Phytochemistry, 1969, Vol. 8, pp. 285 to 293. Pergamon Press. Printed in England

THE FATTY ACIDS OF THE ALGA *BOTRYOCOCCUS BRAUNII*

A. G. DOUGLAS,* K. DOURAGHI-ZADEH† and G. EGLINTON‡

Chemistry Department, The University, Glasgow, W.2

(*Received* 27 *July* 1968)

Abstract—A natural bloom of *Botryococcus braunii* Kützing, although rich in lipid, contains only a small quantity (0·014 per cent) of fatty acids. The most abundant fatty acids are palmitic, oleic and an octacosenoic acid; small amounts of α,ω-dicarboxylic acids are also present. These acids have been characterized by gas–liquid chromatography and by combined gas chromatography–mass spectrometry of their methyl esters. Hydrogenation of the methyl esters shows that the monocarboxylic acids range from n-C_{14} to n-C_{30}, the even numbered acids, especially n-C_{18}, n-C_{16} and n-C_{28}, predominating. Small quantities of branched acids are also present. The rubber-like material, obtained when *Botryococcus* is exposed to the atmosphere, has been examined; preliminary data suggest that it has a fatty acid pattern qualitatively similar to that of the fresh alga.

INTRODUCTION

Botryococcus braunii first described by Kützing[1] in 1849 is a genus of autotrophic algae which is planktonic in fresh water and is found in Scandinavia, Africa, Asia, America and Australia. According to Fritsch[2] *Botryococcus* exists in Britain as four species of which *B. braunii* is the most abundant. (In the following discussion *Botryococcus* refers to *B. braunii*.) The taxonomic position of *Botryococcus* has been the subject of some controversy. Kützing[1] placed it in the Palmellaceae near *Palmella*, while Chodat[3] placed it in the Chlorophyceae and Pascher[4] in the Xanthophyceae (Heterokontae). From pigment analysis, Blackburn[5] and, more recently, Belcher and Fogg[6] have shown that this genus should be included in the Chlorophyceae.

Botyrococcus has also attracted some attention in the controversy over the origin of so-called Boghead coals (e.g. torbanite) and the naturally occurring rubbery deposit coorongite. Boghead coal, which is widely held to have been formed from the organic remains of algae and plant debris in pools in the swamp forests of Carboniferous times,[7] was first discovered in Scotland (see for example, Stewart[8]) and later in New South Wales.[9] Microscopic studies of torbanite and coorongite (the latter is alleged to be the precursor of the former[10]), show the presence of closely packed minute yellow globules (called "yellow

* Present address: Organic Geochemistry Unit, Geology Department, The University, Newcastle upon Tyne, NE1 7RU.

† Present address: Chemistry Department, University of Arya Mehr, Teheran, Iran.

‡ Present address: Organic Geochemistry Unit, School of Chemistry, The University, Bristol, 8.

[1] F. T. KÜTZING, *Species Algarum. Lipsiae*, (1849), cited from Ref. 5.
[2] F. E. FRITSCH, *The Structure and Reproduction of the Algae*, Vol. 1, Cambridge University Press (1935).
[3] M. R. CHODAT, *J. Bot. Paris* **10**, 333 (1896).
[4] A. PASCHER, *Die Susswasserflora Deutschlands Österreichs und der Schweiz* **11**, 86 (1925).
[5] K. B. BLACKBURN, *Trans. R. Soc. Edin.* **58**, 841 (1936).
[6] J. H. BELCHER and G. E. FOGG, *New Phytologist* **54**, 81 (1955).
[7] W. J. SKILLING, *Oil Shale and Cannel Coal*, p. 32, Institute of Petroleum, London (1938).
[8] D. R. STEWART, *Mem. Geol. Survey Scotland* (1906); cited from Ref. 11.
[9] J. E. CARNE, *Mem. Geol. Survey N.S.W.* 333 (1903).
[10] H. R. J. CONACHER, *Oil Shale and Cannel Coal*, p. 42, Institute of Petroleum, London (1938).

bodies"). These observations led early to the postulate that both coorongite and torbanite are of algal origin and that the "yellow bodies" are the remains of the algal colonies (see, for example, Blackburn[5] and Temperley[11] and references therein). In a search to justify this "algal theory", a number of workers put forward limited evidence in its favour. Thus, Zalessky in a series of papers[12] concluded that the "yellow bodies" in both coorongite and torbanite are the remains of *Botryococcus*. Thiessen[13] studied coorongite and the alga which formed it but he named the latter *Elaeophyton coorongiana*, having failed to identify it with *Botryococcus*; this misnomer persists in the recent literature.[14] Temperley, in a more critical study, ends with the statement that "the 'yellow bodies' of torbanite are the remains of colonies of an alga which does not differ in any material respect from the living alga *Botryococcus*". The occurrence of *Botryococcus* in lignites and other Tertiary deposits has been the subject of a recent study by Traverse.[15]

