Cyclopropane Ring Biosynthesis*

T. Y. LIU AND K. HOFMANN

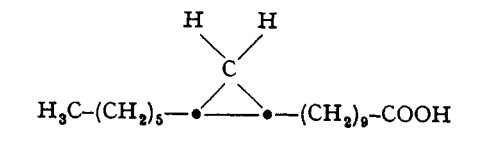
From the Biochemistry Department, University of Pittsburgh, School of Medicine

Received July 26, 1961

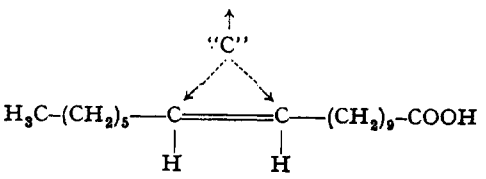
The specific radioactivity of lactobacillic acid isolated from *Lactobacillus arabinosus* was 92% that of *cis*-vaccenic acid-1-C^{14} added to the culture medium. Addition of methionine-methyl-C^{14} and of formate-C^{14} to the medium of the same organism led to incorporation of radiocarbon into lactobacillic acid. Degradation of such lactobacillic acid samples gave iodoform-C^{14} exhibiting 80 and 84%, respectively, of the specific radioactivity of the starting material. These results demonstrate conclusively that lactobacillic acid biosynthesis in *L. arabinosus* involves addition of a one-carbon fragment across the double bond of *cis*-vaccenic acid.

Since their discovery in the lipids of *Lactobacillus arabinosus* (Hofmann and Lucas, 1950), fatty acids of the cyclopropane series have been shown to be of rather widespread distribution in bacterial lipids. Lactobacillic acid (*d*-, or *l*-*cis*-11,12-methyleneoctadecanoic acid) occurs in *L. arabinosus* (Hofmann and Lucas, 1950), *Lactobacillus casei* (Hofmann and Sax, 1953), and *Agrobacterium tumefaciens* (Hofmann and Tausig, 1955), and indirect evidence points to its presence in *Lactobacillus delbrueckii* lipids (Hofmann *et al.*, 1955). On the basis of studies with radioactive tracers O'Leary (1959b) suggested the presence of a C_{17} cyclopropane fatty acid in the lipids of an *Escherichia coli* mutant, and an acid of this structure was isolated from this organism by Dauchy and Asselineau (1960). The position of the cyclopropane ring in this substance remains to be established.

In previous communications (Hofmann *et al.*, 1957, 1959) we have presented indirect evidence in support of our concept that the biosynthesis of lactobacillic acid (I) may involve addition of a one-carbon fragment across the double bond of *cis*-vaccenic acid (II).



(I)



(II)

In the present study we present unequivocal evidence for this rather novel biochemical reaction with *L. arabinosus*. This evidence stems from studies on the distribution of radioactivity in individual fatty acids isolated from *L. arabinosus* grown on various radioactive precursors (*cis*-vaccenic acid-1-C^{14}, methionine methyl-C^{14}, and formate-C^{14}), and on the distribution of radiocarbon in certain samples of lactobacillic acid.

* The authors wish to express their appreciation to the U. S. Public Health Service for generous support of this investigation. Preliminary reports of parts of this investigation have appeared: Hofmann, K., and Liu, T. Y. (1960), *Biochim. et Biophys. Acta 37*, 364, and Liu, T. Y., and Hofmann, K. (1960), *Fed. Proc. 19*, A-227.

EXPERIMENTAL

Materials. *Cis*-Vaccenic acid-1-C^{14} (1.35×10^7 cpm/mmole) was prepared as described previously (Hofmann and Sax, 1953), with potassium cyanide-C^{14} (Volk Radiochemical Company) as the source of radioactive carbon. L-Methionine-methyl-C^{14} (Nuclear Chicago) and sodium formate (Volk Radiochemical Company) were employed. Tween 40 was a gift from the Atlas Powder Company, and the vulcanized Hevea rubber, Mealorub, was obtained through the courtesy of Mr. J. F. Frank, 120 Wall Street, New York 5, N. Y.

Microbiologic Procedures.—Lactobacillus arabinosus, ATCC 8014, strain 17–5, was obtained from the American Type Culture Collection. The bacteria were maintained in stab cultures and were grown in 3.5 liters of synthetic media essentially as previously described (Hofmann *et al.*, 1952, 1959). Tween 40 (1.2 g) was added per liter of basal medium. For the experiments with *cis*-vaccenic acid-1-C^{14}, the radioactive fatty acid (1.92×10^6 cpm) was added per liter of medium. For the experiments with methionine-methyl-C^{14}, the radioactive amino acid (2.69×10^7 cpm) and nonradioactive *cis*-vaccenic acid (40 mg) were added per liter of medium. Formate-C^{14} (7.57×10^6 cpm), and nonradioactive *cis*-vaccenic acid (40 mg) were added per liter of medium.

