Biosynthesis of Alkanes in *Nostoc muscorum*

Jerry Han, Henry W.-S. Chan,[1] and Melvin Calvin

Contribution from Department of Chemistry, Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory and Space Sciences Laboratory, University of California, Berkeley, California 94702.[2] *Received March 28, 1969*

Abstract: The hydrocarbon composition of the blue-green algae is apparently simple. The pattern shows normal hydrocarbons in the C_{15} to C_{18} range with a predominance of n-C_{17}. *Nostoc* contained significant amounts of a 50:50 mixture of 7- and 8-methylheptadecane. An initial investigation of hydrocarbon biosynthesis in *Nostoc muscorum* was performed by measuring the amount of ^{14}C and ^{3}H incorporated into the hydrocarbons from suitable substrates. Palmitic-ω-^{14}C acid and stearic-ω-^{14}C acid were converted into hydrocarbons *via* an enzymatic decarboxylation. The evidence indicates that the labeled hydrocarbons were the end products of cell metabolism. The incubation of L-methionine-methyl-^{14}C and tritiated *cis*-vaccenic acid to form 7- and 8-methylheptadecane indicated that the methionine methyl group was specifically added to the double bond of vaccenic acid. This was followed by decarboxylation to yield the 7- and 8-methylheptadecanes.

In a previous communication[3] we reported results of a study in the distribution of aliphatic hydrocarbons found in a variety of microorganisms. Of all the species examined, the simplest mixture of hydrocarbons was found in the blue-green algae, *e.g.*, *Nostoc*, which has only four components in the hydrocarbon fraction of extracts of the algae. The major components of the hydrocarbons are n-heptadecane (n-C_{17} hydrocarbon) and a "branched-chain C_{18} alkane" which has since been shown[4] to be an equimolar mixture of 7-methyl and 8-methylheptadecane. In addition to these hydrocarbons which constitute 99% of the hydrocarbon content of the algae, two minor components are also present, *viz.* n-pentadecane (n-C_{15} hydrocarbon) and n-hexadecane (n-C_{16} hydrocarbon).[5]

The 50:50 mixture of 7- and 8-methylheptadecane[3,4] appeared to occur uniquely in the blue-green algae and was absent from other photosynthetic and nonphotosynthetic bacteria.[3] The percentage of this mixture in the total alkanes of *Nostoc muscorum* and *Phormidium luridum* are 16 and 4%, respectively.[4] This mixture of hydrocarbons may be of considerable significance since blue-green algae may be among the most primitive organisms, and since the mixture occurs uniquely as the second largest peak among the alkanes of blue-green algae.

The predominance of n-heptadecane and the methylheptadecanes found in the blue-green algae is unique among all the microorganisms whose hydrocarbon content has been examined. The possible derivation of oil shales from organic sources such as algal oozes and the distribution of hydrocarbons in these shales as markers in geological time are currently topics of intensive study.[6] Johns, *et al.*,[7] found that n-heptadecane was the major component in the normal alkane fraction of the Green River Shale (*ca.* 50×10^6 years old), the hydrocarbons of which are considered to have been derived from fresh water algae. The occurrence of these hydrocarbons in the blue-green algae apparently at a critical stage of evolutionery diversification led us to examine their biological origins and possible metabolism in the living cell. In this paper we report results of experiments in the biosynthesis and metabolism of methyl-branched octadecane, n-heptadecane, and the minor constituent n-pentadecane of *Nostoc*. We have suggested a biosynthetic pathway for the formation of these branched C_{18} alkanes in an earlier paper.[4] This paper presents experimental evidence which involves fixation of a methyl group to *cis*-vaccenic acid followed by a decarboxylation reaction.

