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27 May 1968

Olefins of High Molecular Weight in Two Microscopic Algae

Abstract. *The hydrocarbon composition of two algae, a golden-brown* (Botryococcus braunii) *and a blue-green* (Anacystis montana), *has been investigated by gas chromatography–mass spectrometry. Both show distributions of aliphatic hydrocarbons of odd carbon numbers in the medium and high ranges of molecular weight, with maxima at n-C_{17} and n-C_{29} for* B. braunii *and n-C_{17} and n-C_{29} for* A. montana. *With the exception of the* n*-heptadecane of* A. montana *all the hydrocarbons are monoenes, dienes, or trienes. Since certain continental sediments and oils show similar distributions of alkanes with respect to carbon number, these organisms may be the precursors of the hydrocarbons in these formations.*

There is very little data on the biogenesis of hydrocarbons by microorganisms (*1*). Most of the few microscopic algae and related organisms analyzed thus far have shown only small amounts of aliphatic hydrocarbons of relatively low molecular weight, with a maximum at about C_{17} (*2–5*). 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 (*6, 7*), which have been usually considered the source of the paraffin wax in certain continental shales and petroleum crudes (*8*).

In a continuation of our studies on the distribution and genesis of hydrocarbons in nature (*1–3, 6, 9*), we have now found two algae, a golden-brown (*Botryococcus braunii*) and a blue-green (*Anacystis montana*) which, in addition to the common C_{17} aliphatic hydrocarbons, biosynthesize relatively large amounts of hydrocarbons of higher molecular weight.

We have selected *B. braunii* because it has been implicated in the formation of oil in tertiary sediments (*10*) and *A. montana* because it is considered a typical representative of one of the earliest forms of terrestrial life (*11*). Thus the new observations presented here may have significance not only on the formation of precursors of petroleum paraffins, but also on the interpretation of the alkane distributions reported for microfossil-bearing Precambrian rocks (*12, 13*).

In essence the experimental method followed consisted in growing the algae in the laboratory, extracting and fractionating their lipids, and analyzing the aliphatic hydrocarbon fraction by combined gas chromatography–mass spectrometry. The experimental details and analytical results are summarized below.

Botryococcus braunii and *Anacystis montana* were grown autotrophically in the light at 28°C. Bacteria-free cultures were employed and each culture was grown in three liters of D medium (*14*) and aerated continuously with filtered air. All cells were harvested by centrifugation, washed with a saline solution, and dried over P_2O_5 under vacuum.

The methods of extraction and fractionation, used to obtain the aliphatic hydrocarbon content of the organisms, have been reported previously (*3, 6, 9, 12*). Gas chromatographic analyses were performed on an F & M 810 gas chromatograph equipped with a flame ionization detector. An electronic digital integrator (Infotronics CRS 11/AB/H/41) provided an accurate quantitative analysis of the samples at the same time that the gas chromatographic pattern was obtained. Gas chromatographic–mass spectrometric analyses of the hydrocarbon fraction were carried out on an LKB 9000 gas chromatograph–mass spectrometer (*15*).

After these procedures the aliphatic hydrocarbons of *Botryococcus braunii* (Fig. 1) were identified as alkenes, with one, two, or three double bonds, ranging from C_{17} to C_{33}. The C_{27}, C_{29}, and C_{31} diolefins were predominant, the major component being the C_{29} diolefin. *Anacystis montana* (Fig. 2) shows a similar distribution with some particular differences. The olefins are mainly monoenes ranging from C_{19} to C_{29}, the major peak being the C_{27} mono-olefin. In this case heptadecane represents the only paraffin present.

Proper controls were run and necessary precautions were taken to exclude any possible source of contamination. Moreover, the unique nature of the patterns by themselves tends to minimize the possible contribution of extraneous material. Table 1 shows the relative percent composition of hydrocarbons in the cells. In the case of *A. montana*, only 85 percent of the total hydrocarbon content is reported in the table. The remaining 15 percent is made up by the unlabeled hydrocarbon peaks that can be seen in Fig. 2 which existed in small amounts and were not identified.

