Biosynthesis of Hydrocarbons in *Anabaena variabilis*. Incorporation of [*methyl*-^{14}C]- and [*methyl*-$^{2}H_3$]Methionine into 7- and 8-Methylheptadecanes*

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ABSTRACT: The hydrocarbon fraction of *Anabaena variabilis*, which accounts for about 0.09% of the cellular dry weight, consists of *n*-heptadecane and a 50:50 mixture of 7-methylheptadecane and 8-methylheptadecane. The relative amount of the methylheptadecane mixture varies from 22% in young cultures to 84% in cultures grown for 9 days. Incorporation of [1-^{14}C]acetate into heptadecane was at least 20 times as efficient as incorporation into the methylheptadecanes. On the other hand, [*methyl*-^{14}C]methionine was incorporated into the methylheptadecanes, and the incorporation efficiency increased with the age of the culture.

Mass spectrometry of the methylheptadecane mixture formed in cultures grown in the presence of DL-[*methyl*-$^{2}H_3$]methionine showed that the CD_3 group had been incorporated intact, ruling out cyclopropane intermediates in biosynthesis.

The hydrocarbon composition of blue-green algae is relatively simple, with *n*-heptadecane being the principle component in most cases (Winters *et al.*, 1969). A branched octadecane found in *Nostoc muscorum* has been identified as a 50:50 mixture of 7-methylheptadecane and 8-methylheptadecane by comparison with a synthetic mixture on capillary gas–liquid partition chromatography and mass spectrometry (Han *et al.*, 1968). Lesser amounts of this branched C_{18} mixture were found in *Phormidium luridum*, *Anacystis nidulans*, and *Chlorogloea fritschii*, while *C. fritschii* also contained 4-methylheptadecane (Han *et al.*, 1968). Han *et al.* (1969) have observed the incorporation of [16-^{14}C]palmitic acid, [18-^{14}C]stearic acid, [^{3}H]-vaccenic acid, and [*methyl*-^{14}C]methionine into the hydrocarbons of *Nostoc*. They suggested that 11,12-methyleneoctadecanoic acid was the precursor of the methylheptadecane mixture.

This report describes studies on the biosynthesis of a mixture of *n*-heptadecane and 7- and 8-methylheptadecanes by *Anabaena variabilis*. Incorporation experiments using [*methyl*-$^{2}H_3$]methionine indicate that the methyl group of the methylheptadecanes arises by transfer of an intact CD_3 unit.

Experimental Section

A. variabilis (Kützing), originally from J. Myers, Austin, Texas, was obtained from Professor H. Gaffron, Florida State University. The organism was maintained on slants of composition similar to medium D of Kratz and Myers (1955) but with added sodium citrate dihydrate (0.165 g/l.), Difco yeast extract (1.0 g/l.), and 2% agar. Cultures of *Anabaena* were grown in 3 l. of medium D contained in glass tubes 3.5 in. in diameter and gassed with air (100 ml/min) and CO_2 (5 ml/min) through a fritted-glass bubbler. The cultures were maintained at 27 to 30° under fluorescent and incandescent light (1300 ft-candles) with alternating 12-hr periods of light and dark. Smaller cultures (125 ml of medium D in 500-ml erlenmeyer flasks) grown under similar lighting conditions to a Klett reading of 250 (540 mμ) were used as inocula for the 3-l. cultures in all experiments cited here except one. The culture grown in the presence of [*methyl*-$^{2}H_3$]methionine was inoculated directly from a culture slant.

Sodium [1-^{14}C]acetate (radiopurity, 99%) and L-[*methyl*-^{14}C]methionine (radiopurity, 98%) were obtained from New England Nuclear. In incorporation experiments with these compounds, 25-ml aliquots of cells were removed from the *Anabaena* cultures at specified times and radioactive substrate in amounts indicated in individual experiments was added directly to the aliquots. Incubations were carried out for 1 hr under a slow stream of air at ambient temperature and light. Cells were then collected by centrifugation and suspended in 6 ml of chloroform–methanol (3:1), and the total suspension was dried under a stream of N_2 and resuspended in 2 ml of hexane. This hexane suspension was applied to a hexane-washed column of 1.0 g of neutral alumina (Alcoa) layered on 0.5 g of silicic acid (Mallinckrodt Silicar CC-4, 100–200 mesh) and the hydrocarbons were eluted with 15 ml of hexane.