Experimental Section

Eight liters of *Nostoc muscorum* were grown in an inorganic medium[8] for 4 days at 25°, under a stream of air–CO_2 (v/v 95:5) and with illumination (450 ft candles) from both sides. The culture (OD 680 $\sim$ 0.8) was centrifuged and resuspended in a 3-l. flask with 1.5 l. of the same medium and the radioactive hydrocarbons or fatty acids were dispersed in the medium by sonication. The suspension was illuminated at 25° and under CO_2 for 2 hr. The cells were harvested by centrifugation (10,000*g*) in the dark, washed by resuspension in 100 ml of distilled water, and centrifuged again. About 1.8 g of radioactive cells was collected each time after the water was removed by freeze drying.

The dry cells were treated with 150 ml of 3:1 benzene and methanol for 15 min with stirring and sonicated for 30 min. Then the total sample and supernatant were transferred to a Soxhlet apparatus and extracted for 8 hr with the same mixture of solvents. There was no radioactivity remaining in the cells after this extraction. The extraction solvents were evaporated to leave a dark green residue.

The total extracted material was transferred on the top of an alumina column (100 g, 30 cm) previously washed with n-heptane (100 ml); it was then eluted with three solvents to give four fractions. The first fraction containing the total aliphatic hydrocarbons was eluted from the column with 100 ml of n-heptane (n-heptane fraction). The second and third fractions were obtained by elution with 100 ml and 150 ml of benzene, respectively. The second fraction (benzene fraction I) contained aromatic and highly unsaturated hydrocarbons as well as nonpolar ketones.[9] Carotenoids are the major components in the third fraction (benzene

(1) Miller Fellow, 1967–1969.

(2) The work described in this paper was sponsored, in part, by the U. S. Atomic Energy Commission and, in part, by the National Aeronautics and Space Administration, Grant No. NGL 05-003-003.

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Table I. Incubation of Stearic-18-^{14}C Acid[a]

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total act. extracted, %
n-Pentadecane	47.7		*n*-Heptane	1.05×10^5	6.2
n-Hexadecane	686	0.7	Benzene I	650	0.4
n-Heptadecane	1.04×10^5	98.7	Benzene II	1.2×10^4	0.7
Methylheptadecanes	674	0.6	Methanol	1.57×10^6	92.7

[a] Time of incubation: 3 hr; amount of stearic acid used: 50 μCi, 284 mg; radioactivity recovered: 0.77 μCi.

Table II. Incubation of Palmitic-16-^{14}C Acid[a]

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total Act. extracted, %
n-Pentadecane	5.32×10^4	44.2	*n*-Heptane	3.17×10^4	67.6
n-Hexadecane	2.3×10^3	1.9	Benzene I	476	1.0
n-Heptadecane	1.8×10^4	15.0	Benzene II	1.44×10^4	30.7
Methylheptadecanes	4.7×10^4	38.8	Methanol	293	0.6

[a] Time of incubation: 3 hr; amount of palmitic acid used: 50 μCi, 20 mg; radioactivity recovered: 0.06 μCi.

fraction II). The fourth fraction, obtained by elution with 150 ml of methanol, contained glycerides, free acids, bases, amino acids, chlorophylls, and other organic compounds with polar functional groups.

An aliquot of the *n*-heptane fraction was injected onto an Aerograph Model A-90-P gas chromatograph equipped with a 25 ft $\times$ 0.25 in. column of 3% SE-30. The hydrocarbons were trapped and their radioactivities determined by a Tri-Carb liquid scintillation spectrometer.

The methanol fraction was used for fatty acid analyses. An aliquot of the methanol fraction was saponified with sodium hydroxide; impurities were removed by extracting with three 10-ml portions of *n*-hexane. Finally, the alkaline solution was acidified, and the fatty acids were extracted into three 10-ml portions of hexane. After drying with $MgSO_4$, the acids were methylated by refluxing the hexane solution with 1 ml of BF_3 in methanol.

The hexane layer was removed, and the methanol layer was extracted with three 5-ml portions of hexane. The combined hexane extract was concentrated with a rotary evaporator. The samples were then analyzed by gas chromatography and mass spectrometry. All mass spectra were taken by a combination of an Aerograph 204 gas chromatograph and an A.E.I. MS-12 mass spectrometer.