In experiments with cell suspensions, bacterial cells grown in 1 liter of basal medium containing nonradioactive *cis*-vaccenic acid (40 mg) were collected by centrifugation and washed twice with 5 volumes of 0.85% sodium chloride solution. The washed cells were suspended in 10 volumes of 0.03 M potassium dihydrogen phosphate (pH 6.8) containing 1% of glucose and of sodium formate-C^{14} (1.20×10^7 cpm). The suspension was incubated at 37° for 24 hours with occasional shaking.

The radioactive cells were collected by centrifugation in an International Centrifuge at 4000 rpm in closed containers and were washed repeatedly with distilled water (approximately 2 liters) until the radioactivity of the supernatant was negligible. The washed cells were hydrolyzed immediately.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN FRACTIONS FROM *L. arabinosus* CELLS GROWN ON VARIOUS RADIOACTIVE PRECURSORS

	Cis-Vaccenic Acid-1-C^{14}		Methionine-Methyl-C^{14}		Formate-C^{14a}		Formate-C^{14b}	
	Total cpm	%	Total cpm	%	Total cpm	%	Total cpm	%
Hydrolyzed cells	1.26×10^6	100	3.15×10^7	100	9.22×10^5	100	1.15×10^6	100
Ether-extracted hydrolyzed cells	0.01×10^6	0.8	2.20×10^7	69.8	9.20×10^5	99.7	1.13×10^6	98.3
Mixed fatty acids	1.25×10^6	99.2	0.95×10^7	30.2	0.02×10^5	0.2	0.02×10^8	0.2
Nonsap. material	Trace		Trace		Trace		Trace	

[a] Grown in medium containing formate-C^{14}. [b] Cells suspended in medium containing formate-C^{14}.

TABLE II

INCORPORATION OF LABELED PRECURSORS INTO *L. arabinosus* FATTY ACIDS

	Cis-Vaccenic Acid-1-C^{14}			Methionine-Methyl-C^{14}			Formate-C^{14a}		
Acids	Cellular[b] Acids (%)	cpm/mmole	Distribution of Radioactivity (%)	Cellular[b] Acids (%)	cpm/mmole	Distribution of Radioactivity (%)	Cellular[b] Acids (%)	cpm/mmole	Distribution of Radioactivity (%)
Dihydroxy[b]	15.3	1.10×10^7	24.1	15.3	0.16×10^7	0.7	17.8	3.93×10^4	39.1
Capric	0.2	...[c]	...	0.6	0.56×10^7	0.2	0.2	...[c]	...
Lauric	0.2	...[c]	...	0.8	0.39×10^7	0.2	0.5	...[c]	...
Myristic	0.8	...[c]	...	1.0	0.41×10^7	0.2	0.6	...[c]	...
Palmitic	41.8	...[c]	...	46.0	0.12×10^7	2.0	39.6	...[c]	...
Lactobacillic[b]	31.4	1.25×10^7	69.9	33.8	9.65×10^7	96.5	36.5	2.76×10^4	57.1
Total recovery	89.7	...	94.0	97.5	...	99.8	95.2	...	96.2

[a] Cells suspended in medium containing formate-C^{14}. [b] See Hofmann *et al.* (1955) for interpretation of the various chromatographic fractions. [c] Not significantly above background.

Analytical Methods.—The method of Hofmann *et al.* (1957) was employed for isolation and determination of fatty acids. For isolation of individual fatty acids corresponding to the various chromatographic peaks, the contents of the appropriate tubes were pooled and acidified with hydrochloric acid, and the acetone was removed *in vacuo*. The residues were ether extracted and the fatty acids isolated from the extracts in the usual manner. For determination of specific activity, the fatty acids were dissolved in ethanol and suitable aliquots plated on planchets for counting. All counts were corrected for background and self absorption.

Nonradioactive carrier lactobacillic acid, isolated from *A. tumefaciens* (Hofmann and Tausig, 1955), was added to the radioactive lactobacillic acid fraction from the formate and methionine experiments to yield labeled lactobacillic acid samples with specific activities of 3,467 and 22,350 cpm/mmole respectively. Samples (100 mg) of each of these materials were degraded as described (Hofmann *et al.*, 1958), using the Lemieux oxidation procedure. Hypoiodite oxidations were performed on the total oxidation products, and the resulting iodoform was purified to constant specific radioactivity by repeated sublimation.

RESULTS AND DISCUSSION

Of the added *cis*-vaccenic acid-1-C^{14} (6.72×10^6 cpm), 1.26×10^6 cpm, or 18.7%, was associated with the cells. The distribution of label between various fractions derived from these cells (Table I) shows that 99.2% of the radioactivity was located in the fatty acid fraction; the other cellular constituents were practically inactive. The major portion of the activity was located in the dihydroxy and lactobacillic acid fractions. Negligible activity was found in the capric, lauric, myristic, and palmitic acid fractions and the nonsaponifiable material (Table II). The specific activity of the dihydroxy (1.10×10^7 cpm/mmole) and of the lactobacillic acid fractions (1.25×10^7 cpm/mmole), which corresponds to 82 and 92%, respectively, of that of the added labeled *cis*-vaccenic acid, demonstrates rather conclusively that lactobacillic acid is indeed formed from *cis*-vaccenic acid. The results show further that there was little degradation or redistribution of *cis*-vaccenic acid carboxyl-carbon under the conditions of our experiments. These findings are in complete accord with previous observations (O'Leary, 1959a).