For assay of radioactivity and hydrocarbon content the hexane eluent was partially evaporated under a stream of N_2, made up to 3.0 ml with hexane, and a 0.5-ml aliquot was counted in a Tri-Carb Model 3214 liquid scintillation counter using 15 ml of scintillator solution (4 g of diphenyloxazole and 50 mg of 1,4-bis-2(5-phenylazolyl)benzene per l. of toluene). Counting efficiency was 70%. The remainder of the sample was evaporated to about 20 μl and two portions of 3–10 μl each were analyzed by gas–liquid partition chromatography on a Research Specialties Co. instrument containing an argon ionization detector. A glass column (6 ft × 0.25 in.) packed with 4% SE-30 on 100–120 mesh Gas Chrom Q was operated at 160 or 172°. Peak areas were estimated by multiplying width at half-height times height.

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For determining radioactivity in specific peaks of the hydrocarbon fraction, effluent from the column was collected in U tubes cooled in an acetone–Dry-Ice bath. Collection efficiency was 92%. Samples were rinsed from the tubes with scintillator solution directly into scintillator bottles.

DL-[*methyl*-$^2H_3$]Methionine was prepared from DL-homocysteine (Nutritional Biochemicals) and trideuteriomethyl iodide (Brinkman, 99% pure) by a procedure suggested by Dr. J. Law. DL-Homocysteine (2 g) in 100 ml of 50% water–dioxane solution was brought to pH 10 with 4 N NaOH. A 5% excess of trideuteriomethyl iodide was added slowly to the stirred solution. Stirring was continued for 10 min after the trideuteriomethyl iodide dissolved. The reaction mixture was then freeze dried, the dry residue was added to 100 ml of boiling 95% ethanol, and the hot ethanol was filtered and allowed to cool slowly to form methionine crystals. Three recrystallizations resulted in a product with no ninhydrin-positive or iodine-staining impurities detected by thin-layer chromatography on silicic acid (developing solvent; 80 ml of butanol, 20 ml of glacial acetic acid, and 20 ml of water). The mass spectra of DL-methionine (Nutritional Biochemicals) and synthetic DL-[*methyl*-$^2H_3$]methionine, both three-times recrystallized, were obtained on a Nuclide low-resolution mass spectrometer at 70 eV. The DL-methionine spectrum was identical with that reported by Junk and Svec (1963), and the DL-[*methyl*-$^2H_3$]methionine spectrum was that expected for the trideuterated compound from the peak fragment assignment of Junk and Svec (1963). A maximum content of 2.2% nondeuterated methionine, 1.1% DL-[*methyl*-$^2H_1$]methionine, and 2.2% DL-[*methyl*-$^2H_2$]methionine was calculated from the relative intensities of the parent ion (*m/e* 152) and the ions at *m/e* 149, 150, and 151.

In order to obtain a level of incorporation of the [*methyl*-$^2H_3$]methionine into the branched-chain hydrocarbons which would be sufficient for mass spectral analysis, isotope dilution was minimized by using a small inoculum and a relatively high methionine concentration. A 3-l. culture containing 1.8 mM DL-[*methyl*-$^2H_3$]methionine was inoculated from a slant grown for 14 days under standard conditions and harvested at a Klett reading (540 mμ) of 125. Growth was slower than in the other experiments reported here, probably as a result of both the small inoculum and the high methionine concentration. Cells were extracted as described above with 70 ml of chloroform–methanol (3:1). The suspension was dried on a rotary evaporator, taken up in 6 ml of hexane, and placed onto a hexane-washed column of 1.5 g of neutral alumina layered over 0.7 g of silicic acid. The hydrocarbon fraction was eluted with 24 ml of hexane.

Mass spectrometry of the hydrocarbons was carried out by Professor Charles C. Sweeley at the Mass Spectrometer Facility, Department of Biochemistry, Michigan State University. Separation of the hydrocarbons by gas–liquid partition chromatography was achieved on a glass column ($^1/_8$ in. × 6 ft) packed with 2% OV-1 operated at 160°. Mass spectra were obtained on material eluted directly from the gas chromatograph using an LKB 9000 combined instrument with the ion source at 290°. Spectra were obtained at 70 eV with an accelerating voltage of 3500 V and an ionizing current of 60 μA.

