as thin films on NaCl. There was an insufficient amount of alcohols recovered, less than 0.1% of the total lipid, to do any further characterization.

Discussion

The large proportion of monounsaturated hydrocarbons in *S. lutea* during early stationary phase made it possible to obtain information not otherwise possible on the nature of the aliphatic groups to either side of the double bond. In the case of FD-533 and the *Micrococcus*, ATCC-533, over 94% of these aliphatic groups have branched chains. This fact coupled with the fact that 96% of the fatty acids in both organisms have branched chains strongly suggests that the hydrocarbons are synthesized by condensation of two fatty acids. Differences in the proportions of certain fatty acids found in the oxidation products and total lipids, particularly the occurrence of even-carbon-numbered branched-chain acids in the former, suggest that decarboxylation of one of the fatty acids participating in the condensation occurs. This is clearly supported in *S. lutea* by the fact that the two major fatty acids obtained by oxidation of the hydrocarbons were branched-chain C-14 and C-15 and the latter was the major fatty acid found in the lipids. Condensation of 2 moles of the C-15 branched-chain fatty acids with each other and with decarboxylation of one of them would give a C-29 hydrocarbon with the double bond between C-13 and C-14 of the main chain. This is the structure of the major, monounsaturated hydrocarbon.

No such correspondence occurs with ATCC-533. Here again the major fatty acids are branched-chain C-15 but oxidation of the monounsaturated hydrocarbons yields primarily C-12, C-13, and C-14 branched-

chain fatty acids. The fact that most of the aliphatic groups are branched is contrary to what would be expected if the hydrocarbons are synthesized by the elongation of fatty acids. With this mechanism, at least half of the fatty acids obtained by oxidation of the hydrocarbons should be normal or unbranched unless the methyl branches are introduced subsequently to the elongation. An alternative explanation which is consistent with head-to-head condensation is that in ATCC-533 there is selectivity as to which of the fatty acids participates in the condensation and the selectivity extends to whether the fatty acid undergoes decarboxylation. The selectivity most consistent with the data presented would be condensation of *iso*-C-13 fatty acid (with or without decarboxylation) with itself or with *anteiso* and *iso*-C-15 fatty acid (with obligatory decarboxylation) to yield the major hydrocarbons, C-25 and C-27. This requires that the branched-chain C-13 fatty acids, which occur in very low concentrations in the total lipid, play a major role in hydrocarbon synthesis; but it can be argued that the selectivity for C-13 branched-chain fatty acids in hydrocarbon synthesis accounts for its low level in the lipids.

The occurrence of long-chain ketones and secondary alcohols in the lipids of *S. lutea* is also consistent with the synthesis of hydrocarbons by head-to-head condensation of fatty acids. These compounds would be expected as intermediates in addition to the monounsaturated hydrocarbons which in 48-hr cultures are the major form present. On the other hand, no fatty acids in the range of C-22 to C-32 were detected by gas–liquid partition chromatography although if synthesis occurred by way of elongation, they would be expected intermediates. Kolattukudy (1967) proposed that in *Brassica* long-chain fatty acid intermediates remain enzyme bound until converted into hydrocarbon and are therefore not isolated. Data to be presented in subsequent papers of this series on the *in vivo* and *in vitro* incorporation of labeled intermediates into the hydrocarbons of *S. lutea* support a mechanism involving head-to-head condensation; however, long-chain ketones and secondary alcohols do not appear to be direct intermediates and a new mechanism for hydrocarbon synthesis consistent with these labeling experiments will be presented.

References

Albro, P. W., and Huston, C. K. (1964), *J. Bacteriol. 88*, 981.

Amenta, J. S. (1964), *J. Lipid Res. 5*, 270.

Breed, R. S., Murray, E. G. D., and Smith, N. R., Ed. (1957), Bergely's Manual of Determinative Bacteriology, 7th ed, Baltimore, Md., Williams and Wilkins, p 470.

Channon, H. J., and Chibnall, A. C. (1929), *Biochem. J. 23*, 168.

Chapman, D. (1965), *J. Am. Oil Chemists' Soc. 42*, 173.

Cheronis, N. D. (1954), *in* Micro and Semimicro Methods, Techniques of Organic Chemistry, Vol. VI, Weissberger, A., Ed., New York, N. Y., Interscience, pp 474, 504.

