JOURNAL OF BACTERIOLOGY, June 1967, p. 1811-1818
Vol. 93, No. 6

Aliphatic Hydrocarbons and Fatty Acids of Some Marine and Freshwater Microorganisms

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Received for publication 27 February 1967

Gas chromatography and combined gas chromatography-mass spectrometry have been used to study the fatty acids and hydrocarbons of a bacterium from the Pacific Ocean, *Vibrio marinus*, a freshwater blue-green alga, *Anacystis nidulans*, and algal mat communities from the Gulf of Mexico. Both types of microorganisms (bacteria and algae) showed relatively simple hydrocarbon and fatty acid patterns, the hydrocarbons predominating in the region of C-17 and the fatty acids in the range of C-14 to C-18. The patterns of *V. marinus* were more comparable to those of the algal populations than to patterns reported for other bacteria. An incomplete correlation between fatty acids and hydrocarbons in both types of organisms was observed, making it difficult to accept the concept that the biosynthesis of hydrocarbons follows a simple fatty acid decarboxylation process.

The nature and composition of the hydrocarbons and fatty acids found in the lipid extract of *Sarcina lutea* have been reported recently by Albro and Huston (1, 8, 9). The hydrocarbon pattern shows no odd carbon preference and little or no relationship to the fatty acid distribution between C-8 and C-20; most of the hydrocarbons are between C-16 and C-36. This finding is in contrast to the popular concept that hydrocarbons are derived by a loss of carbon dioxide from fatty acids. To our knowledge, *Serratia marinorubra* and *Vibrio ponticus* (W. G. Meinschein, *unpublished data*) are the only other bacteria for which straight chain hydrocarbons have been reported in the lipid extract.

This report presents evidence on the hydrocarbon and fatty acid distribution of *V. marinus*, a marine bacterium isolated from the Pacific Ocean (4); a freshwater blue-green alga, *Anacystis nidulans* (22); and of at least three different species of algae as found in algal mat communities (23; D. W. Nooner, Ph.D. Thesis, Univ. of Houston, Houston, Tex., 1966) from the Texas coast of the Gulf of Mexico. This has been done primarily to compare the composition of the above lipids in different microorganisms and, in addition, to obtain some understanding of the biogenesis of hydrocarbons by marine and freshwater microorganisms.

MATERIALS AND METHODS

Organisms. V. marinus MP-1 was cultivated at 15 C with heavy aeration to the late exponential phase of growth in an S. D. B. medium previously described (6). The cells were sent to us in the frozen state, immediately after harvesting, as a gift from R. Morita. The frozen culture was dried over P_2O_5 under vacuum.

A culture of *A. nidulans* was obtained from D. S. Hoare, and was grown autotrophically in the light at about 28 C on medium D_M of Van Baalen (27). Cultures were grown in 3-liter batches aerated continuously with filtered air. At the early stationary phase of growth, the cells were harvested by centrifugation, washed with a saline solution, and dried over P_2O_5 as above.

The algal mats, collected on the Texas Gulf Coast, were furnished in a dried form by C. C. Smith, Tenneco Oil Co., Houston, Tex. The algal mat sample SM-7 was collected on 23 February 1964 southwest of San Luis Pass on the southeast of Oyster Bay. Sample SM-8 was collected on 1 August 1964 west of Horse Triangulation Station and south of Harlingen Channel through Laguna Atascosa National Wildlife Refuge. Sample SM-9 was collected on 1 August 1964 from the Laguna Madre mud flat on the lagoonal side of Padre Island. The algal species found in sample SM-7 were identified as *Microcoleus chthonoplastes* (Mert.) Zanard, *Lyngbya aestaurii* (Mert.) Lyngb., and, in lesser amounts, *Schizothrix calcicola* (ag.) Gom.

A 3-g amount of the dried bacterial cells, 0.26 g of *A. nidulans*, and samples of 4 to 5 g of the dried algal mats were analyzed.

Extraction procedure. The method used to extract, fractionate, and analyze the hydrocarbons and fatty acids from the bacterium and algae has been thoroughly tested and was essentially the same which we have used for the analysis of meteorites, sediments, and materials of biological origin (15, 18-21). All the glassware used in this experiment was cleaned with hot chromic acid and rinsed with distilled water.