Methyl esters of the *Anabaena* fatty acids from a 9-day culture were prepared by transesterification of the chloroform–methanol extract with BF_3–methanol (Morrison and Smith, 1964). The methyl octadecenoate fraction was purified by preparative gas–liquid partition chromatography on a 6 ft × 0.25 in. column containing 5% ethylene glycol adipate on 100–120 mesh Gas Chrom Q. The methyl ester (about 50 μg), collected from the effluent of several injections, was oxidized with permanganate–periodate (Scheuerbrandt and Bloch, 1962), and the resulting dicarboxylic acids were analyzed as their methyl esters on the same column. The analysis showed the presence of both dimethyl azelaic and dimethylundecanedioic esters and hence demonstrated that the octadecenoic acid fraction from this culture contained about 35% of Δ-11 and 65% of the Δ^9 isomers.

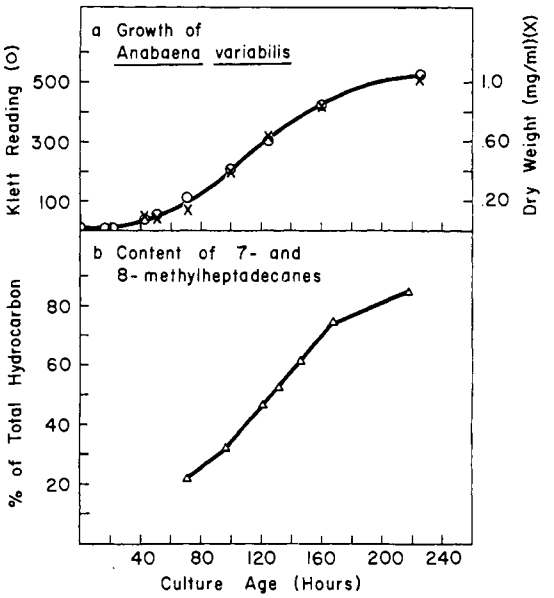

FIGURE 1: Growth of *A. variabilis* and the variation of hydrocarbon composition with the age of culture. Culture conditions are described in the Experimental Section. (a) Aliquots of the culture were removed for the determination of turbidity (○, Klett reading at 540 mμ) or dry weight of cells (×) in milligrams per milliliter of culture. (b) Aliquots (25 ml) of the culture were removed, and analyzed for hydrocarbon as described in the Experimental Section. Peaks corresponding to *n*-heptadecane and branched octadecane accounted for about 99% of the total hydrocarbon. The branched octadecane peak was shown by its mass spectrum to consist of a mixture of 7- and 8-methylheptadecanes. The quantity of this methylheptadecane fraction is plotted as per cent of total hydrocarbon (△). Duplicate determinations agreed within 5%.

Results

Figure 1a illustrates the growth of *A. variabilis* under the conditions employed in this study. The hydrocarbon content of the culture was analyzed at various stages of growth and found to be qualitatively similar to that reported for *N. muscorum* (Han *et al.*, 1968). About 99% of the hydrocarbon from *Anabaena* was found in two peaks with retention times corresponding to *n*-heptadecane and a branched octadecane. Trace amounts of material with retention times corresponding to *n*-pentadecane, *n*-hexadecane, and *n*-octadecane were observed. The mass spectral data discussed below indicate that the branched octadecane fraction of *Anabaena* consists of a 50:50 mixture of 7- and 8-methylheptadecanes comparable with the isomeric mixture identified in *Nostoc* by Han *et al.* (1968).