Chibnall, A. C., and Piper, S. H. (1934), *Biochem. J. 28*, 2209.

Demaecker, J., and Martin, R. H. (1954), *Nature 173*, 266.

Dittmer, J. C. and Dawson, R. M. C. (1961), *Biochem. J. 81*, 535.

Folch, F., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Harrison, J. S. and Trevelyan, W. E. (1963), *Nature 200*, 1189.

Herbert, D. (1955), *Methods Enzymol. 1*, 753.

Hornstein, I, Alford, J. A., Elliot, L. E., and Crowe, P. F. (1960), *Anal. Chem. 32*, 540.

Huston, C. K., and Albro, P. W. (1964), *J. Bacteriol. 88*, 425.

Jones, L. A., Holmes, J. C., and Seligman, R. B. (1958), *Anal. Chem. 28*, 191.

Kaneda, T. (1967), *Biochemistry 6*, 2023.

Kaneda, T. (1968), *Biochemistry 7*, 1194.

Kawada, T., Mookherjee, B. D., and Chang, S. S. (1966), *J. Am. Oil Chemist's Soc. 43*, 237.

Kolattukudy, P. E. (1966), *Biochemistry 5*, 2265.

Kolattukudy, P. E. (1967), *Biochemistry 6*, 2705.

Letters, R. (1966), *Biochim. Biophys. Acta 116*, 489.

Metcalfe, L. D., and Schmidtz, A. A. (1961), *Anal. Chem. 33*, 363.

Miwa, T. K. (1963), *J. Am. Oil Chemists' Soc. 40*, 309.

Mold, J. D., Stevens, R. K., Means, R. E., and Ruth, J. M. (1963), *Biochemistry 2*, 605.

Morris, L. J. (1962), *Chem. Ind. (London)*, 1238.

O'Connor, J. G., and Norris, N. S. (1960), *Anal. Chem. 32*, 701.

O'Connor, R. T. (1960), *in* Encyclopedia of Spectroscopy, Clark, G. L., Ed., New York, N. Y., Reinhold, pp 420–444.

Pietrzyk, D. J., and Belisle, J. (1966), *Anal. Chem. 33*, 1508.

Purdy, S. J., and Truter E. V. (1963), *Proc. Roy. Soc. (London) B158*, 536, 544, 553.

Rao, C. N. R. (1963), Chemical Applications of Infrared Spectroscopy, New York, N. Y., Academic.

Sahai, P. N., and Chibnall, A. C. (1932), *Biochem. J. 26*, 403.

Schwartz, D. P., Haller, H. S., and Keeney, M. (1963), *Anal. Chem. 35*, 2191.

Schwartz, D. P., Parks, O. W., and Keeney, M. (1962), *Anal. Chem. 34*, 669.

Stone, R. W., and ZoBell, C. E. (1952), *Ind. Eng. Chem. 44*, 2564.

Stumpf, P. K. (1965), *in* Plant Biochemistry, Bonner, J., and Varner, J. E., Ed., New York, N. Y., Academic, pp 323–345.

Szymanski, H. A. (1967), A Systematic Approach to the Interpretation of Infrared Spectra, Buffalo, N. Y., Hertillon.

Tornabene, T. G., Bennet, E. O., and Oró, J. (1967b), *J. Bacteriol. 94*, 344.

Tornabene, T. G., Gelpi, E., and Oró, J. (1967a), *J. Bacteriol. 94*, 333.

von Rudloff, E. (1956), *Can. J. Chem. 34*, 1413.

Wanless, G. G., King, W. H., and Ritter, J. J. (1955),

Biochem. J. 59, 684.
Wells, M. A., and Dittmer, J. C. (1965), *Biochemistry* *4*, 2495.
Wheeler, D. H. (1954), *Progr. Chem. Fats Lipids 2*, 268.