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Proper analytical control runs were performed on all instruments and materials. The dried cells were placed in an all-glass Soxhlet-type apparatus and were extracted with 50 ml of a benzene-methanol mixture (3:1) for 8 hr. The extracts from the Soxhlet apparatus were transferred to beakers, and the solvent was removed by evaporation at 40 C under a stream of purified nitrogen.

Column fractionation. The organic residue was separated on a glass column (1 by 30 cm) provided with a sintered-glass filter disc and filled to a depth of 18 cm with silica gel that had been heat-activated for 24 hr at 410 C. The column was washed with 15 ml of *n*-heptane. The residue was added to the column and then eluted into three fractions. The first fraction containing the aliphatic hydrocarbons was eluted from the column with *n*-heptane. The second fraction was obtained by elution with benzene and was saved for future analysis, and the third fraction was obtained by elution with methanol. This fraction contained the glycerides and other lipids. With *A. nidulans*, the *n*-heptane eluate recovered from the silica gel column was further purified by passing it through a 10-cm column of heat-activated alumina. The aliphatic hydrocarbons were recovered by eluting with 10 ml of *n*-heptane.

Preparation of derivatives. The fatty acids were liberated from the glycerides of the methanol fraction by alkaline hydrolysis (16). Methyl esters of the fatty acids were prepared for gas-liquid chromatography analysis as previously described (8).

Gas chromatography. The solutions of hydrocarbons and fatty acid methyl esters were dried under a stream of purified nitrogen. The residues were dissolved in benzene, and samples were taken for the gas chromatographic and mass spectrometric analyses. The hydrocarbon and fatty acid methyl ester chromatograms were obtained on a stainless-steel column (0.076 cm by 91 meters) coated with Polysev [*m*-bis *m*-(phenoxyphenoxy)-phenoxy benzene] at a nitrogen pressure of 700 g/cm^2 and on a column (0.076 cm by 155 meters) coated with Igepal Co-990 (nonyl phenoxy polyoxyethylene ethyl alcohol) at a nitrogen pressure of 933 g/cm^2, by use of an F. & M. 810 gas chromatograph (F. & M. Scientific Corp., Avondale, Pa.) equipped with a flame ionization detector.

Gas chromatography-mass spectrometry. The combined gas chromatographic-mass spectrometric analysis was performed by use of an LKB 9000 gas chromatograph-mass spectrometer (24, 26) and similar capillary columns and conditions as described above. Mass spectra of the major components of a given mixture were taken as each of the corresponding individual compounds emerged from the gas chromatographic column. The components entered the ion source of the mass spectrometer and were ionized by electron impact at 70 ev for fatty acids. An ionizing potential of 20 ev was used for the long-chain hydrocarbons since the molecular ion peak tends to be low. The ionizing current was set at 125 μa, and the accelerating field, at 3.5 kv. The electron multiplier voltage was adjusted between 1.7 and 2.5 kv according to the size of each peak. Each peak was scanned within the range of 10 to 400 mass units in approximately 10 sec. The spectra were recorded by means of an oscillograph recorder.

RESULTS

The gas chromatographic pattern for the hydrocarbons of *V. marinus* is given in Fig. 1A. By measuring relative retention times, the individual hydrocarbons were identified as alkanes and alkenes ranging from C-15 to C-18, with *n*-C-17 and *n*-Δ-C-17 being the two major components. The other two major peaks were identified as another *n*-heptadecene (peak 1) and an *n*-heptadecadiene (peak 2). It is possible that there was a higher quantity of the lower molecular weight hydrocarbons, since small amounts may be expected to be lost by the drying procedure and the evaporation of the extract under a stream of nitrogen. The results cannot be due to any artifact or contamination during our studies, since no measurable hydrocarbons were found in the procedure as shown by the controls and solvent blanks (*see* Fig. 1B). It is obvious from the pattern shown in Fig. 1A that there was a predominance of hydrocarbons with an odd number of carbons.

The gas chromatographic pattern for the fatty acids is given in Fig. 1C. The components were identified by relative retention time mainly as fatty acids with an even number of carbons, ranging from C-12 to C-18, the major components being palmitoleic and oleic acids.