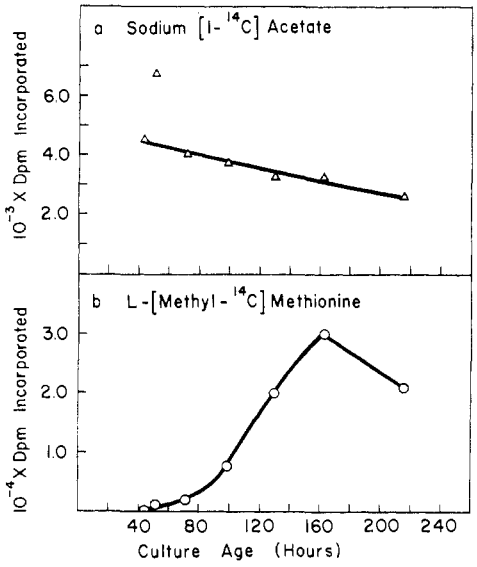

FIGURE 2: Incorporation of radioactive precursors into the hydrocarbon fraction of *A. variabilis*. Growth of cultures, incubation of aliquots with the radioactive precursors, and isolation and chromatography of the hydrocarbons are described in the Experimental Section. Values plotted are disintegrations per minute incorporated into the hydrocarbon fraction in 1 hr by 25 ml of cells of the indicated culture age. (a) Incubation mixtures contained 1.5 μCi of sodium [1-^{14}C]acetate which had a specific activity of 57 μCi/μmole. (b) Incubation mixtures contained 10 μCi of L-[*methyl*-^{14}C]methionine which had a specific activity of 14.9 μCi/μmole.

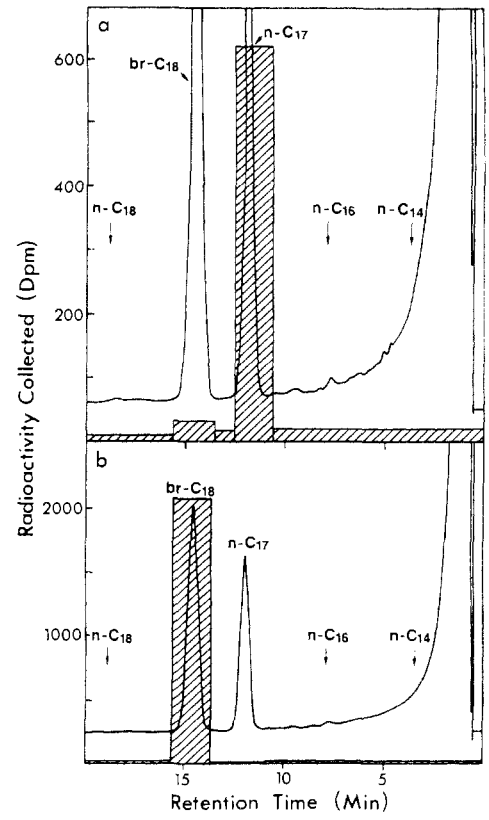

FIGURE 3: Gas–liquid partition chromatography of hydrocarbons obtained from cells incubated with [1-^{14}C]acetate (a) or L-[*methyl*-^{14}C]methionine (b). Experimental conditions are described in the Experimental Section. In the examples shown 25 ml of cells from a 163-hr culture was incubated for 1 hr with the radioactive substrate. An aliquot of the extracted hydrocarbon fraction was injected onto the gas–liquid partition chromatography column operated at 160° and the column effluent was collected and counted. The height of the bars indicates the radioactivity (corrected to decompositions per minute) collected during the time interval covered by the bar. The mass of the *Anabaena* hydrocarbons is indicated by the recorder tracing, and the retention times of standard hydrocarbons are given as reference points. (a) Cells were incubated with 1.5 μCi of sodium [1-^{14}C]acetate, specific activity 57 μCi/μmole. A duplicate analysis of this extract as well as duplicate analyses from 51- and 100-hr cultures all showed that more than 95% of the collected radioactivity eluted with *n*-heptadecane. (b) Cells were incubated with 10 μCi of L-[*methyl*-^{14}C]methionine, specific activity 14.9 μCi/μmole. A duplicate analysis of this extract as well as duplicate analyses from 51- and 100-hr cultures all showed that more than 99% of the collected radioactivity eluted with the methylheptadecane (br-C_{18}) fraction.

Quantitatively, the hydrocarbon content of *Anabaena* varies with culture age as illustrated in Figure 1b. The proportion of the methylheptadecane fraction is low in young cultures but increases throughout the growth period. From the data used to obtain Figure 1b, it was estimated that the total hydrocarbon content was about 0.09% of the cellular dry weight throughout the growth curve, increasing slightly in older cultures. Errors in measuring evaporated and injected volumes make the estimates of total hydrocarbon much less accurate than the relative composition.

