PALEOBIOCHEMISTRY OF ALGAE

A Dissertation

Presented to

the Faculty of the Department of Biology

College of Arts and Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Howard J. Schneider

August 1969

To Lorraine

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ABSTRACT

Simple morphological as well as molecular fossils have been found in ancient and recent sediments. In most cases these fossils show the typical morphological characteristics of prokariotic algae. Algae have been implicated in the biogenesis of the hydrocarbons found in sediments, a fact which appears to be supported by the nature and distribution of their hydrocarbons.

Fifteen different species of algae, including seven Cyanophycophyta (Blue greens), six Chlorophycophyta (greens), one Chrysophycophyta (golden-brown) and a macroscopic marine alga from the coast of Hawaii were analyzed for their hydrocarbon and fatty acid content by gas chromatography and combined gas chromatography-mass spectrometry. All of the algae had significant amounts of hydrocarbons (100 - 3000 ppm) in both the high and medium molecular weight ranges.

Normal hydrocarbons ranging from C_{15} to C_{19} have been identified in several cases and n-C_{17} has been found to be predominant in all forms of algae. Some blue green algae contain substantial portions of methyl substituted alkanes. Significant amounts of high molecular weight straight chain olefins and paraffins with maxima at C_{23}, C_{27}, C_{29} were also identified. Squalene is the only isoprenoid found in these organisms. A triterpene has been tentatively identified in three blue greens.

The contemporary representatives of algae found in the sediments analyzed include: seven blue greens, six greens, and a golden brown. A few of these algae show a bimodal distribution of aliphatic hydro-

carbons with maxima at C_{17} and C_{23}, C_{27}, or C_{29}. Similar bimodal distributions of saturated hydrocarbons have been observed in both Tertiary and Precambrian sediments. These observations add support to the interpretation of the biological origin of hydrocarbons found in these sediments.

The fatty acid content was very similar in all of the algae analyzed, varying mostly in the degree of saturation. They ranged from 14:0 to 18:4 with the C_{16} fatty acids, either saturated or unsaturated, predominating in the blue greens, while the C_{18} fatty acids predominated in the green algae.

A macroscopic marine alga from Hawaii and the seed and seed coats of a plant were also analyzed by the same method as the algae. The hydrocarbon distribution of the marine alga was very similar to several of the other algae, as was its fatty acid content. However, the hydrocarbon content of Plantago ovata, similar to higher plants in the overall distribution of high molecular weight alkanes, was different in that one of the major components was an anteiso-C_{18}. P. ovata's fatty acids were similar to those of the algae.

Inferences from the distributions of hydrocarbons and fatty acids, plus radioactive labelling studied of algae, suggested something more than a simple decarboxylation or elongation of a fatty acid to produce the hydrocarbons found in these microorganisms. However, labelled stearate appears to serve as a precursor to the n-C_{17} in Chlorella.

Attempts of phylogenetic positioning of the algae based on hydrocarbon distributions alone are of little value. On the other hand, there may be some value in using fatty acids in limited taxonomical groups.

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I. INTRODUCTION

INTRODUCTION

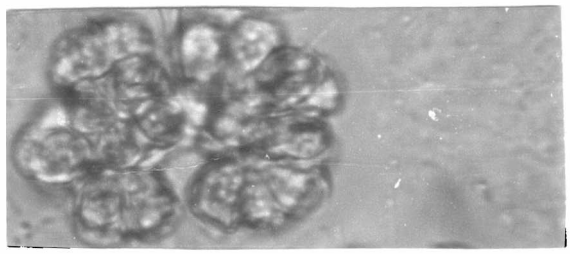
A. Morphological Fossils

One of the paramount questions of biology has been when and where life began. In the past, the only records of ancient life have been well preserved fossils. From these fossils the paleobiologist has constructed a record of life on earth dating back to the beginning of the Cambrian geological period, about 6×10^8 years. Micropaleontologists have extended this period well into the Precambrian, about 3×10^9 years, by the identification of microscopic remains of simple morphological entities.

Geologists, paleobiologists, and micropaleontologists have sought to record and categorize these fossilzed microscopic remains according to their morphological structures (1-27). In many instances their findings have been complemented by the paleobiochemist and organic geochemist with the detection of biological markers or molecular fossils of biological origin from the same geological time period (28-48).

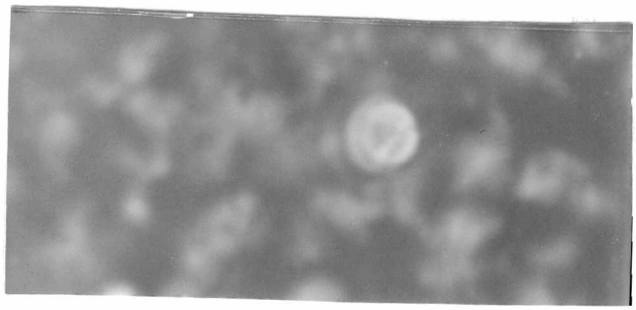
As shown in Figure 1, an examination of the microfossil content in a variety of sediments containing organic compounds shows algal remains to be one of the major materials. Algae have been considered the major contributor of the organic remains found in rocks from these sediments (1-27).

Barghoorn and Tyler (8) noted that the gross physical alteration of Precambrian microfossils has been extraordinarily small in view of the fossil's geological history. They attribute this to the manner in which the microorganisms were entropped in amorphous silica which dehydrated



Botrycoccus Braunii

Anacystis Nidulans



Chlorella Pyrenodosa

Figure 1. Micrographs of Some Fossil and Contemporary Algae

to opal and provided a structure resistant to distortion. This allowed preservation of a three-dimensional entity for long periods of time.

Barghoorn and Tyler's (8) explanation on how simple organisms, without skeletal parts, can withstand geological time allows one to trace a morphological history of life. The earliest known fossils of biological origin occur in the Onverwacht Series (3.2×10^9 years old) of South Africa. They consist of spheroidal and cup-shaped, carbonaceous bodies resembling algal remains (18). Somewhat near the same age as the Onverwacht Series, and overlying it, occur the Fig Tree Series (3.1×10^9 years old) (14, 15). Minute, bacterium-like, rod-shaped organisms were found organically and structurally preserved in black chert. Further studies of the Fig Tree Series carbonaceous chert uncovered spheroid-like microfossils that were interpreted as relics of unicellular algal-like organisms (15). The algal-like organisms reported in the Fig Tree Series appear to resemble, in size and shape, members of the modern blue-green order Chroococcales (15). A small but varied assemblage of primitive plants, representing both blue-green algae and simple forms of fungi have been identified in the nonferruginous cherts of the Gunflint iron formation of Southern Ontario (4, 13). One of the structures identified as algae appeared to have free, unbranched filaments devoid of heterocysts and spores. This algal-like organism could be compared with some species of the contemporary Lyngbya or Oscillatoria. These plant fossils were found in dense black cherts from the lower "algal" members of the Gunflint. It is estimated that the age of algal-like forms is approximately two billion years (4, 13). Precambrian

"coals" in Michigan have oval-shaped graphitized structures which have been interpreted as compressions of organisms. It is thought that these oval-shaped structures arose from colonial blue-green algae little different than the present day blue-green alga Nostoc (1, 3, 6). Spheroidal spore-like bodies that are distributed irregularly throughout the chert resemble the living form of Chroococcus (8).

The billion-year old shale from the Nonesuch formation contains microfossils and porphyrins (6). The fact that microfossils and porphyrins are found in this Precambrian sediment indicates the presence of photosynthetic organisms such as algae (6).

The Bitter Springs formation of Central Australia, which can be dated as Precambrian, contains an assemblage of structurally and organically intact microorganisms (12). These assemblages have been defined as both green and blue-green algae. This is the earliest known occurrence of green algae in the fossil record (12). There are spherical-to-subspherical yellow amber bodies whose morphology appears to be like that of resting zygotes of several modern green algae (12). Blue-greens of the Oscillatoriaceal and Nostocaceae have also been implicated in the Bitter Spring formations; however, the green algae are among "the oldest known multicellular fossils exhibiting distinct historiological preservation" (12).

The Precambrian period from the Onverwacht Series (3.2×10^9 years old) through the Bitter Springs formation (1×10^9 years old) contains countless identifications of fossil algae (1-27). These range from

simple prokaryotes, both bacteria and cyanophycean algae in the Fig Tree and Gun Flint formations (13, 14, 15, 16), to more advanced eukaryotes (green algae) in the Bitter Springs formations (12). These algae are known to occur in fairly large numbers. It has been estimated that in the more densely packed areas of the matrix, several thousand may occur in a one cubic inch of chert (17).

Great interest has been centered on the much younger Cretaceous and Tertiary sediments. The interest in these geological time periods is not centered so much on the question when did life originate on earth, but rather what specific organisms are responsible for the tremendous amount of organic compounds entrapped in the rocks of those periods. As with the Precambrian sediments, micropaleontologists have implicated algae as one of the major contributors of organic deposits found in younger sediments (19-27).

In the oil shale of the Lower Cretaceous Fusion formation, it has been calculated that there are about three hundred thousand algae per cubic centimeter (20). The Green River shale of Colorado, Utah, and Wyoming contains several types of algae that appear no different from contemporary algae. The lakes that formed these sediments existed for several million years during the Eocene epoch, which is early in the Tertiary period (19). The lake beds are considered to be compacted and lithified lacustrine oozes. The organic matter contained there is a result of microscopic organisms that inhabited the lake.

Algae very similar to Tetraedron sp. and Coelastrum sp. have been identified in the Green River formations (19). These two green algae

and a blue-green alga, much like Chroococcus, have been associated with the richest beds of oil shale, or those areas of the sediments that contain the greatest amount of organic matter (19). There have been reports of the green alga Pediastrum occurring in the Waltman Shale Member of Central Wyoming (26) and in the Subathu formation of Himachal Pradesh, India (24).

The alga most implicated in either the Tertiary or Cretaceous periods has been the golden-brown alga Botryococcus braunii (20-23, 25-27). Botryococcus braunii has existed with little change since the Paleozoic (27). This organism is said to be an outstanding example of an oil producing alga (22). "Yellow bodies" in several channels, bogheads and torbanite are thought to be the remains of B. braunii (20-22). A comparison of the extant Botryococcus alga from a Swedish lake with that of the fossil algae found in a Tertiary black clay of France leaves little doubt that the latter belongs to the genus Botryococcus (20).

To summarize, the morphological fossil record, from the first simple prokaryotes found in the Precambrian Fig Tree formations to the more phylogenetically advanced eukaryotes of the Tertiary sediments is indeed heavily influenced by the presence of algae.

B. Molecular Fossils

The possible role of the biogenic molecular fossils detected in geological samples is well documented (28-45). Organic compounds were most probably formed early in the history of the earth (30); therefore, both the organic geochemist and paleobiochemist are faced

with the task of distinguishing the abiological from the biological chemical fossils. Modern analytical techniques have left very little doubt as to the identification of these so-called "biological markers" (38, 40, 43). Eglinton and Calvin (28) describe "biological markers" as a term being applied to organic substances that show pronounced resistance to chemical change and whose molecular structure gives a strong indication that they could have been created in significant amounts only by biological systmes. The most abundant organic material found in sediments is in an insoluble amorphous material known as kerogen. Kerogen appears to be formed due to the combination of a variety of reactive unsaturated compounds (28).

Amino acids, carbohydrates, porphyrins, fatty acids, and hydrocarbons all have been detected in these old sediments (29, 33-37, 41, 42, 43). Porphyrins and hydrocarbons account for the widest variety of molecular fossils (11, 28, 41, 43). Diagenesis of the biogenic pigments produces hundreds to thousands of different porphyrins in sediments (43). The presence of vanadyl porphyrins in certain Precambrian sediments suggest that photosynthetic organisms have existed for more than a billion years (11). Sediments containing porphyrins are thought not to have been subjected to high temperatures since they would have decomposed (11, 28). Chemical fossils such as the prophyrins could not have persisted for more than 100 years in a lithologic environment with temperatures of 250° C. However, it appears that those soluble lipid compounds consisting only of carbon and hydrogen, such as aliphatic hydrocarbons and isoprenoids,

can withstand a wide variety of geological conditions. This is due to the strong carbon-carbon bonds and their resistance to reactions with water (28).

Oro, et al. (36) analyzed cuts of black chert from the Precambrian Gunflint formations that had been examined microscopically. The sections of chert contained micro-fossils, with blue-green algae being the most abundant. Gas chromatographic and combined gas chromatographic-mass spectrometric analysis of the chert showed that it contained normal paraffinic hydrocarbons ranging approximately from C_{16} to C_{32} with the distribution being bimodal but showing no predominance of C-odd over C-even alkanes. The chert sample also contained pristane and phytane and the assumed chemical evidence of life (presence of pristane and phytane) correlates with the morphological evidence provided by the fossilized organisms (4, 36). Meinschein (37) observed the presence of pristane, phytane, and n-paraffins in the Precambrian Soudan formation. These compounds are widely believed to indicate a biological origin and their presence in these samples could indicate that life on earth existed 2.7 billion years ago (37). Sedimentary rock 3.2×10^9 years old from the Fig Tree series of the Swaziland system, eastern Transvaal, and South Africa have been analyzed for aliphatic hydrocarbons in our laboratory (38). Aliphatic hydrocarbons are present in the Fig Tree rocks at extremely low levels (0.003 - 0.15 ppm). Because of the compact nature of some of these rocks and the fact that internal samples were analyzed in most cases, it is probable that

the hydrocarbons are indigenous to the rock. This, coupled with the fact that microfossils have been found in some of these rocks (14, 15), suggests a biochemical origin for these hydrocarbons which took place about 3.2×10^9 years ago (38).

The Green River Shale, a sediment of the Eocene Age, constitutes the major oil-shale reserve of the United States. This shale yields an average of 40 gallons of crude oil per ton (46). Bradley (47) presents evidence that the bulk of this organic matter is of algal origin. Normal alkanes, exhibiting an odd over even carbon chain length, have been identified in this particular shale. This odd over even hydrocarbon pattern is observed in contemporary organisms (48). The range of normal alkanes in the Green River formation is from C_{13} - C_{33} (47). The evidence for the biological origin of the extractable organic matter from this shale lies in the fact that the saturated hydrocarbon fractions contain aliphatic, isoprenoidal, steroidal, triterpenoidal, and tetraterpenoidal alkanes (49).

In addition to the hydrocarbons found in these sediments, one would expect organic acids to be present. The organic acids of the Green River shale have been extensively reviewed by Burlingame, et al. (46). Blumer and Cooper (45) isolated phytanic acid, pristanic acid, and 4,8,12-trimethyltridecanoic acid from three recent sediments and found that the ratio of palmitic to pristanic acid is similar to marine acids, a fact which is indicative of a biochemical origin. They did not detect the C_{11} to C_{22} isoprenoid acids found in ancient sediments.

Leo and Parker (50) report the finding of branched-chain acids in sediments. They maintain that these branched-chain fatty acids most probably originate from bacteria (51).

The analysis of ancient sediments has indicated that the alkanes contained therein are related to the long-chain carbon compounds of lipids in present day organisms (28). The isoprenoids are branched chains of five carbon units assembled in a regular order. These units are assembled in a head to tail fashion and the methyl branch is attached to every fifth carbon. Chlorophyll is the most widely distributed molecule with a isoprenoid side chain (11, 28, 33, 41-43). It is a reasonable assumption that isoprenoids occurring in sediments have been formed by a biological process. Gelpi (31), in our laboratory, generated hydrocarbons abiotically in an open flow Fischer-Tropsch system, and he has shown that the accepted biologically significant molecules such as the isoprenoids are not generated by this process (31). Saturated hydrocarbons related to phytol have been found in both crude oil and ancient sediments (41). Tetramethylheptadecane is a chemical fossil, implanted in sediments, that has possibly arisen from carotenoids or other isoprenoids of biological origin (41).

There is always the possibility that these hydrocarbons could have migrated from some other source. The C^{13}/C^{12} ratios of the lipid soluble hydrocarbons has been compared to the C^{13}/C^{12} ratios of kerogen from the same sediment. Kerogen, because of its solubility properties, is believed not to have migrated from its origin. The C^{13}/C^{12} isotope

ratios are very similar both in hydrocarbon and kerogen functions (42). The literature supports the view that organic compounds with ordered structures can indeed be indices of ancient life.

C. Contemporary Counterparts of Fossil Algae

Designation of algae implicated in both recent and ancient sediments has been based on morphological comparisons with contemporary counterparts (1-27). Tables 1A and 1B, based on recent literature, presents a list of 28 fossil forms identified on the comparison basis. The organisms have been arranged into taxonomic groupings of division, order and family.

Seventeen of the organisms are members of the cyanophycophyta, the blue-green algae. Blue-green algae are considered to be closely related to bacteria, possessing the primitive characteristic of a poorly defined membranous system. This is reflected in their prokaryotic condition, the absence of a membrane-bound chloroplast and the absence of normal mitochondria, golgi bodies, endoplasmic reticulum and lysosomes. Blue-green algae differ from bacteria primarily in their photosynthetic pigmentation, photosynthetic reactions and occasionally demonstrated thylakoids. Within the cyanophycophyta, four families are listed in order of increasing complexity. The chroococaceae is composed of unicellular or colonial organisms which lack specialized reproductive structures, reproducing only by simple cell division or fragmentation. The remaining families of the Cyanophycophyta represented all consist of organisms characterized by uniseviate filaments. The oscillatoriaceac

TABLE IA

CONTEMPORARY COUNTERPARTS OF ANCIENT ALGAE

Organism	Division	Order	Family
Anacystis montana	Cyanophycophytce	Chrococcales	Chroococaceae
Anacystis niculans	Cyanophycophytce	Chrococcales	Chroococaceae
Anacystic cyanea	Cyanophycophytce	Chrococcales	Chroococaceae
Anacystis menegh	Cyanophycophytce	Chrococcales	Chroococaceae
Chroococcus macrococcus	Cyanophycophytce	Chrococcales	Chroococaceae
Chroococcus turgidus	Cyanophycophytce	Chrococcales	Chroococaceae
Arthrospira sp.	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Lyngbya aestaurii	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Microcoleus vaginatus	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Oscillatoria chilkensis	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Oscillatoria grunowiana	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Oscillatoria vaucher	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Spirulina meneghiniana	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Spirulina platensis	Cyanophycophytce	Oscillatoriales	Oscillatoriaceae
Anabaenopsis sp.	Cyanophycophytce	Oscillatoriales	Nostocaceae
Gloetrichia sp.	Cyanophycophytce	Oscillatoriales	Rivulariaceae
Homoethrix sp.	Cyanophycophytce	Oscillatoriales	Rivulariaceae

TABLE IB

CONTEMPORARY COUNTERPARTS OF ANCIENT ALGAE

Organism	Division	Order	Family
Chlorosphaera sp.	Chlorophycophyta	Tetrasporales	Coccomyxaceae
Pleurococcus sp.	Chlorophycophyta	Ulotrichales	Protococcaceae
Microspora sp.	Chlorophycophyta	Ulotrichales	Microsporaceae
Chlorococcum sp.	Chlorophycophyta	Chlorococcales	Chlorococcaceae
Chlorella pyrenoidosa	Chlorophycophyta	Chlorococcales	Oocystaceae
Tetraedron sp.	Chlorophycophyta	Chlorococcales	Oocystaceae
Coelastrum microsporum	Chlorophycophyta	Chlorococcales	Coelastraceae
Pediastrum sp.	Chlorophycophyta	Chlorococcales	Hydrodictyaceae
Phacus caudata	Euglenophycophyta	Euglenales	Euglenaceae
Phacus pleuronectes	Euglenophycophyta	Euglenales	Euglenaceae
Botryococcus braunii	Chrysophycophyta	Heterococcales	Botryococcaceae

contains simple filamentous organisms which reproduce by division or fragmentation (hormogonia). The Nostocaceae, on the other hand, contains filamentous organisms with heterocysts and akinetes, specialized reproductive structures. The Rivulariaccae resemble the Nostocaceae in this respect, but additionally are characterized by more specialized and organized filaments.

Eight of the listed species belong to the Chlorophycophyta, or green algae. Organisms within this division have well-developed membranous structure within the cell, usually show an alternation of generations and have pigmentation similar to that in higher plants. Complexity within the Chlorophycophyta can best be discussed on the order level. The Tetrasporales is composed of unicellular or colonial algae whose vegetative cells are immobile but possess the unique ability to become directly mobile. Vegetative division is common, multinucleate cells are absent and a palmella stage frequently appears in the life cycle. The evolution of the Ulotrichales from the Tetrasporales is evidenced by the occasional presence of a palmella stage. Organisms of this order are filamentous, branched or unbranched, with totally immobile vegetative cells, sexual reproduction characterized by bi- or quadriflagellate gametes and occasional occurrence of multinucleate cells. Within the Chlorococcales, organisms are unicellular or colonial. Vegetative division is absent, division always giving rise to reproductive cells. Non-motile autospores are common and there is a tendency towards a multinucleate condition.

Two of the reported species belong to the Euglenophycophyta, whose members exhibit intercellular structure and pigmentation similar to the Chlorophycophyta, but most of the organisms in this division exist as unicellular, free-swimming organisms which lack a typical cell wall. Nutrition is variable, including holozoic, and sexual reproduction is questionable. The presence of colorless forms in this division has led to postulations of common ancestry between animals and plants.

Botryococcus braunii is one reported species which has been difficult to place taxonomically. The tendency is to place this organism in the Xanthophyceae of the Chrysophycophyta on the basis of pigmentation and cell wall structure. However, this organism also possesses features which wouldd correlate with the Chlorococcales. The photosynthetic storage product is starch and reproduction is through autospore formation. The morphology of the organism is a free-floating amorphous colonial form. Botryococcus probably represents a transitional organism.

D. Aliphatic Hydrocarbons and Fatty Acids in Algae and Bacteria

Aliphatic hydrocarbons have been reported in the lipid extracts of algae and bacteria (31, 32, 53-73). Serratia marinorubra (53), Vibrio ponticus (55, 56) and many other microorganisms that are common terrestrial species (31) have been shown to have measurable amounts of hydrocarbons. Normal paraffins were shown to be present in various samples of benthic and planktonic algae (54).

Most of the few microscopic algae and related organisms analyzed thus far have shown aliphatic hydrocarbons of relatively low molecular

weight, with a maximum at about C_{17} (70-73, 84). This is in contrast to the fact that higher plants synthesize substantial amounts of alkanes of high molecular weight in the C_{23} to C_{33} range (48, 76). Gelpi, et al. have now shown high molecular weight hydrocarbons to be present in amounts of 0.25 percent of the dry cell weight in B. braunii and 0.1 percent of the dry cell weight in A. montana (61). Clark and Blumer (54) found that the range of hydrocarbons in the benthic and planktonic algae exhibited only a slight odd-carbon predominance. All of the algae had a major maximum at n-C_{17} and a secondary maximum around n-C_{27}. Oro, et al. (84) studied the hydrocarbons of a bacterium from the Pacific Ocean, Vibrio marinus, a fresh water blue-green alga, Anacystis nidulans and algal mat communities from the Gulf of Mexico.

The microorganisms investigated showed relatively simple hydrocarbon patterns predominating in the region of the n-C_{17}. An analysis of four blue-green algae by Han, et al. (70) showed that the n-C_{17} hydrocarbon is the predominant component of the hydrocarbon mixtures. Han (70) also reported the finding of methyl branched alkanes which were not iso- or anteiso-structures. Two species of blue-green and two species of green algae analyzed by Han, et al. (72) revealed a presence of normal hydrocarbons ranging in carbon number from n-C_{15} to n-C_{20}. In all four species the n-C_{17} was the major constituent. Winters, et al. (73) found that the hydrocarbon composition of 11 species of blue-green algae was simple and qualitatively similar. The monoenoic and dienoic C_{19} hydrocarbons were the only hydrocarbon

constituents present in three marine coccoids. The remaining eight species had a range of hydrocarbons from C_{15} to C_{18} with n-C_{17} as the major component.

The hydrocarbon distribution of Chlorella vulgarsis has been shown to shift with changing culture conditions. The C_{25} and C_{27} predominate when C. vulgarsis is grown hetrotrophically (74). It has been well established that the fatty acid composition of microorganisms is affected both quantitatively and qualitatively by the nature of the medium and by the conditions under which they are grown (59, 62, 77, 81). The fatty acids of bacteria have been reviewed extensively by O'Leary (77) and Kates (78). Lauric, myristic, palmitic and stearic acid occur frequently in bacteria as well as the unsaturated forms (77). However, other workers (31, 60, 85, 86) have shown that branched-chain fatty acids having one methyl group at the iso or anteiso position occur in bacteria even though they are rare in nature. Work in our laboratory has shown that in Sarcina lutea, Staphylococcus aureus, and Bacillus species the iso and anteiso pentadecanoic acids are major components (31, 60). Kaneda (79) investigated ten species of the genus Bacillus and found that in all cases the branched-chain fatty acids comprised over 50 percent of the total fatty acids. Parker, et al. (85) reported that the iso plus the anteiso C_{15} fatty acid accounted for 47.0 and 57.0 percent in two out of four marine bacteria analyzed.

It appears that algal fatty acids are made of even numbered straight chains in the C_{14} to C_{18} range (80-85). Algal fatty acids, whether

derived from either prokaryots or eukaryots, differ little in their distribution or relative amounts (87). Analysis of fatty acids by Oro, et al. (84) have shown the blue-green alga A. nidulaus to contain large amounts of palmitic and palimtoleic acid. The same was true of the three algal mats analyzed. However, all of the algae analyzed contained odd chain length fatty acids. No methyl-branched fatty acids were found. The green algae C. pyrenoidosa has a range of even numbered fatty acids from C_{14} to C_{20} with the major acids being the saturated and unsaturated C_{18} series (82, 83). A. nidulans was shown to have palmitic acid and a hexadecanoic acid, presumed to be palmitoleic, that totaled approximately 90 percent of the fatty acids present (81). Holton, et al. (80) suggests that algae containing more highly unsaturated acids are usually morphologically more complex. An exception to this correlation in the blue-green algae they analyzed was in the thermophile Hapalosiphon laminosus. Han, et al. (72) found that fatty acids of all of the algae they analyzed have their dominant molecules among the lower molecular weights (C_{16} to C_{18}).

The hydrocarbons of algae represent a diverse group of homologous molecules ranging from C_{15} to C_{31} with a predominance of odd numbered chain lengths (31 and the references therein). Methyl-branched hydrocarbons, as well as isoprenoids, are found (72). It appears that the n-C_{17} either saturated or unsaturated is a major constituent of the hydrocarbon fraction of algae (31, 32, 65-68, 70-73, 84). High molecular weight hydrocarbons are present in some algae (54, 66, 74). However,

the high molecular hydrocarbons are not present in such a wide variety of algae as the lower molecular weights. The fatty acids of algae, in contrast to the hydrocarbons, exhibit a simple pattern of even-numbered chain lengths (80-82, 87). The greatest variation in the fatty acids of algae is the degree of saturation (80). Algae contain substantial amounts of fatty acids and hydrocarbons which find their way into the environment (54) and could very well give rise to these lipid components found in sediments.

E. Biosynthesis of Hydrocarbons

There is very little data on the biogenesis of hydrocarbons by microorganisms (31, 58, 88-91). The first adequate investigation into the biosynthetic pathways of hydrocarbons was the tracer experiment reported by Sanderman and Schweers (88), which showed that acetate-^{14}C incorporation into n-heptane was the result of a condensation of four acetate units with an apparent decarboxylation. Since this report, there have been attempts to understand the biosynthesis of aliphatic hydrocarbons through acetate-^{14}C and glucose-^{14}C incorporation into the paraffin, nonacosane, of surface wax of cabbage leaves (Brassica oleracea) (89, 91). In the case of Brassica oleracea Kolattukudy has shown that fatty acids act as precursors for long-chain hydrocarbons (90). Fatty acids of chain length C_{14}, C_{16} and C_{18} were presumably elongated to long-chain fatty acids with subsequent decarboxylation. Stumpf (92) has proposed a mechanism whereby even-carbon fatty acids undergo decarboxylation, producing an odd-numbered alcohol or aldehyde, which is then reduced to yield the corresponding hydrocarbon.

An investigation into the mechanism of hydrocarbon biosynthesis in Sarcina lutea was performed by measuring the amounts of ^{14}C-incorporation into hydrocarbons and fatty acids with a combination gas chromatograph and high temperature gas flow ionization apparatus (58). The following observations were made (58): 1) L-iso-leucine-(UL)-^{14}C was predominantly incorporated into the anteiso-branched chains; 2) that the palmitate-16-^{14}C gave evidence that a direct correlation may exist between the non-polar end of the palmitate and the biosynthesis of hydrocarbons and carotenoids; 3) that the lable from palmitate-1-^{14}C was incorporated into the various hydrocarbon groups as a compound, derived from the polar-end of the palmitate, consisting of more than two carbon atoms; 4) that the palmitate-16-^{14}C and 1-^{14}C gave no detectable evidence that transformed products were incorporated into other fatty acids; and 5) that the sodium acetate-2-^{14}C gave evidence of non-specific incorporation into both the aliphatic hydrocarbons and fatty acids of Sarcina lutea. Laseter (91), in an attempt to elucidate the precursor-product role of long-chained fatty acids, supplied C_{14}, C_{16} and C_{18} fatty acids to cabbage leaf disks. He found that in all cases, no matter where the labelled carbon appeared in the acids, the paraffins, ketones and other wax fractions became radioactive. He concluded that this gives support to the theory that the acids enter the wax molecules intact or that there is complete degradation of the submolecule to C_2 units which are reincorporated. Work is now being carried out, using C^{14} labelled fatty acids that are normal constituents of algae, to ascertain the fate of such fatty acids, and if they are eventually incorporated into algal hydrocarbons (93).

Morphological remnents of organisms which resemble certain contemporary algae have been found in both ancient (3.1×10^9 years) and recent sediments. This fact is well supported in the literature. Organic molecules that have been determined to be of biological origin by means of modern analytical techniques have been found in these same sediments. The contemporary counterparts of these fossil algae still exist today, apparently unchanged by geological time. Analysis of algae and related microorganisms gives evidence that they contain substantial amounts of the organic molecules found in those old sediments. It has been shown by various investigators that microorganisms have several possible pathways for the biosynthesis of hydrocarbons from fatty acids. The biosynthesis of fatty acids has been well characterized and need not be discussed. It is apparent from the literature that microorganisms have the capability of producing substantial amounts of fatty acids and hydrocarbons, and these stable organic molecules can survive along with the fossilized remains of their originators for geological periods of time.

II. STATEMENT OF THE PROBLEM

STATEMENT OF THE PROBLEM

There are numerous reports on the presence of morphological entities in sediments as old as 3.2×10^9 years. It has also been established that these same sediments contain organic compounds. These organic compounds are usually the remains of an array of organisms. If attempts are made to compare morphological fossils with chemical fossils (Figure 2), it must be assumed that the biochemistry of ancient organisms was similar to that of present day organisms (28).

The literature indicates that algae appear to be the predominating organisms in many of these old sediments. It is well established that certain algae contain substantial amounts of hydrocarbons and fatty acids. It is of interest to see if a correlation between the hydrocarbons adn fatty acids of the contemporary counterparts of the algae specifically implicated in sediments and of the hydrocarbons and fatty acids found in such sediments exist.

The purpose of this study is to (i) determine if the hydrocarbons and fatty acids found in certain sediments are the remains of algae no different from present day algae, (ii) determine the possible relationships of fatty acids with hydrocarbon production, and (iii) determine if aliphatic hydrocarbon or fatty acid distributions can be employed to ascertain the phylogenetic positions of certain blue-green and green algae.