Identifications were supported in all cases by mass spectrometric data, and although there is little doubt concerning the values obtained for the molecular masses of these compounds (Table 1), it could be argued that they may correspond to monocycloalkanes and monocycloalkenes instead of to straight

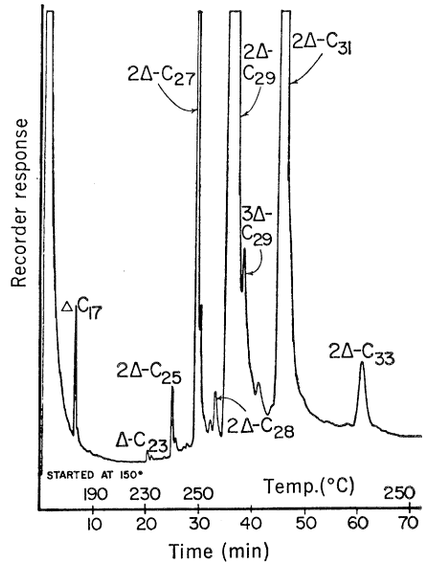

Fig. 1. Gas chromatographic separation of hydrocarbons of *Botryococcus braunii*, by use of an F & M 810 gas chromatograph equipped with a flame ionization detector. The glass column, 1.7 m by 0.3 cm inside diameter, was packed with OV-1 (methyl silicone fluid). The nitrogen pressure was 703 g/cm²; range was 10^2; and attenuation was 2.

chain olefins and diolefins. This argument is not supported by the mass spectral patterns which in all cases show a smooth distribution of olefin ions without the maxima that would characterize the cleavage of the cyclic moiety.

Since blue-green algae, which are considered to be among the most primitive organisms on earth (*11*), are procaryots, one would expect that they would exhibit hydrocarbon patterns similar to the majority of procaryots analyzed thus far (*2–5*). Indeed, this is the case with *Anacystis nidulans* and other related organisms (*2*). However, *Anacystis montana,* besides showing such simple distribution of hydrocarbons of low molecular weight, also resembles higher plants in its distribution and substantial concentrations of olefins of high molecular weight (*16*), with an obvious predominance of odd-carbon-numbered chain lengths (*6, 7*); the same is true of *Botryococcus braunii*. The hydrocarbon patterns displayed by these two species of algae are in part typical of higher plants. Using these two types of patterns as indices of evolution in the biosynthesis of hydrocarbons, one might label these algae as transitional forms.

The observed predominance of the odd numbered C_{17}, C_{27}, C_{29}, and C_{31} olefins in the contemporary golden-brown algae shows a marked similarity to the distribution of the normal alkane fraction from the Cenozoic Green River shale (*4*). Although this genus in particular has not been observed in the shale, the presence of other algae has been reported (*17*). Also, the occurrence of *Botryococcus braunii* has been noted in the olive-green shales belonging to Subathu Series of Himachal Pradesh, India (*18*), as well as in lignites and other tertiary sediments (*10*). Recently, large amounts of alveolar "yellow bodies" thought to be the remains of an alga that appears no different than the living *B. braunii* have been found in the carboniferous limestone series of the Scottish Lothiane (Torbanite) (*19*). The pyrolysis of the high organic content of Torbanite produces an oil yield of 90 to 130 gallons per ton (370 to 540 liters per metric ton) (*19*).

This appears to support the theory that algal ooze may be a precursor of the oil of shales such as the Green River formation. The isoprenoids reported present in the branched fraction of this type of shale (*4*) could be derived diagenetically from the chlorophyll of the algal population and in fact it has been shown (*20*) that the phytane content decreases with increasing shale depth concurrent with a proportional increase of the C_{19}, C_{18}, and C_{16} isoprenoids. This supports the assumption that the phytyl group of the chlorophyll 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 is thought to have taken place.

Table 1. Relative percent content of hydrocarbons of two algae.