The extent of [1-^{14}C]acetate incorporation into hydrocarbon as a function of culture age is illustrated in Figure 2a. The incorporation values plotted are for 25 ml of culture medium. Since the dry weight of cells per ml of culture is increasing with age (Figure 1a), the acetate incorporated per unit weight of cells falls considerably as the culture gets older. A separate experiment showed that for cells from a given culture age, the acetate incorporation into hydrocarbon is proportional to the number of cells in the incubation. These observations indicate that isotopic dilution of the acetate is more extensive in the older cultures, since the total hydrocarbon per cell remains constant or increases slightly. Figure 3a illustrates that more than 95% of the radioactivity from [1-^{14}C]acetate incorporated into the hydrocarbon fraction is accounted for in *n*-heptadecane.

The incorporation of [*methyl*-^{14}C]methionine into hydrocarbon is a function of culture age as illustrated in Figure 2b. The extent of incorporation is greater in older cultures, and parallels the rise in methylheptadecane content shown in Figure 1b. Gas–liquid partition chromatographic analysis of this incorporated radioactivity showed that more than 99% of it is accounted for in the methylheptadecane fraction (Figure 3b).

In order to determine whether the methionine methyl group was incorporated specifically into the branch methyl group of the methylheptadecanes, and to gain some insight into the mechanism of biosynthesis, an experiment with [*methyl*-$^{2}H_3$]-methionine was carried out. Cells were grown from a slant inoculum in the presence of 1.8 mM [*methyl*-$^{2}H_3$]methionine. The *n*-heptadecane and methylheptadecane fractions from these cells, as well as the hydrocarbons from control cells, were analyzed by combined gas–liquid partition chromatography and mass spectrometry.

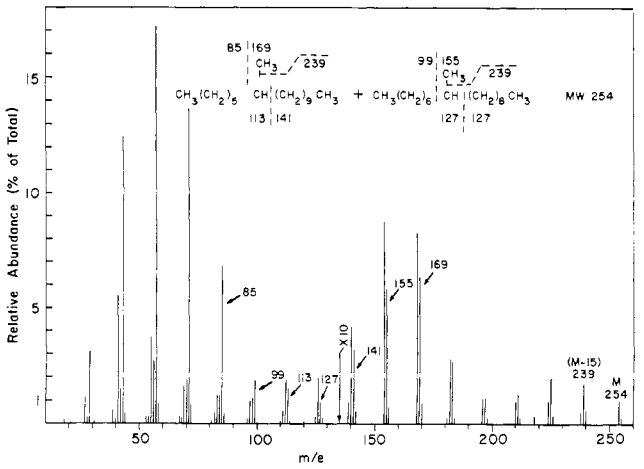

FIGURE 4: Mass spectrum of 7- and 8-methylheptadecane mixture isolated from cultures of *A. variabilis*.

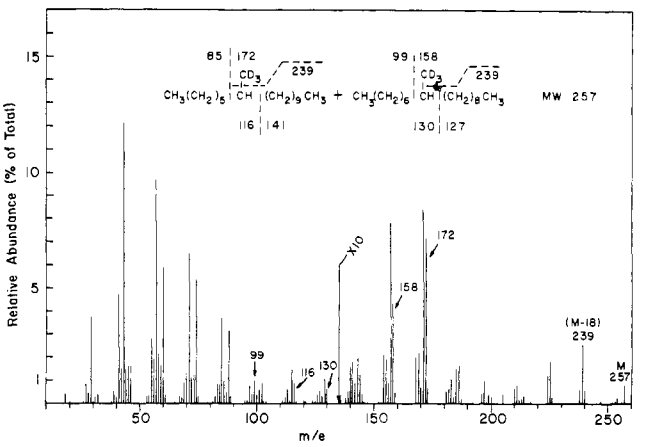

FIGURE 5: Mass spectrum of deuterated 7- and 8-methylheptadecane mixture isolated from cultures of *A. variabilis* grown in the presence of DL-[*methyl*-$^2H_3$]methionine.