FIGURE 2

GEOLOGICAL TIME SCALE OF MORPHOLOGICAL AND CHEMICAL FOSSILS

TIME (YEARS)	GEOLOGICAL FORMATIONS	MORPHOLOGICAL FOSSILS (ALGAE)	MOLECULAR FOSSILS (HYDROCARBONS FATTY ACIDS)
present 50×10^6 1×10^8	tertiary sediments	Botryococcus braunii Chroococcus sp. Tetraedron sp.	n-alkanes isoprenoids methyl alkanes
0.8 1×10^9	bitter springs	Green algae Oscillatoriace Nostoc	olefins n-fatty acids isoprenoid acid
1.8 2×10^9	nonesuch shale gunflint chert 1.8×10^9	Chroococuss Spirulina Oscillatoria Lyngbya	n-alkanes isoprenoids
2.7 3×10^9	soudan shale 2.7×10^9		n-alkanes isoprenoids polycyclic hydrocarbons
3.1 3.2 4×10^9	fig tree system onverwatch series 3.1×10^9	Order Chroocoales	n-alkanes isoprenoids olefins
4.8 5×10^9	formation of earth		

III. EXPERIMENTAL

EXPERIMENTAL

A. List of Algae Analyzed

1. Cyanophycophyta (Blue-greens)
    a. *Anacystis niculaus*
    b. *Anacystis montana*
    c. *Anacystis cyanea*
    d. *Lyngbya aestaurii*
    e. *Spirulina platensis*
    f. *Nostoc sp.*
    g. *Chroococcus turgidus*
2. Chlorophycophyta (Greens)
    a. *Chlorella pyrenoidosa*
    b. *Coelastrum microsporum*
    c. *Scenedesmus quadricauda*
    d. *Scenedesmus obliquus*
    e. *Tetraedron sp.*
    f. *Pediastrum sp.*
3. Chrysophycophyta (Golden-brown) -- *Botryococcus braunii*
4. Macroscopic Marine Alga from Hawaii

1. Cyanophycophyta

a. *Anacystis nidulans* was obtained from Dr. D. S. Hoare, Department of Microbiology, University of Texas, Austin, Texas. The organism was grown in three liters of modified Kratz and Myers

medium (94) described in Table II. The pH of the medium was adjusted to 7.4 and filtered sterilized with a 500 ml millipore filter using an HA 0.45 x 47 mm white grid and a vacuum. Three liters of medium were inoculated with 3 ml of a 7-day old culture, maintained at 23° C under constant aeration (6010 liters/hour), and illuminated with an intensity of 6000 foot candles provided by 4 Ken-Rad "cool white" flourescent lamps. The cells were harvested after 14 days by centrifugation in an RC2 Servall centrifuge, washed with saline solution, then frozen and dried over P_2O_5 under vacuum.

b. Anacystis montana was received as a viable culture (gift) from Dr. M Rodriguez-Lopez, Centro de Investigaciones Biologicas, Instituto Marañon, Madrid, Spain. A. montana was cultured and harvested in the same manner as A. nidulaus.

c. Anacystis cyanea was purchased from Dr. Patrick Echlin (95), Botany School, University of Cambridge, Cambridge, England. The organism was grown in a modification of Kratz and Myers medium (94), Table II. A. cyanea was cultured by aseptic transfer into 200 ml batch cultures in 38 x 300 mm test tubes. Each test tube contained a bubbling tube connected to a manifold system which provided for aeration by 1 percent CO_2 in air at an approximate rate of 25 ml/min/tube. The aeration system employed cotton filtration at several levels to eliminate contamination and introduction of extraneous materials. Illumination was provided by 300 foot c. cool white fluorescent light banks placed at a distance of 10 cm from the test tubes. Growth runs were conducted in thermostatted water baths

TABLE II

COMPOSITION OF KRATZ AND MYERS MEDIUM

Component	Concentration (g/l)
KNO_3	4.0
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.25
Na citrate	0.165
$Ca(NO_3)_2 \cdot 4H_2O$	0.025
$Fe_2(SO_4)_3 \cdot 6H_2O$	0.004

Plus 1 ml Hutner's A-5 microelement solution; pH adjusted to 7.6.

Hutner's A-5 Microelement Solution

Component	Stock Concentration (g/l)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$CuSO_4 \cdot 5H_2O$	0.079
MoO_3 (85%)	0.0177

at ± 0.5° C. Duration of growth, dependent upon species and size of the inoculum, varied between one and two weeks. Sufficient replicate samples were run to provide for 0.5 to 1.0 gms dry weight of cells. Second-stage microscopic examination was performed at the end of the growth runs, verifying typical morphology and pigmentation and a bacterial level of contamination between trace and void. The culture was harvested by centrifugation at 10,000 xg for 10 minutes. The algal pellet was dried over P_2O_5 under vacuum and the dry weight determined.

d. Lyngbya aestaurii was purchased from Echlin (95) culture collection of Cambridge University, Cambridge, England. It was cultured and harvested under the same conditions as A. cyanea, page 25.

e. Spirulina platensis was received in a lyophilized form from Dr. E. Petit, Jardin Botanique National de Belgique, 236 rue Royale, Bruxelles 3, Belgique. It had been collected from pond water.

f. Nostoc sp. was received as a gift from Dr. J. Mann, Department of Biology, University of Houston, Houston, Texas. It was grown and collected in the same manner as A. cyanea.

g. Chroococcus turgidus was purchased from Dr. Richard C. Starr (95), Director, Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Indiana. It was cultured and harvested like A. cyanea.

2. Chlorophycophyta

a. Chlorella pyrenoidosa was purchased from the Carolina Biological Supply House (97). The conditions for culturing and harvesting C.

pyrenoidosa were the same as for A. cyanea, page 25, except for the culturing medium. C. pyrenoidosa and all Chlorophycean algae were grown in a modification of the Scenedesmus medium of Hilton and Trainar (98). The composition of this medium is listed in Table III.

b. Coelastrum microsporum was purchased from the Starr (96) collection of Indiana University. This alga was grown and collected by the method described for C. pyrenoidosa.

c. Scenedesmus quadricauda was purchased from the Starr (96) collection of Indiana University and cultured in the same manner as C. pyrenoidosa.

d. Scenedesmus obliquus was purchased from Merck, Sharp and Dohme of Canada Limited, Isotopic Products Department, 350 Selby Street, Montreal 6, Quebec. It was received in a lyophilized form and had the order number of MD-743, deuterated algae, lot number B-232. This alga was grown in deuterium oxide and was purchased as such.

e. Tetraedron sp. was purchased from the Indiana Algal Collection (96) and grown and collected as previously described for C. pyrenoidosa.

f. Pediastrum sp. was purchased from the Indiana Algal Collection (96) and treated as C. pyrenoidosa.

3. Chrysophycophyta

Botryococcus braunii was purchased from the Indiana Algal Collection and was cultured and harvested like A. nidulaus.

4. A macroscopic alga, similar to Fucus, was collected by Dr. J. Oro,

TABLE III

COMPOSITION OF SCENEDESMUS MEDIUM

Component	Concentration (g/l)
NH_4NO_3	0.3
$MgSO_4 \cdot 7H_2O$	0.3
K_2HPO_4	0.1
KH_2PO_4	0.1
$CaCl_2$	0.04
$FeCl_3 \cdot 6H_2O$	0.01
Na citrate	0.165
$Ca(NO_3)_2 \cdot 4H_2O$	0.025
$Fe_2(SO_4)_3 \cdot 6H_2O$	0.004

Plus 1 ml Hutner's A-5 microelement solution; pH adjusted to 7.6.

Hutner's A-5 Microelement Solution

Component	Stock Concentration (g/l)
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$CuSO_4 \cdot 5H_2O$	0.079
MoO_3 (85%)	0.0177

Department of Biophysical Sciences and Chemistry, University of Houston, Houston, Texas, from the coast of Hawaii. The alga was dried over P_2O_5 prior to sample analysis.

All of the purchased algal cultures were unialgal and verification of all cultures was made using phase microscopy.

B. Other Biological Samples

1. Plantago ovata was obtained through the courtesy of Mr. M. C. Rosenthall, P. O. Box 6157, Phoenix, Arizona, 85005. Both seeds and seed coats were analyzed.

2. Thermophilic Bacteria

Two samples of thermophilic bacteria were received as a gift in a frozen state from Dr. Thomas D. Brock, Indiana University, Department of Microbiology, Jordan Hall 438, Bloomington, Indiana. The bacteria designated Y-IV-51-1 and Y-IV-51-2 were dried over P_2O_5 under vacuum and weighed.

C. Lipid Extration

1. The Algae (A. nidulaus, A. montana, C. pyrenoidosa and B. braunii), the seed and seed coats of P. ovata and the two thermophilic bacteria were extracted using an all-glass soxhlet equipped with a fritted-glass disc. A 50 ml blank of benzene-methanol (3:1) was refluxed for eight hours preceeding the extraction of the dried cells. The dried cells were placed on the disc and extracted for eight hours with 50 ml of fresh benzene-methanol (3:1). The refluxing temperature was controlled by a power-stat connected to an electric heating mantle.

2. All of the remaining samples were extracted by placing the dried cells in a beaker containing 50 ml of a 3:1 mixture of benzene and chloroform for 30 minutes at 50° C, with constant stirring. A second extraction was performed on the cells using 50 ml of n-heptane with the above conditions. The two extracts were combined.

With either method, after extraction the lipid soluble material in the extract was placed in 150 ml beaker and the solvent was evaporated at 40° C under a stream of purified nitrogen. Water-pumped nitrogen was purified by passing it through a Dri-Pac column (Illinois Instrument Group, Des Plaines, Illinois) containing molecular sieves and humidity-indicating silica gel.

D. Column Fractionation

The organic residue remaining after evaporation of the cell extract was separated on a 1 x 30 cm glass column fitted with a sintered-glass filter disc. The column was filled to a depth of 18 cm with heat activated silica gel (410° C for 24 hours). The column containing the silica gel was then washed with 30 ml of n-heptane.

Total extractable lipid was transferred to the top of the column with three 3 ml aliquots of n-heptane. Aliphatic hydrocarbons were eluted with 15 ml of n-heptane and collected in a 1 ounce glass bottle. The same procedure was used to elute the aromatics and ketones with 15 ml of benzene. A third fraction was collected using the same procedure with 15 ml of methanol for the elution of glycerides and other lipids.

The n-heptane and benzene fractions from the silica gel column were evaporated to small quantities under a stream of pure nitrogen at 40° C. The final concentration was achieved by careful agitation in order to prevent loss of low molecular weight compounds. These fractions were diluted to the desired concentrations with benzene and analyzed directly.

E. Preparation of Fatty Acid Methyl Esters

To analyze the fatty acids by gas-liquid chromatography, it was necessary to convert the fatty acids to their methyl esters. The procedures for three methods of methylation used in this investigation are given below. The significance of the three different methods of methylation are discussed in Results.

1. The methanol fraction was taken to dryness, transferred to a refluxing condenser containing 5 ml of 50 percent methanol and 5 ml of 10 percent NaHO (98), and refluxed for three hours at 75° C. The hydrolysate was cooled and the pH adjusted to 1 with hydrochloric acid. The hydrolysate was extracted three times with 5 ml of n-heptane and the extract evaporated to dryness under a stream of nitrogen. The residue was transferred to a refluxing condenser with 5 ml of methanol. Five-tenths percent concentrated sulfuric acid (by weight) and 5.0 percent 2,2-dimethoxypropane (by weight) were added to the methanol and were refluxed for two hours (100). The reaction was stopped with water and the fatty acid methyl esters were extracted three times with 5 ml of n-heptane. The 15 ml of the n-heptane extract was

prepared for analysis in the same manner as the benzene and heptane fractions.

2. Simplified procedures for hydrolysis or methanolysis of lipids by M. Kates (101).

The methanol fraction was taken to dryness in a combination hydrolysis and extraction flask as described by Kates (101) in Journal of Lipid Research (Jan., 1964), Volume (5), pp. 132-135. Only a brief outline will be given here. A 2.5 percent methanolic hydrogen chloride solution was prepared by bubbling hydrogen chloride gas through 4.5 ml of absolute methanol. The methanolic HCl was added to the evaporated methanol fraction in the extraction flask and heated under reflux (calcium chloride tube) on a hot plate for one hour. After refluxing 0.5 ml of water was added to the extraction flask. Then petroleum ether and the fatty acid methyl esters was collected in the ether portion of the two phase system. The petroleum ether extract containing the fatty acid methyl esters contained virtually no water and need not be treated with drying agents prior to evaporation of the solvent for analysis by gas chromatography.

3. Fatty Acid Esterification with BF_3-MeOH (102)

Three ml of reagent (BF-MeOH), purchased from Applied Science Laboratories, Inc., State College, Pennsylvania, was added to 100-200 mg of fatty acids in a 20 x 150 mm test tube and the test tube containing the reaction mixture was put in a water bath and allowed to boil for two minutes. The boiled mixture was transferred

into a separatory funnel containing 30 ml of petroleum ether and 20 ml of water was added. The petroleum ether phase was decanted off (containing the methyl esters) and evaporated under purified nitrogen to a desired volume and analyzed by gas chromatography.

F. Sample Analysis

1. Gas-liquid chromatography (GLC)

a. The models of the gas chromatographs used for sample analyses were 1) a Barber-Colman Series 5000, 2) an F & M Scientific Corporation Model 810, 3) a Varian Aerograph Series 1200, and 4) a Perkin-Elmer Model 900. All of the above gas chromatographs were equipped with flame ionization detectors.

b. Types and Uses of Gas Chromatographic Colums (Table IV)

Two general types of columns were used for the separation of sample components. The separation was achieved by partition of the components of a sample between the phases (103).

The sample was first injected into a metal or glass tube, packed with an inert support, i. e., a fine granular material upon which the thin layer of liquid or stationary phase in a partition column is held. This allows a relatively fast scan of high molecular weight compounds. A portion of the same sample was also injected into a capillary column (open tubular column). This was usually stainless steel tubing of a small diameter 0.25 - 1.0 mm I.D. in which the inner walls of the tube are used to support the stationary phase.

TABLE IV

TYPE OF GAS CHROMATOGRAPHIC COLUMNS USED

Column	Stationary Phase	Packed Column Support	Sample
195 m x 0.076 cm I.D. Stainless Steel Capillary	Igepal CO - 880 [nonyl-phenoxypoly(ethyl-ene-oxy)ethanol]		Hydrocarbons Fatty Acid Methyl Esters
198 m x 0.076 cm I.D. Stainless Steel Capillary	OV - 17 Silicone Methyl-Phenyl (50-50)		Hydrocarbons
30 m x 0.025 cm I.D. Stainless Steel Capillary	10 percent Apiezon L (High Temperature Grease)		Hydrocarbons
91.5 m x 0.076 cm I.D. Stainless Steel Capillary	Polysev [M-Bis-(Phenoxyphenoxy)-Phenoxybenzene]		Hydrocarbons
1.7 m x 0.3 cm I.D. Glass (Packed Column)	OV - 1 (Methyl Silicone Fluid)	Gas-Chrom Q 100/200 Applied Sciences Laboratories State College, Pa.	Hydrocarbons

TABLE IV CONTINUED

TYPE OF GAS CHROMATOGRAPHIC COLUMNS USED

Columns	Stationary Phase	Packed Column Support	Sample
8' x 1/4" I.D. Copper Tubing (Packed Column)	OV - 1 (Methyl Silicone Fluid)	Gas-Chrom Q 100/200 Applied Sciences Laboratories State College, Pa.	Hydrocarbons
155 m x 0.076 cm I.D. Stainless Steel Capillary	Igepal CO - 990 (Monyl phenoxy polyoxy-ethylene ethanol)		Fatty Acid Methyl Esters Hydrocarbons
12' x 1/8" I.D. Stainless Steel (Packed Column)	15% EGS (Ethylene Glycol Succinate)	80/100 Chromosorb W Aw	Fatty Acid Methyl Esters

All gas-chromatographic identifications were made by using known internal standards, by relative retention, which is the ratio of the retention time of a component to the retention time of a second component chosen as a standard, and retention volume, which is the product of the retention time of a component and the volumetric flow rate of the carrier gas.

For quantitative measurements the peak area was calculated by taking the peak height x the width at 1/2 the peak height. To determine relative percent composition, the total area of all the peaks was divided into the area of each individual peak.

$$\frac{\text{Area of A}}{\text{Areas of A + B + C}} \times 100 = \text{relative percent}$$

2. Combined Gas Chromatography-Mass Spectrometry

All of the samples were analyzed by combined gas chromatography-mass spectrometry (104, 105) using the same columns described in Table II.

The validity of all but a few of the identifications reported in this work rests on the gas chromatographic-mass spectrometric analysis of the samples. The theory behind the combination of a gas chromatograph with a mass spectrometer and the wide range of applications of the technique has been discussed in detail by Gelpi (31). An LKB-9000 Gas Chromatograph-Mass Spectrometer was used for all of the hydrocarbon and fatty acid-methyl ester identifications. Typical

operating conditions were the following:

Electron energy:	20 or 70 eV
Ionizing current:	60-125 μA
Accelerating voltage:	3.5 KV
Temperature of ion source:	~250-290° C
Analyzer pressure:	5×10^{-6} - 10^{-7} mm Hg
Electron multiplier voltage	1.7 - 2.5 KV
Scanning speed:	25 millisec/amu

All samples were measured and aliquots (0.1 to 3.0 microliters for capillary columns and 3.0 to 8.0 microliters for packed columns) were injected with a Hamilton microliter syringe (No. 701-A).

3. Thin-Layer Chromatography (TLC)

The methanol extracts, and their derivatives, of the two thermophillic bacteria were analyzed on glass-plates coated with Silica Gel G (according to Stahl). The plates were heat-activated for one hour at 100° C. After spotting (concentrating) the samples and standards on the activated plates, they were equaliberated with the solvent system and then developed by the ascending method. The solvent system used was hexane:diethyl ether:acetic acid in a 90:10:1 ratio. This solvent system will separate out classes of lipids. The components were detected after development by spraying with dichromate-saturated sulfuric acid and charring. Components were identified with appropriate standards and by R_F values.

4. Radio-Active Incorporation

Chlorella pyrenoidosa cultures were grown under asceptic conditions in a ferric potassium EDTA, Knop's (Table V) solution in a 4 liter

COMPOSITION OF KNOP'S MEDIUM

Knop's Medium -- To 1000 ml. distilled water add

Urea	1.86 gms.
KH_2PO_4	1.50 gms.
$MgSO_4 \cdot 7H_2O$	0.50 gm.
EDTA	0.50 gm.
H_3BO_3	0.114 gm.
$CaCl_2$	0.084 gm.
$FeSO_4 \cdot 7H_2O$	0.050 gm.
Na citrate	2.00 gms.

1 ml. Hunter's A-3 micronutrients

pH adjusted to 7.5

Erlenmeyer flask purged with air at the rate of 1 liter per minute. When light grown, the cultures were given continuous light at 1000 f.c. Cultures were supplied with appropriate labelled substrates after the cultures were 10 days old. All substrates were filter sterilized before use. Tween 80 (0.05 ml) was added to the aqueous solution containing the sterate-UL-^{14}C or acetate-1-^{14}C prior to filtration.

Following incubation for 48 hours the cells were collected by low speed centrifugation and washed twice with distilled water to remove media and excess labelled substrates. The cells were then freeze-dried and stored at 5° C until extracted. Initial extractions were made with 100 ml of 3:1 mixture of benzene and chloroform; for secondary extractions, 100 ml of n-heptane was used. Each extraction was for 1 hour at 50° C, with frequent stirring. The combined solvents were decanted and evaporated at 40° C under a stream of purified nitrogen. The residue was taken up in 5 ml portions of n-heptane and transferred to the top of an activated silica gel column (1 x 15 cm). The column was eluted with 20 ml of n-heptane. In order to remove residual pigments, the silica gel eluate was passed through an activated alumina column (1 x 15 cm). This final hydrocarbon fraction was taken to dryness under a stream of purified nitrogen.

All compounds of interest were characterized by gas chromatography (Perkin-Elmer Model 900) and by a gas chromatograph-mass spectrometer (LKB-9000). Chromatographic separation was achieved with a capillary column (50 m x 0.025 cm) coated with 2 percent OV - 1. Determination of label within a given component was accomplished by converting each

compound as it emerged from the gas chromatograph to CO_2 with a Packard combustion furnace, which in turn was connected by a gas flow cell to a Packard liquid-scintillation spectrometer. Radiographic procedures with thin-layer chromatography were also employed to characterize the labelled products.

Nooner and Gelpi (31, 32) have extensively investigated possible contaminates of biological samples. All solvents, chromatographic material and media used in these experiments were analyzed in this laboratory and have been previously reported (31, 32). All glassware and experimental apparatus were cleaned with hot chronic acid and rinsed with distilled water.

IV. RESULTS

RESULTS

A. Algal Lipids

1. Hydrocarbons

The hydrocarbon distribution of the algae will be presented according to the complexity of the patterns rather than on their phylogenetic positions.

Tables VI and VII summarize the data for the hydrocarbon composition of the fifteen species of algae analyzed. The hydrocarbon compositions in both tables show that the n-C_{17} alkane is a predominant constituent of most of the algae (44, 61, 72, 73, 84). Thirteen of the fifteen species have n-C_{17} as a major hydrocarbon whereas four of the fifteen show high molecular weight hydrocarbons as their major components. All identifications of the aliphatic hydrocarbons reported here have been verified by mass spectrometry.

Figure 3 represents the hydrocarbons of C. microsporum and indicates that n-heptadecane accounts for 100% of the aliphatic hydrocarbons and 52 ppm of the dry cell weight. Scenedesmus obliquus and the marine algae from Hawaii as seen in Figures 4 and 5 are very similar to one another in their hydrocarbon distribution, with n-C_{17} accounting for 100% of the detectable aliphatic hydrocarbons. C. pyronoidosa, as shown in Figure 6, displays a simple hydrocarbon pattern similar to the above algae; however, the Δn-C_{17} olefin accounts for 76.9% of the aliphatic hydrocarbon while the n-C_{17} paraffin only accounts for the remaining 18.5%. Chlorella contains 105 ppm hydrocarbons of the dry cell mass.

TABLE VI

HYDROCARBONS FROM CONTEMPORARY REPRESENTATIVES OF ALGAE FOUND IN SEDIMENTS

DIVISION	ORGANISM	SEDIMENT	HYDROCARBONS	
			RANGE	MAJOR
Chlorophycophyta	Coelastrum microsporum	Green River	C_{17}	C_{17}
Chlorophycophyta	Chlorella pyrenoidosa	Bitter Spring	C_{17}	ΔC_{17}
Chlorophycophyta	Scenedesmus quadricauda	--	C_{17} -ΔC_{27}	C_{17},ΔC_{27}
Chlorophycophyta	Tetraedron sp.	Green River	C_{15} - C_{27}	C_{17}, C_{23}
Cyanophycophyta	Anacystis cyanea	Bitter Springs	C_{17}, $7MeC_{17}$, $8MeC_{17}$	C_{17}
Cyanophycophyta	Anacystic nidulans	--	C_{15} - C_{18}	C_{17}
Cyanophycophyta	Spirulina platensis	Bitter Springs	C_{15} - C_{17}	C_{17}
Cyanophycophyta	Lyngbya aestuarii	Bitter Springs	C_{15} - C_{18}, X	C_{17}
Cyanophycophyta	Nostoc sp.	--	C_{15} - C_{18}, X	C_{17}
Cyanophycophyta	Chroococcus turgidus	Green River	C_{16} - C_{19}, X	C_{17}, X
Cyanophycophyta	Anacystis montana	Bitter Springs	C_{17} - C_{29}	ΔC_{25},ΔC_{27}
Chrysophycophyta	Botryococcus braunii	Tertiary	ΔC_{17} - $2\Delta C_{33}$	$2\Delta C_{29}$, $2\Delta C_{31}$

X Compound with mass spectrometric fragmentation pattern corresponding to a polycyclic triterpenoid with empirical formula $C_{30}H_{50}$.

Chlorophycophyta: Green Algae Cyanophycophyta: Blue Green Algae Chrysophycophyta: Golden Brown Alga

TABLE VII

RELATIVE PERCENT HYDROCARBON COMPOSITION OF SEVERAL ALGAE*

	G**				BG**							GB**
HYDROCARBON	COEL. MICRO.	CHLOR. PYR.	SCEN. QUAD.	TETR. SP.	ANA. CYAN.	ANA. NID.	SPIR. PLAT.	LYNG. AEST.	NOST. SP.	CHROO. TURG.	ANA. MONT.	BOTY. BRAU.
C_{15}				1		23	10	2	3			
C_{16}						8	20	6	4	3		
6Me + 7MeC_{16}										1.0		
C_{17}	100	18.5	26	30	87	44	70	35	48	32	11.5	
ΔC_{17}		76.9	0.6			20	<1					1.5
7Me + 8MeC_{17}					13			38	27	22		
C_{18}						2		1.4		3		
2MeC_{18}										<1		
C_{19}			6.8							2		
2Δ-C_{21}											8.9	
C_{23}				40								
ΔC_{23}											8.0	0.14
C_{24}				2.6								

TABLE VII CONTINUED

RELATIVE PERCENT HYDROCARBON COMPOSITION OF SEVERAL ALGAE*

	G**				BG**							GB**
HYDROCARBON	COEL. MICRO.	CHLOR. PYR.	SCEN. QUAD.	TETR. SP.	ANA. CYAN.	ANA. NID.	SPIR. PLAT.	LYNG. AEST.	NOST. SP.	CHROO. TURG.	ANA. MONT.	BOTY. BRAU.
C_{25}				20.0								
Δ-C_{25}											14.6	0.65
Δ-C_{26}											3.8	
C_{27}				5.9								
Δ-C_{27}			43.2								34.7	
2Δ-C_{27}											2.8	11.1
2Δ-C_{29}												50.4
3Δ-C_{29}												5.5
2Δ-C_{31}												27.9
2Δ-C_{33}												2.0
X								16	10	38		
Squalene***										✓		✓

X Compound with mass spectrometric fragmentation pattern corresponding to a polycyclic triterpenoid structure with empirical formula $C_{30}H_{50}$.

TABLE VII CONTINUED

RELATIVE PERCENT HYDROCARBON COMPOSITION OF SEVERAL ALGAE

Footnotes to table:

* Differences to 100 made up by unidentified compounds.

** G: green algae. BG: blue green algae. GB: golden brown alga.

*** In benzene fraction.

FIGURE 3

GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS FROM

COELASTRUM MICROSPORUM

Glass column (1.7 m long by 0.3 cm I.D.) packed with 1 percent OV-1. Barber Colman Series 5000 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 910 g/cm^2; sensitivity x 1; attenuation, 10. Temperature programmed from 140° C to 240° C at 5° C/min.

COELASTRUM MICROSPORUM

HYDROCARBONS

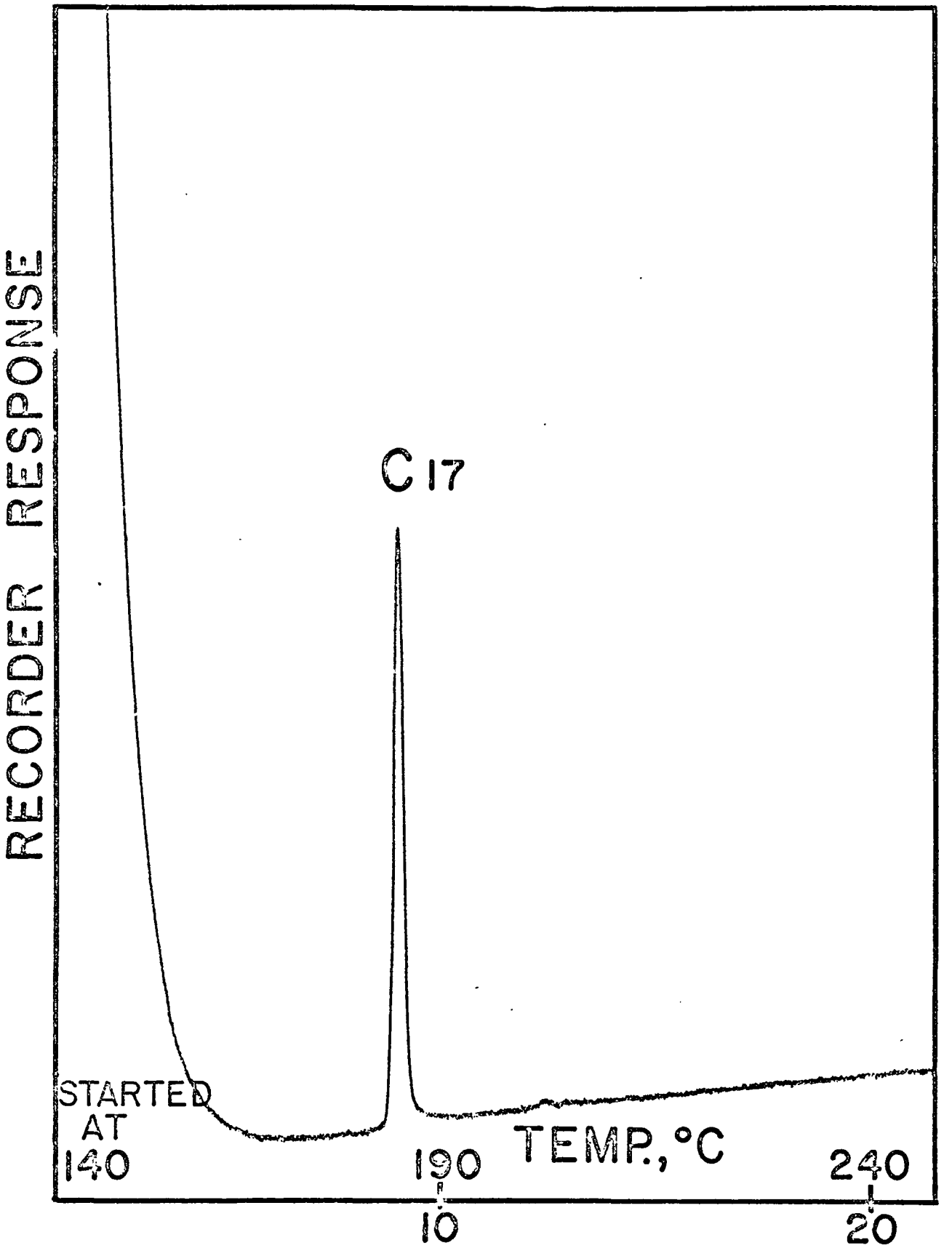


FIGURE 4

GAS CHROMATOGRAPHIC SEPARATIONS OF HYDROCARBONS FROM

SCENEDESMUS OBLIQUUS

All operating conditions were the same as Figure 3 except for the starting temperature, which was 180° C.

SCENEDESMUS OBLIQUUS

PERDEUTEROALKANES

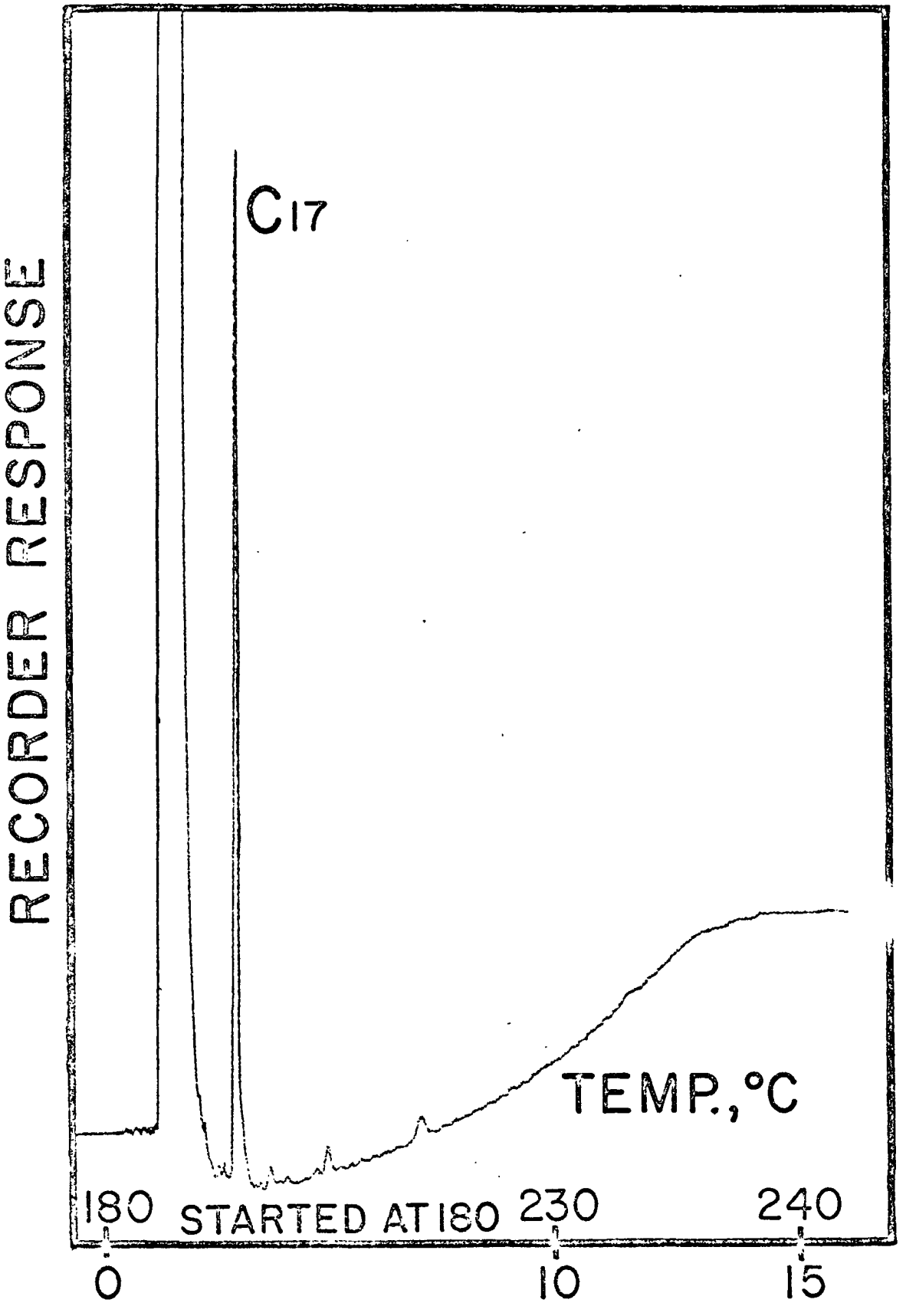


FIGURE 5

GAS CHROMATOGRAPHIC SEPARATION OF HYDROCARBONS FROM A MACROSCOPIC MARINE ALGAE

Stainless steel capillary column (0.076 cm x 155 m) coated with Igepal CO 990. Barber Colman 5000 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; sensitivity x 5; attenuation, 10. Temperature programmed from 140° C to 180° C at 4° C/min.