The gas chromatographic analyses of three different algal mat samples are shown in Fig. 2. A resemblance to the *V. marinus* hydrocarbons was apparent, but the analyses showed a larger number of hydrocarbons, ranging from *n*-C-15 through *n*-C-31, with small amounts above *n*-C-23. The small amounts of hydrocarbons above *n*-C-23 were separated on a stainless-steel capillary column coated with Apiezon L and are not shown here. The components above *n*-C-23 showed a strong predominance of alkanes with odd rather than even numbers of carbons. It could not be determined from these results whether the small amounts of high molecular weight hydrocarbons were produced by the algae or were derived from other sources. The same can be said with regard to the isoprenoid hydrocarbons pristane and phytane which are shown as peaks a and b, respectively, of Fig. 2. The major components were *n*-C-17, *n*-Δ-C-17 plus br-Δ-C-18 (peak 1), and anteiso-C-18 (peak 2).

The fatty acids of the algal mats (Fig. 3, showing chromatograms of the corresponding methyl esters) were in fair agreement with the fatty acids found in algae by Parker and associates (22, 23).

The hydrocarbons and fatty acids of *A. nidulans* are shown in Fig. 4. There was a general resem-

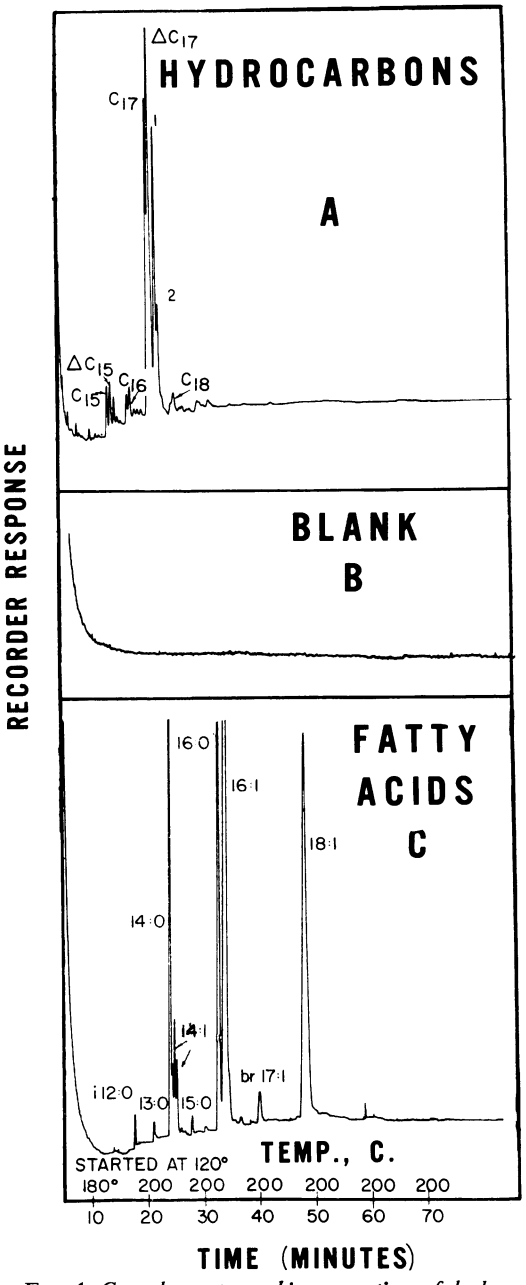

FIG. 1. *Gas chromatographic separation of hydrocarbons and fatty acid methyl esters of Vibrio marinus on a 155 meters by 0.076 cm stainless-steel tubing coated with Igepal CO-990. Nitrogen pressure, 933 g/cm²; no split. F. & M. 810 apparatus equipped with a flame ionization detector was used. Range, 10²; attenuation, 1. Temperature was programmed at approximately 6° per min from 120 to 200 C and was held isothermally at 200 C. From 3 g of extracted cells, (A) 1/10 of the sample was injected, (B) 1/1 of the sample was injected; and (C) 1/1,200 of the sample was injected. The first number indicates the number of carbon atoms in the chain of the fatty acid; the second number indicates the number of double bonds. Symbols: Δ = double bond; i = iso; br = branching.*