Synthesis of Normal Hydrocarbons and Tritium-Labeled Vaccenic Acid

The ω-^{14}C fatty acids (obtained from New England Nuclear Corp.) were decarboxylated by photodecomposition of their acyl iodides followed by zinc dust reduction of the alkyl halides as described by Barton, *et al.*[10] In each case 0.1 mmol of the acid was used and the over-all yield as determined by the radioactivity retained in the product was 60%. The products were purified by chromatography on alumina. Uniform tritium-labeled methyl vaccenite was made in this laboratory by the Wilzbach gas exposure method.[11,12] Methyl vaccenate (10 mg) was deposited evenly on a 100-ml glass reaction tube. After evacuation to 0.1 mm, 7 Ci of tritium gas and 20 ml of oxygen gas at standard condition were introduced into the reaction tube.

Methyl tritiostearate was formed by adding tritium to the double bond, and methyl tritiovaccenate was formed by tritium substitution reactions. Liquid scintillation counting indicated that 3.1 mCi of tritium was incorporated into the total product. After hydrolysis of methyl esters 300 μCi of methyl tritiovaccenic acid was isolated by thin layer chromatography on silica gel G containing 15% of silver nitrate.[13]

Biosynthesis of Normal Hydrocarbons

Although the formation of hydrocarbons in living organisms by decarboxylation of fatty acids has been a subject of conjecture for some time,[14] little is known concerning the derivation of hydrocarbons in algae. Studies in the biogenesis of hydrocarbons in other microorganisms have shed light on their mode of formation but little positive evidence has been produced for the decarboxylation of fatty acids to yield hydrocarbons. Thus Oró, *et al.*,[15] examined the formation of hydrocarbons in *Sarcina lutea* by including a variety of ^{14}C-labeled substrates (*e.g.*, acetate, glucose, leucine, isoleucine, bicarbonate, and both palmitic-1-^{14}C and -16-^{14}C acids) in the culture media. However, the length of incubation (48 hr) and the general nature of metabolites used led to the appearance of label in a wide variety of substances.

In an effort to study the decarboxylation of fatty acids in isolation we have used exclusively ω-^{14}C-labeled acids and comparatively short incubation periods (2 hr). The low level of incorporation into hydrocarbons obtained under these conditions can, however, be detected and the mixture of products analyzed by the techniques of gas chromatography[4] and liquid scintillation counting.

The results of an incubation of stearic-18-^{14}C acid in a culture of *Nostoc* is shown in Table I. An exclusive incorporation of radioactivity into *n*-heptadecane is observed indicating a direct decarboxylation of stearic acid. The *n*-heptane fraction containing the hydrocarbons accounts for 6.2% of the total radioactivity recovered; close to the remainder of the radioactivity

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Table III. Incubation of *n*-Heptadecane-1-^{14}C[a]

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total act. extracted, %
n-Pentadecane	35		*n*-Heptane	4.4×10^4	93.6
n-Hexadecane	644	1.5	Benzene I	2340	5.0
n-Heptadecane	4.15×10^4	94.7	Benzene II	119	0.2
Methylheptadecanes	1660	3.8	Methanol	530	1.1

[a] Time of incubation: 3 hr; amount of *n*-heptadecane used: 10 μCi, 16.3 mg; radioactivity recovered: 2.2×10^{-2} μCi.

Table IV. Incubation of *n*-Pentadecane-1-^{14}C[a]

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total act. extracted, %
n-Pentadecane	5.4×10^5	95.6	*n*-Heptane	6.35×10^5	98.7
n-Hexadecane	2.22×10^4	3.6	Benzene I	6700	1.0
n-Heptadecane	4830	0.8	Benzene II	265	
Methylheptadecanes	338		Methanol	1340	0.2

[a] Time of incubation: 3 hr; amount of *n*-pentadecane used: 15 μCi, 18 mg; radioactivity recovered: 0.295 μCi.