HAWAII ALGAE
HYDROCARBONS

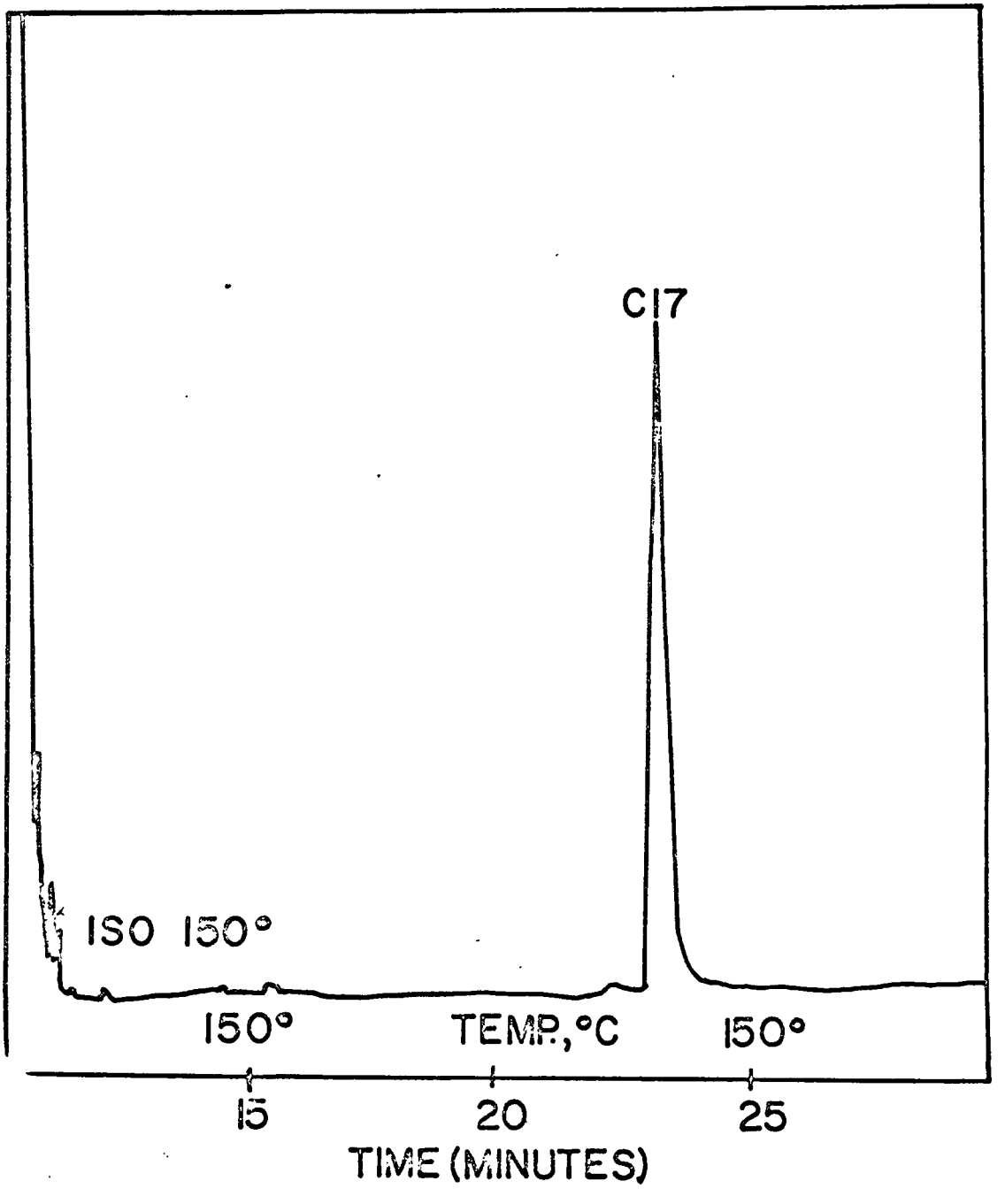
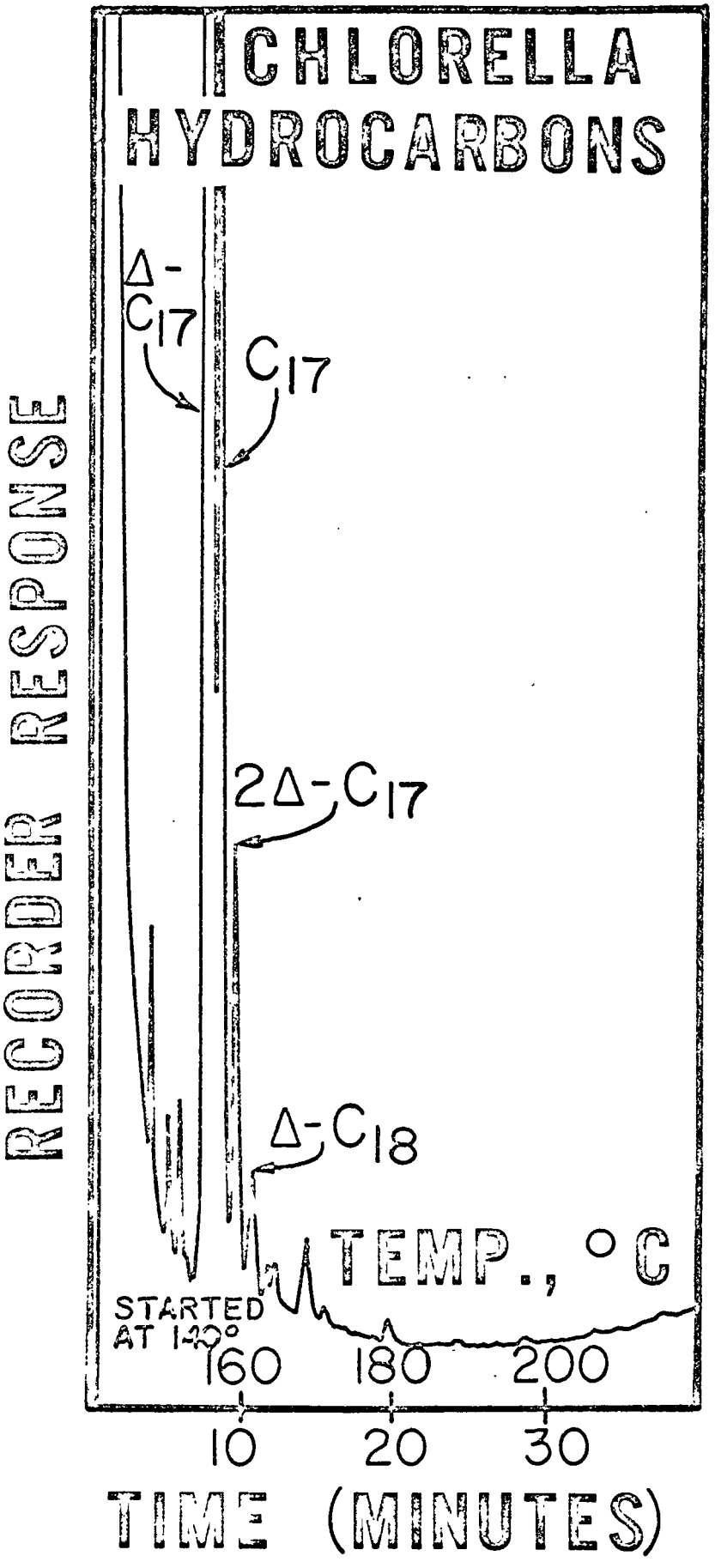


FIGURE 6

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *CHLORELLA PYRONOIDOSA*

Glass column (1.7 m long x 0.3 cm. I.D.) packed with OV-1. F & M Model 810 gas chromatograph equipped with a flame ionization detector. Nitrogen pressure, 910 g/cm^2; range, 10; attenuation, 4. Temperature programmed from 140° C to 240° C at 2° C/min.


CHLORELLA HYDROCARBONS
RECORDER RESPONSE
Δ-C17
C17
2Δ-C17
Δ-C18
TEMP., °C
STARTED AT 140°
160
180
200
10
20
30
TIME (MINUTES)

Anacystis cyanea (see Figure 7) also contains n-C_{17} as the major hydrocarbon. The smaller component, which accounts for 13% of the total aliphatic hydrocarbons, has been identified as a mixture of the 7-methyl, and 8-methyl, heptadecanes. The 8-methyl plus the 7-methyl C_{17} was resolved from the n-C_{17} by a high resolution stainless steel capillary column. It is calculated that A. cyanea has 340 ppm of aliphatic hydrocarbons.

The gas chromatographic pattern for the hydrocarbons of A. nidulans is given in Figure 8. It shows a pattern of alkanes and alkenes ranging from C_{14} to C_{19}. The major peaks are pentadecane, hexadecane, heptadecane, and heptadecene. The peaks labelled a and b have been identified as pristane and phytane. Using a Digital Readout System, Model CRS 11AB/H41 Introtronics, Houston, Texas, it was calculated that the hydrocarbon content of A. nidulans was 0.006% (60 ppm) of the cell mass.

Spirulina plantensis (Figure 9) has a major n-C_{17} which is 70 percent of the aliphatic hydrocarbons, with the n-C_{15} and n-C_{16} accounting for the remaining 30 percent. There is a trace of the C_{17} monoolefin present.

The hydrocarbon patterns for Lyngbya aestuarii, Nostoc sp. and Chroococcus turgidus (Figures 10, 11, 12, and 13) exhibit a great deal of similarity in their distributions. All three of these cyanophycean algae have a n-C_{17} along with substantial amounts of the mixture of 7-methyl and 8-methyl heptadecanes and of compound X, which has a gas chromatographic retention time similar to C_{29}; however, mass

FIGURE 7

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *ANACYSTIS CYANEA*

Stainless steel capillary column (0.076 cm x 155 m) coated with Igepal CO 990. Barber Colman 5000 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; sensitivity x 5; attenuation, 10. Temperature programmed from 140° C to 180° C at 4° C/min.

ANACYSTIS CYANEA

HYDROCARBONS

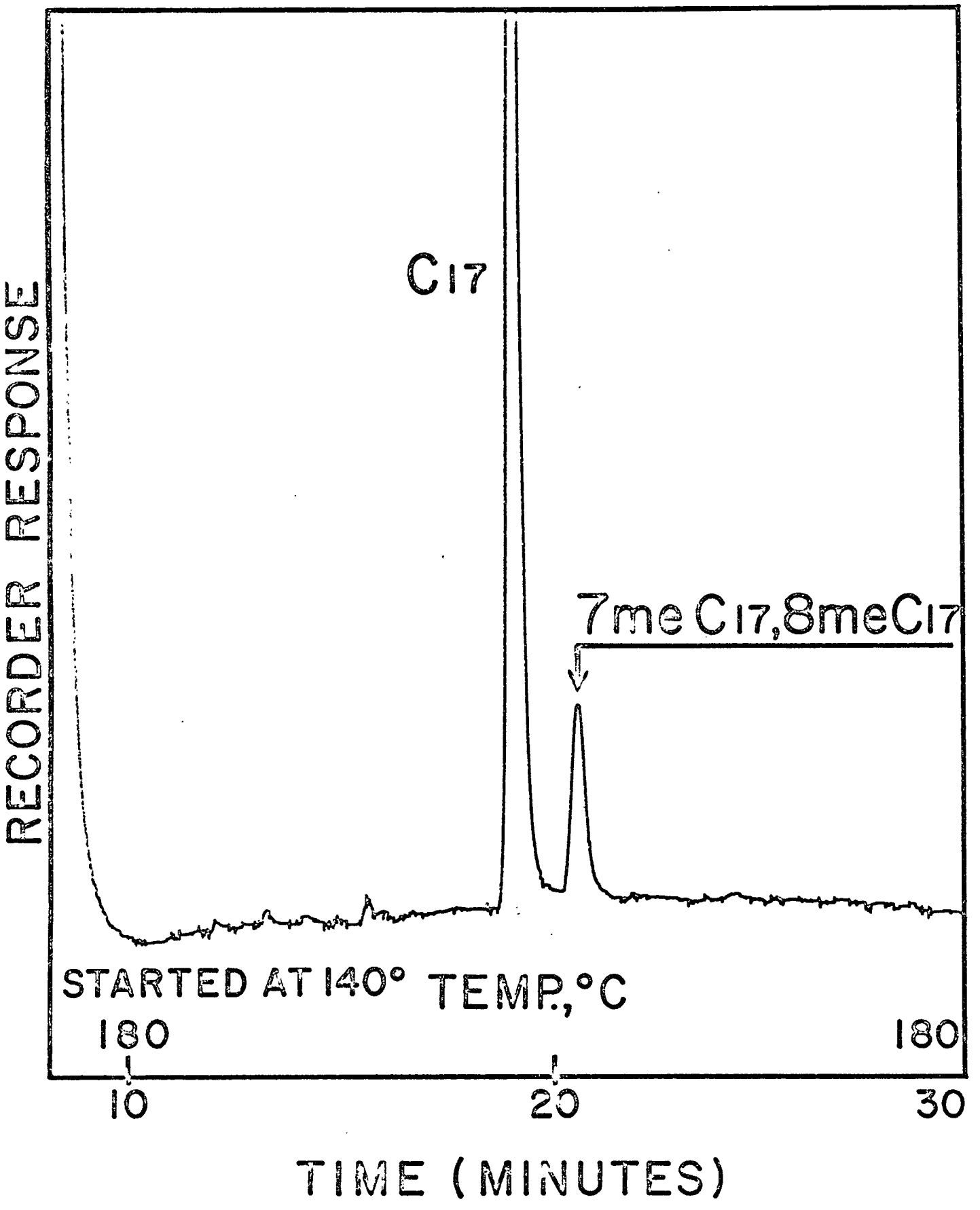


FIGURE 8

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *ANACYSTIS NIDULANS*

Stainless steel capillary column (0.076 cm x 155 m) coated with Igepal CO 990. F&M Model 810 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; range, 10^2; attenuation, 1. Temperature programmed from 125° C to 200° C at 5° C/min.

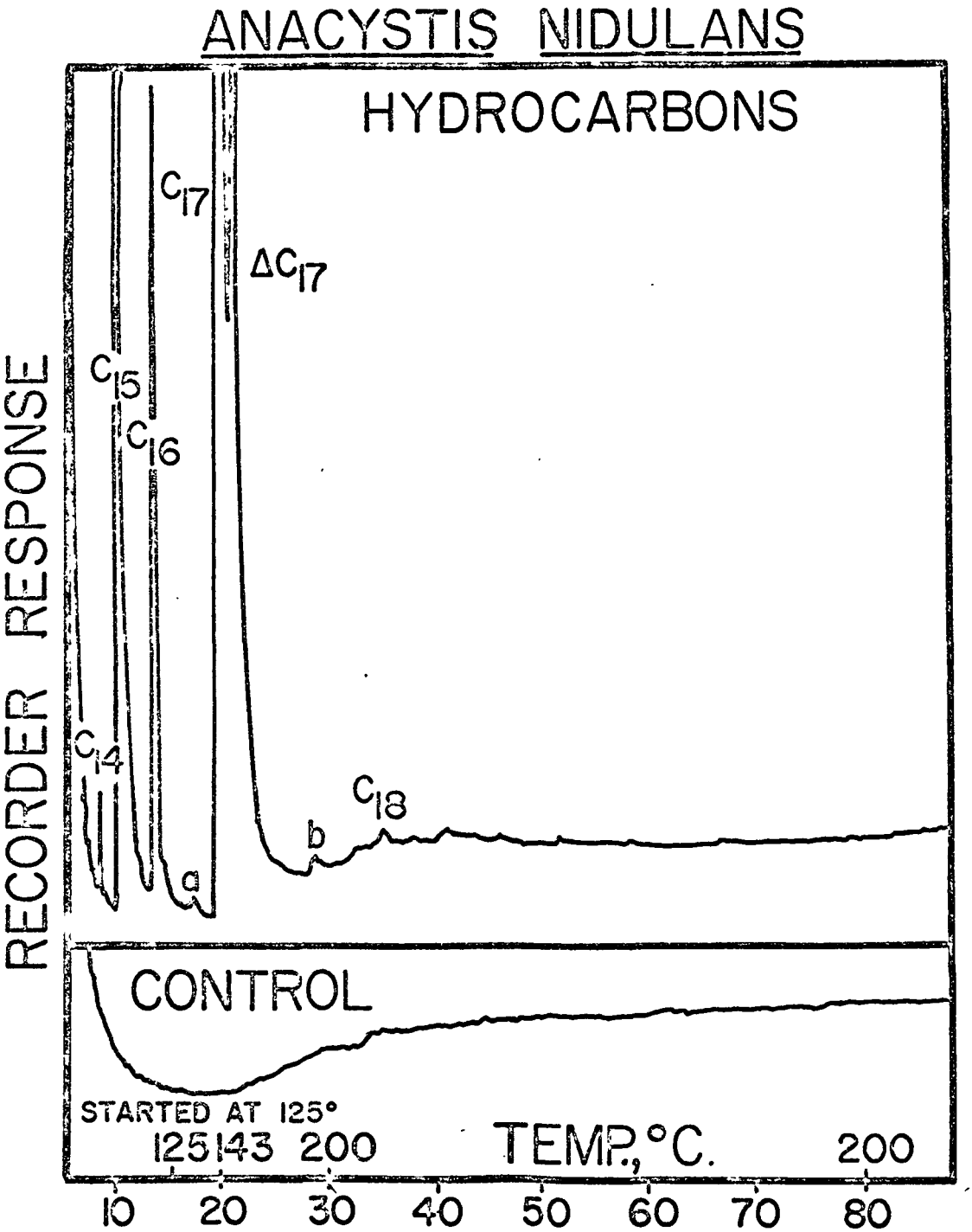

ANACYSTIS NIDULANS
HYDROCARBONS
RECORDER RESPONSE
C17
ΔC17
C15
C16
C14
a
b
C18
CONTROL
STARTED AT 125°
125 143 200
TEMP., °C.
200
10
20
30
40
50
60
70
80

FIGURE 9

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM

SPIRULINA PLATENSIS

Glass column (1.7 m x 0.3 cm I.D.) packed with 1 percent OV-1. Varian Aerograph Series 1200 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 910 g/cm^2; range, 1; attenuation, 8. Temperature programmed from 140° C to 240° C at 4° C/min.

SPIRULINA PLATENSIS
HYDROCARBONS

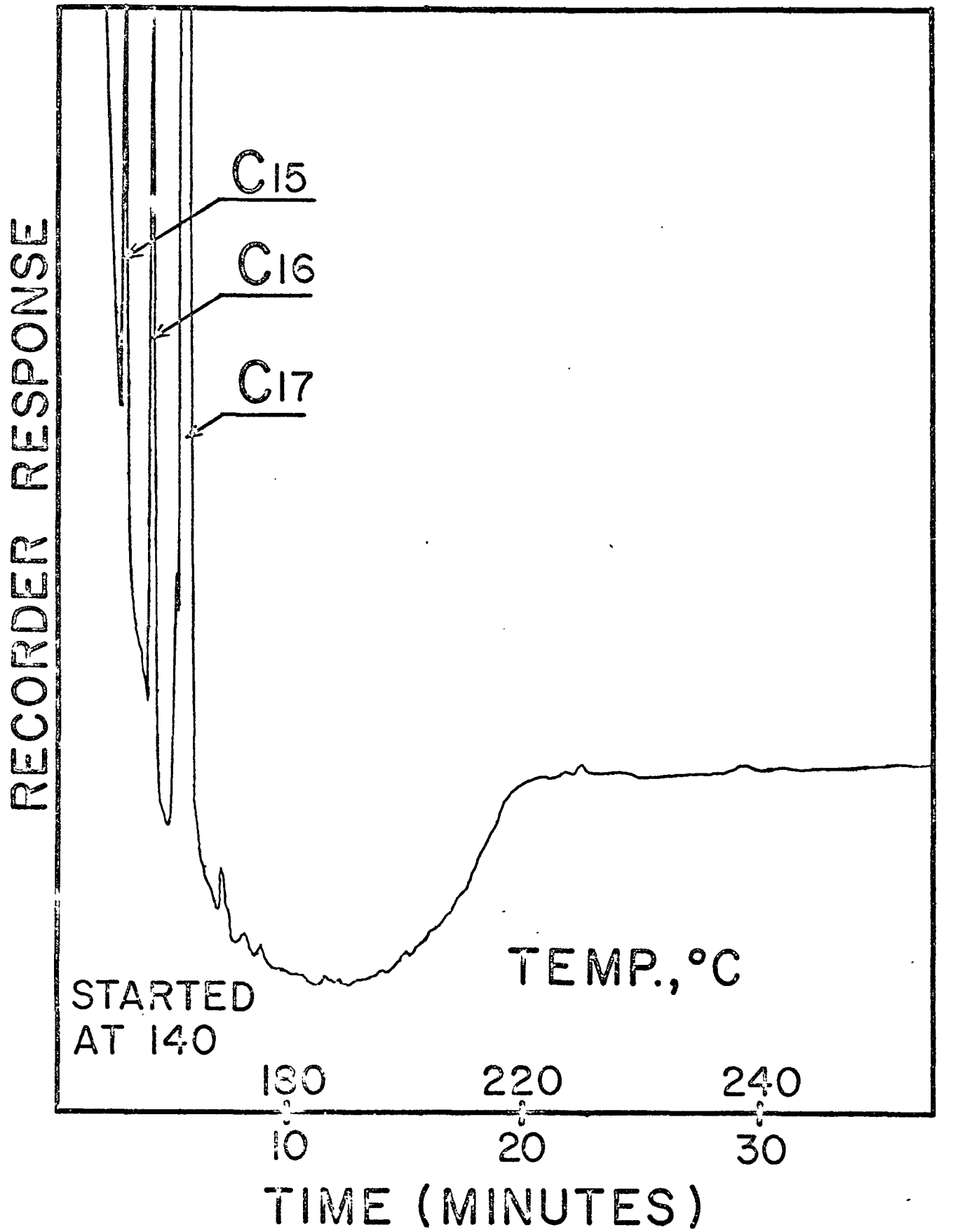


FIGURE 10

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *LYNGBYA AESTAURII*

All of the operating conditions were the same as in Figure 9.

LYNGBYA AESTAURII

HYDROCARBONS

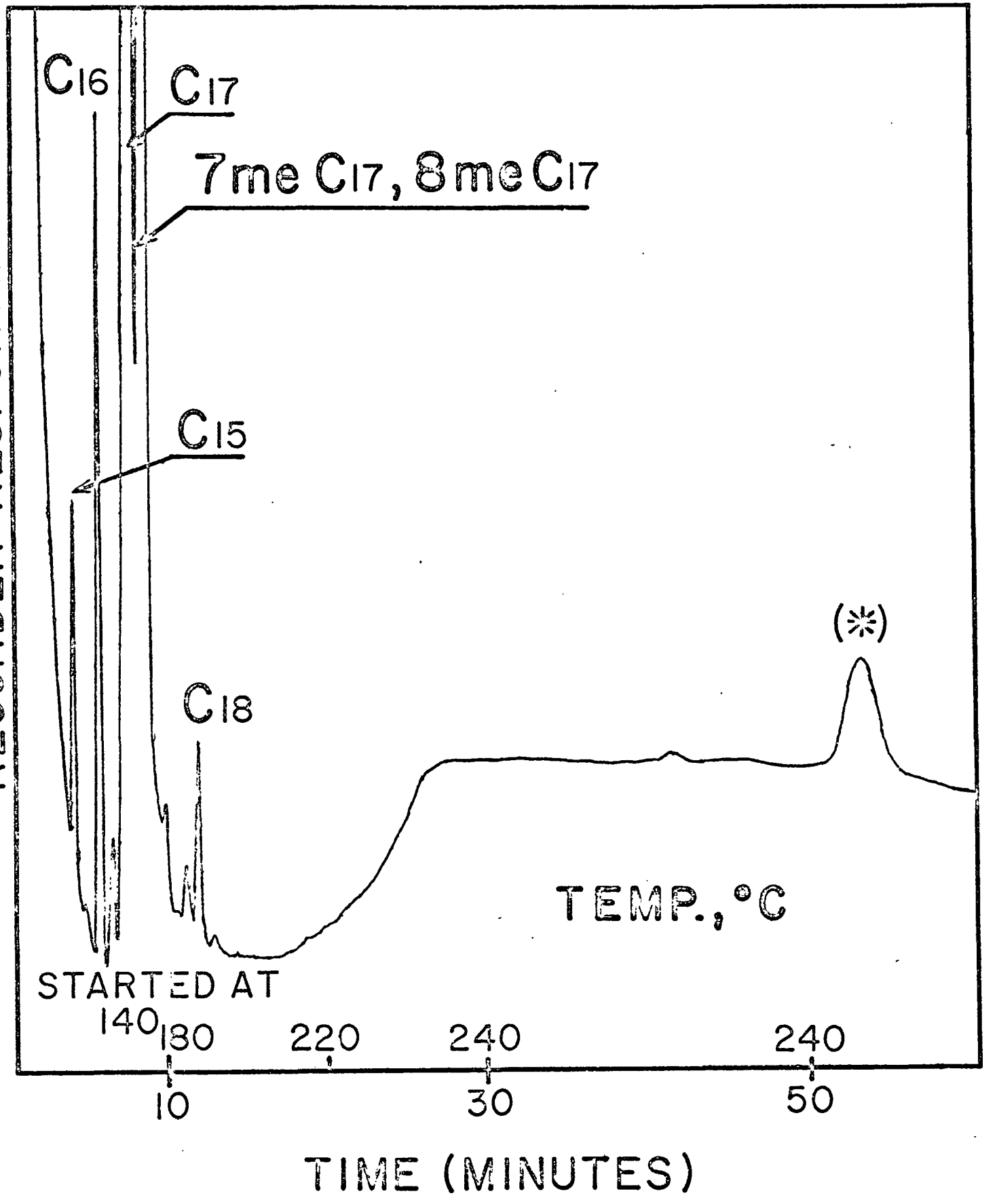


FIGURE 11

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *NOSTOC SP.*

All of the operating conditions were the same as in Figure 9.

NOSTOC SP.

HYDROCARBONS

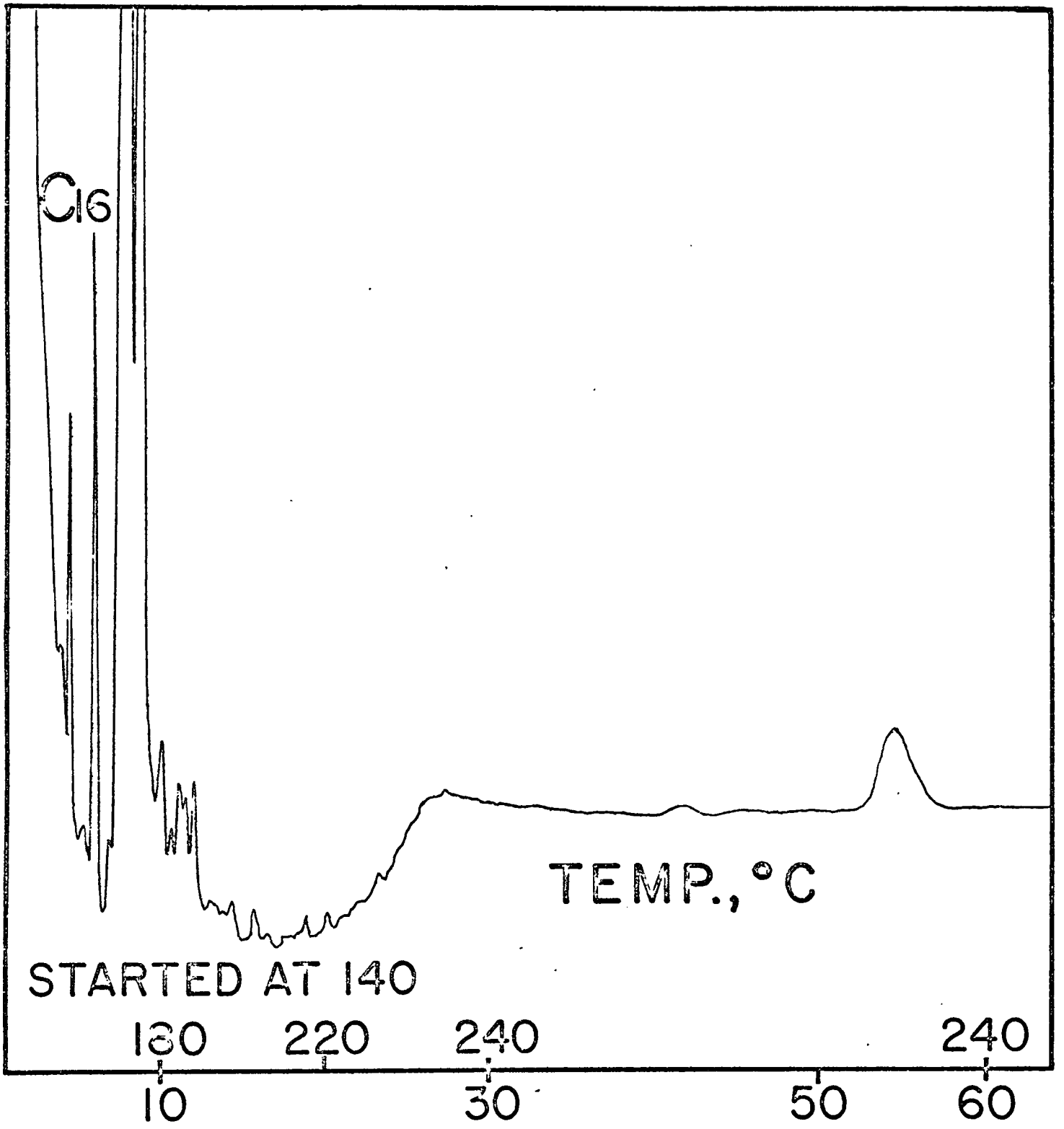


FIGURE 12

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *CHROOCOCCUS TURGIDUS**

All of the operating conditions were the same as in Figure 9.