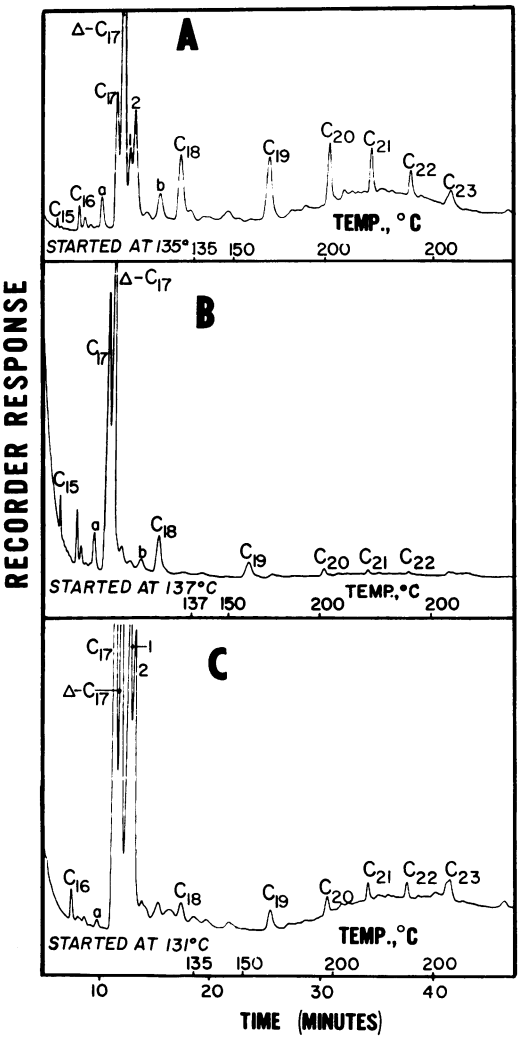

FIG. 2. *Gas chromatographic separation of aliphatic hydrocarbons from algal mats with a 91 meters by 0.076 cm stainless-steel tubing coated with Polysev. F. & M. 810 apparatus equipped with a flame ionization detector was used. Nitrogen pressure, 700 g/cm². Isothermal at 135 C for 18 min, then programmed at approximately 6° per min to 200 C. (A) Algal mat (SM-7) extracted, 4.7 g. About 1/15 of the n-heptane sample eluate was injected. Range, 10²; attenuation, 2. (B) Algal mat (SM-8) extracted, 5.1 g. About 1/20 of the n-heptane eluate was injected. Range, 10²; attenuation, 4. (C) Algal mat (SM-9) extracted, 4.1 g. About 1/12 of the n-heptane eluate was injected. Range, 10²; attenuation, 1. Symbols: Δ = double bond.*

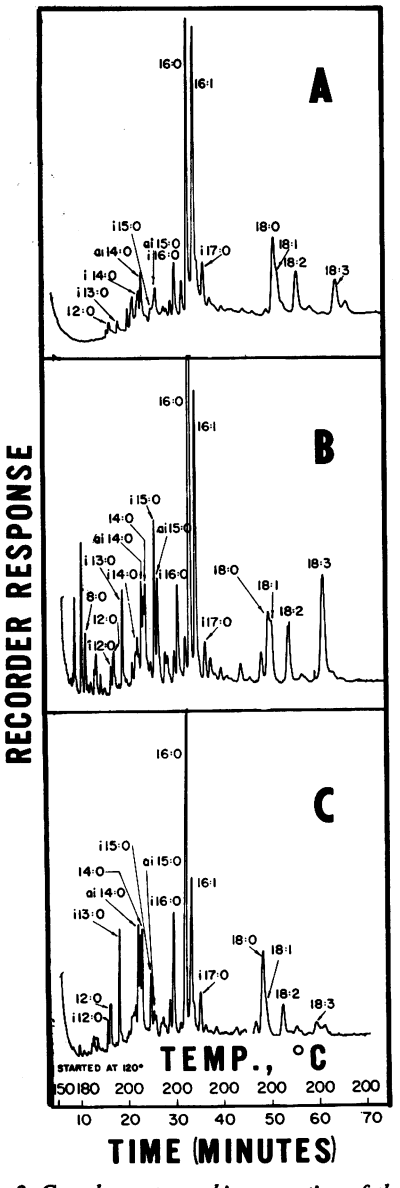

FIG. 3. *Gas chromatographic separation of the fatty acid methyl esters of algal mats. All conditions are the same as in Fig. 1. About $\frac{1}{200}$ of each sample was injected. The first numbers indicate the number of carbon atoms in the chain of the fatty acid; the second number indicates the number of double bonds. Symbols: i = iso; ai = anteiso.*