Hydrocarbon	*Anacystis*	*Botryococcus*
n-C_{17}	11.5	
Δ-C_{17}		1.52
Δ-C_{19}	0.2	
Δ-C_{20}	.1	
2Δ-C_{21}	8.9	
Δ-C_{23}	8.0	0.14
Δ-C_{24}	0.2	
Δ-C_{25}	14.6	
2Δ-C_{25}	0.2	0.65
3Δ-C_{25}		.10
Δ-C_{26}	3.8	
Δ-C_{27}	34.7	
2Δ-C_{27}	2.8	11.10
Δ-C_{28}	0.1	
2Δ-C_{28}		0.65
Δ-C_{29}	0.2	
2Δ-C_{29}		50.40
3Δ-C_{29}		5.54
2Δ-C_{31}		27.90
2Δ-C_{33}		2.00

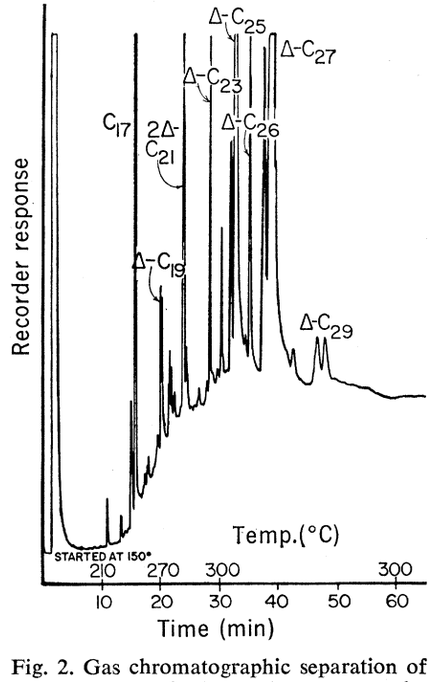

Fig. 2. Gas chromatographic separation of hydrocarbons of *Anacystis montana,* by use of an F & M 810 gas chromatograph equipped with a flame ionization detector. The capillary column was stainless steel tubing, 30 m by 0.025 cm inside diameter, coated with 10 percent Apiezon L (a high-temperature grease). The nitrogen pressure was 1406.0 g/cm^2; range was 10^2; attenuation was 1.

Although fossils of very primitive organisms resembling several contemporary blue-green algae have been recovered from Precambrian sediments (*13*), the hydrocarbon distribution in these old samples in general shows a complex pattern of normal and isomeric alkanes in the range of low and high molecular weights, 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 (*12*) may suggest some degree of diagenetic activity. However, it would be hard to explain the observed distribution of the alkanes of high molecular weight 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 hydrocarbons of high molecular weight, 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 differences in biosynthetic mechanisms and in part by differences in the time available for diagenetic changes.

From the foregoing information it can be concluded that (i) hydrocarbons of high molecular weight (C_{23} to C_{33}) are found in the contemporary counterpart of ancient microscopic organisms, (ii) these hydrocarbons are present in high concentration in the cells (0.1 to 0.3 percent of dry cell weight), (iii) they possess a high degree of unsaturation as an outstanding common characteristic, (iv) they have a marked odd over even predominance, and (v) they show actual and potential similarities with the patterns of ancient and modern sediments and oil shales.

Further work is needed to locate the positions of the double bonds, and to ascertain the biosynthetic pathway of these olefins (*21*).

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21. In addition to *Anacystis montana* and *Botryococcus braunii* a green alga *Chlorella pyrenoidosa* and a marine alga from Hawaii, thought to be of the order Fucales, were analyzed (*2*) and found to contain a C_{17} alkene (76.9 percent) and a C_{17} alkane (100 percent), respectively.
22. After completion of our work we learned about the recent analysis of four species of algae (*Nostoc, Anacystis, Spirogyra*, and *Chlorella*) by another group of investigators [J. Han, E. D. McCarthy, W. Van Hoeven, M. Calvin, W. H. Bradley, *Proc. Natl. Acad. Sci. U.S.* **59**, 29 (1968)]. According to this report the C_{17} alkane is the major component in the molecular weight range C_{15} to C_{20}. These results, including the high content of heptadecene in *Chlorella*, agree with our own findings (*2, 3*). However, with the exception of *Spirogyra*, the algae analyzed by them did not contain any appreciable amount of hydrocarbons of high molecular weight. Recently K. Stransky, M. Streibl, and F. Šorm [*Collection Czecho. Chem. Commun.* **33**, 416 (1968)] have reported, in agreement with our results, the presence of alkanes and alkenes of high molecular weight in *Scenedesmus quadricauda*.