*Fast scan packed column

CHROOCOCCUS TURGIDUS

HYDROCARBONS

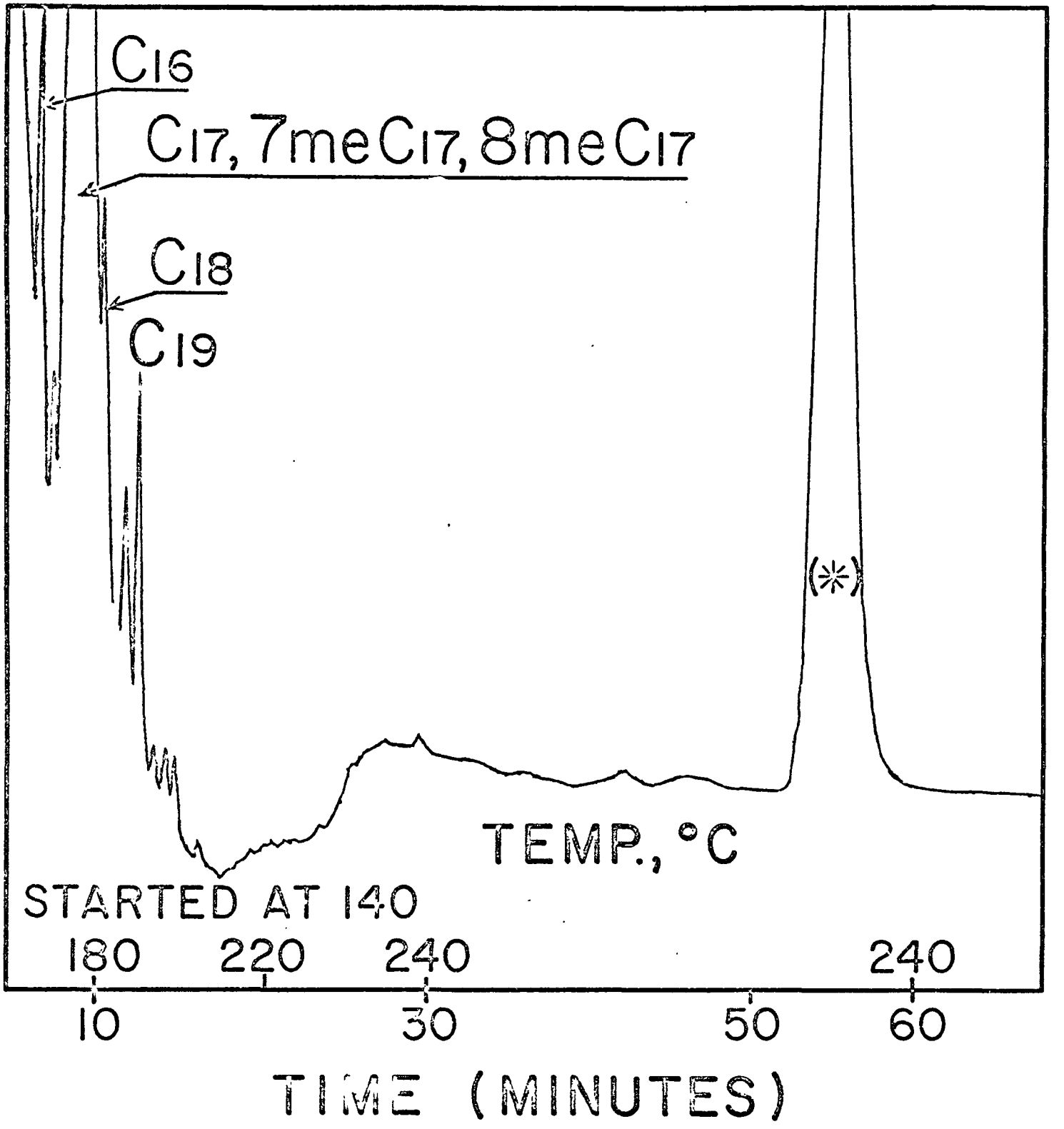


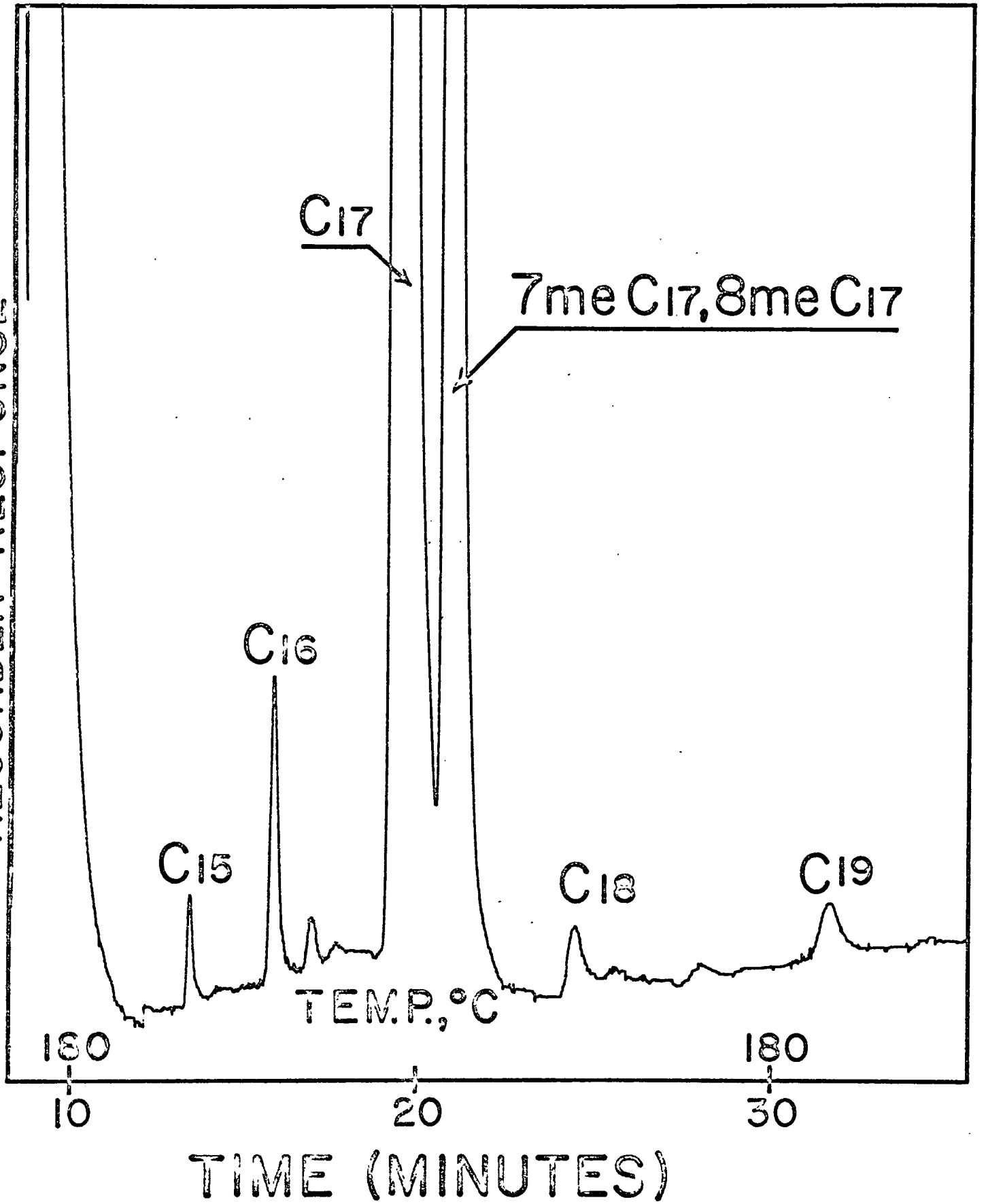
FIGURE 13

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *CHROOCOCCUS TURGIDUS**

All of the operating conditions were the same as in Figure 7.

*High resolution capillary column

CHROOCOCCUS TURGIDUS
HYDROCARBONS



spectrometric analysis showed this compound to have the same molecular weight as squalene but a different mass spectral pattern.

Thirty-five percent of the aliphatic hydrocarbon content in L. aestuarii is the n-C_{17} while 38 percent is the 7-methyl plus 8-methyl C_{17}. Compound X accounts for another 16% and the n-C_{15}, n-C_{16} and n-C_{18} make up the remaining concentrations. There are 568 ppm of aliphatic hydrocarbons in the dry cell mass of Lyngbya. Nostoc sp. has 48 percent of the C_{17} alkane. The 7-methyl plus 8-methyl C_{17} is 9 percent less than in Lyngbya and Compound X is 6 percent less than in Lyngbya. The remaining hydrocarbons are the n-C_{15} and n-C_{16}. C. turgidus has less n-C_{17} and 7-methyl plus 8-methyl C_{17} than either Lyngbya or Nostoc, i.e., a total of 32 percent and 22 percent, respectively. However, C. turgidus has well over twice as much of Compound X as Lyngbya or Nostoc. In addition to n-C_{16}, n-C_{18} and n-C_{19}, C. turgidus shows trace amounts of an iso-C_{19} alkane (see Figure 14). Squalene was also found, but its relative concentration has not been calculated. The amount of hydrocarbons per dry cell weight has been calculated at 812 ppm for Chroococcus. The mass spectrometric interpretations of the 7-methyl and 8-methyl heptadecane, plus that of compound X, were performed by Dr. Emilio M. Gelpi, Department of Chemistry, University of Houston.

The mass spectra of the two mixtures of monomethylalkanes are shown in Figure 15. The presence of a small amount of an equimolar mixture of 7-methyl and 8-methylheptadecane in Nostoc has already been reported by Han, et al. (70). Their compound gave a mass spectrum consistent with a 7,9 dimethylhexadecane structure (72), having major fragments at

FIGURE 14

THE MASS SPECTRA OF THE ISO C_{19} HYDROCARBON OF

CHROOCOCCUS TURGIDUS

Taken as it emerged from a 155 m by 0.076 mm I.D. capillary gas chromatographic column coated with Igepal CO 990.

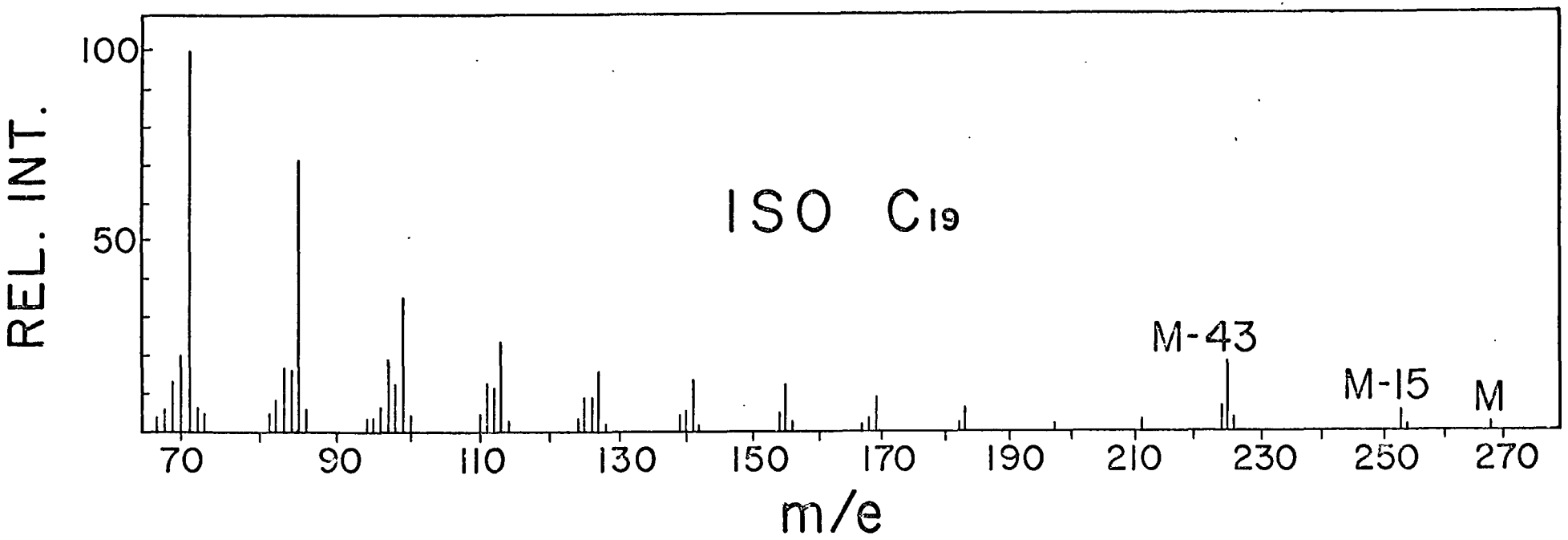
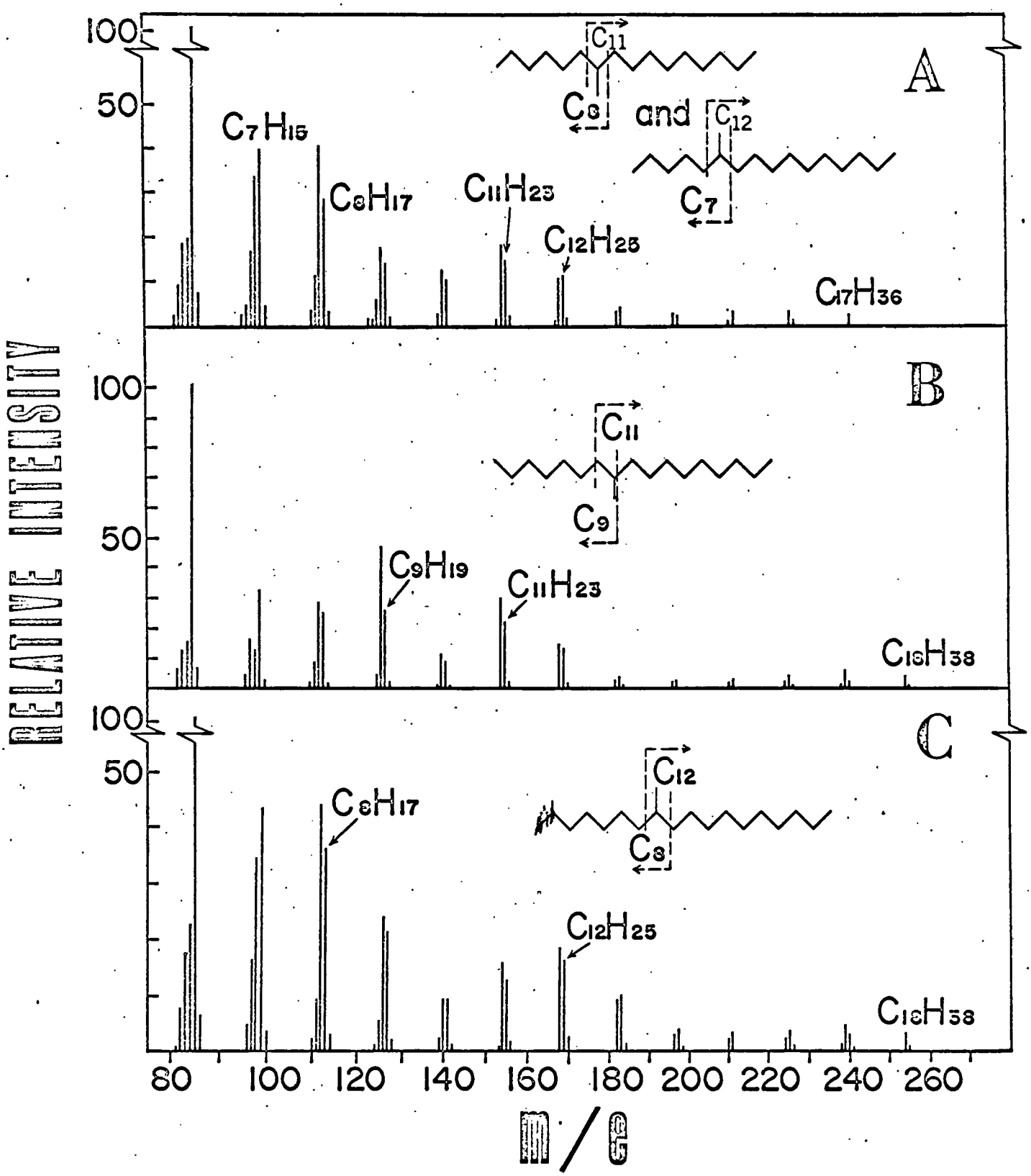

REL. INT.
100
50
ISO C19
M-43
M-15
M
70
90
110
130
150
170
190
210
230
250
270
m/e

FIGURE 15

MASS SPECTRA OF METHYL ALKANES OF CHROOCOCCUS TURGIDUS

Taken as they emerged from a 155 m by 0.076 mm I.D. capillary gas chromatographic column coated with Igepal CO 990.


A
B
C
100
50
RELATIVE INTENSITY
C7H15
C8H17
C11H23
C12H25
C17H36
C11
C8
and
C12
C7
C9H19
C18H38
C9
80
100
120
140
160
180
200
220
240
260
m/e

C_8, C_9, C_{11}, C_{12} and parent ion at C_{18}. However, the synthesis of the pure diastereoisomers of 7,9-dimethyl hexadecane followed by capillary coinjection techniques (71) showed that this was not the proper structure. A synthetic mixture of the two methyl heptadecanes demonstrated that the single chromatographic peak actually consisted of a mixture of two alkanes.

We have proved by a different technique that this is also the case here. Although they cannot be well separated by gas liquid chromatography, repetitive scans of the chromatographic peak in the ascending and descending slopes show very clearly that we are not dealing with a single compound but with a mixture of two isomeric C_{18} alkanes.

The peaks at C_9 and C_{11} predominate over the C_{18} and C_{12} fragments in Figure 15B, which is representative of a mass spectrum taken on the upward slope. On the other hand, the C_8 and C_{12} fragments were found to show an increase in relative intensity along the downward slope of the GC peak, Figure 15C. The mass spectral pattern of Figure 15B is consistent with that of a mixture of 8-methyl and 7-methyl heptadecanes in which the first predominates. The same is true of the pattern in Figure 15C, but with the second compound predominating. Also, the relatively high intensity of the C_7 and C_{13} ions in Figure 15C appears to indicate the presence of smaller amounts of the 6-methyl heptadecane.

The order of elution of these methyl alkanes would be 8-methyl heptadecane followed by the 7-methyl and the 6-methyl heptadecanes. This order is consistent with the apparent distribution of these compounds within the GC peak. Also, their retention times correspond to those of

the monomethylated compounds. The dimethyl substituted isomers, being more volatile, would be eluted in front of them.

All this in the previous paragraph also applies to the mass spectrometric pattern shown in Figure 15A, but in this case repetitive scanning of the peak was not possible due to its small concentration in the sample.

Of particular interest is the mass spectrum of compound X (Figure 16) which shows a fragmentation pattern consistent with that of a polycyclic triterpenoid structure of empirical formula $C_{30}H_{50}$. The base peak at m/e 191 is a characteristic fragment in the mass spectra of pentacyclic triterpenes (75). This is also supported by other ions, especially those at m/e 218, 204, 205, 136, 123, 109. The strong molecular ion at 410 also reflects the higher stability inherent in any polycyclic structure.

A particularly interesting observation made in this study is the relatively large amounts of long chain hydrocarbons (61, 74) in a number of the organisms examined. Scenedesmus quadricauda (Figure 17), besides the typical n-C_{17} peak which accounts for 26 percent of the total hydrocarbons, shows a prominent peak corresponding to a C_{27} monene at a concentration of 43.2 percent. Smaller amounts of the C_{19} paraffin and other hydrocarbons in the C_{16} - C_{31} range also seem to be present, but with the exception of the C_{19}, their structures have not been verified mass spectrometrically.

The aliphatic hydrocarbons of Botryococcus braunii (Figure 18) were identified as alkenes, mainly with one and two double bonds, ranging

FIGURE 16

MASS SPECTRA OF SQUALENE AND COMPOUND X IN CHROOCOCCUS TURGIDUS

Taken as they emerged from a 1.7 m x 3 mm I.D., glass column packed with OV-1 (Refer to text for details).

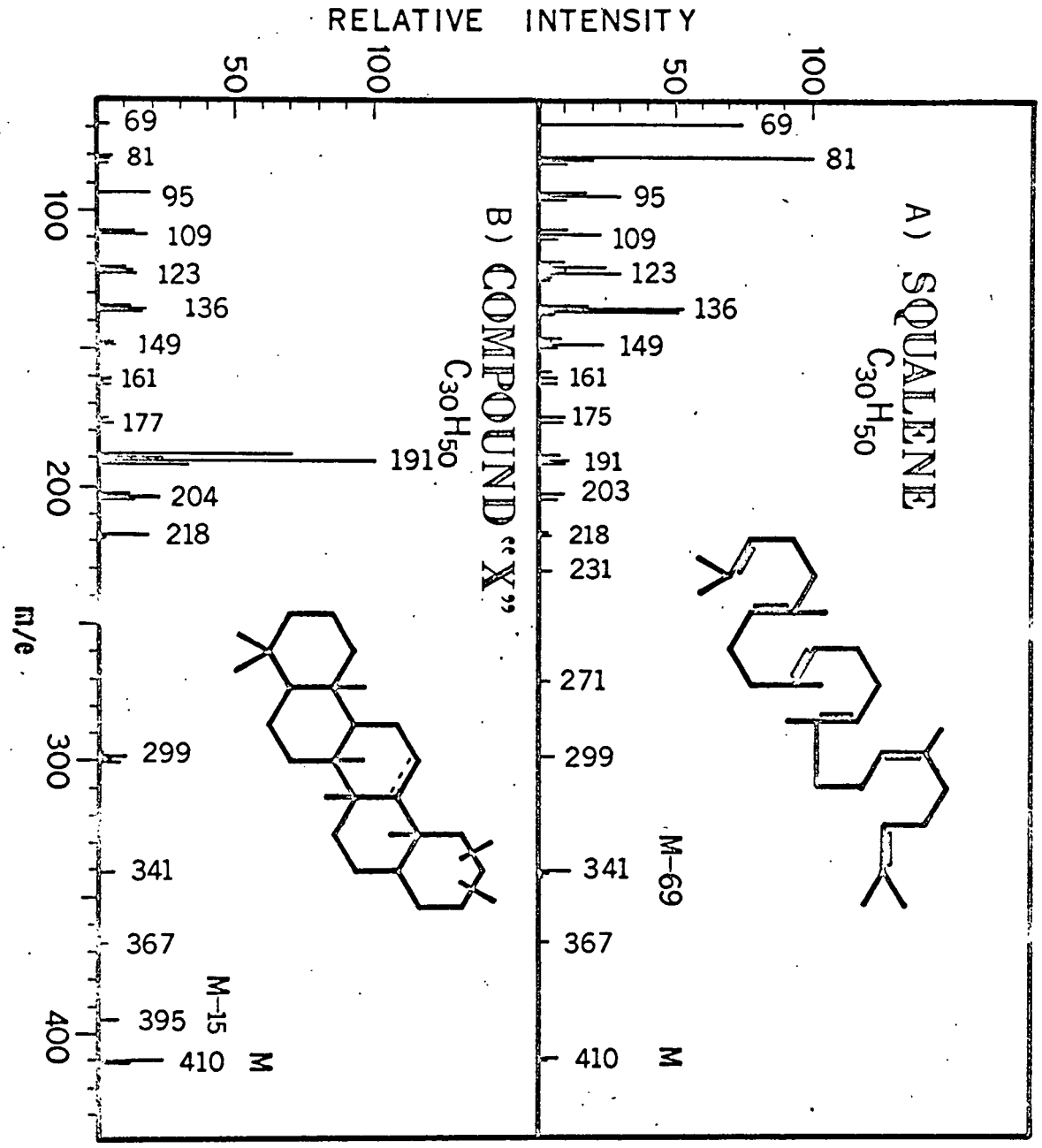

RELATIVE INTENSITY
50
100
A) SQUALENE
$C_{30}H_{50}$
B) COMPOUND "X"
$C_{30}H_{50}$
m/e
100
200
300
400
69
81
95
109
123
136
149
161
175
177
191
203
204
218
231
271
299
341
367
395
410
M
M-15
M-69

FIGURE 17

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *SCENEDESMUS QUADRICAUDA*

All of the operating conditions were the same as in Figure 9.

SCENEDESMUS QUADRICAUDA

HYDROCARBONS

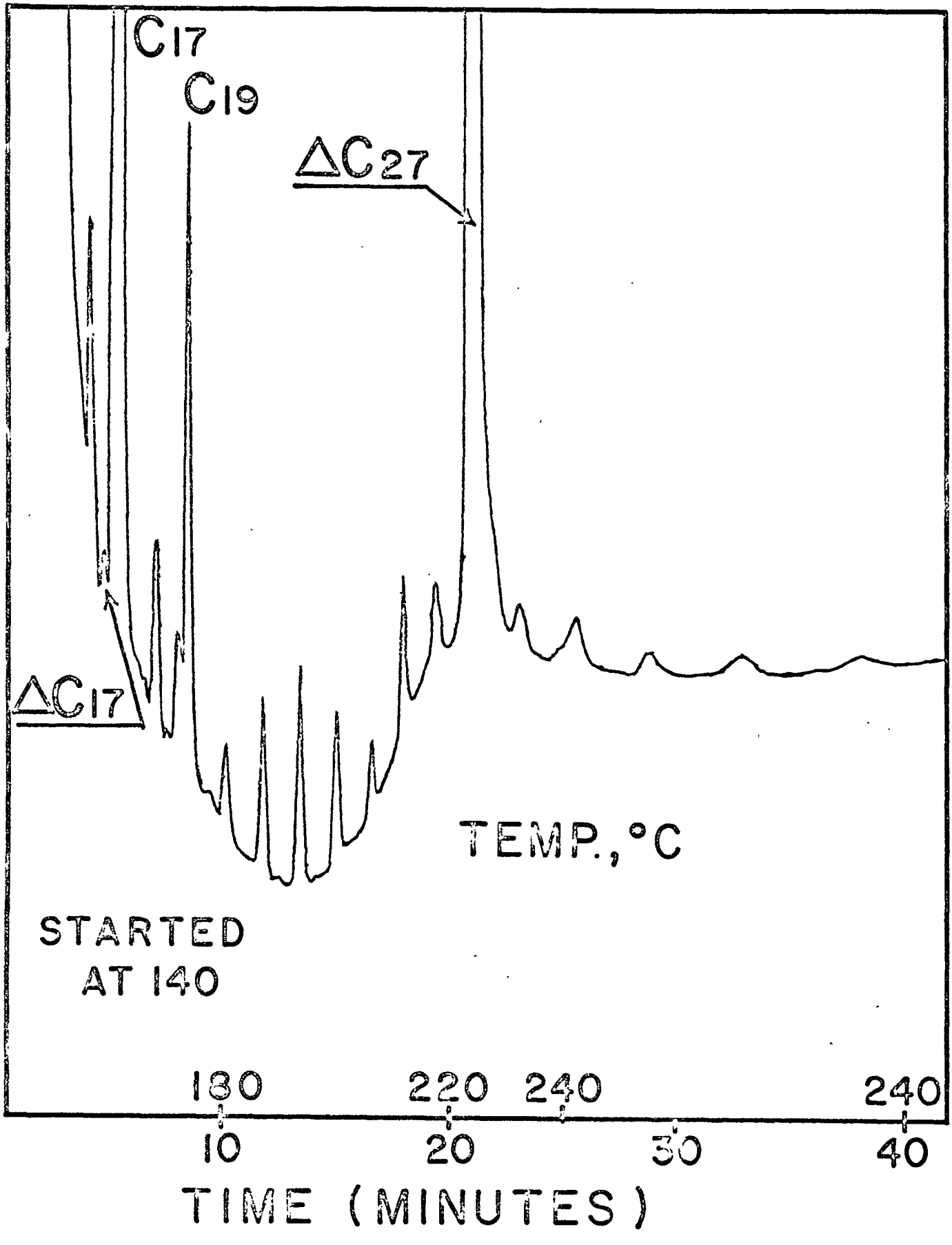
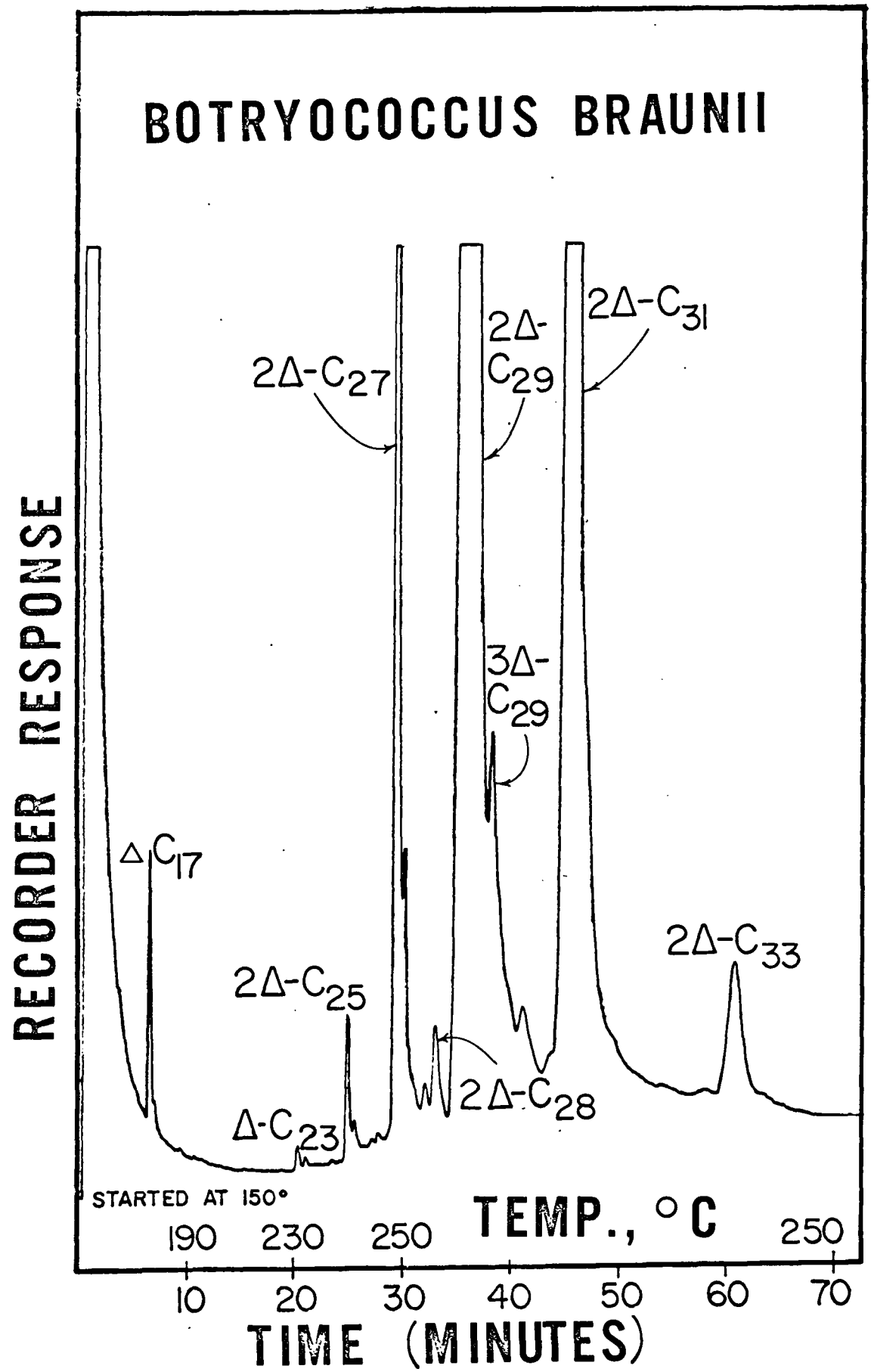


FIGURE 18

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *BOTRYOCOCCUS BRAUNII*

Glass column (1.7 m x 0.3 cm I.D.) packed with 1 percent OV-1. F&M Model 810 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 703 g/cm^2; range, 10^2; attenuation, 2. Temperature programmed from 150° C to 250° C at 4° C/min.


BOTRYOCOCCUS BRAUNII
RECORDER RESPONSE
2Δ-C27
2Δ-C29
2Δ-C31
3Δ-C29
ΔC17
2Δ-C25
2Δ-C33
Δ-C23
2Δ-C28
STARTED AT 150°
190 230 250
TEMP., °C
250
10 20 30 40 50 60 70
TIME (MINUTES)

from C_{17} to C_{33}. The C_{27}, C_{29}, and C_{31} diolefins are predominant, the major component being the C_{29} diolefin. The relative percent concentration of the 2Δ-C_{29} is 50.4 percent, the 2Δ-C_{31} is 27.9 percent and the 2Δ-C_{27} is 11.1 percent. These three compounds account for 89.4 percent of the total hydrocarbons present. The hydrocarbon content of B. braunii is 0.3 percent of the dry cell weight.

Anacystis montana (Figure 19) shows a similar distribution with a somewhat shorter range. The olefin content goes from C_{19} to C_{29}, the major peak being the C_{27} monoolefin. In this case heptadecane represents the only paraffin present, its relative concentration being 11.5 percent. In the dry cell weight basis of A. montana, the hydrocarbons account for about 0.1 percent.

Tetraedron sp. (Figure 20) has a somewhat unique distribution when compared to other algae that contain substantial amounts of high molecular weight hydrocarbons. Tetraedron has major peaks at C_{17}, C_{23}, and C_{25}. The C_{27} which is one of the major high molecular weight components of S. quadricauda, B. braunii, and A. montana is a relatively minor component in Tetraedron sp. All of the hydrocarbons are n-paraffins with n-C_{23} accounting for 40 percent of the hydrocarbons while the n-C_{17} and n-C_{25} account for 30 percent and 20 percent, respectively.