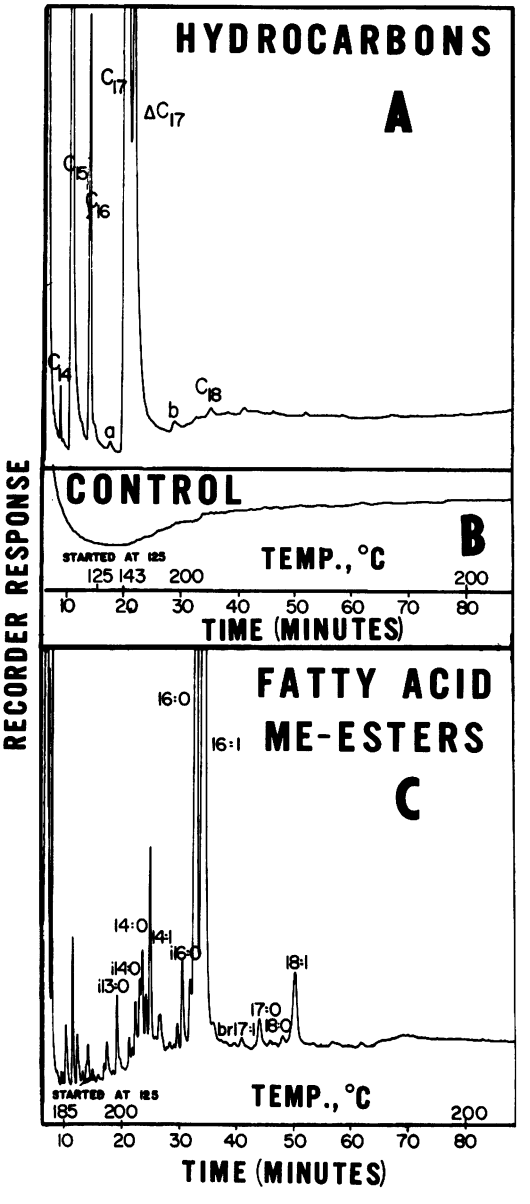

FIG. 4. *Gas chromatographic separation of hydrocarbons and fatty acid methyl esters of Anacystis nidulans. All conditions are the same as in Fig. 1, except for the starting temperature at 125 C. From 0.26 g of extracted cells, (A) approximately $\frac{1}{5}$ of the sample was injected; (B) $\frac{1}{100}$ of the sample was injected.*

blance to the hydrocarbon and fatty acids of marine organisms just described. The distribution of fatty acids in *A. nidulans* was in fair agreement with those published recently by Parker et al. (22).

Mass spectrometric analysis made on major hydrocarbon and fatty acid methyl ester peaks as they emerged from the columns confirmed most of the above assignments of compounds. The assigned identities and the relative estimation of the percentage composition for *V. marinus*, *A.*

nidulans, and algal mats are summarized in Tables 1 and 2. The summary in Table 2 notes which fatty acids were identified by both gas chromatography and mass spectrometry and which were assigned an identity only on the basis of their retention time. Unmarked compounds in the gas chromatograms (Fig. 1–4) were in amounts too small to be properly identified, or gave complex mass spectral data. Mass spectra, identical to those described in the literature (19, 25), were obtained for all the hydrocarbons listed in Table 1 and for most of the fatty acid methyl esters listed in Table 2 (*see* Fig. 5 for representative mass spectra).

Mass spectra showing the M-32, M-74, and M-116 maxima were observed for the methyl oleate and other monoenoic fatty acid methyl esters. In general, the mass spectra of the positional isomers of monounsaturated carboxylic acids are very much alike (7) when the double bonds are at position 6, 7, or higher in the chain. Usually, a peak corresponding to the loss of methanol (m/e = M-32) is found in their specta together with other characteristic peaks at m/e = M-74 and m/e = M-116. The peak at M-74 is produced by β cleavage with concerted rearrangement of a γ-hydrogen atom to the carbonyl oxygen of the ester group (3, 14). The peak at M-161 arises from a 5,6-cleavage. Similar fragmentation mechanisms were observed in the monoenoic fatty acid methyl esters.

Sightly different characteristic fragmentation patterns were observed with the C-18 di- and trienoic fatty acid methyl esters (Fig. 5). Since these two important acids, linoleic and linolenic, have only been analyzed once before (7, 25), their mass spectra are shown in Fig. 5 as confirmation of this work and as a representative example of our analyses. With methyl linoleate and methyl linolenate (Fig. 5), a peak appears at m/e = M-31 instead of the M-32 peak corresponding to the monoenoic esters. Two other peaks appear for linolenate at m/e = M-56 and m/e = M-69. It is possible that these peaks originate through single-bond cleavage favored by resonance stabilization of the alkyl cation and concerted rearrangement of hydrogen atoms.