Bile Acids. XXVI. The Metabolism of 12α-Hydroxycholanoic Acid-24-^{14}C in the Rat*

Robert C. Sonders,† S. L. Hsia,‡ E. A. Doisy, Jr., John T. Matschiner, and William H. Elliott§

ABSTRACT: 12α-Hydroxycholanoic acid-24-^{14}C was prepared by a nitrile synthesis from the norbromide and administered intraperitoneally to each of three rats with bile fistulas. Within 24 hr most of the administered ^{14}C was recovered in bile. After alkaline hydrolysis of the conjugated bile acids, the free bile acids were separated by partition chromatography. Of the chromatographed ^{14}C, 12% was identified as unchanged 12α-hydroxycholanoic acid, 26% as 7α,12α-dihydroxycholanoic acid, 18% as deoxycholic acid, 15% as cholic acid, and a small amount of 6β,12α-dihydroxycholanoic acid, a new bile acid. Methods of preparation and data for characterization of the new acid are provided. These results extend earlier observations on the ability of the rat to oxygenate the nucleus of cholanoic acid at position 7 in the absence of a 3α-hydroxyl group and suggest a limited ability to oxygenate 12α-hydroxycholanoic acid in the 6β position.

The ability of the rat to hydroxylate bile acids at positions 6β and 7α has been demonstrated in several previous studies. For example, lithocholic acid is metabolized to chenodeoxycholic acid, 3α,6β-dihydroxycholanoic acid, and α- and β-muricholic acids (Thomas *et al.*, 1964); and deoxycholic acid is metabolized mainly to cholic acid (Bergström *et al.*, 1953) with 3α,6β,12α-trihydroxycholanoic acid as a minor product (Ratliff *et al.*, 1959).

Hydroxylation at positions 6β and 7α does not seem to be limited to the natural occurring bile acids which contain hydroxyl groups at position 3α. In a previous study at this laboratory cholanoic acid was found to form 7α-hydroxycholanoic, chenodeoxycholic, and α- and β-muricholic acids. Since lithocholic acid was not detected among the metabolites, the identification of 7α-hydroxycholanoic acid suggested that the metabolic process might be initiated by 7α hydroxylation rather than 3α hydroxylation (Ray *et al.*, 1961). In an attempt to determine what effects, if any, hydroxylation at the 12α position of the steroid nucleus may have on the course of bile acid metabolism, we have studied the metabolism of 12α-hydroxycholanoic acid in the rat. This study necessitated the preparation of a series of new bile acids. Methods of preparation and data for characterization of these new acids are also included with results of metabolic studies in this report.

Experimental Procedure[1]

Chromatography. Bile acids were chromatographed on an acetic acid partition column as previously described (Matschiner *et al.*, 1957). The fractions have been designated according to the percentage of benzene in hexane. For example, fraction 20-1 represents the first fraction of the eluent containing 20% benzene in hexane. The procedure of reversed-phase partition column chromatography of Bergström and Sjövall

* From the Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104. *Received August 15, 1968.* This work was supported in part by the National Institutes of Health (Grants TiGM 446 and HE-07878) and the Nutrition Foundation, Inc. (Grant 277). For the preceding paper in this series, see Ziller *et al.* (1968). A preliminary report of some studies contained in this paper has been made (Sonders and Elliott, 1964). The material presented herein is taken in part from the dissertation submitted to the Graduate School of St. Louis University by R. C. S. in partial fulfillment for the degree of Doctor of Philosophy in Biochemistry. Cholanoic acid and all derivatives referred to in this report are derivatives of 5β-cholanoic acid; *e.g.*, 12α-hydroxycholanoic acid is 12α-hydroxy-5β-cholanoic acid.

† Present address: Department of Pharmacology, Abbott Laboratories, North Chicago, Ill. 60601.

‡ Present address: Departments of Biochemistry and Dermatology, University of Miami School of Medicine, Miami, Fla. 33136.

§ To whom inquiries should be addressed.

[1] Melting point determinations were taken on a Fisher-Johns apparatus and are reported as read. Infrared spectra were determined in Nujol with a Perkin-Elmer spectrophotometer, Model 21, equipped with rock salt optics and ordinate scale expansion. Ultraviolet spectra were determined with a Hitachi Perkin-Elmer spectrophotometer, Model 139. Optical rotations were determined in methanol at a concentration of 1% unless stated otherwise with a Rudolph photoelectric spectropolarimeter, Model 200-S. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Radioactivity was measured with a Packard Tri-Carb liquid-scintillation spectrometer, Model 3314, as previously reported (Ray *et al.*, 1961).