2. Fatty Acids

Table VIII gives the fatty acid ranges of the algae analyzed and the sediments in which the fossil forms are found. All patterns appear similar and are in close agreement with other data on micro-

FIGURE 19

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *ANACYSTIS MONTANA*

Stainless steel capillary column (0.025 cm x 30 m) coated with Apiezon L. F&M Model 810 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; range, 10^2; attenuation, 1. Temperature programmed from 150° C to 300° C at 6° C/min.

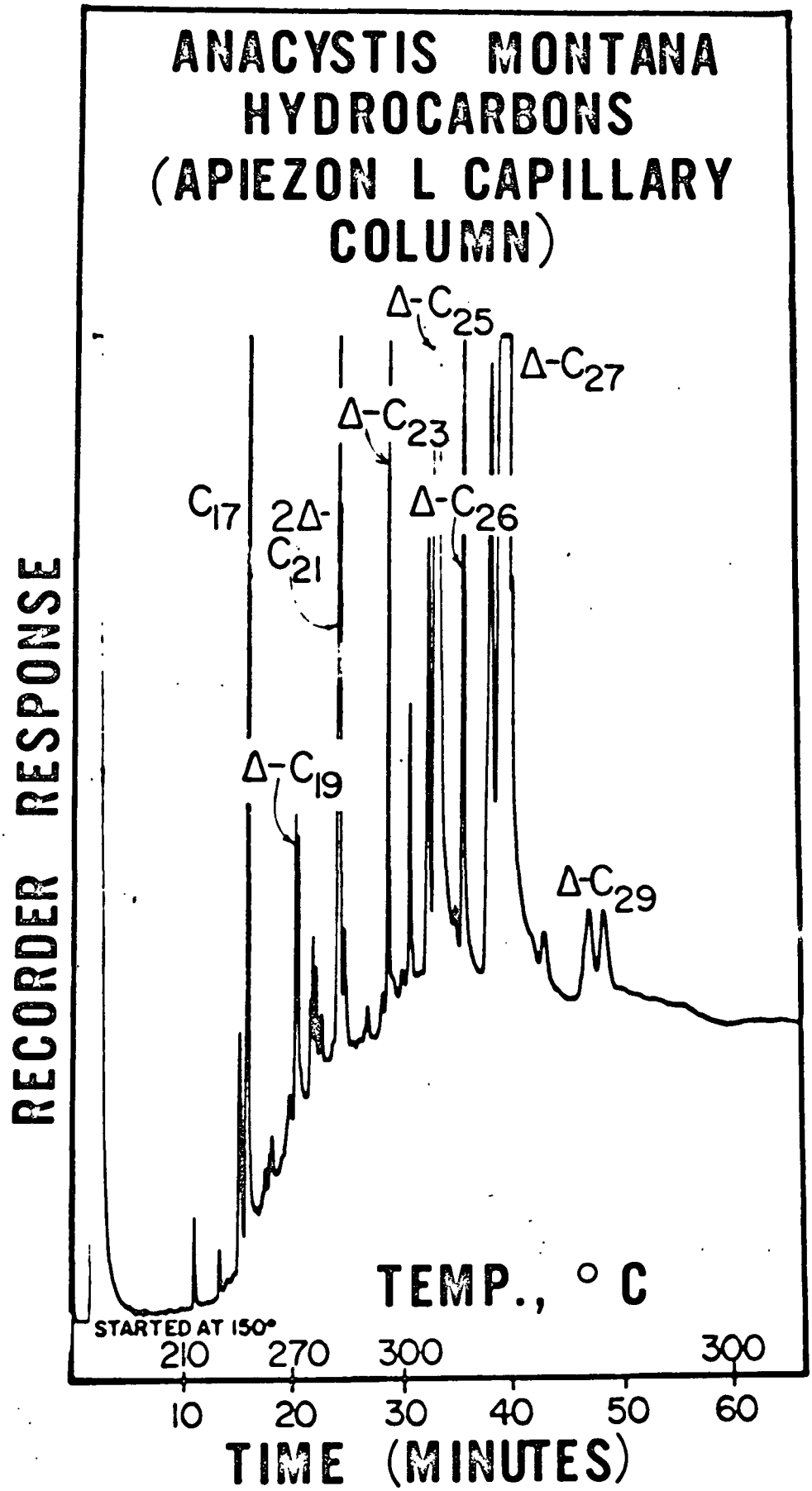

ANACYSTIS MONTANA
HYDROCARBONS
(APIEZON L CAPILLARY
COLUMN)
Δ-C25
Δ-C27
Δ-C23
C17
2Δ-
C21
Δ-C26
Δ-C19
Δ-C29
RECORDER RESPONSE
TEMP., ° C
STARTED AT 150°
210
270
300
300
10
20
30
40
50
60
TIME (MINUTES)

FIGURE 20

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS FROM *TETRAEDRON SP.*

Glass column (1.7 m x 0.3 cm I.D.) packed with 1 percent OV-1. Barber Colman gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 703 g/cm^2; sensitivity x 1; attenuation, 10. Temperature programmed from 140° C to 250° C at 4° C/min.

TETRAEDRON SP.

HYDROCARBONS

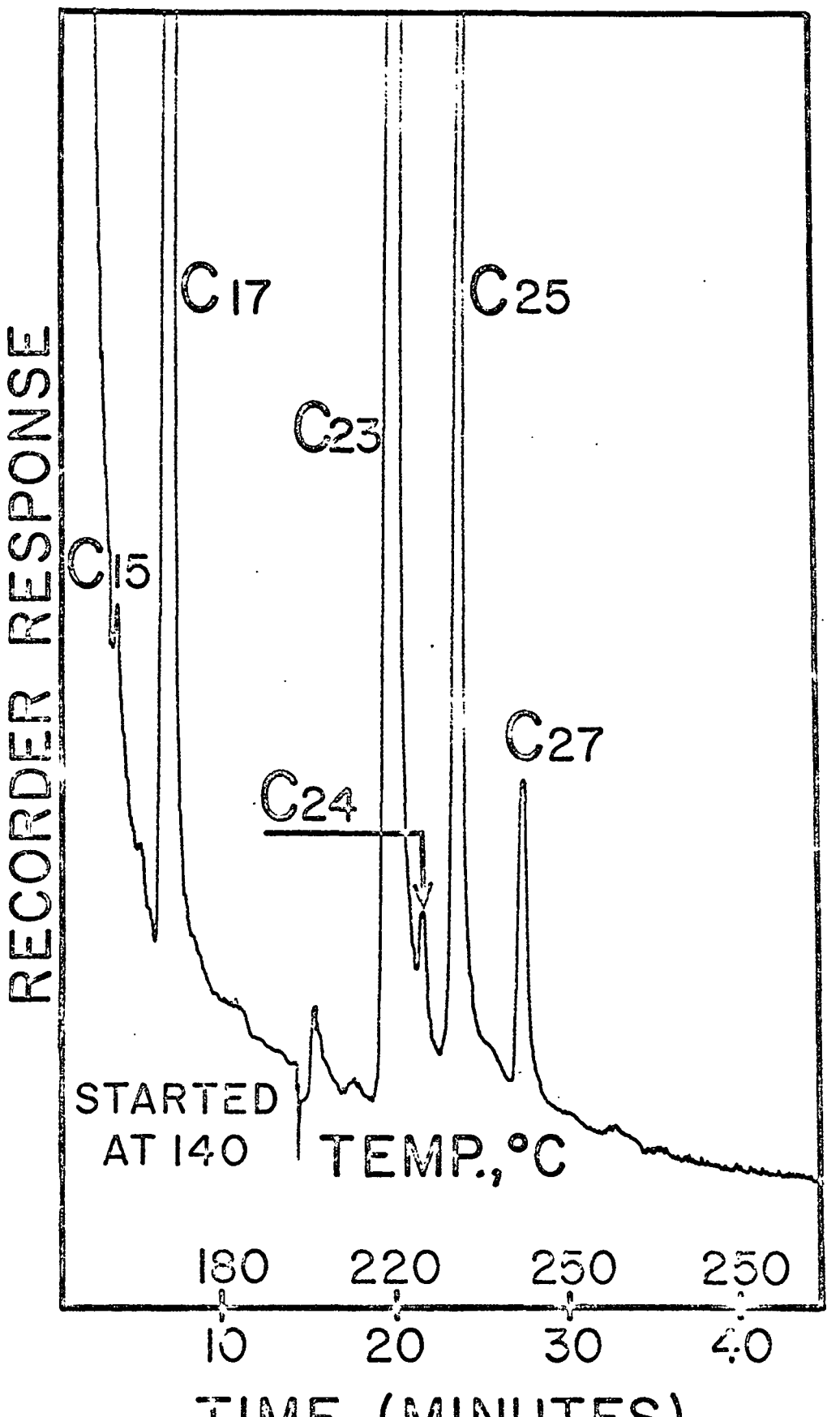


TABLE VIII

FATTY ACIDS FROM CONTEMPORARY REPRESENTATIVES OF ALGAE FOUND IN SEDIMENTS

			FATTY ACID Me ESTERS	
DIVISION	ORGANISM	SEDIMENT	RANGE	MAJOR
Chlorophycophyta	Coelastrum microsporum	Green River	14:0 - 18:4	18:1
Chlorophycophyta	Chlorella pyrenoidosa	Bitter Springs	14:0 - 18:3	18:3
Chlorophycophyta	Scenedesmus quadricauda	---	16:0 - 18:3	18:3
Chlorophycophyta	Tetraedron sp.	Green River	16:0 - 18:4	18:1
Cyanophycophyta	Anacystis cyanea	Bitter Springs	15:0 - 18:3	16:0, 16:1
Cyanophycophyta	Anacystis nidulans	---	14:0 - 18:1	16:0, 16:1
Cyanophycophyta	Spirulina platensis	Bitter Springs	14:0 - 18:3	16:0
Cyanophycophyta	Lyngbya aestuarii	Bitter Springs	16:0 - 18:3	16:0
Cyanophycophyta	Nostoc sp.	---	14:0 - 18:3	16:0
Cyanophycophyta	Chroococcus turgidus	Green River	16:0 - 18:3	16:0, 18:3
Cyanophycophyta	Anacystis montana	Bitter Springs	16:0 - 18:3	18:1

organisms presented in the literature (77, 81, 82, 84). The major variations in patterns relate to the relative amounts and degrees of saturation. Fatty acid patterns of a number of cyanophycean algae have been reported (80). The identities of all of the fatty acid methyl esters reported in Tables VIII and IX have also been confirmed by mass spectrometry. Their mass spectrometric fragmentation patterns have already been discussed in the literature (84).

Table VIII shows the predominant fatty acids of each organism. The widest range is in the green algae Coelastrum microsporum (see Figure 21), 14:0 to 18:4. All of the algae analyzed fall within this range. From Table VIII and Figures 21, 22, 23, 24, and 25 it can be seen that the green algae have a C_{18} unsaturate as their major fatty acid, while all of the blue green algae (Table VIII and Figures 26, 27, 28, 29, 30, and 31) except for Anacystis montana have either a 16:0 or 16:1 as their major component. Table IX gives the relative percent composition of each individual fatty acid. Table X shows a ratio of total C_{16} acids to total C_{18} acids. From Table X it is shown that in the total C_{16}/C_{18} fatty acids, the ratio is greater in all of the blue greens except for A. montana and is about equal in C. turgidus. Conversely, the ratio of C_{16}/C_{18} fatty acids in the green algae shows the C_{18} fatty acids to predominate. All of the algae except A. nidulans have a ratio of saturated to unsaturated C_{16}, in which the saturated form predominates. The reverse is seen in the C_{18} fatty acids. Also, the C_{18} fatty acids show a greater variety of poly unsaturated acids in all of the algae except A. nidulans.

TABLE IX

RELATIVE PERCENT FATTY ACID COMPOSITION OF ANALYZED ALGAE*

	GREEN ALGAE				BLUE GREEN ALGAE						
FATTY ACID Me ESTERS	CHLOR. PYR.	TETR. SP.	COEL. MICRO.	SCEN. QUAD.	ANA. NID.	ANA. CYAN.	SPIR. PLAT.	NOST. SP.	LYNG. AEST.	CHROO. TURG.	ANA. MONT.
14:0	1.6		0.7		3.0		1.9	2.3			
14:1					4.0						
15:0						3.0					
16:0	21.4	18.3	13.1	13.0	30.7	23.0	45.0	36.3	25.8	28.2	21.3
16:1	2.0	2.6	1.3	4.0	33.3	20.0	6.0	10.0	15.4	12.3	3.1
16:2	5.8	2.7	4.6					1.0	3.2	3.8	
17:0					1.2						
17:1							2.0				
18:0		1.7			.4	2.0		3.0			
18:1**	16.7	33.5	30.0	18.0	3.6	7.0	10.1	19.8	8.3	7.3	25.3
18:1***			7.0	14.0							
18:2	18.7	11.1	13.0	7.0		10.0	16.0	8.0	16.8	16.4	17.8
18:3	26.4	18.3	16.0	29.0		5.0	13.0	6.5	18.2	25.7	19.4
18:4		6.3	7.0								
Total C_{16}/C_{18}	0.47	0.33	0.26	0.25	16.0	1.92	1.31	1.27	1.02	0.92	0.39

TABLE IX CONTINUED

RELATIVE PERCENT FATTY ACID COMPOSITION OF ANALYZED ALGAE

Footnotes to table:

* Differences to 100 percent made up by unidentified compounds.

** 9-cis-octadecenoic acid (oleic)

*** 9-trans-octadecenoic acid (elaidic)

TABLE X

TOTAL C_{16}/C_{18} FATTY ACIDS

Blue Greens	C_{16}/C_{18}	Greens	C_{16}/C_{18}
Anacystis nidulans	16.0	*Chlorella pyrenoidosa*	0.47
Anacystis cyanea	1.92	*Tetraedron sp.*	0.33
Spirulina platensis	1.31	*Coelastrum microsporum*	0.26
Nostoc sp.	1.27	*Scenedesmus quadricauda*	0.25
Lyngbya aestuarii	1.02		
Chroococcus turgidus	0.92		
Anacystis montana	0.39		

FIGURE 21

GAS CHROMATOGRAPHIC SEPARATIONS OF THE FATTY ACID METHYL ESTERS OF COELASTRUM MICROSPORUM

Stainless steel column (1/8" x 12') packed with 15 percent E.G.S. Barber Colman 5000 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 910 g/cm^2; sensitivity x 5; attenuation, 10. The temperature was isothermal at 180° C.

COELASTRUM MICROSPORUM

FATTY ACID Me-ESTERS

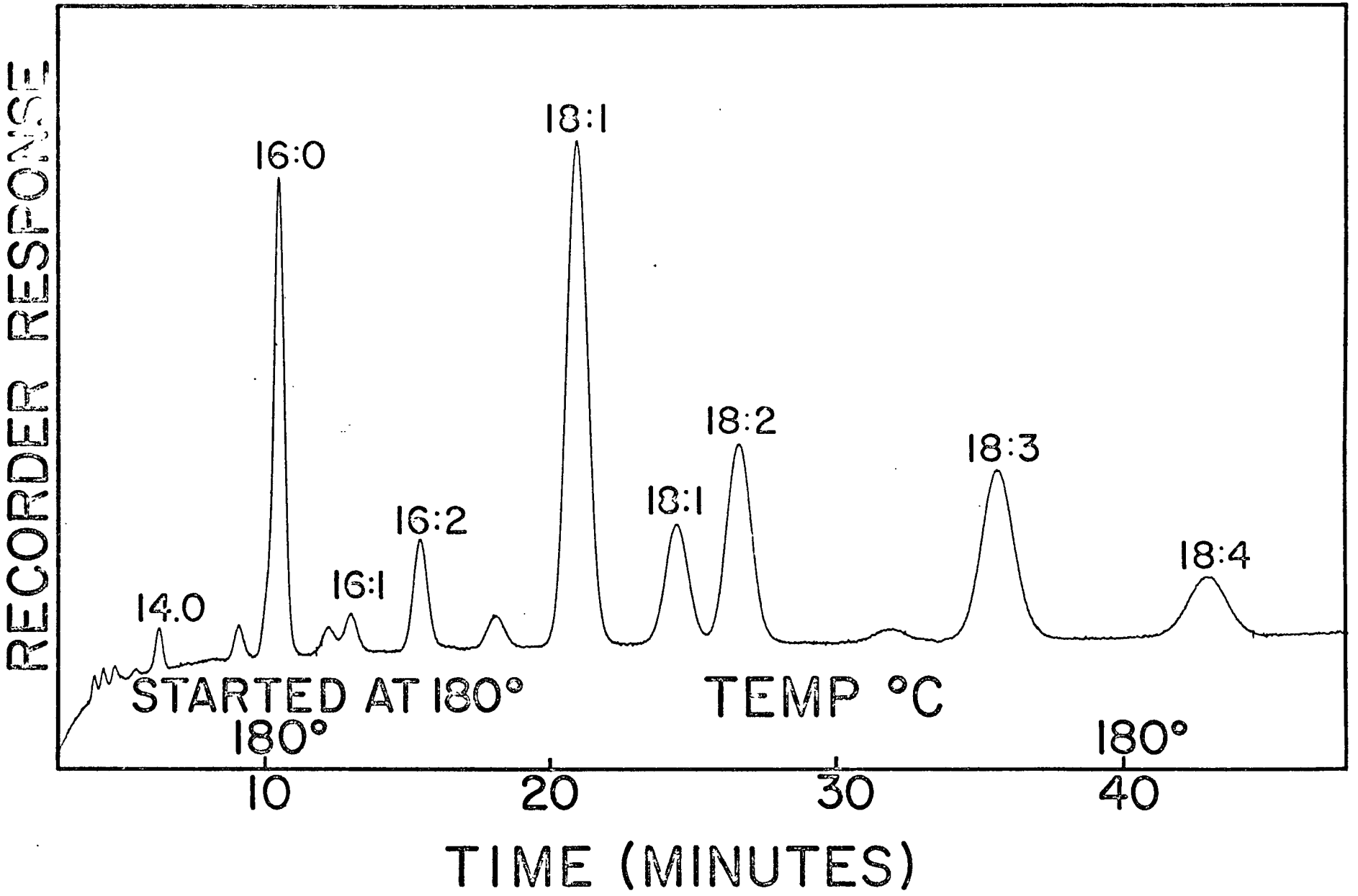


FIGURE 22

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM SCENEDESMUS QUADRICAUDA*

All of the operating conditions were the same as in Figure 21 with the exception of the temperature, which was 190° C isothermally.

* One half of the total methanol fraction was methylated with BF_3 (102)

SCENEDESMUS QUADRICAUDA
FATTY ACID Me-ESTERS

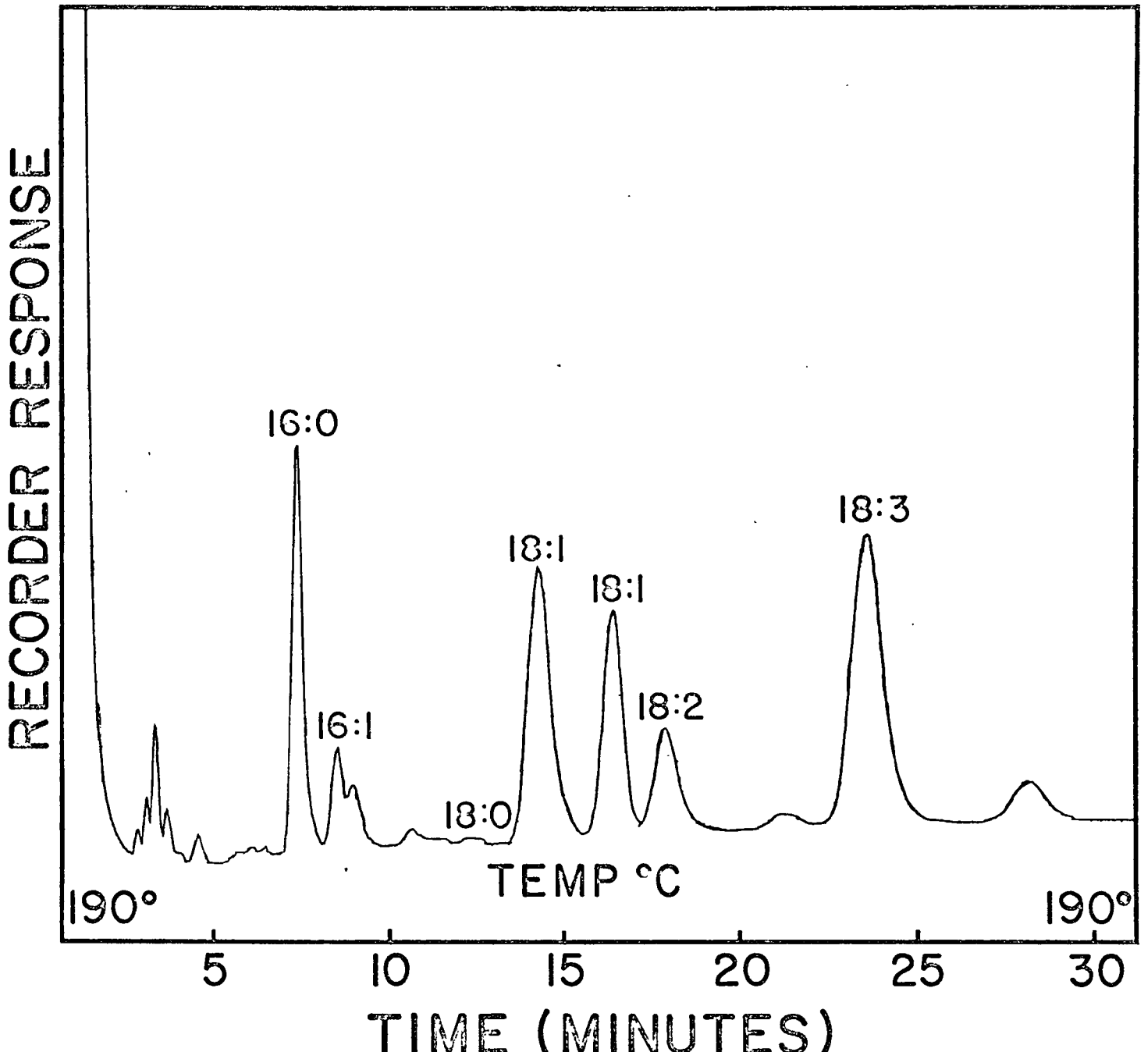


FIGURE 23

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM TETRAEDRON SP.

All of the operating conditions were the same as in Figure 21.

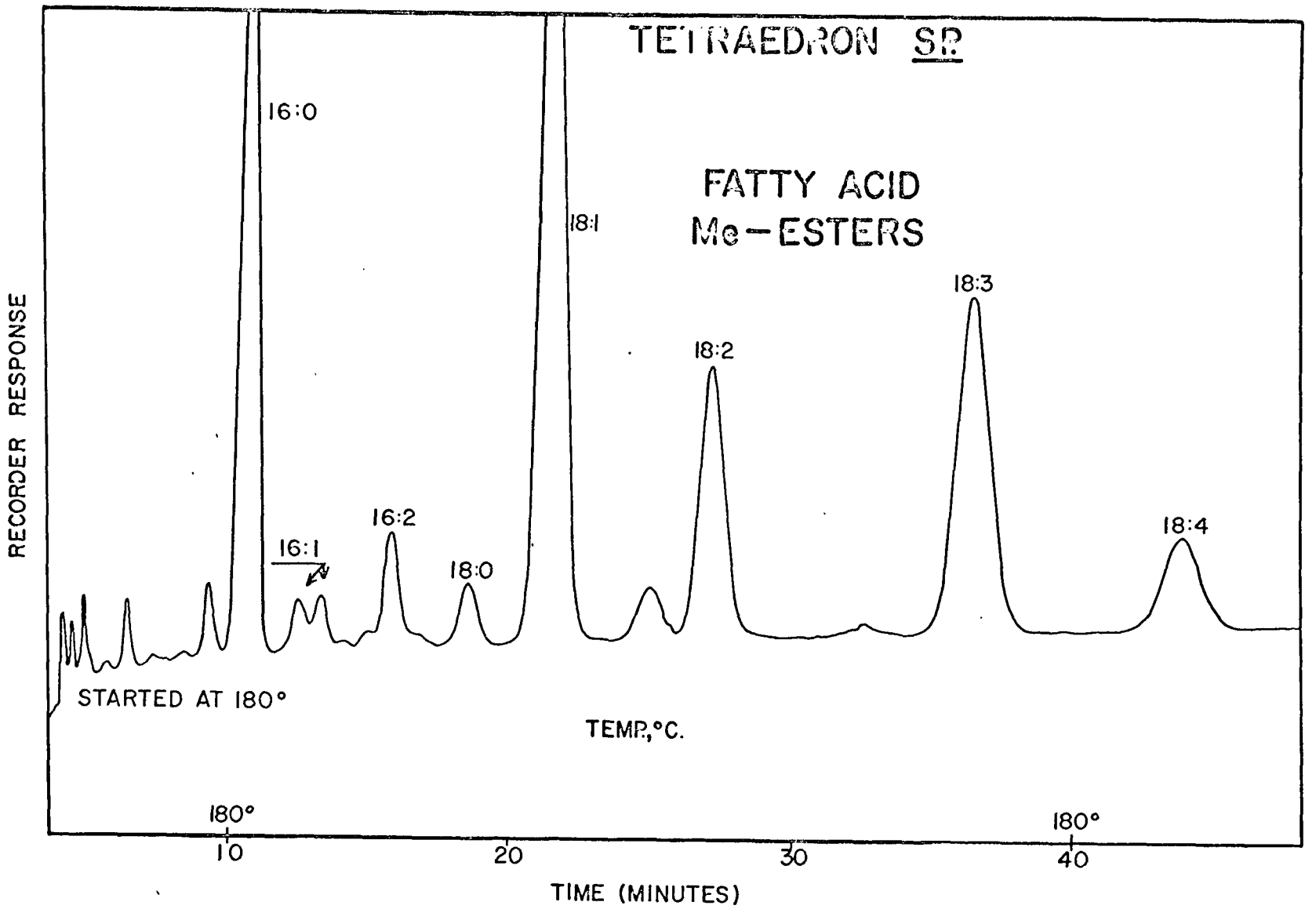

TETRAEDRON SP
FATTY ACID
Me-ESTERS
16:0
18:1
18:3
18:2
16:2
16:1
18:0
18:4
STARTED AT 180°
RECORDER RESPONSE
TEMP.,°C.
180°
180°
10
20
30
40
TIME (MINUTES)

FIGURE 24

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM CHLORELLA PYRENOIDOSA

All of the operating conditions were the same as in Figure 21.

CHLORELLA PYRENOIDOSA

FATTY ACID Me-ESTERS

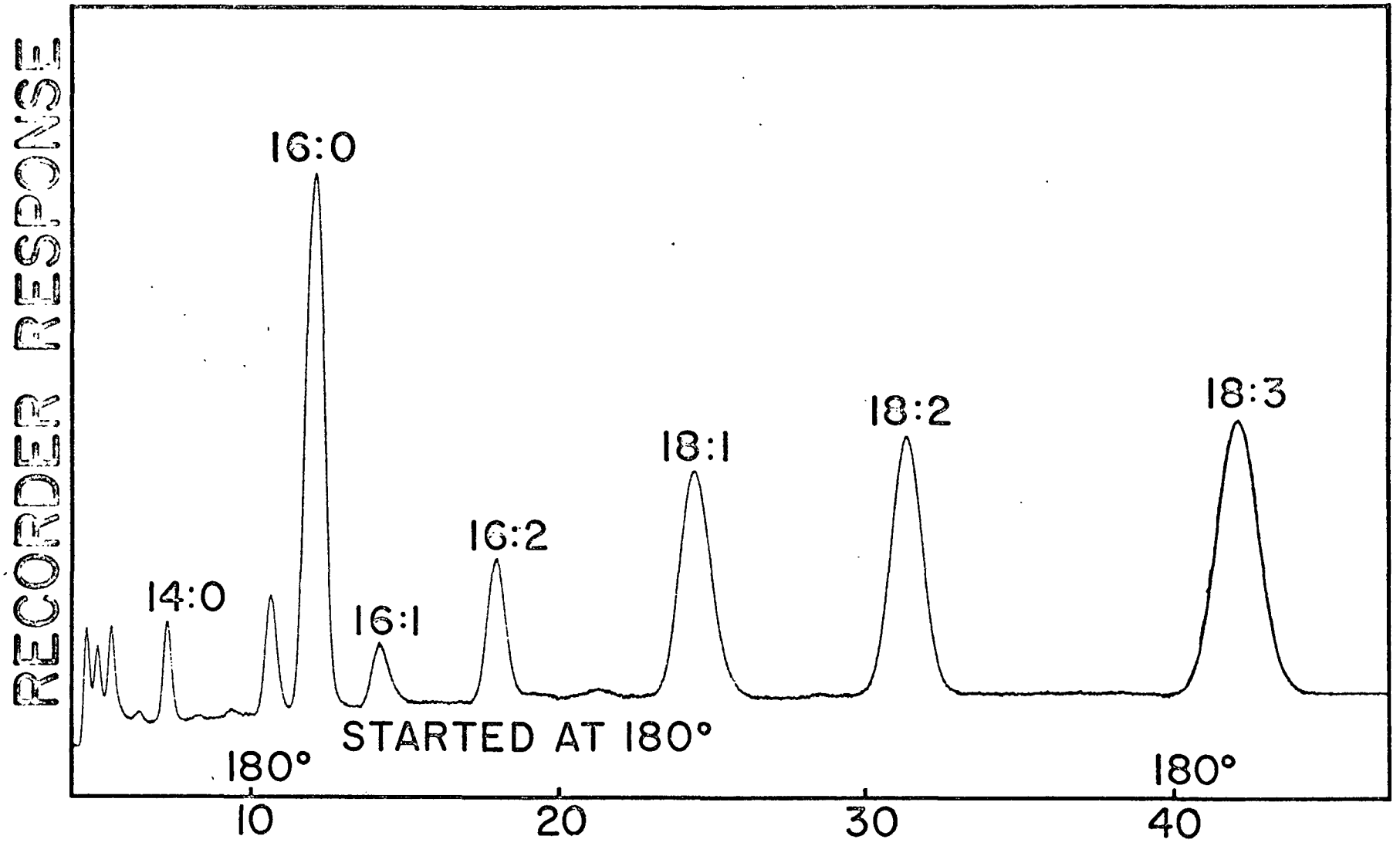


FIGURE 25

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM *SCENEDESMUS OBLIQUUS*

All of the operating conditions were the same as in Figure 21.

C. microsporum, Figure 21, and S. quadricauda, Figure 22, contain two components having different retention times, but were found to have the mass spectral characteristics of a monounsaturated C_{18} fatty acid. By comparing the gas chromatographic peaks' retention times with that of the cis and trans C_{18} acids, they were identified as cis and trans isomers. A similar situation applies to the monounsaturated C_{16} acid in the same two species of algae.

Two of the green algae, C. microsporum and Tetraedron sp., Figure 23, have tetra unsaturated C_{18} fatty acids. C. pyrenoidosa, Figure 24, has the largest C_{16}/C_{18} ratio of the green algae and has a distribution more similar to the blue green algae.

A. nidulans, Figure 26, is the only blue green alga found in this study that does not contain polyunsaturated acids. A. nidulans, A. cyanea (Figure 27) and S. platensis (Figure 28) each have an odd numbered chain length fatty acid, whereas the rest of the algae, both greens and blue greens, have only even-numbered chain lengths.

C. turgidus (Figure 29) and L. aestuarii (Figure 30) appear very similar in their fatty acid distribution. Lyngbya has a slightly larger C_{16}/C_{18} value. Nostoc sp., as seen by Figure 31, has a very small peak at 18:3 but still resembles most of the other blue greens in distribution. A. montana has a C_{16}/C_{18} ratio in the range of the green algae as shown in Figure 32. The 16:0 to 16:1 ratio of A. montana is similar to the green algae also. The fatty acids of the Hawaii Marine Alga (Figure 33) present in major amounts were myristic, palmitic, palmitoleic, oleic and other unsaturated C_{18} fatty acids. The total lipid content was calculated to be 30.7 percent.

FIGURE 26

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM ANACYSTIS NIDULANS

Stainless steel capillary column (0.076 cm x 155 in) coated with Igepal CO 990. F&M Model 810 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; range, 10^2; attenuation, 1. Programmed at 6°/min from 120° C to 200° C and held isothermally at 200° C.