23. Parts of this work were presented at the International Summer School, Biology in Space, Cambridge, England, 8–20 July 1967 and at the meeting of the Geological Society of America, New Orleans, 20–22 Nov. 1967. Supported in part by research grants NSG 257 and NGR 44-005-020 from NASA. H.J.S. was partially supported by NDEA Title IV Fellowship No. 66-8250. We thank Dr. M. Rodriguez-Lopez, Centro de Investigaciones Biologicas, Instituto Marañon, Madrid, Spain, for supplying us with a sample of *Anacystis montana*. *Botryococcus braunii* was obtained from Dr. R. C. Starr, Indiana University.

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Vascular Injury and Lysis of Basement Membrane in vitro by Neutral Protease of Human Leukocytes

Abstract. *Frozen and thawed granules of human, peripheral-blood leukocytes rapidly produce hemorrhage when injected into animal tissues. The effect is blocked by inhibitors of proteolysis. The granule extract can digest vascular basement membrane in vitro at neutral* p*H. In addition, basement membranes of blood vessels damaged in vivo by the leukocyte fraction are found to be attenuated when examined by electron microscopy. The proteases of human leukocyte granules differ in several important respects from known lysosomal cathepsins and trypsin-like esterases. Polymorphonuclear neutrophils are a major source of the neutral proteases present in circulating white cells, and release these enzymes during phagocytosis of immune complexes.*

Recently the role of leukocyte proteases in inflammation has been reexamined (*1*). Attention has been focused on the acid cathepsins of these cells as a result of studies in rabbits indicating that such granule-bound enzymes participate in immunological vascular injury of the Arthus type (*2, 3*). For example, cathepsins D and E degrade vascular basement membrane in vitro (*3*) at the *p*H optima (2.5 to 3.0) of these enzymes. However, injection of such proteases into rabbit tissues fails to induce comparable vascular injury, probably because proteolysis is prevented by the neutral *p*H along endothelial surfaces. Therefore vessel damage mediated by acid cathepsins requires that tissue *p*H be considerably lowered in order for these hydrolases to act. The concentration of acid needed for intracellular functions of cathepsins can be attained within phagocytic vacuoles, but it is not certain that *p*H at the blood-tissue interface can be sufficiently lowered during inflammation to permit cathepsins D and E to degrade basement membrane or other vascular components extracellularly.

On the other hand, significant differences exist between the proteolytic enzymes of rabbit and human white blood cells, including the presence in the latter of trypsin-like enzyme (leukoprotease) which is active at physiological *p*H (*4*). This enzyme is found in polymorphonuclear leukocytes (PMN) of many species in addition to man; some of its properties and possible actions in infection have been reviewed (*4, 5*). Our experiments deal with lysosomal proteases of human leukocytes as potential mediators of vascular damage. Our observations not only confirm the presence of marked neutral proteolytic activity in human PMN, but show that this activity is also present in the lysosomal fraction of these cells and that it is released during phagocytosis. With regard to inflammation, our experiments show that human PMN granules can degrade vascular basement membrane in vitro at neutral *p*H and can rapidly produce vascular injury when injected into normal animal tissues (an effect which is blocked by inhibitors of proteolytic enzymes. Studies with synthetic substrates and with inhibitors of serine and thiol-dependent enzymes reveal important differences between human leukocyte granule proteases and the known cathepsins and trypsin-like esterases. A preliminary report of these findings has been presented (*6*).

Human white cells were obtained from freshly drawn, citrated venous blood of normal male subjects after sedimentation of erythrocytes with dextran. The washed leukocytes were homogenized, and their cytoplasmic granules were obtained essentially as described before (*7*). Granule fractions were then resuspended in 0.15*M* sodium phosphate buffer (*p*H 8.0) and disrupted by freezing and subsequent thawing. Membranes of the ruptured granules were sedimented by high-speed centrifugation; the clarified supernatants were dialyzed against cold, normal saline buffered with 0.01*M* phosphate to *p*H 7.4; the final extracts were stored at −60°C until used.

Granule protein (50 to 100 μg) was injected into the skin or bowel wall of rabbits or passed via tracheal catheter into the lungs of mice. As markers of vessel leakage, the animals were given intravenous Evans blue (4 ml of a 1 percent solution in isotonic NaCl per kilogram of body weight) and carbon suspension (Pelikan C11/1431a, 1 ml per kilogram of body weight). Accumulation of both labels was visible within the injected tissues 30 minutes after administration of the extract. The lesions reached maximum size by 1 to 2 hours, becoming 1.5 to 2.0 cm in