The percentage composition of the hydrocarbons and the fatty acids shown in Tables 1 and 2 was calculated on the basis of their gas chromatographic peak areas, which were obtained by multiplying the peak heights by the widths at half the peak heights. For *V. marinus*, it was estimated that the total lipid fraction accounted for 9.8% and the total hydrocarbons for 0.01% (or 100 ppm) of the cell mass. For *A. nidulans*, the lipids constituted 12.2% of the cell mass, whereas the hydrocarbons accounted for 0.006% (60 ppm).

TABLE 1. *Relative percentage composition of hydrocarbons*

Hydrocarbon[a]	*Vibrio marinus*	Alga and algal mats			
		Anacystis nidulans	Sm-7	Sm-8	Sm-9
n-C-14	—	0.63	—	—	—
n-C-15	3.5	23.31	0.88	3.0	—
n-Δ-C-15	3.1	—	—	—	—
n-C-16	2.3	7.88	2.0	—	1.56
n-C-17	23.8	43.85	11.9	28.7	23.92
n-Δ-C-17	30.8	19.95	30.6	43.0	21.84
n-Δ-C-17 (a)	18.0	—	—	—	—
n-2Δ-C-17 (b)	7.5	—	—	—	—
br-Δ-C-18 plus *n*-Δ-C-17 (c)	—	—	6.28	—	21.32
anteiso-C-18 (d)	—	—	10.1	—	15.6
n-C-18	1.5	2.52	6.7	4.5	1.2
n-C-19	—	—	6.6	2.1	1.3
n-C-20	—	—	4.84	1.1	1.04
n-C-21	—	—	3.96	0.8	0.91
n-C-22	—	—	2.4	0.7	0.78
n-C-23	—	—	2.4	—	1.1
Pristane (a)	—	0.32	2.52	3.75	0.26
Phytane (b)	—	0.50	2.5	1.5	—
Others	9.5	1.04	6.32	10.85	9.17

[a] All the hydrocarbons listed by specific name or abbreviated formula were identified by mass spectrometry and by comparison with the retention of authentic compounds. (a) Peak 1 (Fig. 1); (b) peak 2 (Fig. 1); (c) peak 1 (Fig. 2); (d) peak 2 (Fig. 2).

TABLE 2. *Relative percentage composition of fatty acids*

Fatty acid[a]	*Vibrio marinus*	Alga and algal mats			
		Anacystis nidulans	Sm-7	Sm-8	Sm-9
8:0	—	—	—	2.01[b]	—
i12:0	1.1[b]	—	—	0.91[b]	1.5[b]
12:0	—	—	1.05[b]	1.56[b]	2.2[b]
i13:0	0.7[b]	1.58[b]	0.9[b]	3.58[b]	6.4[b]
i14:0	—	1.71[b]	2.55[b]	1.50[b]	—
ai14:0	—	—	4.05[b]	3.58[b]	6.6[b]
14:0	14.4	2.97	—	3.45	6.1
14:1	3.7 (2.4)	4.05	—	—	—
i15:0	—	—	1.1	5.72	3.4
ai15:0	—	—	2.70	3.71	1.4
15:0	0.72	—	—	—	—
i16:0	—	2.16	4.35	3.45	6.94
16:0	19.6	30.74	24.15	16.9	21.5
16:1	32.1	33.35	22.65	9.88	8.8
i17:0	—	—	3.75[b]	1.37[b]	2.5[b]
br17:1	1.5	0.36	—	—	—
17:0	—	1.17	—	—	—
18:0	—	0.36	6.45	2.6	9.4
18:1	22.8	3.56	3.45	2.28	?
18:2	—	—	3.6	3.2	2.6
18:3	—	—	3.70	7.15	2.0
Others	0.98	17.99	15.55	27.15	18.66

[a] Symbols: br, branching; i, iso; ai, anteiso.
[b] Identified by retention time alone. All the other fatty acids were identified by retention time and mass spectrometry.