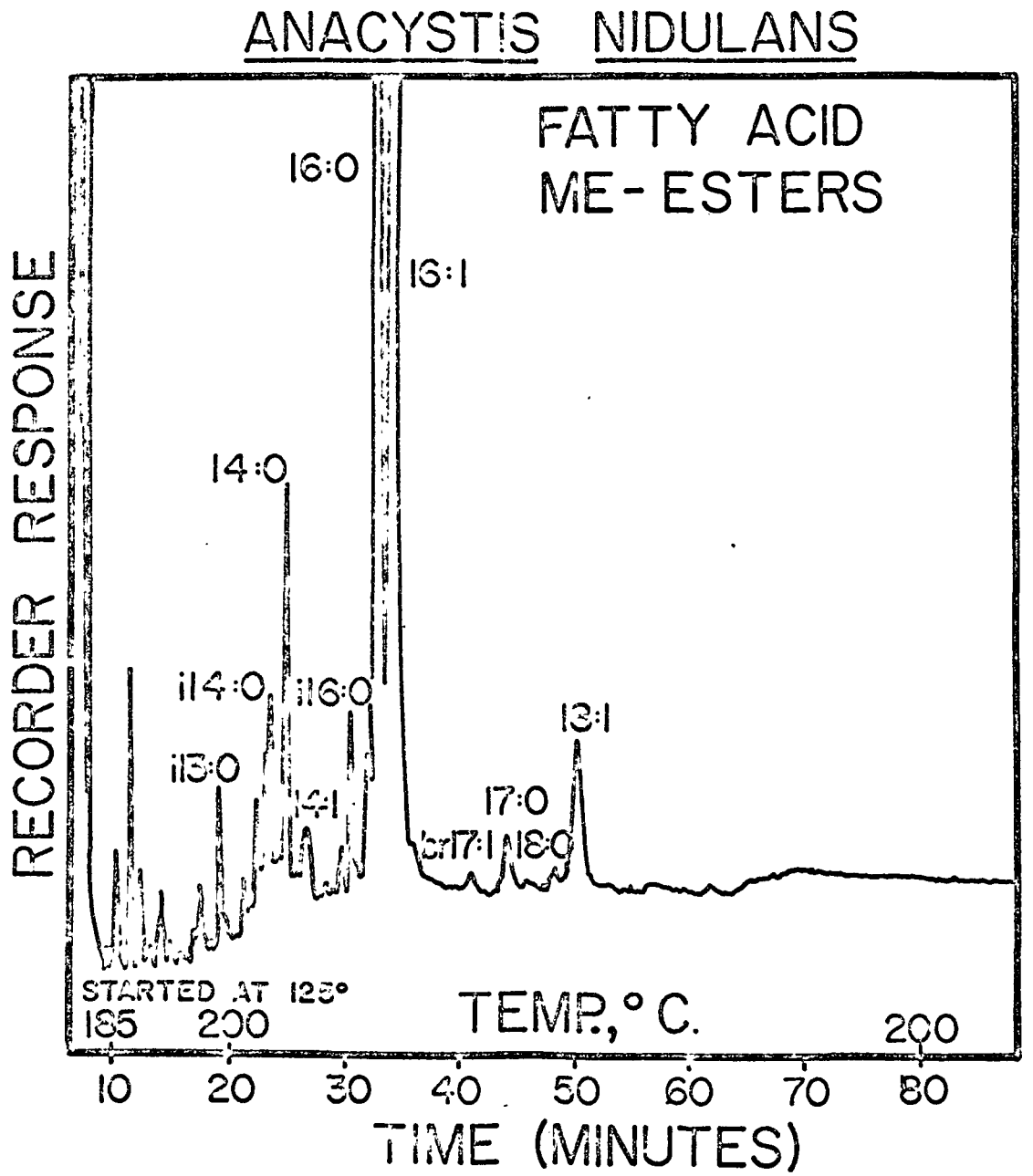

ANACYSTIS NIDULANS
FATTY ACID
ME-ESTERS
16:0
16:1
14:0
i14:0
i16:0
i13:0
14:1
17:0
br17:1
18:0
13:1
RECORDER RESPONSE
STARTED AT 125°
185
200
TEMP., °C.
200
10
20
30
40
50
60
70
80
TIME (MINUTES)

FIGURE 27

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM *ANACYSTIS CYANEA*

All of the operating conditions were the same as in Figure 21 with the exception of the temperature which was 190° isothermally.

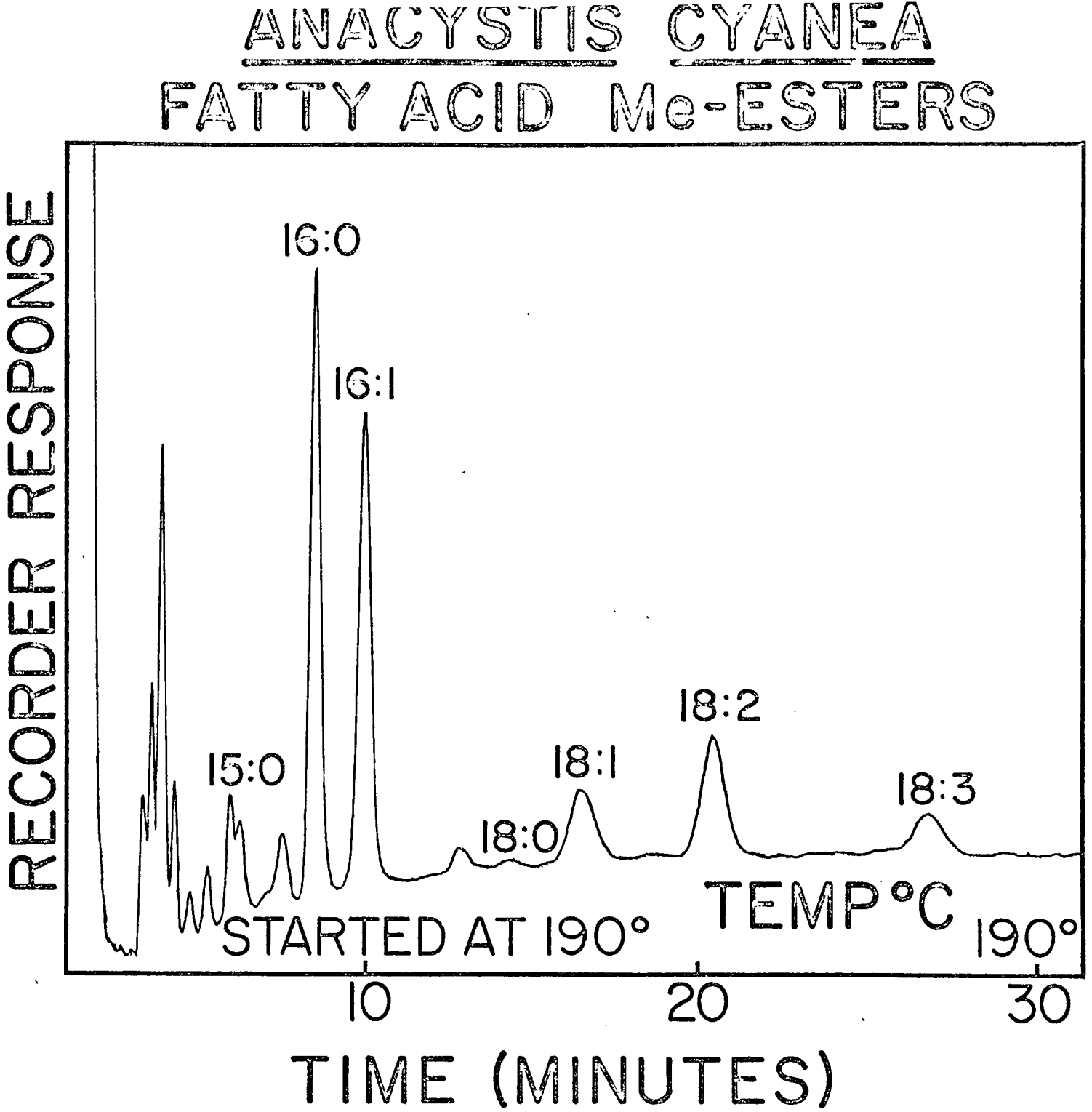

ANACYSTIS CYANEA
FATTY ACID Me-ESTERS
RECORDER RESPONSE
16:0
16:1
15:0
18:0
18:1
18:2
18:3
STARTED AT 190°
TEMP °C
190°
10
20
30
TIME (MINUTES)

FIGURE 28

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM SPIRULINA PLATENSIS

All of the operating conditions were the same as in Figure 21 with the exception of the temperature which was 190° isothermally.

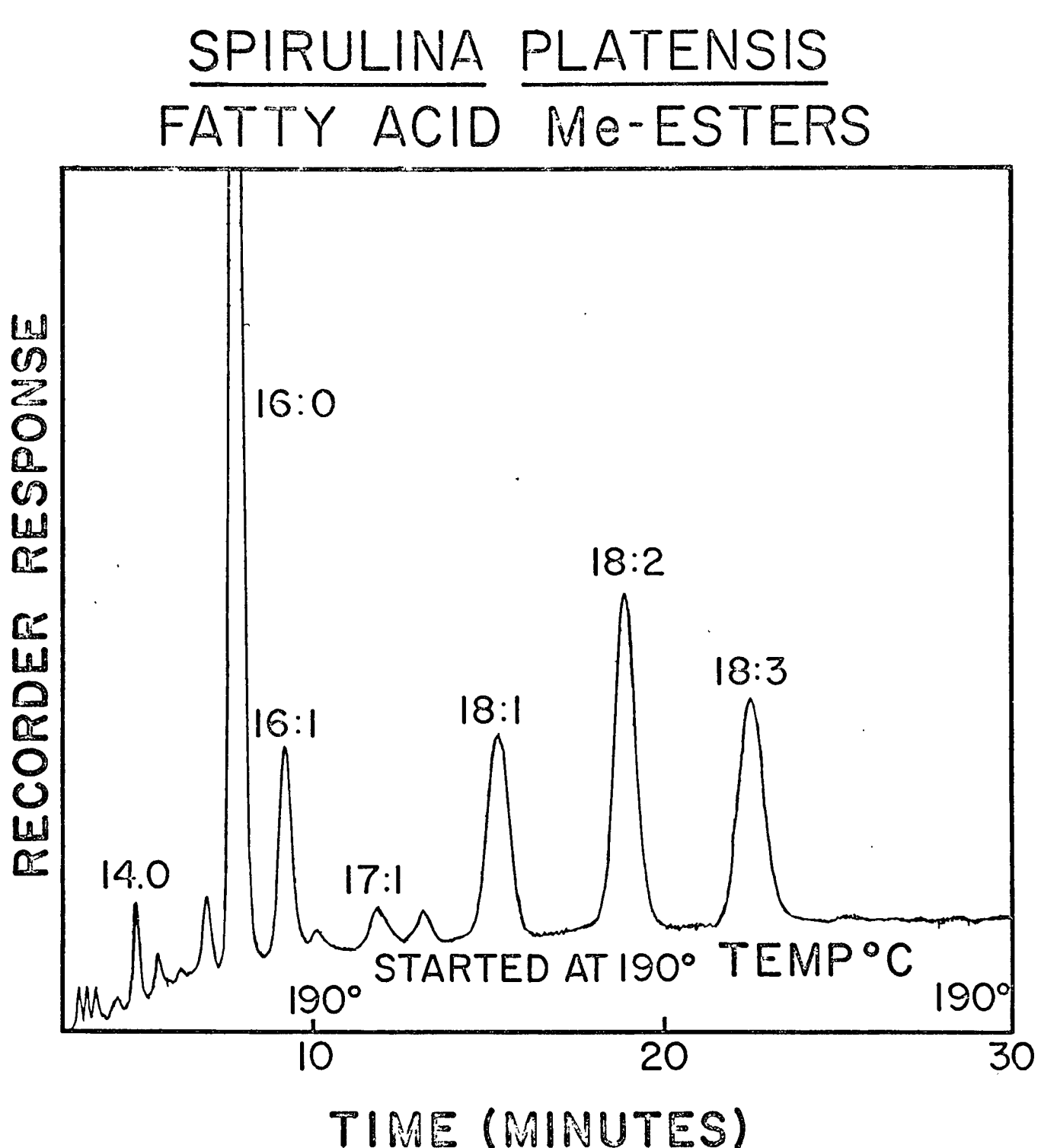

SPIRULINA PLATENSIS
FATTY ACID Me-ESTERS
RECORDER RESPONSE
16:0
18:2
18:3
16:1
18:1
14.0
17:1
STARTED AT 190° TEMP °C
190°
190°
10
20
30
TIME (MINUTES)

FIGURE 29

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM CHROOCOCCUS TURGIDUS

All operating conditions were the same as in Figure 21 with the exception of the temperature which was 190° isothermally.

CHROOCOCCUS TURGIDUS
FATTY ACID Me-ESTERS

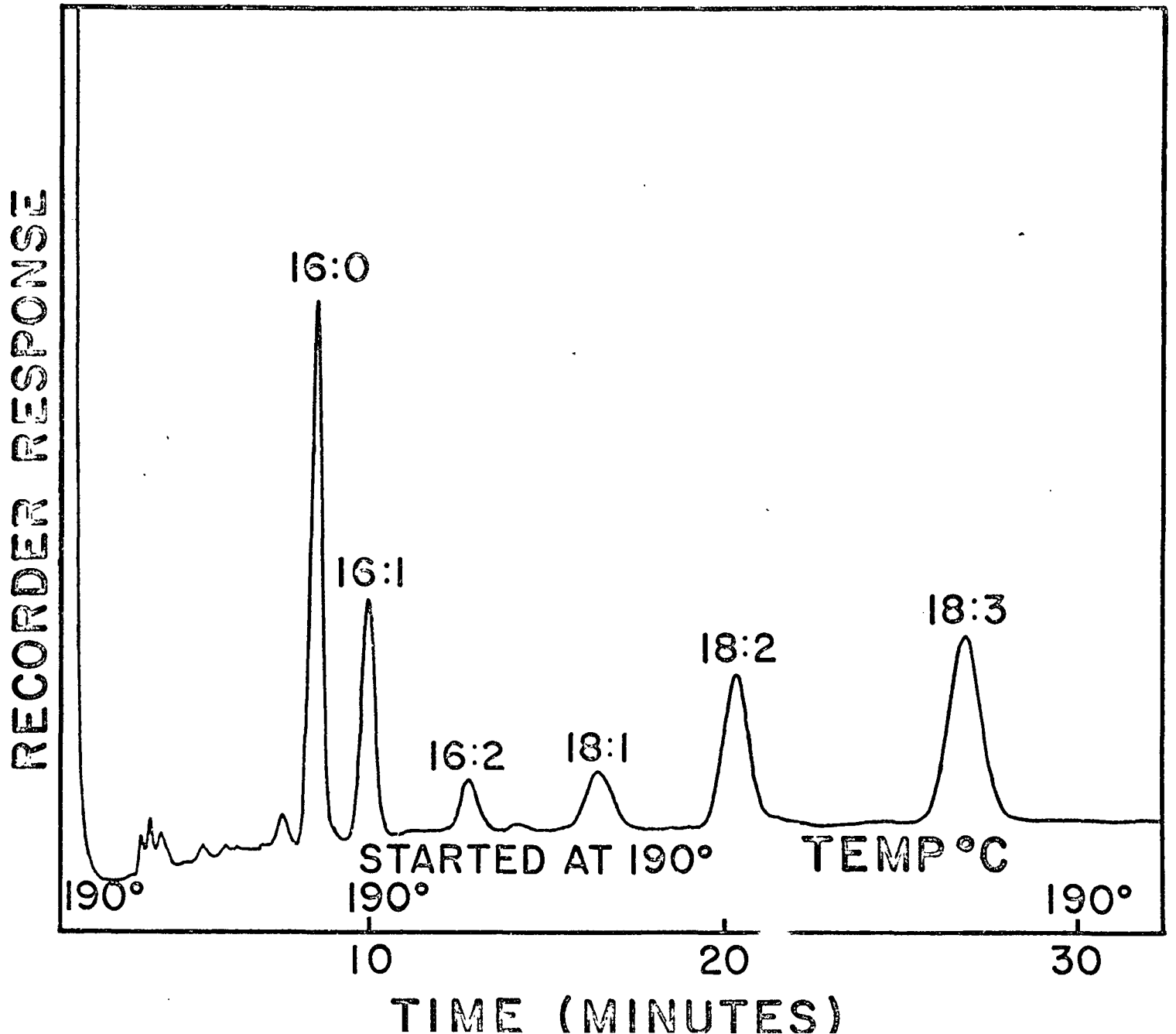


FIGURE 30

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM LYNGBYA AESTUARII

All of the operating conditions were the same as in Figure 21.

LYNGBYA AESTUARII

FATTY ACID Me-ESTERS

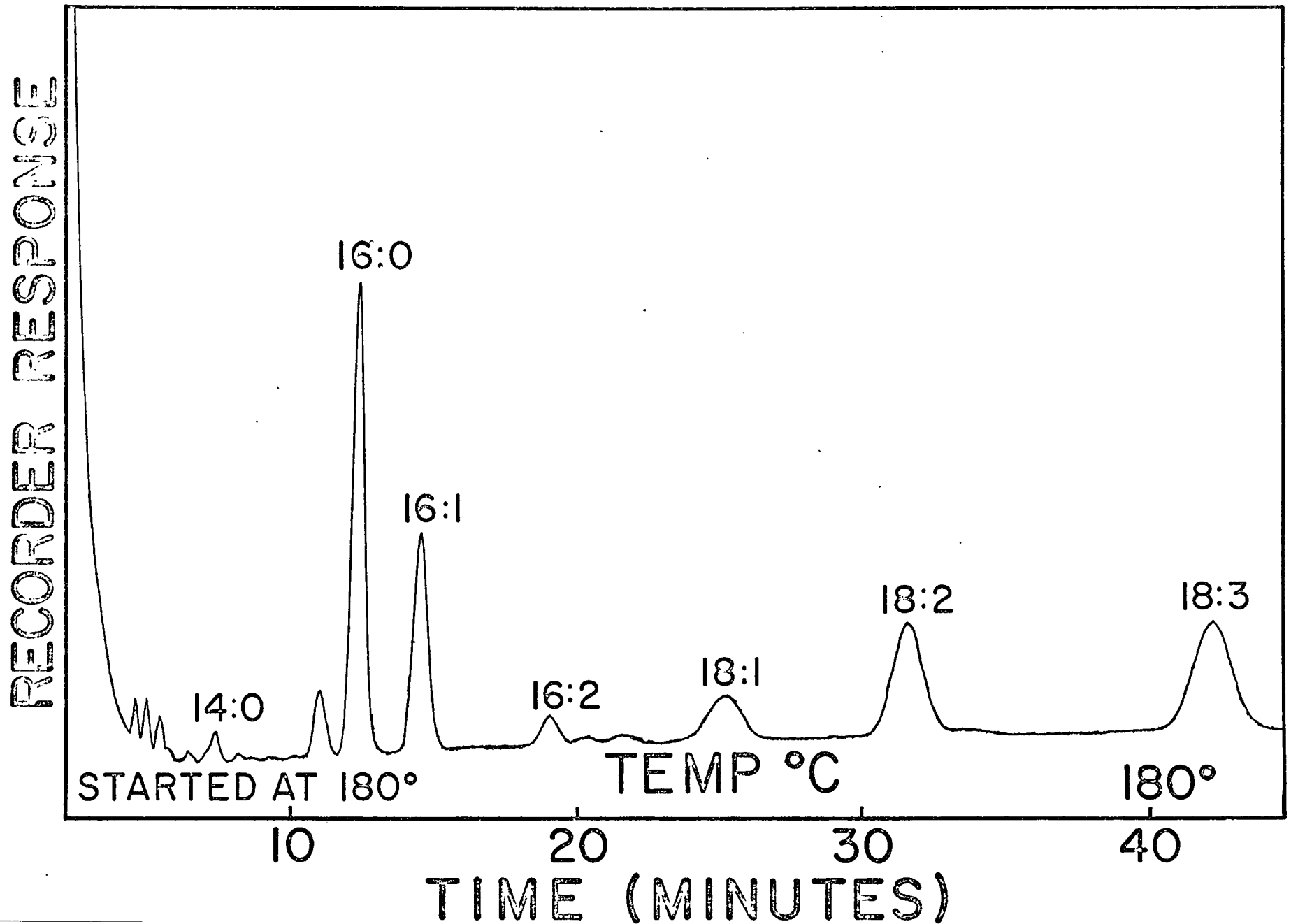


FIGURE 31

GAS CHROMATOGRAPHIC SEPARATIONS OF THE FATTY ACID METHYL ESTERS FROM *NOSTOC SP.*

All of the operating conditions were the same as in Figure 21.

NOSTOC S.P.
FATTY ACID Me-ESTERS

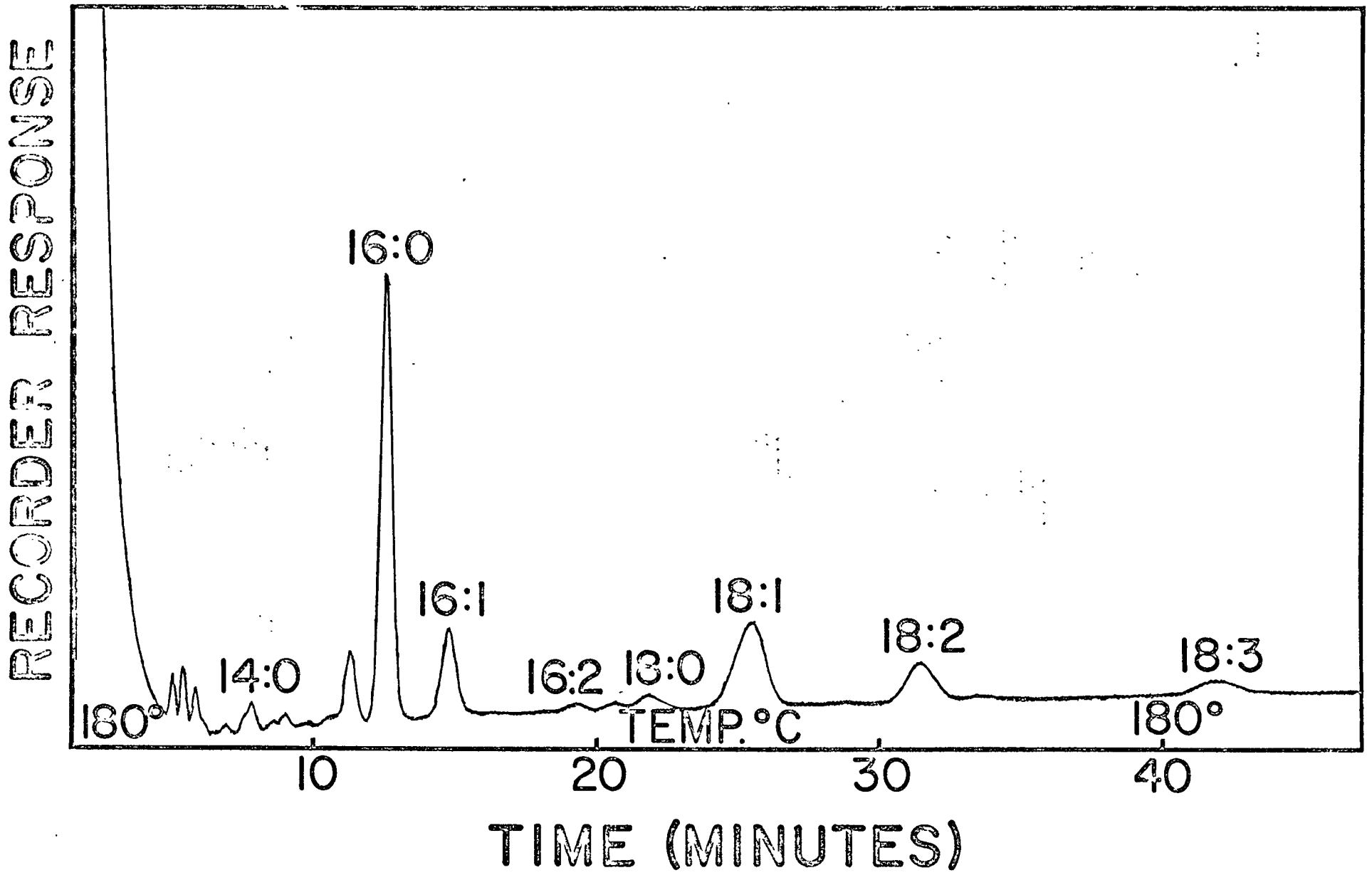


FIGURE 32

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM *ANACYSTIS MONTANA*

All of the operating conditions were the same as in Figure 21.

ANACYSTIS MONTANA
FATTY ACID Me-ESTERS

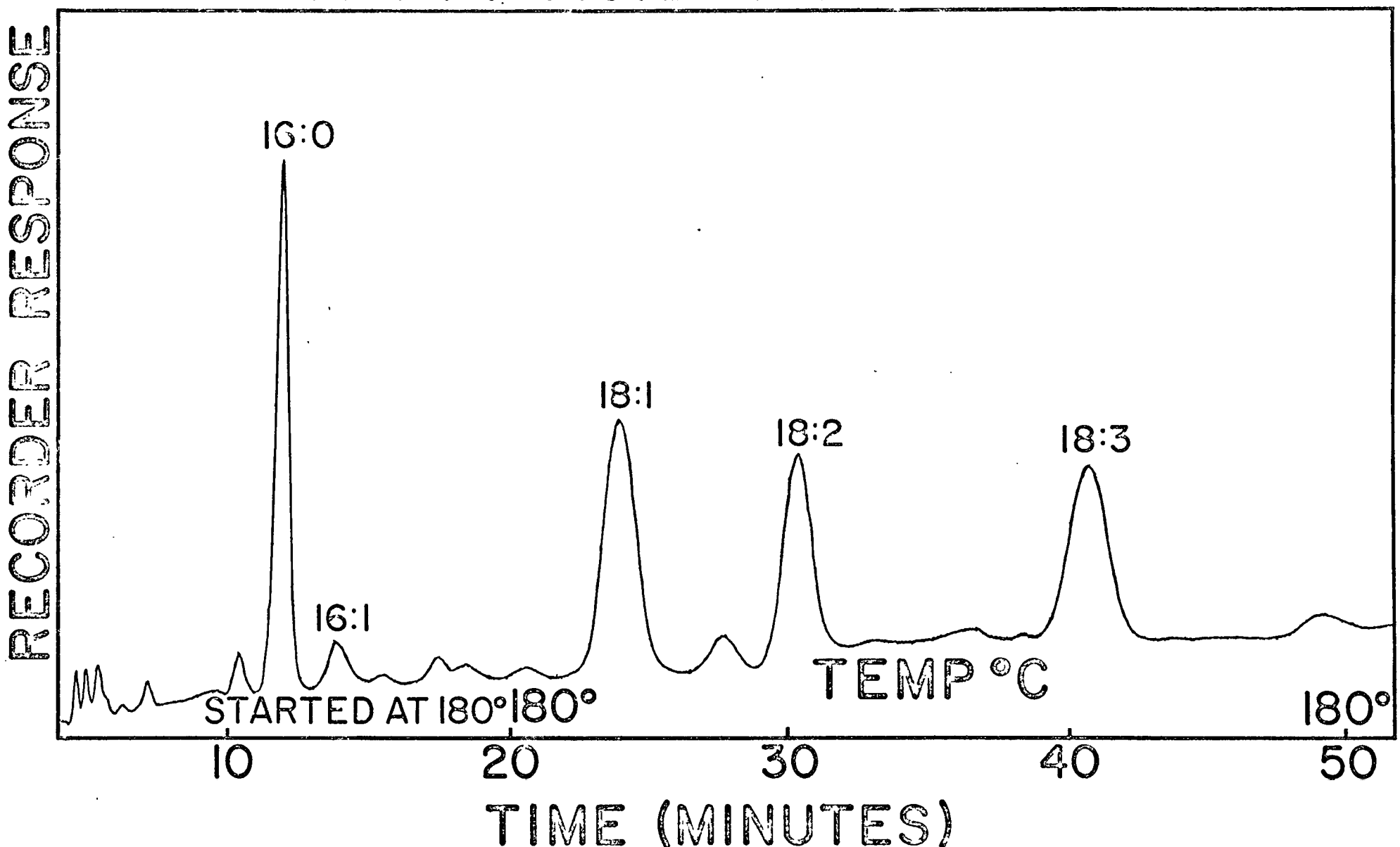
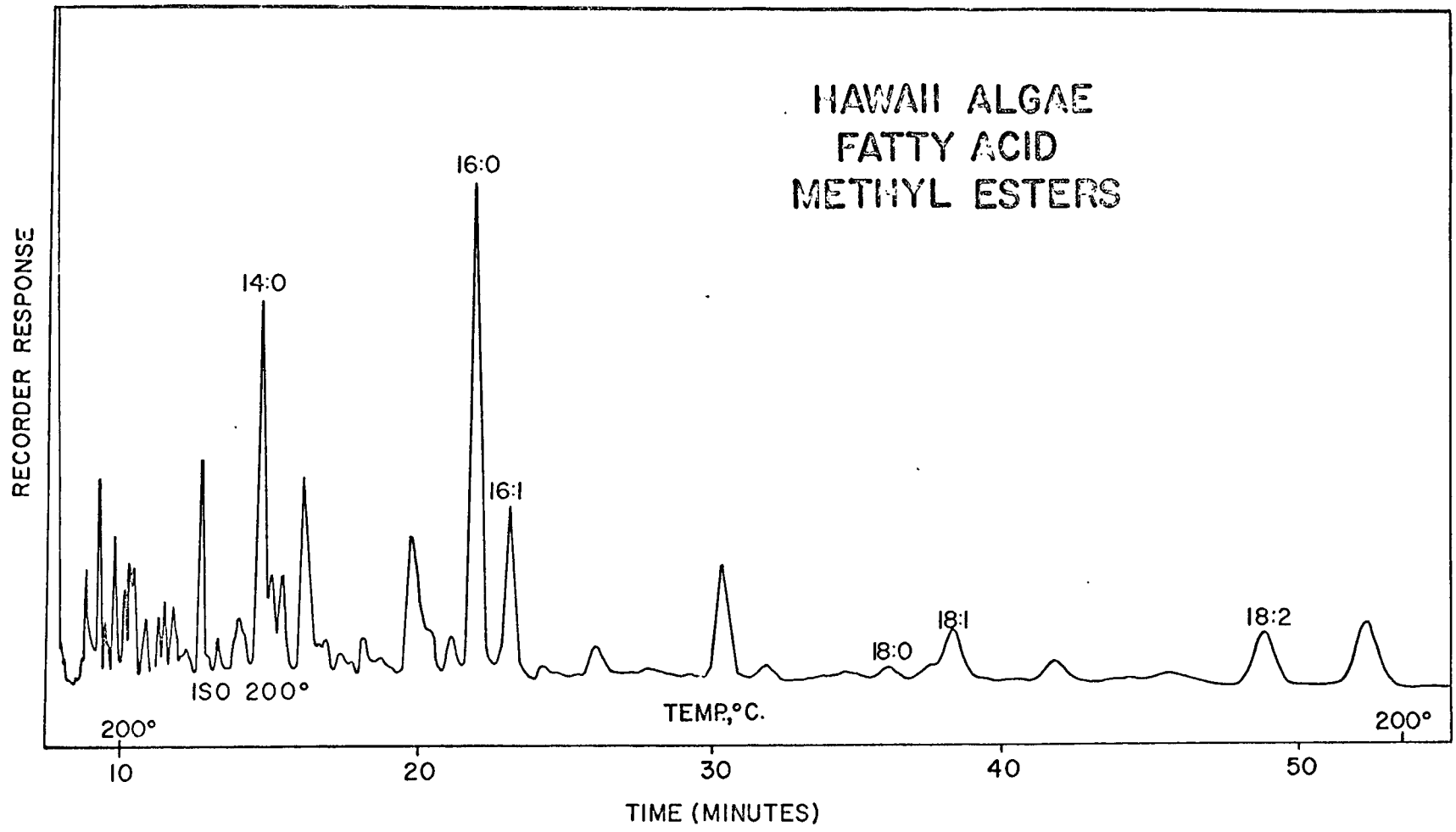


FIGURE 33

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM THE MACROSCOPIC MARINE ALGA FROM HAWAII

All of the operating conditions were the same as in Figure 26 with the exception of the temperature which was 200° C isothermally.