Methionine-methyl-C^{14} was also effectively incorporated into the bacterial cells, which contained 3.15×10^7 cpm, or 33.4% of the 9.42×10^7 cpm which was added. In contrast to the observations with *cis*-vaccenic acid, 69.8% of the label was located in the nonlipid fractions, the remaining radioactivity being present in the mixed fatty acids (Table I). As may be seen from Table II, 96.5% of the radioactivity of the mixed fatty acids was present in the lactobacillic acid fraction, the other fatty acids exhibiting a very low degree of labeling. A series of experiments with growing cells and with suspensions of resting cells were performed with formate-C^{14}. Incorporation of formate carbon into the bacterial cells was low under both sets of experimental conditions; *i.e.*, 3.5% was incorporated into the growing and 9.6% into the suspended cells. It will be noted from inspection of Table I that little label was present in the mixed fatty acids, in contrast to other cellular constituents, which contained between 98.3 and 99.7% of the radioactivity. The label present in the mixed fatty acids was distributed between the lactobacillic and dihydroxy fractions (Table II). Samples of biosynthetically labeled lactobacillic acid isolated from both the methionine and formate experiments

TABLE III

SPECIFIC ACTIVITY OF IODOFORM ISOLATED FROM RADIOACTIVE LACTOBACILLIC ACID

	Formate-C^{14} Experiment cpm/mmole	Methionine-Methyl-C^{14} Experiment cpm/mmole
Lactobacillic acid	3467	22,350
Iodoform	2771	18,797

were degraded (Hofmann *et al.*, 1958), and the radioactivity of the resulting iodoform is recorded in Table III. As discussed previously (Hofmann *et al.*, 1958), the iodoform derived from cyclopropane fatty acids according to our scheme of degradation is derived exclusively from the methylene bridge carbon of these acids and thus provides a direct and unequivocal measure of the radioactivity of this particular carbon atom in lactobacillic acid samples. The observation that the specific activity of the iodoform was 84% that of the lactobacillic acid in the methionine experiment and 80% that of the lactobacillic acid in the formate experiment demonstrates conclusively that the methylene bridge carbon of lactobacillic acid can derive from one-carbon fragments.

O'Leary (1959a,b), working with *L. arabinosus*, has demonstrated incorporation of *cis*-vaccenic acid-1-C^{14} and of methionine-methyl-C^{14} into the lactobacillic acid molecule, but he did not elucidate the position of the label.

In the present study we have shown for the first time that one-carbon fragments which are incorporated into the mixed fatty acid fraction of *L. arabinosus* are localized almost exclusively in the methylene bridge carbon of the lactobacillic acid molecule. It seems likely that addition of one-carbon fragments to carbon-carbon double bonds provides a general biochemical mechanism for formation of branched-chain compounds. Methionine methyl and formate are efficient sources for carbon atom number 28 in the ergosterol molecule (Danielson and Bloch, 1957; Alexander and Schwenk, 1957). The incorporation of the methyl group, which in this sterol is the sole carbon atom derived from one-carbon sources, may involve similar mechanisms.

REFERENCES

Alexander, G. S., and Schwenk, E. (1957), *J. Am. Chem. Soc. 79*, 4554.
Danielson, H., and Bloch, K. (1957), *J. Am. Chem. Soc. 79*, 500.
Dauchy, S., and Asselineau, J. (1960), *Compt. rend. 250*, 2635.
Hofmann, K., Henis, D. B., and Panos, C. (1957), *J. Biol. Chem. 228*, 349.
Hofmann, K., Hsiao, C. Y., Henis, D. B., and Panos, C. (1955), *J. Biol. Chem. 217*, 49.
Hofmann, K., and Lucas, R. A. (1950), *J. Am. Chem. Soc. 25*, 4328.
Hofmann, K., Lucas, R. A., and Sax, S. M. (1952), *J. Biol. Chem. 195*, 473.
Hofmann, K., Marco, G. J., and Jeffrey, G. A. (1958), *J. Am. Chem. Soc. 80*, 5717.
Hofmann, K., O'Leary, W. M., Yoho, C. W., and Liu, T. Y. (1959), *J. Biol. Chem. 234*, 1672.
Hofmann, K., and Sax, S. M. (1953), *J. Biol. Chem. 205*, 55.
Hofmann, K., and Tausig, F. (1955), *J. Biol. Chem. 213*, 425.
O'Leary, W. M. (1959a), *J. Bacteriol. 77*, 367.
O'Leary, W. M. (1959b), *J. Bacteriol. 78*, 709.