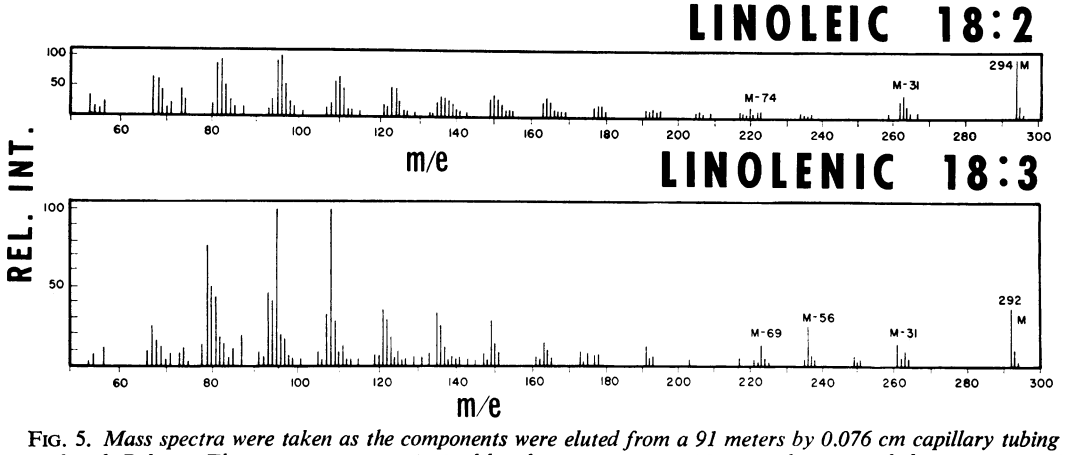

FIG. 5. *Mass spectra were taken as the components were eluted from a 91 meters by 0.076 cm capillary tubing coated with Polysev. The components were ionized by electron impact at 70 ev as they entered the ion source of the LKB 9000 gas chromatograph-mass spectrometer. Each peak was scanned within the range of 0.0 to 400 mass units in 10 sec. The spectra were recorded by means of an oscillograph recorder, and the chart speed was set at 4 inches per sec.*

The respective values for the algal mats varied from 2.7 to 5.8% for the lipid fraction and from 0.001 to 0.01% for the hydrocarbons. The percentages obtained for the algal mats should be considered only as lower-limit values because of possible loss of volatile components by extensive drying (in situ) before the samples were analyzed.

DISCUSSION

The range and nature of the hydrocarbons and fatty acids of *V. marinus* are relatively simple as compared with the bacterial hydrocarbons (1; W. G. Meinshein, *unpublished data*) and with bacterial lipids in general (8–10, 12, 13, 17). The

patterns obtained for *V. marinus* compare closely with those of algal mats and the blue-green alga *A. nidulans*, showing a predominance of fatty acids with even numbers of carbons and hydrocarbons with odd numbers of carbons. It would be unwarranted to attempt to draw a biochemical and ecological relationship between the algae and *V. marinus*, since very little information is available concerning the lipid-extractable content of marine microorganisms and freshwater blue-green algae.

In line with previous proposals (5, 11), it may be safely concluded that there are several possible pathways for the production of hydrocarbons and that the decarboxylation of a fatty acid resulting in a hydrocarbon is not an exclusive one. This conclusion is based on the fact that only some of the hydrocarbons of *V. marinus* (*n*-C-15, *n*-Δ-C-15, and *n*-Δ-C-17) and algal mats *n*-C-17 and *n*-Δ-C-17) show a possibility of correlating to the corresponding fatty acids.

On the other hand, no apparent direct correlation with the corresponding fatty acids was shown by the following hydrocarbons: *n*-C-17, *n*-heptadecadiene, and *n*-C-18 of *V. marinus*; br-Δ-C-18, anteiso-C-18, *n*-C-18, and other hydrocarbons of the algal mats; and some of the blue-green algae hydrocarbons. A more marked lack of correlation has been observed in other cases (1, 5, 8, 11), yet no other pathways for hydrocarbon formation have been adequately demonstrated. Work with radioactive precursors, which is being carried out in our laboratory with bacteria, algae, and other microorganisms, may provide some insight about other possible routes of hydrocarbon biogenesis.

ACKNOWLEDGMENTS

We are indebted to R. Morita, Oregon State University, for the *V. marinus* sample, to C. C. Smith, Tenneco Oil Co., for the algal mats, and to D. S. Hoare, University of Texas, for the *A. nidulans* culture.

This investigation was supported by grants NsG-257 and NGR-44-005-020 from the National Aeronautics and Space Administration.

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