Mass spectra of the *n*-heptadecane fraction from both the control culture and the [*methyl*-$^2H_3$]methionine grown culture were identical and corresponded to the published spectrum of this compound (Budzikiewicz *et al.*, 1967). This analysis confirmed the identity of *n*-heptadecane in *Anabaena*. The mass spectrum of the methylheptadecane mixture from the control culture is shown in Figure 4, and appears to be identical with the spectra published by Han *et al.* (1968), both for the *Nostoc* branched C_{18} hydrocarbon and for the synthetic mixture of 7- and 8-methylheptadecanes. The ions above *m/e* 169 are not clear in their published spectra, however. Figure 5 shows the mass spectrum of the deuterated methylheptadecane mixture. The parent ion peak has clearly shifted by three mass units to *m/e* 257, showing incorporation of three deuterium atoms into each component of the mixture. Cleavage of the branch methyl group still produces the ion at *m/e* 239 which represents M − 18 and corresponds to the M − 15 peak in the nondeuterated spectrum. This shows that all of the incorporated deuterium is in the branch methyl group. The ion at *m/e* 240 is even smaller relative to *m/e* 239 than in the nondeuterated spectrum, and so is not likely a result of CD_2H loss. The presence of an ion at *m/e* 256, however, indicates the possibility of up to 20% of the deuterated molecules being dideuterated. If dideuterated molecules are present, they could result from exchange of the methionine hydrogens. In the biosynthesis of methyltuberculostearic acid, which involves a transfer of a CD_2 group, about 12% of the deuterated molecules contained only one atom (Jauréguiberry *et al.*, 1965).

Fragmentation of 7-methylheptadecane at the branch methyl group produces an ion at *m/e* 169 and another at *m/e* 168 resulting from the loss of a proton. A similar pair of ions is produced at *m/e* 155 and 154 from cleavage at the branching position of 8-methylheptadecane. The deuterated sample shows both of these pairs displaced by three mass units to *m/e* 172 and 171, and *m/e* 158 and 157, respectively, which shows that these fragments also contain three deuterium atoms. Furthermore, the intensity ratio of trideuterated to undeuterated ions for these pairs is similar to that of the parent ions, almost 3:1. Examination of the mass spectrum also shows other ions of mass C_nH_{2n} and C_nH_{2n+1} which have been shifted by three mass units.

Discussion

There have been two basic mechanisms proposed for the biosynthesis of long-chain nonisoprenoid hydrocarbons. Kolattukudy (1968) has presented evidence in favor of an elongation, decarboxylation pathway for the biosynthesis of the *n*-C_{29} alkane in leaves from the plant *Brassica oleracea*. On the other hand, Albro and Dittmer (1969) have obtained a cell-free preparation from the bacterium *Sarcina lutea* which catalyzes the biosynthesis of long-chain hydrocarbons, principally C_{27} and C_{29}, from [2-^{14}C]acetate. Characteristics of this preparation favor a head to head condensation of two intermediate chain length fatty acids, with the two acids arising from different pools. Further evidence for and against each pathway has been discussed extensively by both groups and need not be summarized here. It is possible that both pathways exist depending upon the system studied.

We felt that the blue-green algae would provide an interesting additional system for studying hydrocarbon biosynthesis since the principle hydrocarbon present is *n*-heptadecane (Winters *et al.*, 1969). Han *et al.* (1968) have also identified a branched C_{18} component of *N. muscorum* as a 50:50 mixture of 7- and 8-methylheptadecanes. The same group (Han *et al.*, 1969) found a low level of incorporation of several precursors into the hydrocarbons of *Nostoc:* [18-^{14}C]stearic acid principally into *n*-C_{17} alkane; [16-^{14}C]palmitic acid principally into *n*-C_{15}, *n*-C_{17}, and branched C_{18} alkanes; [^{3}H]vaccenic acid principally into *n*-C_{17} and branched C_{18} alkanes; and [*methyl*-^{14}C]methionine into the branched C_{18} alkanes. They found a small amount of 11-octadecenoic acid (vaccenic acid) in the C_{18}-monounsaturated fatty acid fraction. They proposed that methylation of this acid could produce 11,12-methyleneoctadecanoic acid as demonstrated in *Lactobacillus arabinosus* by Liu and Hofmann (1962), and that reduction and decarboxylation would lead to the 7- and 8-methylheptadecane mixture. Thus their evidence favors a direct fatty acid decarboxylation mechanism over a head to head condensation of shorter fatty acids.