The present investigation represents part of an examination of the theory that coorongite and torbanite originate from *Botryococcus*. We intend to report on the chemical composition of the three materials, the living alga (*Botryococcus*), the Recent deposit (coorongite) and the Carboniferous sediment (torbanite) and the correlation, if any, which exists between them. This paper reports our findings on the total fatty acid composition of the alga *Botryococcus*,* and of *Botryococcus* "rubber", obtained by exposure of the alga to the atmosphere. The *Botryococcus* rubber discussed below was obtained by allowing an aqueous suspension of living alga to evaporate in the air from a large surface; in a few weeks a black elastic-like material was deposited which was not unlike a sample of coorongite examined by us. It is interesting to note that the formation of coorongite was witnessed by Broughton[16] and De Hautpick,[17] the former author stating "...a thick scum, like green paint, is forming and drying to a semi-elastic substance, every stage from the green, liquid, paint-like substance to the tough, elastic, sand-containing coorongite may be observed...".

RESULTS AND DISCUSSION

Botryococcus

The alga was concentrated (centrifuge) and dried azeotropically using a benzene–methanol mixture. The residue was hydrolysed with methanolic potassium hydroxide and most of the neutral lipids were removed by liquid–liquid extraction (*n*-hexane). Acidification of the residue and extraction with ether furnished a crude fatty acid fraction which was purified via an acid-base extraction. Although the procedure was repeated, the unsaturated hydrocarbon contaminant botryococcene† (cf. Ref. 18) persisted. A gas–liquid chromatogram of the methyl esters of this fraction (Fig. 1A) shows methyl oleate, methyl palmitate,

* The alga used in this investigation occurred in winter as a rust-coloured bloom floating on the surface of a freshwater lake at Oakmere, Cheshire; its authenticity was confirmed by microscopic examination (see Experimental).

† Botryococcene is the name given to the high mol. wt. poly-unsaturated hydrocarbon which comprises more than 70 per cent of the dry weight of *Botryococcus*.

[11] B. N. TEMPERLEY, *Trans. R. Soc. Edin.* **58**, 855 (1936).
[12] M. B. ZALESSKY, *Bull. Comité Geol. Pétersbourg* **33**, 495 (1914); *Ann. Soc. Palaéont. Russia* **1**, 25 (1916); *Bull. Soc. Géol. France* **17**, 373 (1917); *Rev. Gén. Bot.* **38**, 31 (1926).
[13] R. THIESSEN, *U.S. Geol. Survey, Prof. Papers* No. 32 (1925).
[14] I. A. BREGER, *J. Am. Oil Chemists' Soc.* **43**, 197 (1966).
[15] A. TRAVERSE, *Micropaleontology* **1**, 343 (1955).
[16] A. C. BROUGHTON, *Proc. R. Soc. South Australia* **44**, 386 (1920).
[17] E. DE HAUTPICK, *Bull. Soc. Géol. France* **26**, 61 (1926).
[18] J. R. MAXWELL, A. G. DOUGLAS, G. EGLINTON and A. MCCORMICK, *Phytochem.*, in press.

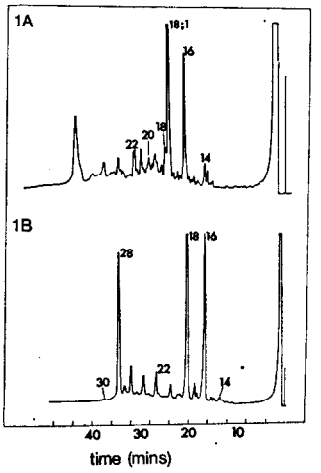

FIG. 1. GAS CHROMATOGRAMS OF FATTY ACIDS (AS METHYL ESTERS) ISOLATED FROM *Botryococcus braunii*.

Conditions: Column 7 ft × 0·040 in. (i.d.) containing 4·6% JXR on 100–120 mesh silanized Chromosorb G. (A) Total crude fatty acid fraction, temperature programmed from 100–300° at 5°/min, nitrogen 30 p.s.i. (B) Hydrogenated fatty acids, temperature programmed from 130–300° at 5°/min, nitrogen 30 p.s.i.