HAWAII ALGAE
FATTY ACID
METHYL ESTERS
RECORDER RESPONSE
14:0
16:0
16:1
ISO 200°
18:0
18:1
18:2
200°
TEMP.,°C.
200°
10
20
30
40
50
TIME (MINUTES)

3. Methyl Ester Preparations

Figures 22 and 34 show fatty acid methyl ester gas chromatographic distributions of S. quadricauda. The fraction from the column separation that contained the fatty acids was divided into equal parts. One part was treated with BF_3 (102) and the other part was treated as described by Kates (101) in the Experimental Section of this study. After methylation, equal amounts of the two samples were injected into the gas chromatograph and gave identical results.

B. Other Biogenic Lipids

1. Plantago ovata Hydrocarbons and Fatty Acids

The distribution pattern of hydrocarbons in the seed coat of P. ovata is shown in Figure 35 and the percentages are reported in Table XI. The various peaks were identified by gas chromatography and mass spectrometry. An example of the mass spectrometric identification of the iso-C_{17} and the anteiso-C_{18} is shown in Figure 36.

In addition to the hydrocarbons in the range C_{25} to C_{35} predominant in most plant sources (125), the seeds of P. ovata also show the presence of relatively large amounts of lower molecular weight hydrocarbons. Among them, the anteiso-C_{18} is the major component (10.1 percent). Only two other components, the n-C_{31} and iso-C_{33} occur in higher concentrations (see Table XI).

A close study of the pattern illustrated in Figure 35 reveals a kind of trimodal distribution for the straight chain hydrocarbons. The first mode (C_{16} - C_{19}) has its maximum at the n-C_{17}, the second

FIGURE 34

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM SCENEDESMUS QUADRICAUDA*

All of the operating conditions were the same as in Figure 21 with the exception of the temperature which was 190° C isothermally.

* One half of the total methanol fraction was methylated according to Kates (101)

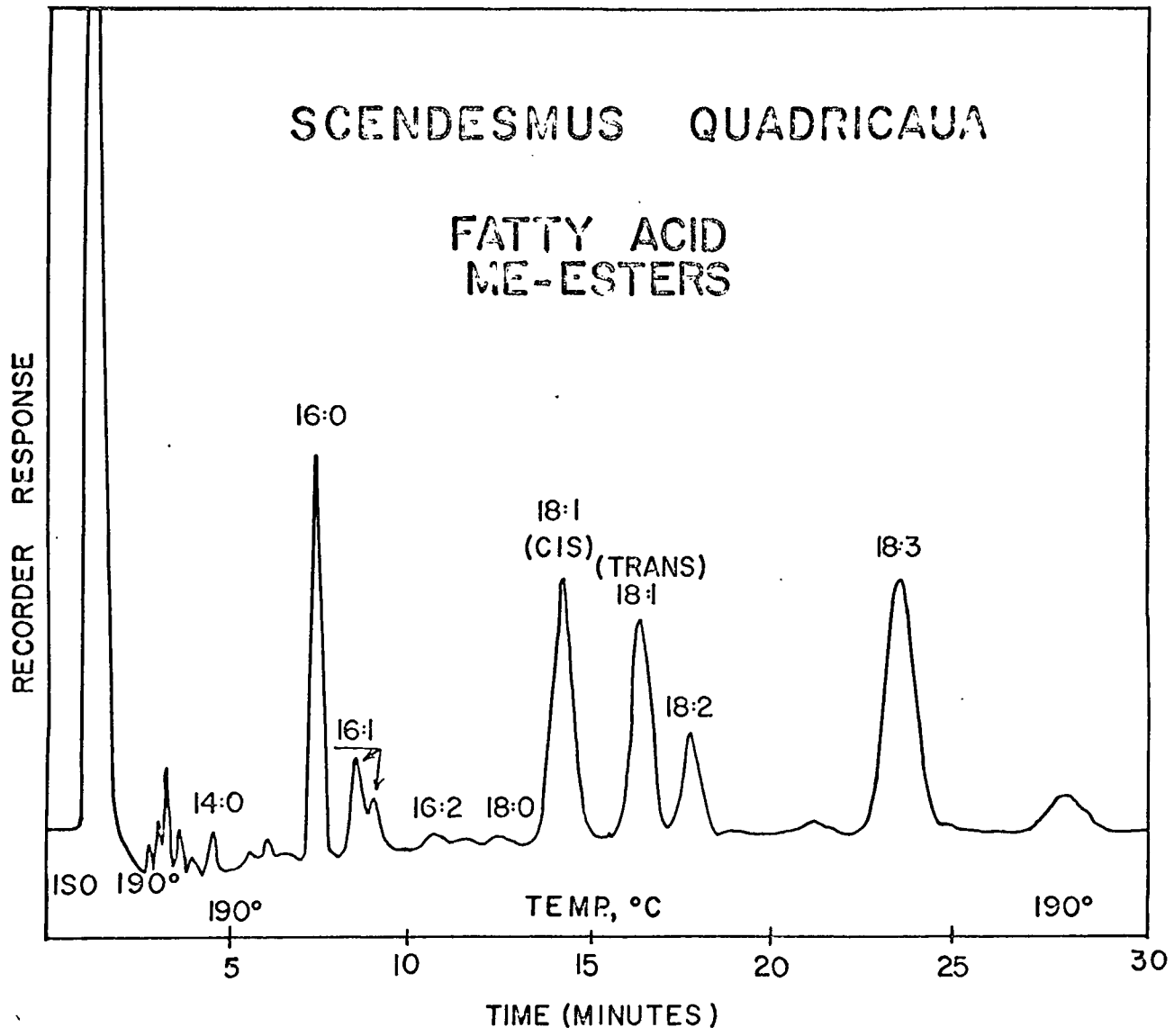

SCENDESMUS QUADRICAUA
FATTY ACID
ME-ESTERS
RECORDER RESPONSE
16:0
18:1
(CIS)
(TRANS)
18:1
18:3
18:2
16:1
14:0
16:2
18:0
ISO
190°
190°
TEMP, °C
190°
5
10
15
20
25
30
TIME (MINUTES)

FIGURE 35

GAS CHROMATOGRAPHIC SEPARATION OF THE HYDROCARBONS OF *PLANTAGO OVATA* SEED COAT

Copper column (3 m x 0.6 cm I.D.) packed with 1 percent OV-1 on Gas Chrom Q. Nitrogen pressure, 700 g/cm^2. Barber Colman Series 5000 Gas Chromatograph equipped with a flame ionization detector. Range x 1; attenuation, 10.

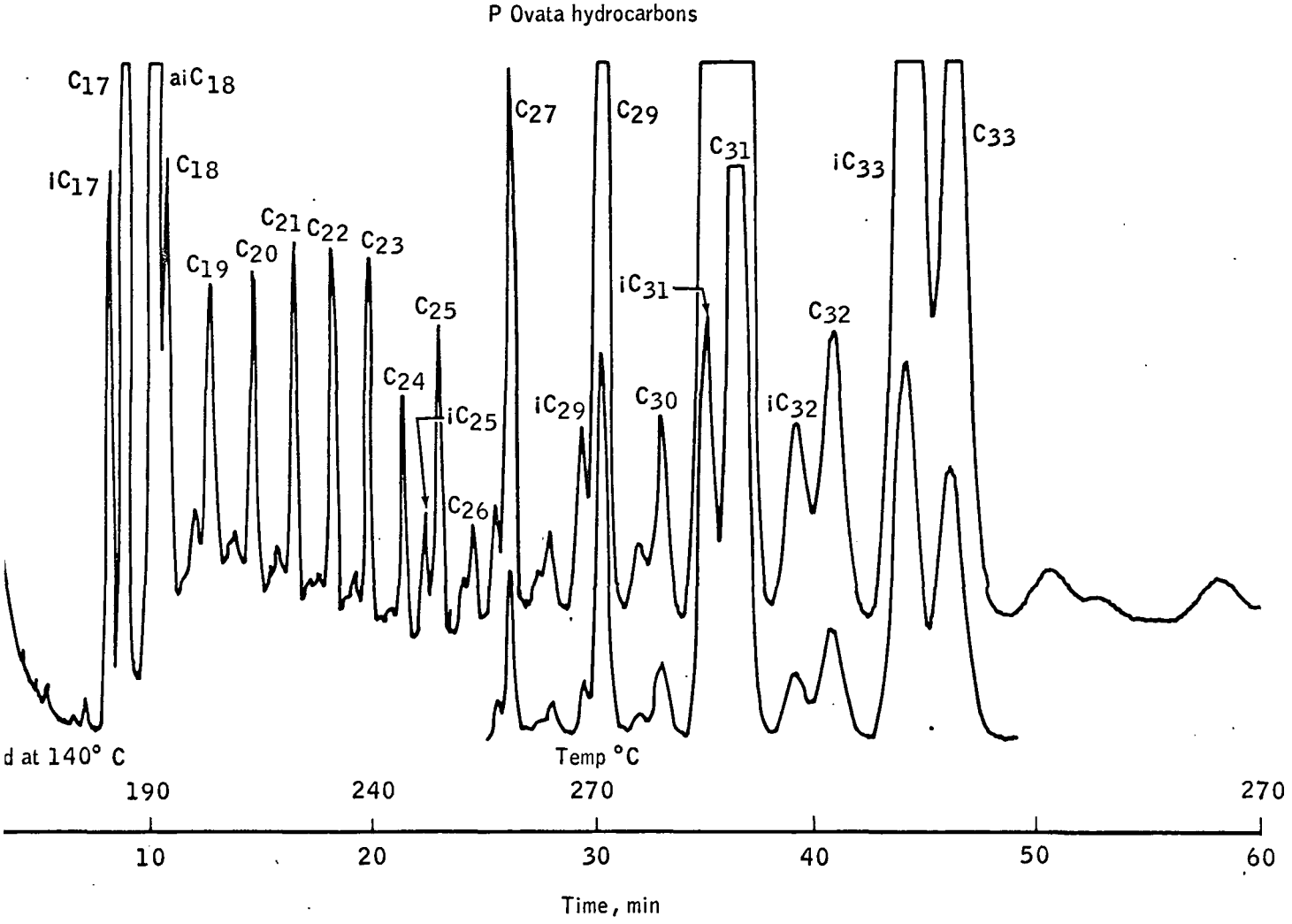

P Ovata hydrocarbons
C17
aiC18
iC17
C18
C19
C20
C21
C22
C23
C24
C25
iC25
C26
C27
iC29
C29
C30
iC31
C31
iC32
C32
iC33
C33
d at 140° C
Temp °C
190
240
270
270
10
20
30
40
50
60
Time, min

FIGURE 36

MASS SPECTRA OF ISO-C_{18} OF PLANTAGO OVATA

Capillary gas chromatographic column (180 m x 0.076 cm) coated with Polysev. For details of the mass spectrometer conditions, refer to the text.

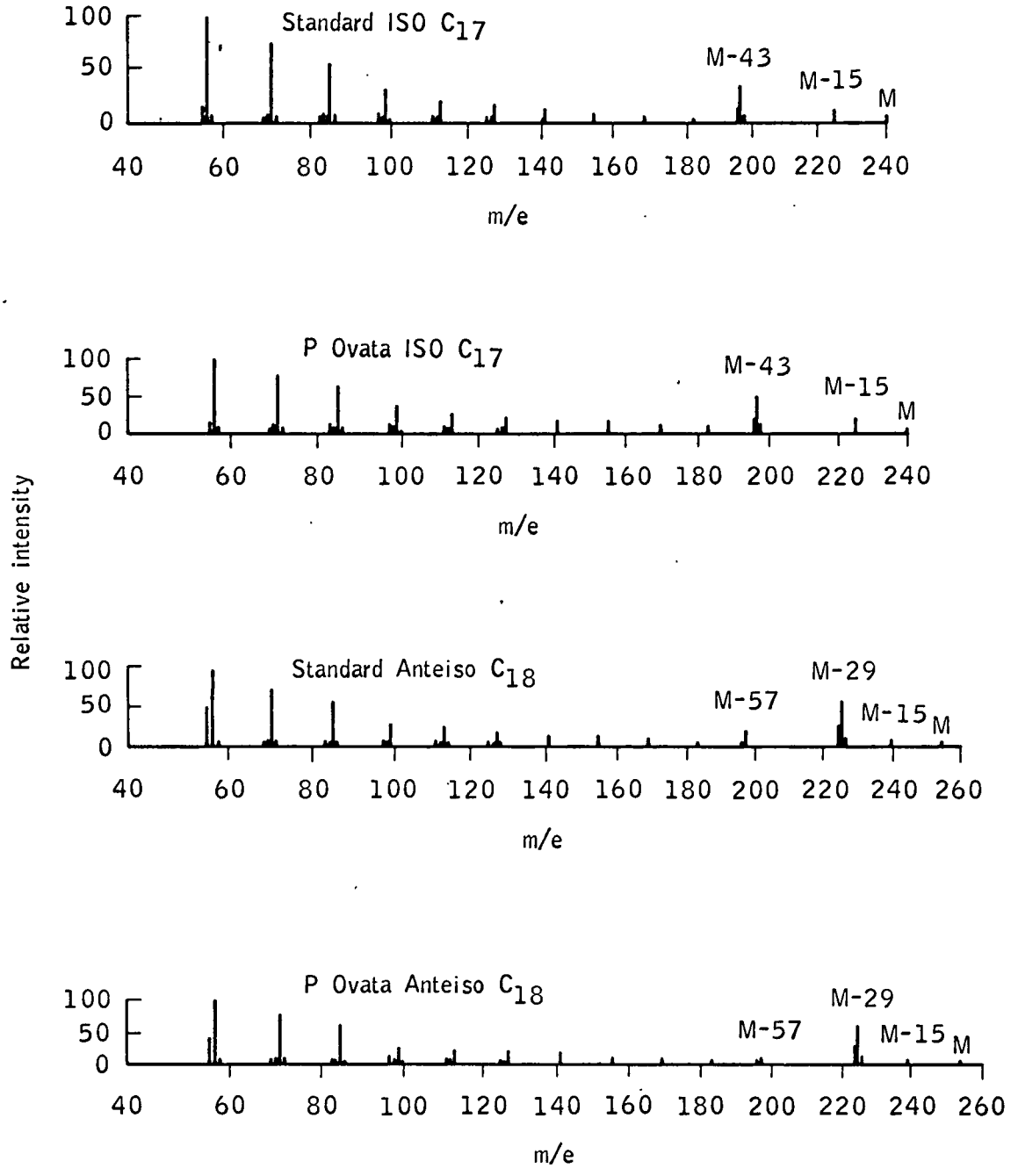

Standard ISO C_{17}
M-43
M-15
M
P Ovata ISO C_{17}
M-43
M-15
M
Standard Anteiso C_{18}
M-29
M-57
M-15
M
P Ovata Anteiso C_{18}
M-29
M-57
M-15
M
100
50
0
40
60
80
100
120
140
160
180
200
220
240
260
m/e
Relative intensity

TABLE XI

RELATIVE PERCENTAGE COMPOSITION HYDROCARBONS

IN THE SEED COAT OF PLANTAGO OVATA

HYDROCARBON*	CONTENT (Percent)	HYDROCARBON*	CONTENT (Percent)
i-C_{16}	tr.	i-C_{25}	0.4
ai-C_{16}	0.5	n-C_{25}	1.0
n-C_{16}	0.5	n-C_{26}	0.1
i-C_{16}	1.9	n-C_{27}	1.3
n-C_{17}	5.1	i-C_{29}	0.7
i-C_{18}	tr.	n-C_{29}	4.9
ai-C_{18}	10.1	n-C_{30}	1.2
n-C_{18}	1.6	i-C_{31}	8.2
n-C_{19}	1.1	n-C_{31}	20.0
n-C_{20}	2.2	i-C_{32}	1.6
n-C_{21}	2.2	n-C_{32}	2.7
n-C_{22}	1.5	i-C_{33}	10.9
n-C_{23}	1.2	n-C_{33}	9.1
n-C_{23}	0.7		

*The hydrocarbons were identified by their gas chromatographic retention times and by their respective mass spectrometric fragmentation patterns. Symbols: n-normal; i-iso; ai-anteiso. The percent composition of the hydrocarbons was calculated on the basis of their gas chromatographic area, which was obtained by multiplying the peak heights by the widths at half peak heights. The total hydrocarbon content was 400 ppm.

(C_{19} - C_{24}) is centered around the n-C_{21}, while the third (C_{24} - C_{33}) shows a maximum at n-C_{31} as well as a marked predominance of the odd carbon numbered alkanes. In relation to the methyl substituted hydrocarbons, the first mode is clearly dominated by the anteiso-alkanes, while the iso-alkanes predominate within the third mode.

The seeds contain trace amounts of alkanes as compared to the seed coat, but the pattern is essentially the same. The distribution pattern of fatty acids (Figure 37) in the seed coat of P. ovata is much less complex than that of the hydrocarbons, as indicated by the identifications and relative percentages reported in Table XII. However, the total hydrocarbon content of this sample (400 ppm) was lower in comparison with the amount of fatty acids (2610 ppm).

2. Thermophilic Bacteria I and II Hydrocarbons and Fatty Acids

The heptane fraction showed insignificant amounts of aliphatic hydrocarbons, less than 1 ppm in both samples. The methyl-ester fraction was evaporated to 15 microliters for Thermophile I and 20 microliters for Thermophile II. The methyl-esters were injected into the gas chromatograph with a 650' x .03" CO 990 column. Thermophile I and Thermophile II gave similar, if not identical, patterns (Figures 38 and 39). It appeared by retention volume that both samples contained myristic acid and several other lower weight acids. It was calculated that Thermophile I had a fatty acid content of 300 ppm while Thermophile II had a fatty acid content of 600 ppm. This is a relatively low fatty acid content. The patterns were quite different from other

FIGURE 37

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM PLANTAGO OVATA

Stainless steel capillary column (0.02" x 680') coated with CO 990. Barber Colman 5000 gas chromatograph equipped with a flame ionization detector. The nitrogen pressure was 1406.0 g/cm^2; sensitivity x 5; attenuation, 10. Temperature was 200° C isothermally.

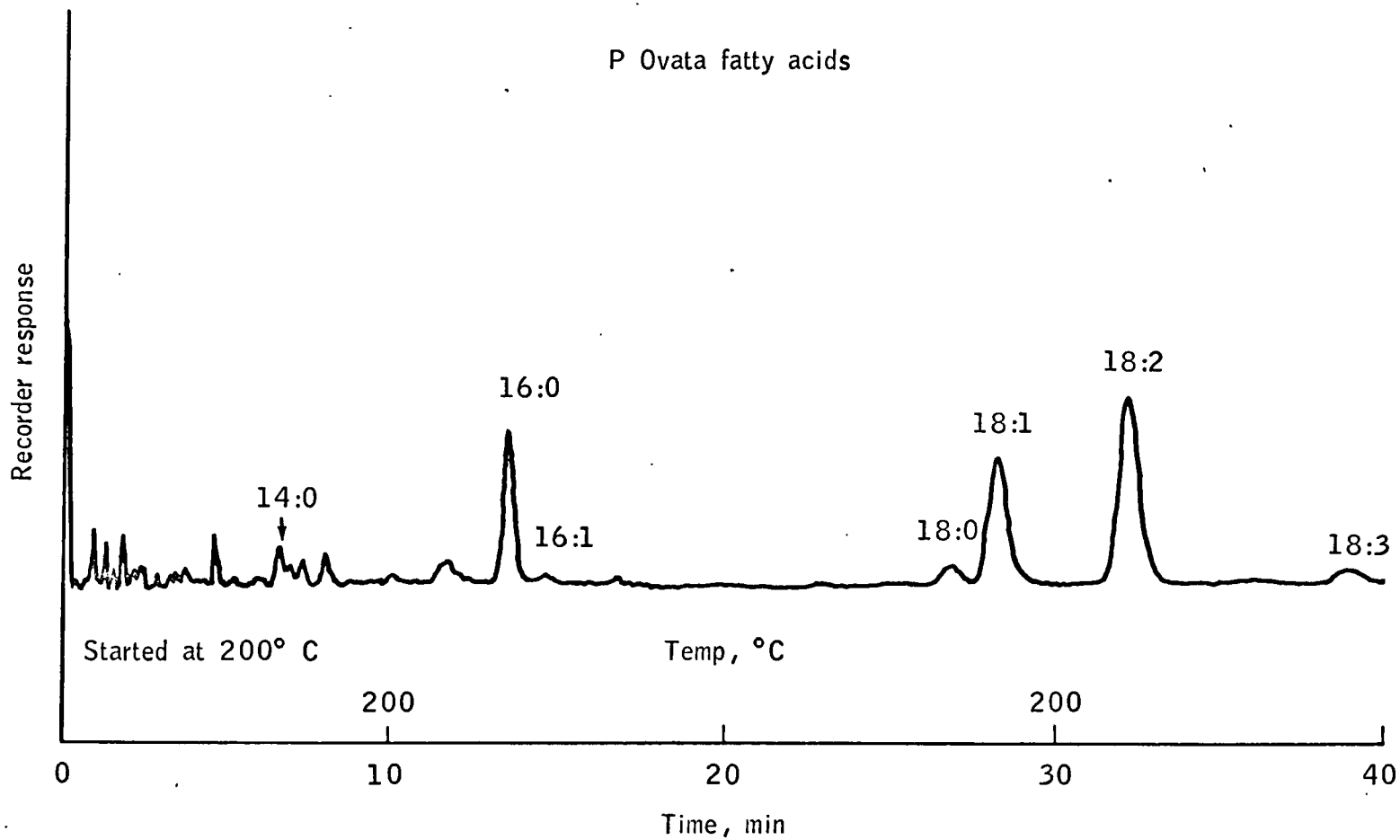

P Ovata fatty acids
Recorder response
14:0
16:0
16:1
18:0
18:1
18:2
18:3
Started at 200° C
Temp, °C
200
200
0
10
20
30
40
Time, min

TABLE XII

RELATIVE PERCENTAGE COMPOSITION OF FATTY ACIDS

IN THE SEED COAT OF PLANTAGO OVATA

Fatty Acid*	Content (Percent)
Myristic	2.2
Palmitic	10.9
Stearic	2.5
Oleic	16.0
Linoleic	26.3
Linolenic	3.0

*The fatty acids were identified as methyl esters by mass spectrometry and by comparing their retention time with that of authentic samples. The percent composition of the fatty acids was calculated on the basis of their gas chromatographic areas which were obtained by multiplying the peak heights by the widths at half peak heights. The total lipid content of the seed coat was 2.5 percent while the methyl esters of fatty acids were 2610 ppm.

FIGURE 38

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM THERMOPHILE NO. I

All of the operating conditions were the same as in Figure 37.

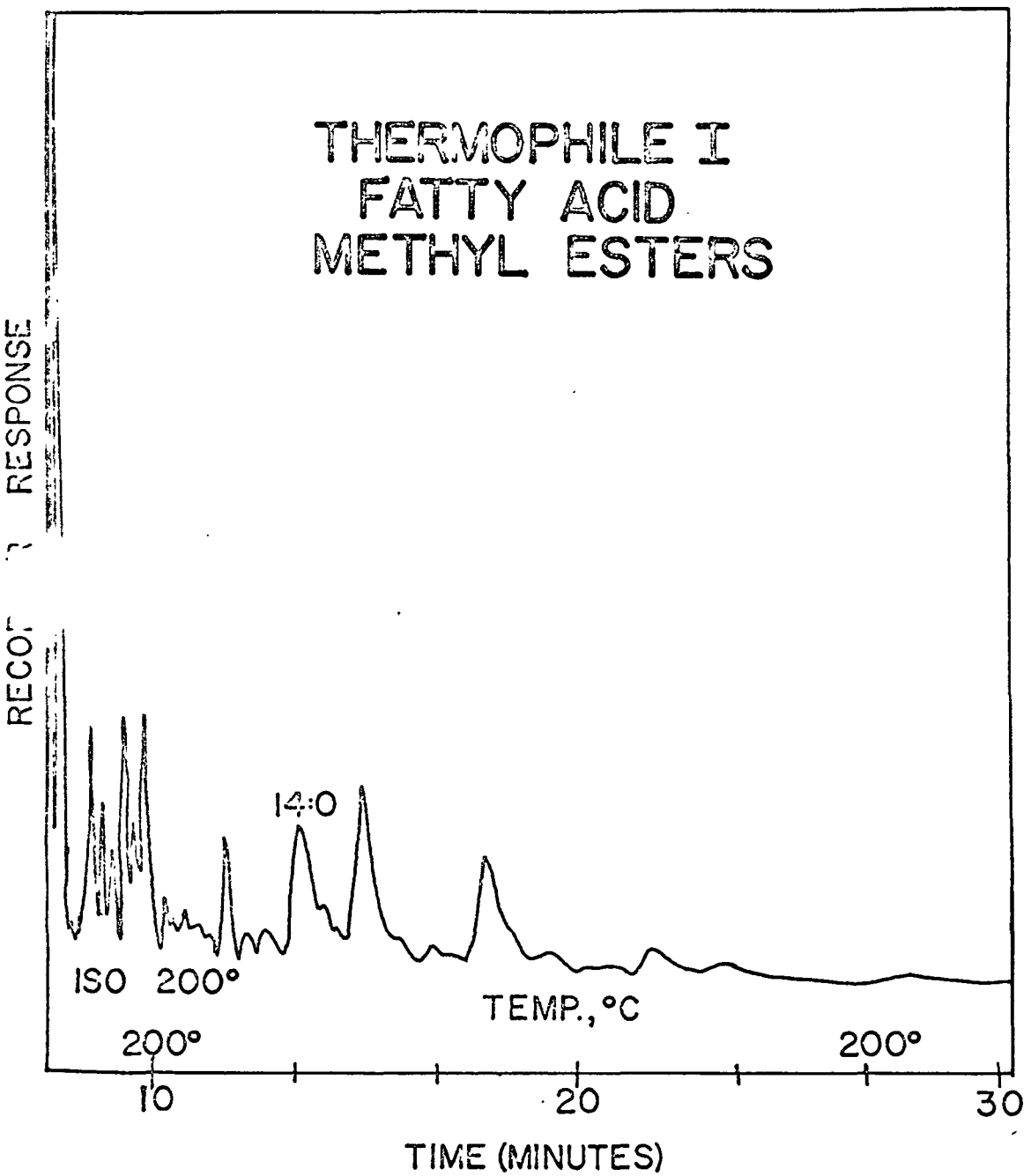
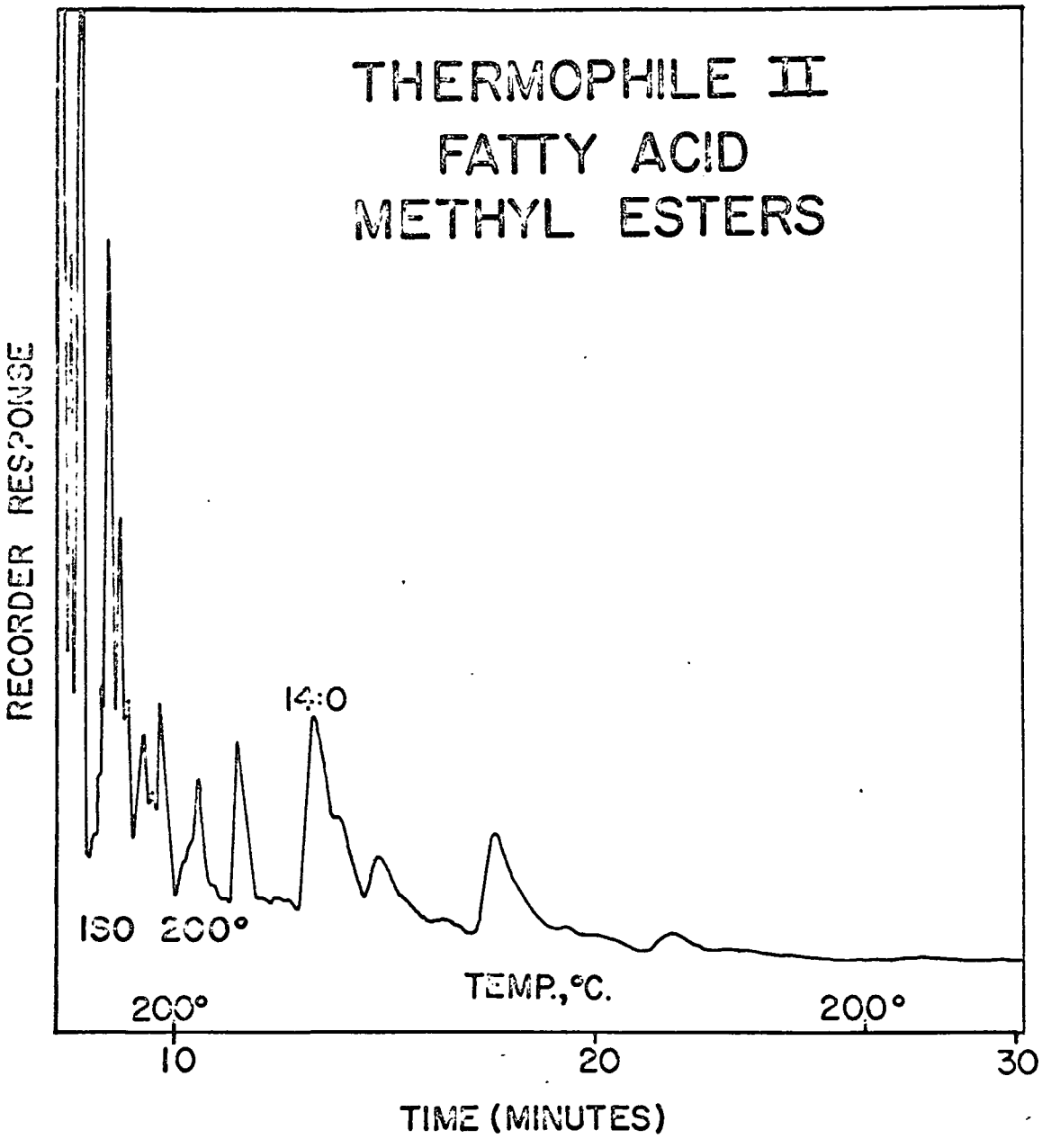

THERMOPHILE I
FATTY ACID
METHYL ESTERS
RESPONSE
RECOT
14:0
ISO 200°
TEMP., °C
200°
200°
10
20
30
TIME (MINUTES)

FIGURE 39

GAS CHROMATOGRAPHIC SEPARATION OF THE FATTY ACID METHYL ESTERS FROM THERMOPHILE NO. II

All of the operating conditions were the same as in Figure 37.


THERMOPHILE II
FATTY ACID
METHYL ESTERS
RECORDER RESPONSE
14:0
ISO 200°
200°
TEMP., °C.
200°
10
20
30
TIME (MINUTES)

bacteria including both psychrophilic and mesophilic organisms (62). Thin-layer chromatography was performed on the methyl ester fraction of Thermophiles I and II, and both gave identical patterns. Three spots appeared on each. The uppermost spots appeared with the solvent front and in the hexane; diethyl ether: acetic acid solvent system; this would be interpreted as a hydrocarbon. The other spots for Thermophiles I and II gave R_f values of .88 and .196. These spots did not appear in the vicinity of methyl esters found in Vibrio marinus. The methyl ester fraction of Vibrio marinus did not contain a spot that was hydrocarbon in nature.

C. Incorporation of Acetate-1-^{14}C and Stearate-UL-^{14}C into the Hydrocarbons of Chlorella Pyrenoidosa

Table XIII shows the incorporation of acetate-1-^{14}C into the hydrocarbon fraction. By thin-layer chromatography of the n-heptane eluate only a single spot was found which contained radioactivity. It possessed an R_f value corresponding to n-alkanes. Figure 40 shows the radioactive monitor recording of the labelled product found in the n-heptane eluate of C. pyrenoidosa after being incubated for 48 hours with uniformly labelled stearic acid (sp. act. 90 mc/mM). Figure 41 is the gas chromatographic separation of the same fraction showing the n-C_{17} and the Δ-C_{17} hydrocarbons. Allowing for the necessary three minute time delay in the radioactive monitor, recording the radioactive peak corresponds to the n-C_{17} gas chromatographic peak. The C_{18} fatty acid, therefore, does serve as a precursor of the n-C_{17}.

TABLE XIII

INFLUENCE OF LIGHT ON THE INCORPORATION

(20 μc) OF ACETATE-1-^{14}C * INTO THE

HYDROCARBONS OF CHLORELLA PYRENOIDOSA

Experiment	Time (Hrs.)	Conditions	Total Radioactivity (cpm/mg dry wt.) Heptane Eluate
1	48	Light	1.7
2	48	Light	2.0
3	48	Dark	0.17

* Purchased from New England Nuclear Corporation with a sp. act. of 30 mc/mM.