It is possible to write mechanisms of the second type; for example, head-to-head condensation of a C_8 and a C_{10} fatty acid could produce a double bond or other functional group in the proper position of the chain for methylation. Alterna-

tively, methylation of palmitoleic acid (9-hexadecenoic acid) followed by condensation with a C_2 unit (head to head or head to tail) and decarboxylation could also lead to the observed methylheptadecanes. Current evidence, while favoring one mechanism, cannot yet exclude all other possibilities.

In this report we have found that the hydrocarbon content of *A. variabilis* is similar to that of *Nostoc*. The effect of culture age on the biosynthesis of 7- and 8-methylheptadecanes in *Anabaena* is reminiscent of cyclopropane fatty acid biosynthesis in bacteria (Law *et al.*, 1963), which occurs mainly in the late logarithmic and stationary phases. We have also observed that the octadecenoic acid fraction in a 9-day culture of *Anabaena* contains about 35% of the Δ-11 isomer.

The incorporation of an intact CD_3 group from [*methyl*-$^2H_3$]-methionine into the methylheptadecanes of *Anabaena* excludes any mechanism involving a cyclopropane intermediate, however. The possibility still exists that a branch methyl fatty acid, either C_{17} or C_{19}, serves as an intermediate. If so, the mechanism of methylation must be different from that observed for the biosynthesis of 10-methylstearic acid in *Mycobacterium smegmatis*. In that case the methyl group is transferred with only two of its hydrogen atoms (Jauréguiberry *et al.*, 1965) and 10-methylenestearate is an intermediate (Toubiana *et al.*, 1967). Furthermore the methylation occurs specifically at position 10, rather than producing a 50:50 mixture of positional isomers as would be required for synthesis of the algal hydrocarbons.

Decarboxylation of an unsaturated fatty acid followed by methylation of the unsaturated hydrocarbon is also a possibility. Unsaturated hydrocarbons have not been observed in *Nostoc* or *Anabaena*, but have been detected in other blue-green algae (Winters *et al.*, 1969; Oro *et al.*, 1967).

[1-^{14}C]Acetate is incorporated at least 20-fold more efficiently into heptadecane than the methylheptadecanes in a 1-hr incubation. One might expect that the normal and branched hydrocarbons have a common precursor somewhere in the biosynthetic pathway. If so, there must be at least one intermediate between this common precursor and the methylheptadecane product to account for the additional dilution of isotope. Alternatively, pathways to the two products might be compartmentalized and completely independent of each other. In the absence of any compartmentalization, the acetate incorporation is still consistent with most of the mechanisms mentioned above as well as a mechanism in which *n*-heptadecane is itself a precursor of the methylheptadecanes. Only a mechanism involving a concerted methylation reduction of 7-heptadecene, in which 7-heptadecene is the common intermediate, can be ruled out by the acetate incorporation data.

Biological methylation at carbon was reviewed several years ago by Lederer (1964). Loss of one hydrogen atom from the methyl group was observed in the biosynthesis of ergosterol in *Neurospora crassa* as well as 10-methylstearic acid mentioned above (Jauréguiberry *et al.*, 1965), and it was felt that this might be the general mechanism for methylation of non-activated aliphatic double bonds. Since then, however, at least two cases in addition to the current one have been reported in which transfer of an intact CD_3 unit to an aliphatic double bond is involved. Synthesis of the ethyl side chain of stigmast-22-en-3β-ol in *Dictyostelium discoideum* involves two methionine methyl groups which retain five of their six deuterium atoms (Lenfant *et al.*, 1969). The mechanism depends upon the organism, since the ethyl side chain of poriferasterol from *Ochromonas malhamensis* retained only four of six deuterium atoms (Smith *et al.*, 1967). Smegmamycolic acid from *M. smegmatis* also retained three deuterium atoms in the methyl group (Jauréguiberry *et al.*, 1966). Jauréguiberry *et al.* (1966) have discussed methylation mechanisms which lead to either the CD_2 or the CD_3 result depending on the fate of a proposed carbonium ion intermediate.

Acknowledgment

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