(92.7%) is present in the methanol fraction which contains the starting material. When palmitic-16-^{14}C acid is substituted for the radioactive stearic acid, radioactivity was incorporated into *n*-heptadecane as well as into *n*-pentadecane (Table II) in the extracted hydrocarbons. The formation of *n*-heptadecane is likely to be a result of chain elongation to stearic acid prior to decarboxylation. However, there appears to be appreciable incorporation (15%) into fractions of the

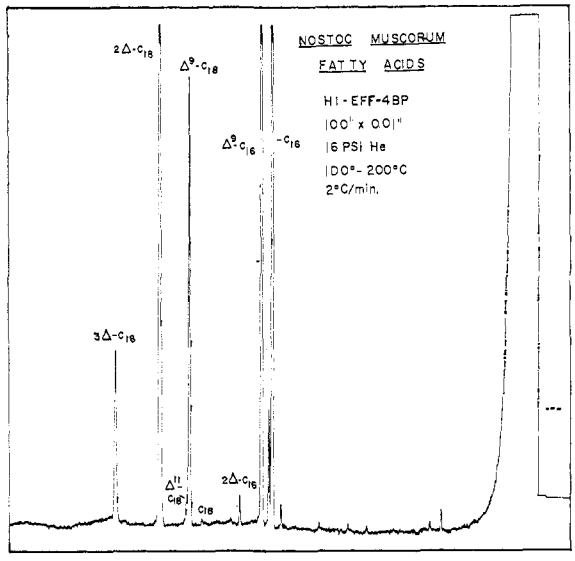

Figure 1.

extracts other than those containing the hydrocarbons and the fatty acids. This would indicate that the synthesis of other compounds from fragments such as acetyl-CoA would give rise to radioactivity in the benzene II fraction. Unlike the case of stearic-18-^{14}C acid there was considerable breakdown of the palmitic-16-^{14}C acid during the period of incubation.

As a means of studying the possible further metabolism of the normal hydrocarbons, synthetic *n*-heptadecane-^{14}C and *n*-pentadecane-^{14}C were also incubated with cultures of *Nostoc*. In contrast to the incubations of ^{14}C fatty acids, no appreciable metabolism of the radioactive hydrocarbons was detected after the same period of incubation (Tables III and IV). The recovered radioactivity remains essentially in the hydrocarbon fractions. Analysis of these showed the retention of radioactivity only in the peaks of the gas chromatogram corresponding to the starting materials. The small amounts of radioactivity present in other peaks of the chromatograms are due to tailing of the large quantities of the ^{14}C hydrocarbons present in each chromatogram. This was shown by injection of a similar amount of starting material and observing a similar radioactive distribution of the peaks in each case. Within experimental error therefore, the results above may be regarded as evidence for the absence of metabolism of the hydrocarbons. However, the presence of trace amounts of hydrocarbon remaining outside the algal cells even after washing with water subsequent to harvesting them cannot be excluded. The results may, therefore, only indicate the failure of the hydrocarbons to enter the algal cells.

Biosynthesis of 7- and 8-Methylheptadecanes

The first part of this work was a search for the presence of possible intermediates such as vaccenic acid in *Nostoc*. Holton, *et al.*,[16] reported 7.4% octadecenoic acid in the total acids of *Nostoc muscorum*. They reported it as Δ^9-C_{18} acid. However, the double bond position isomers were not separated by their packed gas–liquid chromatographic column.

Our gas chromatographic pattern for the methyl esters is given in Figure 1. The components were identified by glpc coinjections of standard esters and their mass spectra. Methyl oleate and methyl vaccenate had identical mass spectra but could be separated by a capillary column coated with Hi-Eff-4BP (polybutane-1,4-diol succinate). The fatty acid mixture contained mainly

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Table V. Fatty Acid Composition (%) of Total Lipids of *Nostoc muscorum*

Saturated acids		Monounsaturated acids		Polyunsaturated acids	
n-C_{16}	23.4	Δ^9-C_{16}	29.8	2Δ-C_{16}	1.5
n-C_{18}	0.6	Δ^9-C_{18}	13.8	2Δ-C_{18}	18.3
		Δ^{11}-C_{18}	1.2	3Δ-C_{18}	8.6

ment 8% (3.6×10^6 dpm) of the radioactivity was incorporated into the cell. The distribution of radioactivity in the various column chromatographic fractions of these experiments is given in Tables VI and VII.