TABLE 1. CONSTITUENT FATTY ACIDS (AS METHYL ESTERS) OF THE ALGA *Botryococcus braunii* AND OF *Botryococcus* "RUBBER"

	Monocarboxylic acids in fresh alga (and "rubber")				Fresh alga. Esters after hydrogenation		
	Saturated		Unsaturated				
Acid carbon number	*n*-	Branched	Monoene	Diene	α,ω-	*n*-	Branched
11	(†)						
12	(†)						
13	(† *)						
14	† * († *)				*	*	
15	* († *)	*			*	*	*
16	† * († *)		* (*)		*	*	
17	* († *)	*	* (*)		*	*	*
18	† * († *)		* († *)	*[1]	*	*	*
19	(†)				*	*	
20	† * (*)		* (*)			*	
21						*	
22	† * (*)		* (*)			*	
23						*	
24	*					*	
25							
26			*			*	
27							
28			*	*		*	
29							
30						*	

Values for *Botryococcus* "rubber" are given in parentheses.
* Determined by GC–MS.
† Determined by GLC co-injection.
[1] Two $C_{18:2}$ acids are present.

and a compound with the approximate retention time of methyl octacosanoate, as the predominant esters. The positions of the saturated n-C_{16}, n-C_{18}, n-C_{20} and n-C_{22} methyl esters and of methyl oleate, indicated in the chromatogram, were established by co-injection with standard esters. The total ester fraction was subsequently separated by preparative TLC on silver nitrate–silica gel,[19] into several bands which were examined by analytical TLC, gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS). A number of the peaks shown on the above gas chromatogram were then identified (Table 1).

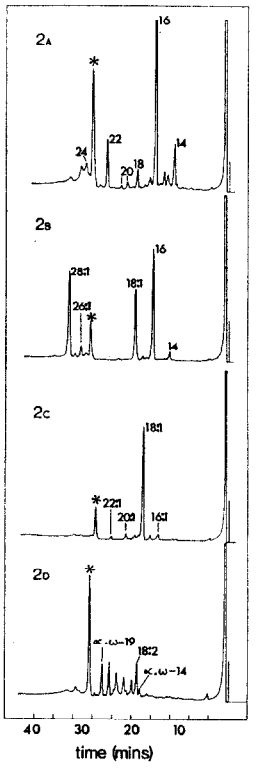

FIG. 2. GAS CHROMATOGRAMS OF FATTY ACIDS (AS METHYL ESTERS) ISOLATED FROM *Botryococcus braunii* AFTER TLC.

Conditions: Column 7 ft × 0·040 in. (i.d.) containing 4·6% JXR on 100–120 mesh silanized Chromosorb G, programmed from 130–300° at 5°/min, nitrogen 30 p.s.i. (A) Band I. (B) Band II. (C) Band III. (D) Band V from TLC plate (cf. text). The peak marked with an asterisk is due to botryococcene.

Band I (Fig. 2A) was a mixture of saturated fatty acid methyl esters with methyl palmitate the predominant component. The esters were characterized by co-injection with n-C_{14}, n-C_{16}, n-C_{18}, n-C_{20}, n-C_{22} and n-C_{24} methyl esters and also by GC–MS. Their mass spectra showed the pattern expected for saturated normal methyl esters.[20] In Band II (Fig. 2B) GC–MS examination of the methyl esters showed that the saturated n-C_{14}, n-C_{16} and n-C_{18} acids were present, with only traces of the n-C_{15} and n-C_{17} acids; similarly the methyl esters of unsaturated n-C_{18}, n-C_{26} and n-C_{28} acids were identified. Band III (Fig. 2C) contained

[19] L. J. MORRIS, *J. Lipid Res.* **7**, 717 (1966).
[20] R. RYHAGE and E. STENHAGEN, *Ark. Kemi* **13**, 523 (1959).

a series of methyl esters of the mono-unsaturated acids *n*-C_{16}, *n*-C_{18}, *n*-C_{20} and *n*-C_{22} and some botryococcene, as determined by GC–MS. The predominant components were the $C_{18:1}$ ester and botryococcene, other components being present in small quantity. Co-injection of methyl oleate with this fraction enhanced the $C_{18:1}$ peak. The mass spectra of these unsaturated methyl esters are more complex than those of the corresponding saturated esters.[21,22] Hallgren *et al.*[23] have demonstrated that the structures of mono-unsaturated acids cannot be deduced directly by mass spectrometric analysis of their esters; thus, they report that methyl oleate, elaidate, petroselinate and petroselaidate give indistinguishable mass spectra. In agreement, we were unable to determine the position of the double bonds of the esters by mass spectrometry. Band IV contained mainly botryococcene and a $C_{28:2}$ ester; a small amount of a $C_{18:1}$ ester was also present. Band V (Fig. 2D) consisted mainly of a series of methyl esters of *n*-C_{14}–*n*-C_{19} α,ω-dicarboxylic acids together with botryococcene and a $C_{18:2}$ ester as major constituents. The mass spectra of the α,ω-di-esters showed the expected peaks[24] viz: *m/e* at M-31 (loss of OCH_3), M-64, M-73 (loss of CH_2COOCH_3), M-92, M-105, M-123, M-146 and also *m/e* = 59, 74 ($CH_2{:}C(OH)OCH_3$), 84, 87, 98, 101 and 112. The mass spectrum of the *n*-C_{15} di-ester is shown in Fig. 3 and is in good agreement with that of dimethyl pentadeca-1,15-dioate, the only authentic di-ester available to us.