FIGURE 40

RADIOACTIVITY MONITOR OF GAS CHROMATOGRAPHIC EFFLUENTS OF C. PYRENOIDOSA

Radioactivity monitor of gas chromatographic effluents separated on a 50 m x 0.025 cm stainless steel capillary column coated with 2 percent OV-1. No split. Helium pressure at 560 g/cm^2. About 1/20 of the sample injected. Programmed at 1° C per minute from 100° C to 200° C and held isothermally at 200° C. Time constant setting at 30 and linear range at 300. Chart speed set at 6 in. per hour.

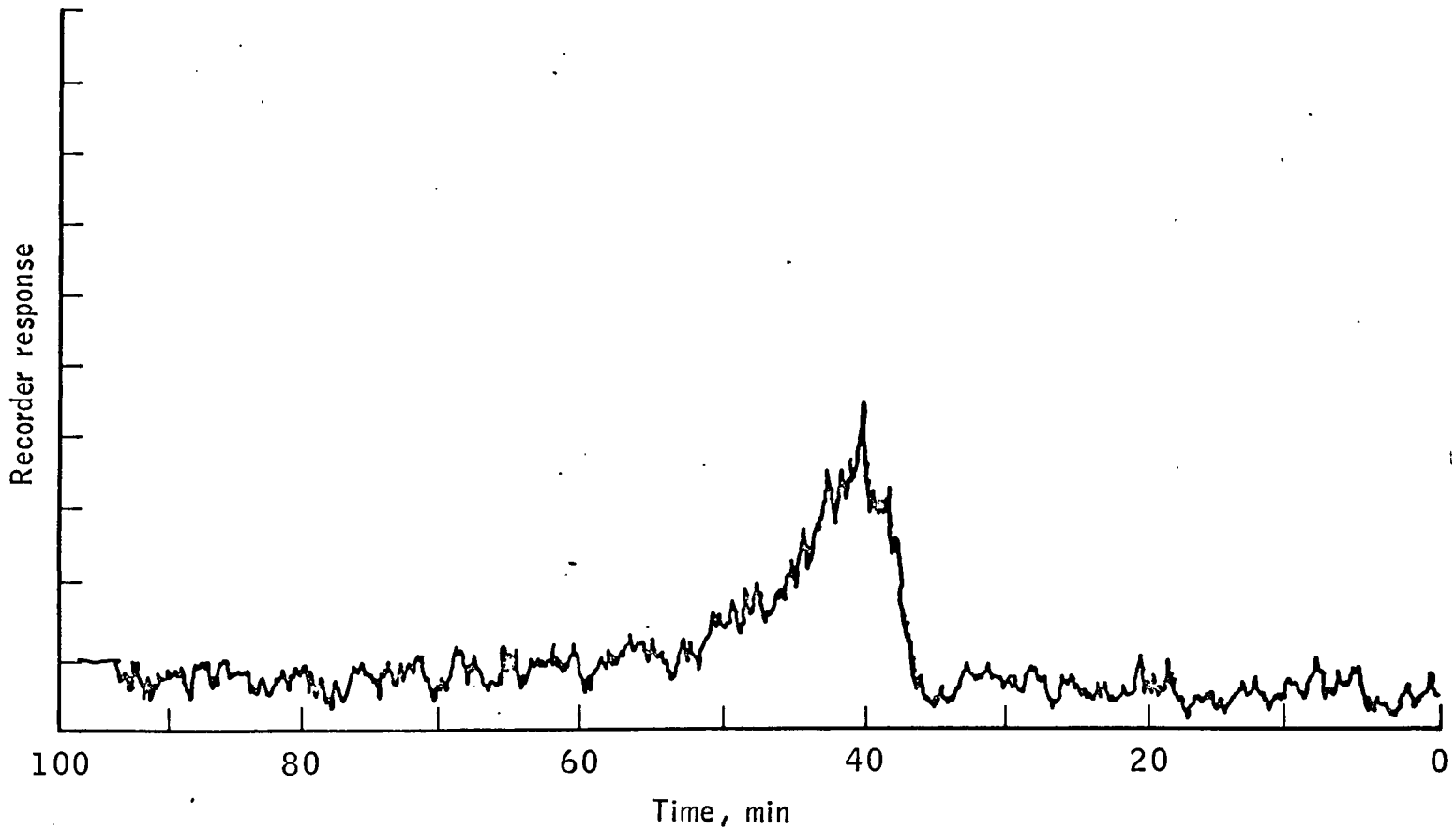
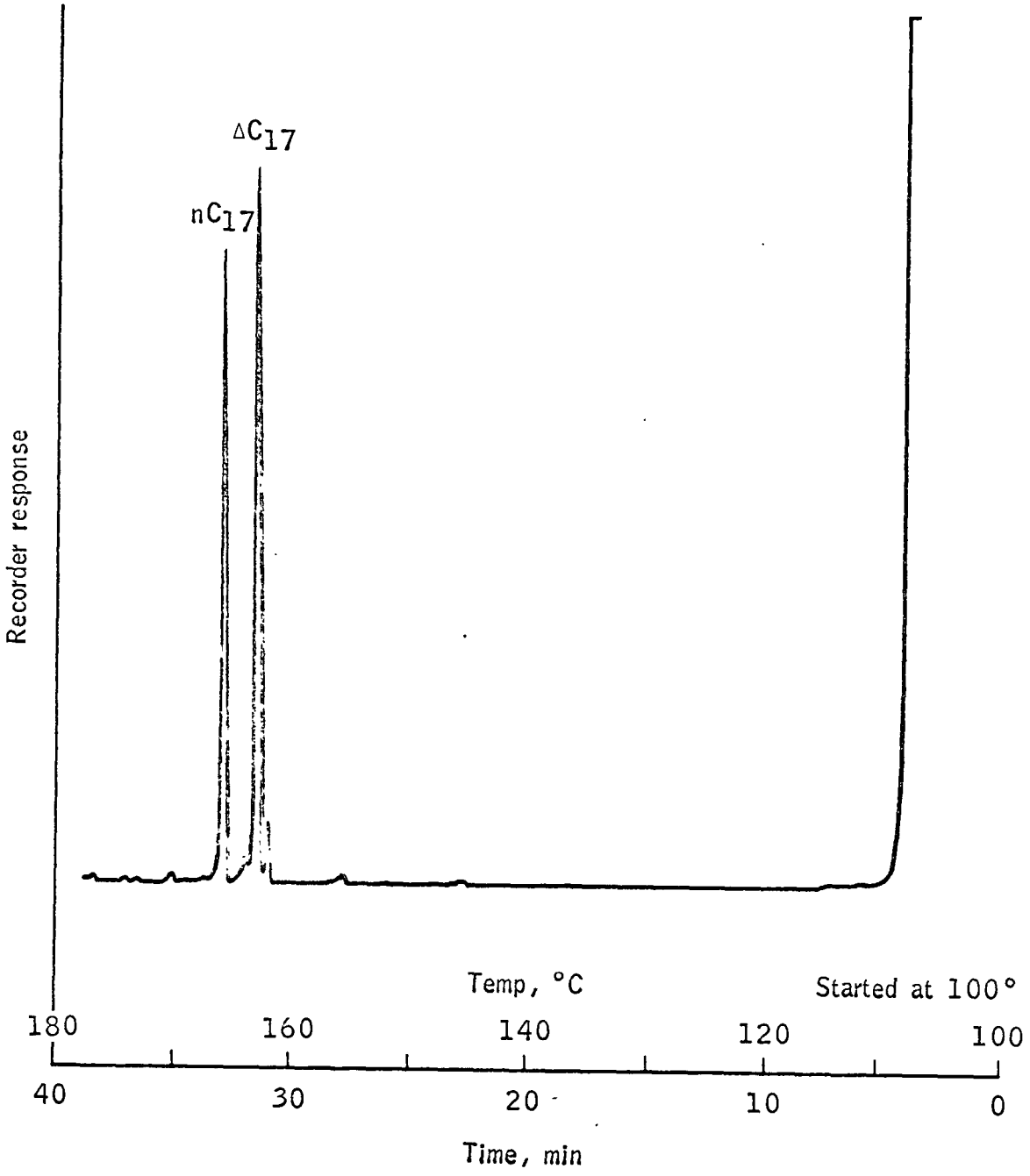

Recorder response
100
80
60
40
20
0
Time, min

FIGURE 41

GAS CHROMATOGRAPHIC SEPARATIONS OF RADIOACTIVE LABELLED HYDROCARBONS OF C. PYRENOIDOSA

Gas chromatographic separation of n-heptane fraction under the same conditions as described in Figure 40. Approximately 1/20 of the sample injected. No split. Attenuation, 128; range, 1. A total of 1.52 g (dry weight) material extracted.


ΔC17
nC17
Recorder response
Temp, °C
Started at 100°
180
160
140
120
100
40
30
20
10
0
Time, min

V. DISCUSSION

DISCUSSION

A. Hydrocarbon Distributions of Contemporary Algae

1. Geochemical Significance

The cyanophycean algae Anacystic cyanea, Spirulina platensis, Lyngbya aestuarii, Anacystis montana, Nostoc sp., and Chroococcus turgicus have been implicated in Precambrian sediments (1, 3, 6, 8, 12). Their hydrocarbon distribution ranges from the n-C_{17} in Anacystic cyanea to the bimodal distribution of medium and high molecular weight hydrocarbons seen in Anacystic montana.

Although fossils of very primitive organisms resembling the above contemporary blue-green algae have been recovered from Precambrian sediments (8, 14), the hydrocarbon distribution in these old samples in general shows a complex pattern of normal and isomeric alkanes in the low and high molecular weight range, with no odd over even predominance. The absence of an odd carbon predominance in the Precambrian sediments, and the presence of isoprenoid structures such as pristane and phytane (36), may suggest some degree of diagenetic activity. However, it would be hard to explain the observed distribution of high molecular weight alkanes solely by the diagenesis of a single n-C_{17} alkane as found in most algae. Therefore, the existence of some varieties of algae, such as those discussed here, which are also able to produce high molecular weight hydrocarbons, could offer an explanation for the distribution of the alkanes in ancient as well as in recent sediments. The difference in the distributions of the hydrocarbons in recent and Precambrian sediments could be

accounted for in part by difference in biosynthetic mechanisms and in part by differences in the time available for diagenetic changes.

Along the same line, the chlorophycean Coelastrum microsporum, Tetraedron sp. and Pediastrum sp., along with the blue-green alga C. turgidus and the golden brown algae Botryococcus braunii have all been implicated in tertiary sediments (19, 20, 24, 26). Again these species represent a range in complexity from the single n-C_{17} component in C. microsporum and Pediastrum sp. to the more complex distributions in Tetraedron sp. and B. braunii.

The aliphatic hydrocarbon distribution of one of these tertiary sediments, the Green River shale, displays a pattern which can be easily correlated to the hydrocarbon pattern of B. braunii, which has a prediminance of the odd-numbered C_{17}, C_{27}, C_{29} and C_{31} olefins. Although this alga is not listed among the species found in this particular shale (19), it has more recently been observed in swamp sediments of the lowest parts of the Green River formation (26), in the olive green shales belonging to Subathu Series of Himachal Pradesh, India (106), and in lignites and other tertiary sediments (20). Recently, large amounts of alveolar "yellow bodies," thought to be the remains of an algae that appears no different than the living B. braunii, have been found in the carboniferous limestone series of the Scottish Lothiane (Torbanite) (107). The pyrolysis of the high organic content of Torbanite produces an oil yield of 90-130 gallons per ton (107).

Likewise Tetraedron, with its distribution of high molecular weight alkanes, could also play a major role in the deposition of sedimentary hydrocarbons. The marked odd/even predominance typical of the algal distributions finds its counterpart in the hydrocarbon distributions of recent sediments (42). The gradual disappearance of such a predominance with the increasing age of the sediment (Precambrian sediments) has been explained as a consequence of the longer time allowed for diagenetic changes of the originally deposited organic matter (61).

This evidence appears to support the theory that algal ooze may be a precursor of oil shales such as the Green River formation. The isoprenoids reported present in the branched fraction of this type of shale (42) could be derived diagenetically from the chlorophyll of the algal population. In fact, it has been shown (108) that the phytane content decreases with increasing shale depth, concurrent with a proporitonal increase of the C_{19}, C_{18}, and C_{16} isoprenoids. This supports the assumption that the phytol group of the cholorphyll is the common precursor. Given a proper reducing environment, the olefins present in these forms of algae could give rise to the saturated hydrocarbons found in the shale in much the same manner as the double bond saturation step in the conversion of phytol to phytane.

The attempted morphological and geochemical correlations, based before almost exclusively on the nature and distributions of the normal hydrocarbons, can be developed now along new lines of evidence. As our knowledge of the algal lipids increases it is becoming clear that the

extreme morphological simplicity exhibited by most species of algae is not readily reflected in the simplicity of their lipid constituents. However, it should be pointed out that culturing conditions can play a significant role in the observed distributions of algal lipids (74, 81), especially in heterotryphic microorganisms.

In any case, the results of the present study seem to indicate that these algae are capable of biosynthesizing a more complex array of organic compounds than was previously thought. Examples are the methylalkanes, squalene and the triterpenoid structure indicated on Tables VI and VII. Recent literature has also shown the presence of steroids in various species of algae (109).

Although it has been found in very small amounts, it is important to evaluate the presence of the iso-C_{19} hydrocarbon in algae against the wide-spread occurrence of iso and anteiso alkanes in petroleum crudes, oil shales, and precambrian sediments.

Our positive identification of squalene in two species of algae, coupled with the recent reports on the identification of steroids (109) in blue greens, demonstrates that this particular channel of the isoprenoid biosynthetic pathways is active in the contemporary algae. With this in mind, the possible presence of polycyclic triterpenes in some species of algae would not be too surprising. Thus, the triterpenoid structure tentatively identified in three blue-green algae (compound x) could be directly derived from squalene. Squalene has not been reported in sediments, but there is tentative gas chromatographic evidence of the presence of its fully saturated

counterpart, squalane, in petroleum (110). Also, polycyclic triterpanes, as well as steranes, have been found in petroleum, shales, and sediments (111). Again, (61) reductive diagenesis could account for the saturation of squalene to squalane, steroids to steranes, and triterpenes to triterpanes.

Concerning the mixture of 7-methyl and 8-methyl heptadecanes, their presence in algal extracts constitutes a unique example of their occurrence in nature. This mixture first was detected by Han, et al. (72) in an extract of Nostoc sp. It was reported as a minor component not amounting to more than 19 percent of the heptadecane. However, in our case (see Table VII), this mixture accounts for a substantial proportion of the total amount of hydrocarbons in the cells. To establish a comparison, the culture of Nostoc sp. grown in our laboratory contains an amount of the two, possibly three, methyl alkanes close to 50 percent of the C_{17} peak. This variation could simply reflect, as indicated before, different growth parameters (74, 81). At present, it is hard to attach any geochemical significance to these internally methyl-substituted hydrocarbons, since there are no reports of their presence in geological samples. However, from our particular experience in the high resolution GLC of hydrocarbon mixtures, we feel that the lack of reports may simply be a consequence of the difficulties involved in their proper isolation and identification. Petroleum crudes have been shown to contain methyl alkanes ranging progressively from 2-methyl to the more internally substituted 5-methyl alkanes (111). This is also true in meteorites

extracts (112), Precambrian sediments (113), and Fischer Tropsch products (31).

Considering now the overall pattern of the organisms analyzed, perhaps the most salient feature common to all of them is the high amounts of the n-C_{17} hydrocarbons (see Table VII). In general, one would expect the contribution of algae to the hydrocarbons in geological samples to be most strongly felt in the C_{17} content of these samples. Although with time, and due to its higher vapor pressure relative to the heavier hydrocarbons, C_{17} would be preferentially lost. Thus the amount of C_{17} hydrocarbon would decrease with time in relation to the higher molecular weight hydrocarbons. Eventually this could lead to a shift of the distribution mode towards the higher hydrocarbons.

The data presented here clearly shown that algae of different taxonomic classifications produce significant amounts of paraffins and olefins in both the medium and high molecular weight ranges, and that some contain methyl branched hydrocarbons as well as acyclic and cyclic triterpenes. The majority of these organisms have been implicated in ancient and/or recent sediments.

2. Phylogenetic considerations

There has been a tendency in the literature (80, 114) to correlate molecular complexity with evolutionary development, with more complex molecules representing advanced phylogeny. Such interpretations must be considered suspect, however, when one considers the inconsistencies

in distributions found in the hydrocarbons. If one considers the prokaryotic cyanophycean algae A. cyanea, S. plantesis, L. aestuarii, and A. montana, the first three exhibit simple hydrocarbon distribution whereas A. montana gives a pattern similar to higher plants (115). This would imply that A. montana is a transitional organism. However, C. pyrenoidosa, an eukaryotic chlorophycean alga, displays a very simple hydrocarbon pattern, as do the chlorophycean algae, C. microsporum, S. obliquus, and Pediastrum sp. One is hard pressed to make phylogenetic conclusions solely on the basis of hydrocarbon distribution in the light of such discrepancies. Phylogenetic comparisons appear to be justified only within a limited taxonomic grouping.

B. Fatty Acid Distributions of Contemporary Algae

1. Possible roles as hydrocarbon precursors

The data obtained on algae so far does not suggest a direct biosynthetic relationship between hydrocarbons and fatty acids. Rather, if the hydrocarbons in the algal cells arise from the regular fatty acids, a variety of complex intermediate processes must be involved.

For instance, the marked predominance of the n-C_{17} hydrocarbon could be a point in favor of either of two direct pathways: elongation of the C_{16} fatty acids or decarboxylation of the C_{18} acids. However, depending on the prevailing mechanism, one would also expect to see the corresponding substantial amount of pentadecane, if the C_{16} fatty were to undergo decarboxylation, or nonadecane if the C_{18} acid were to elongate. The absence of significant amounts of these two hydrocarbons

implies a more complex mechanism than simple elongation of decarboxylation of corresponding fatty acids. The same would apply to the high molecular weight hydrocarbons found in some species of algae (61) since their corresponding fatty acids are absent. In this case, the mechanisms proposed for the condensation of two fatty acids (with decarboxylation of one of them) involving the participation of alcohols and ketones (48) or aldehydes (119) as intermediates do not seem to explain the facts either. An alternative mechanism would be something on the line of the elongation-decarboxylation complex recently proposed by Kolattukudy (120).

A number of reports have recently appeared in the literature (90, 91, 116) on the biosynthesis of long-chain alkanes in plant systems. In the case of the straight-chain alkanes, long-chain fatty acids appear to be the preferred precursors, whereas the branched-chain amino acids serve as initial precursors for the iso- and anteiso-alkanes. No information, however, is available on the formation of the common short-chain hydrocarbons. Stumpf (92) has proposed a mechanism whereby even carbon fatty acids undergo decarboxylation producing an odd-numbered carbon alcohol or aldehyde, which is then reduced to yield the corresponding hydrocarbon. In order to test this hypothesis, both labelled acetate and stearate were used as precursors for the hydrocarbons of Chlorella. The evidence obtained from the incorporation of labelled acetates (see results page 99 part C) may be interpreted to mean that acetate is incorporated into the hydrocarbon products intact; however, because acetate is

readily incorporated into free fatty acids, it was not possible to determine whether the hydrocarbons were synthesized via fatty acids or from acetate directly.

It has been demonstrated (117) that dark will greatly diminish the synthesis of free fatty acids from acetate in chloroplasts, even with a full complement of cofactors. Kolattukudy's (118) work, on the other hand, showed that the synthesis of very long-chain fatty acids and hydrocarbons are light insensitive. It therefore must be assumed that, in the case of hydrocarbon synthesis in Chlorella, the acetate is entering the light sensitive fatty acid synthetase system as a two carbon unit and participating in the formation of free fatty acids, some of which are converted into hydrocarbons.

If such is the case, then stearic acid should serve as an immediate precursor of the low-molecular-weight hydrocarbons in Chlorella. The data in the result section page 99 suggest that C_{18} fatty acid does serve as a precursor of the n-C_{17}. However, no trace of label could be found in the Δ-C_{17}, and it must be assumed that it was formed by a different mechanism. A logical precursor for the monounsaturated compound would be oleic acid by a similar decarboxylation and reduction as that observed for the normal C_{17}. This possibility is undergoing study at the present time. Investigations into the nature of the decarboxylation and reduction are also under way to determine if the mechanisms involved are merely a reduction of an intermediate found in the α-oxidation pathway, or whether a special pathway exists for the generation of odd-numbered hydrocarbons from even-numbered fatty acids.

2. Phylogenetic Considerations

Considering the fatty acid distributions, any attempt made to correlate the variations in these distributions to phylogenetic position is in our opinion still premature. Our patterns do not show significant and consistent variations between species, or in some cases genera (Tables VIII and IX).

Among the fatty acid patterns there are considerable differences in the degree of saturation of the C_{16} and C_{18} fatty acids and, as shown by recent literature, changes in growth parameters such as temperature can lead to shifts in the degree of saturation of fatty acids (81). However, in our case, all cultures were grown under controlled conditions which would tend to minimize any shifts in degree of saturation. In addition, phylogenetic interpretation of fatty acid patterns should correlate with observed variations in hydrocarbon distributions. We cannot at present decipher any meaningful correspondence along the two lines of evidence. The only marked difference we have noted that could be related to phylogenetic position or morphological complexity is the total C_{16}/C_{18} ratio which shown (Table X) the blue-green (prokaryots) to have a predominance of C_{16} fatty acids, and the greens (eukaryots) to have a predominance of C_{18} fatty acids. If this were really an indication of morphological complexity, the low value for A. montana (Table X) would seem to indicate that this organism is a transitional form (61) between the blue-green and green algae.

3. Geochemical Considerations

In relation to their geochemistry, the specific involvement of algal fatty acids in sediments does not seem to be as well substantiated as that of the aliphatic hydrocarbons. However, fatty acids are among the most abundant components of living systems and in general have been regarded as the possible precursors of petroleum and sedimentary hydrocarbons (121, 1-2).

Normal fatty acids are present in recent as well as Precambrian sediments and, considering the large number of algae implicated in these sediments, the data presented here would certainly suggest that algae must have contributed in one way or another to the fatty acid distributions found in our lithosphere (46, 121, 123). However, it is hard to evaluate the extent of their contributions.

The absence of the unsaturated acids in sediments can be explained by their lower stability in the geological environment. Likewise, the appearance of significant amounts of odd-numbered normal fatty acids with geological time, which parallels the appearance of even carbon numbered hydrocarbons can also be related to diagenetic changes of the originally deposited algal debris.

On the other hand the fatty acid distributions of contemporary algae fail to account for the presence in sediments of branched chain acids (50, 121, 123) and of acids higher than C_{18} (121 - 123). Neither do they account for the presence of odd fatty acids in recent muds and soils.

Isoprenoid fatty acids, which have been reported in recent sediments (45, 124), have not been detected in the algae studied in our laboratory. This, however, would not be unexpected since they most likely arise from slow diagenetic degradation of the algal chlorophylls.

Consideration of the contributions of bacteria and higher plants to the fatty acid patterns observed in Tertiary and Cambrian sediments would fill the gap left by algae. Concerning the Precambrian, the data available in the literature appears to support the role of algae as their direct source, except for the C_{20} and C_{22} acids (121).

Contrary to the hydrocarbons, it seems that the correlation of algal fatty acids with the fatty acids in sediments is less apparent, although nevertheless valid.

Plantago ovata is an annual caulescent herb native to Asia and the Mediterranean countries. This plant is extensively cultivated in India, and of late in Arizona, U.S.A., for its medical applications.

In general, the overall distribution pattern of its seed hydrocarbons (see Fig. 35) agrees with the expected predominance of the odd numbered n-alkanes which is common in higher plants (125). However, it is remarkable that while higher plants contain only traces of low molecular weight hydrocarbons the major portion of the P. ovata seed coat contains significant amounts of hydrocarbons in the C_{16}-C_{22} range.

Similar distributions of lower molecular weight hydrocarbons have been reported in lower plants and microorganisms (44, 61, 63, 72, 84).

However, the presence of a large amount of the anteiso-C_{18} alkane constitutes an unuaual finding in the plant kingdom.

An alternating pattern in the concentrations of the iso and anteiso components has been reported by Mold, et al. (126, 127) in wool and tobacco wax where the iso homologs with the odd number of carbon atoms predominated over the corresponding anteiso alkanes and vice versa. This is also the case here as indicated in Table XI. Analogous results were reported by Eglington, et al. (128) for the high molecular weight alkanes (C_{25}-C_{35}) in the surface wax of the leaves of certain species of Aconium; however, the present study appears to be the first reported evidence of significant concentrations (20.8%) of hydrocarbons in the C_{16}-C_{19} weight range, with maximum at the anteiso C_{18} alkane, in higher plants.

It is probable that one route to the biosynthesis of plant hydrocarbons involves decarboxylation of the corresponding long chain fatty acids or their immediate precursors. Mazliar (129) reported that the non-crystalline fraction of the apple cuticle wax contains fatty acids ranging from C_{16} to C_{30}, and the alkanes range from C_{15} to C_{29}. In the present study the results show that one of the pathways for the hydrocarbon synthesis may be the decarboxylation route because C_{18} fatty acids and C_{17} hydrocarbons are both present in significant concentrations. On the other hand, the presence of anteiso-C_{18} alkane (10.1%) cannot be explained on this assumption because the P. ovata seed coat does not contain branched C_{19} fatty acids. Presumably other mechanisms, viz, condensation or the elongation

route which have been discussed by Elington and Hamilton (130), or some other possible mechanism may be involved. This aspect of the study deserves further investigations using tracer metabolites as precursors of hydrocarbon biogenesis.

C. Thermophilic Bacteria Lipids

Only a very limited interpretation can be made on the data available from the lipid analysis of the thermophilic bacteria. The hydrocarbon content was below the level of our detection. There were, however, other unidentifiable lipid components that accounted for approximately 300-600 PPM of the dry cell weight. Thin layer chromatography indicated that these lipids were not fatty acids or fatty acid methyl esters. One might say, because of the unusual environment of these organisms, they might indeed have less volatile, i.e. more stable, molecules than those lipids that we now readily recognize. These might possibly be higher molecular weight ethers or alcohols. Because of the limited availability of these bacteria, only preliminary analysis of the lipids could be performed.

VI. SUMMARY

SUMMARY

Algae have been identified, on the basis of morphology by the micropaleontologist, in sediments as old as 3.2×10^9 years. The fossil record dating from the early Precambrian to the more recent Tertiary sediments has been heavily influenced by the presence of algae. In fact, they are the most numerous microorganisms present in these old formations.

Organic geochemists have shown that very old sediments such as the Precambrian have organic molecules which are indigenous to the rock. The paleobiochemist, using modern analytical techniques, has shown some of these organic molecules to be of a biogenic origin.

In the more recent sediments, there is no doubt as to whether the organic substances contained therein are indigenous or not; it is a question of what is the precursor of the organically rich materials found in those sediments. Table XIV summarizes accounts from the literature concerning the microorganisms and chemical fossils found in different geological formations.

All of the algae analyzed here have been implicated in both ancient and recent sediments. They have significant amounts of aliphatic hydrocarbons present. In several cases the hydrocarbon patterns of the algae differ little from those of the sediments. There is a preponderance of the C_{17} in all of the algae and, in many, it is the only hydrocarbon present. Both ancient and recent sediments

TABLE XIV

MORPHOLOGICAL AND CHEMICAL FOSSILS OF GEOLOGICAL FORMATIONS

Microfossil Algae	Geologic Formation	Time	Chemical Fossils
Algal-like forms	Onverwacht series	3.2×10^9	?
Blue green	Fig tree	3.1×10^9	A, B, F
	Soudan shale	2.7×10^9	A, B, C
Blue green and green algae	Gunflint chert	1.9×10^9	A, B
Algal-like bodies	Nonesuch shale	1×10^9	A,B,C,D,E,F
Blue green and green	Bitter Springs	$\simeq 0.8 \times 10^9$	?
Blue green and green	Green River shale	50×10^9	A,B,C,G,H,I,J

A: n-alkanes
B: isoprenoids
C: polycyclic hydrocarbons
D: cycloalkanes
E: methyl alkanes
F: olefins
G: n-fatty acids
H: isoprenoid acids
I: polycyclic acids
J: other acids

show the presence of the C_{17} hydrocarbon. Besides the C_{17}, several of the algae have higher molecular weight paraffins and olefins present, C_{23} - C_{31}. In addition, some of the blue green algae have methyl substituted alkanes along with substantial amounts of a triterpenoid-like structure.

Both eukaryots and prokaryots display simple patterns of a single n-C_{17} to a complex array of olefinic hydrocarbons in the low and high molecular weight ranges. On the basis of the hydrocarbon patterns obtained from the algae analyzed, phylogenic considerations could be justified only within a limited taxonomic group.

The fatty acid patterns of the algae analyzed range from 14:0 to 18:4. Odd numbered fatty acids are a very minor component, and branched fatty acids appear to be non-existent in algae.

The major differences between the greens and blue greens is the C_{16}/C_{18} ratio. In most cases, it is larger in the blue greens. The major differences within all of the algae analyzed are in the degree of saturation rather than in the series of acids present.

The apparently consistent fatty acid patterns displayed by the algae investigated do not support a simple decarboxylation or elongation in the production of hydrocarbons from fatty acids. This assumption is made in view of the wide variety of hydrocarbons produced by the same alga.

Because of the large amounts of fatty acids produced by algae, they must have contributed to the fatty acids found in sediments. However,

the algal fatty acid distributions do not correlate with those of the sediments as well as the hydrocarbon distributions.

VII. CONCLUSIONS

CONCLUSIONS

The data presented in this work, that is, the hydrocarbon and fatty acid analyses of certain contemporary representatives of ancient algae, clearly complement the findings of micropaleontologists, organic geochemists, and paleobiochemists (Figure 42).

The lipid compositions of the algae analyzed show (i) distributions of odd-numbered aliphatic hydrocarbons in the medium and high ranges of molecular weight, (ii) that strongly resemble the distributions obtained from certain Tertiary sediments, (iii) these same hydrocarbon producing algae offer an explanation for the distribution of the alkanes found in ancient sediments, (iv) the differences in the distributions of the algae and the Precambrian sediments could be accounted for, in part, by differences in biosynthetic mechanisms and in part by the time available for diagenetic changes.

If fatty acids are the precursors to hydrocarbons, the data presented here (i) indicates that a variety of complex intermediate processes must be involved, (ii) to generate the wide variety of hydrocarbons produced.

The distributions of hydrocarbons within the algae are so diverse on one hand and so similar on the other that no meaningful taxonomical or phylogenetic criteria can be given at this time. The fatty acid patterns are so similar among the algae that only limited taxonomical and phylogenetic interpretations can be made.

This work presents chemical and morphological evidence that organisms which existed early in the earth's history still exist today, apparently unchanged either morphologically or biochemically.

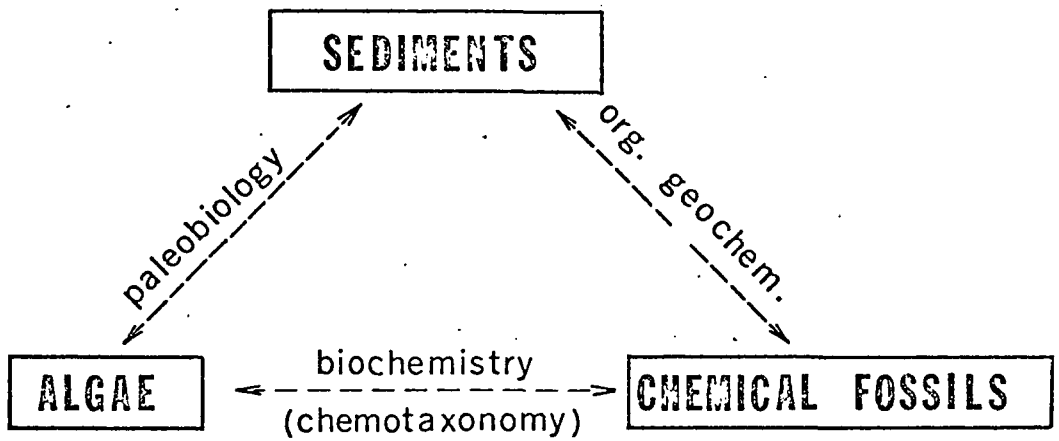


FIGURE 42. PALEOBIOCHEMISTRY (INTRA-RELATIONSHIP DIAGRAM)

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LITERATURE CITED

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