When methionine-methyl-^{14}C was used, essentially all the radioactivity in the *n*-heptane fraction was incorporated in 7- and 8-methylheptadecanes. As shown in Table VI this mixture contains 9.05×10^5 dpm or 99% of the radioactivity. However, only 80.1% of the radioactivity of the *n*-heptane fraction was found in the branched C_{18} when tritiated vaccenic acid was used as a starting material. The incorporation of radioactivity into *n*-heptadecane is presumably a result of hydrogenation followed by decarboxylation.

Table VI. Incubation of Methionine-methyl-^{14}C

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total act. extracted, %
n-Pentadecane	15	1.6×10^{-3}	*n*-Heptane	9.52×10^5	26.8
n-Hexadecane	80	8.8×10^{-3}	Benzene I	7×10^3	0.2
n-Heptadecane	9×10^3	0.85	Benzene II	2.49×10^4	0.7
Methylheptadecanes	9.05×10^5	99	Methanol	2.56×10^6	72.3

Table VII. Incubation of Tritiated Vaccenic Acid

Distribution of radioactivity					
In the *n*-heptane fraction			In different fractions of the extraction		
Hydrocarbon	Dpm	Act. in fraction, %	Fraction	Dpm	Total act. extracted, %
n-Pentadecane	360	0.05	*n*-Heptane	7.15×10^5	6.5
n-Hexadecane	2.2×10^4	3.1	Benzene I	2.2×10^4	0.2
n-Heptadecane	1.09×10^5	15.3	Benzene II	8.8×10^4	0.8
Methylheptadecane	5.37×10^5	80.1	Methanol	1.01×10^7	92.5

C_{16} and C_{18} acids. The percentages of fatty acids are given in Table V.

This is the first observation of vaccenic acid (*cis*-Δ^{11}-C_{18}) in blue-green algae. Bloch, *et al.*,[17,18] considered that two different pathways exist for the synthesis of monounsaturated fatty acids. According to Bloch:[18] "among the C_{18} monosaturated fatty acids, the anaerobic bacterial pathway is apparently capable of producing both the Δ^9 and the Δ^{11} isomers, whereas the aerobic process invariably yields the Δ^9 acid." In our case, Δ^{11} is presumably produced by an aerobic process. Vaccenic acid (*cis*-Δ^{11}-C_{18}) could be formed as a minor product from stearic acid by a oxidative mechanism. Other possible precursors of the branched alkane such as acids with a methyl branch or cyclopropyl ring have not been found in *Nostoc*.

The confirmation of the presence of vaccenic acid in *Nostoc* led us to investigate its possible role in the biosynthesis of the methylheptadecanes. When tritium-labeled vaccenic acid was incubated with *Nostoc*, about 10% (1.1×10^7 dpm) of the radioactivity was incorporated into the cell and 90% remained in the supernatant solution. In the ^{14}C-labeled methionine experi-

The data in Tables VI and VII also confirm the fact that the methionine methyl group is specifically added to the double bond of vaccenic acid. If a methylene group is inserted into the double bond, followed by decarboxylation and then ring opening, it could produce the 50:50 mixture of 7- and 8-methylheptadecanes.

By using methionine-methyl-^{14}C and *cis*-vaccenic-^{14}C acid, Liu and Hofmann[19] were able to demonstrate that lactobacillic acid (11,12-methyleneoctadecanoic acid) biosynthesis in *Lactobacillus arabinosus*, an anaerobic bacterium, involved the addition of a methylene group into the double bond of *cis*-vaccenic acid. In the methionine-methyl-^{14}C and formate-^{14}C experiments, their results showed most of the radioactivity of lactobacillic acid was on the cyclopropyl site. It is suggested that the 11,12-methyleneoctadecanoic acid could be the intermediate of the branched C_{18} hydrocarbon. We are at present investigating this possibility.

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