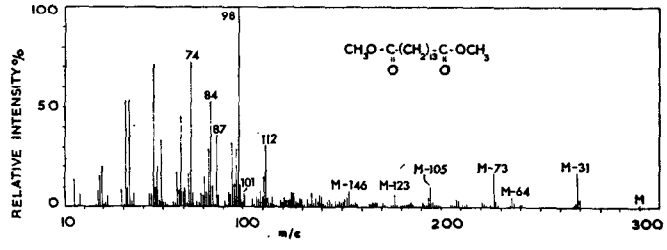

FIG. 3. MASS SPECTRUM OF DIMETHYL PENTADECA-1,15-DIOATE ISOLATED AS THE FREE ACID FROM *Botryococcus braunii*.

In order to further characterize the esters of the unsaturated fatty acids a portion of the total methyl ester fraction was hydrogenated using 10% palladium charcoal. The resulting hydrogenated esters were then purified by TLC and characterized by GLC (Fig. 1B) and GC–MS. In the gas–liquid chromatogram, the largest peaks were methyl stearate, palmitate and octacosanoate, respectively. Further, the normal fatty acids exhibit an even/odd predominance which is customary for fatty acids obtained from biological sources.[25] The mass spectra of all the hydrogenated normal mono-carboxylic acid methyl esters were in agreement with those reported in the literature.[20] Very small quantities of branched-chain fatty acid methyl esters were present in the hydrogenated fraction as shown by GLC and GC–MS[26] but they have not been fully characterized.

Parker *et al.*[27] have studied the fatty acid content (in the C_{10}–C_{20} range) of eleven species

[21] J. ASSELINEAU, R. RYHAGE and E. STENHAGEN, *Acta Chem. Scand.* **11**, 196 (1957).
[22] R. RYHAGE and E. STENHAGEN, in *Mass Spectrometry of Organic Ions* (edited by F. W. MCLAFFERTY), p. 339, Academic Press, New York (1963).
[23] B. HALLGREN, R. RYHAGE and E. STENHAGEN, *Acta Chem. Scand.* **13**, 845 (1959).
[24] R. RYHAGE and E. STENHAGEN, *Ark. Kemi* **14**, 497 (1959); *Ark. Kemi* **23**, 167 (1964).
[25] F. B. SHORLAND, in *Comparative Biochemistry* (edited by M. FLORKIN and H. S. MASON), Vol. 3, Academic Press, London (1964).
[26] R. RYHAGE and E. STENHAGEN, *Ark. Kemi* **15**, 291 (1960).
[27] P. L. PARKER, C. V. BAALEN and L. MAURER, *Science* **157**, 707 (1967).

of blue–green algae and have also found that the branched fatty acids are present only in minute quantities. However, in bacteria, some species of which undoubtedly play a part in the decomposition of organic matter,[28] branched fatty acids predominate.[27,29,30] Data available[27,31–36] on the fatty acid content of freshwater algae show that palmitic acid is the predominant saturated fatty acid* and *n*-C_{16} and *n*-C_{18} mono- and polyenoic acids are the predominant unsaturated acids. Thus in the green alga, *Scenedesmus obliquus*, these acids account for 99 per cent of the total acid content.[36] Parker *et al.*[27] have stated that "the fatty acid compositions in eleven species of blue–green algae were simple but had a pronounced qualitative variation amongst the different species" (based on the unsaturated C_{18} carboxylic acids). All the algae studied contained saturated and mono-unsaturated C_{18} acids but poly-unsaturated acids were absent from *Anacystis marina* and *A. nidulans*, while *Oscillatoria williamsii* and *Lyngbya lagerhaimii* contained di- but not tri-unsaturated C_{18} acids, and *Anabena variabilis*, *Microcoleus chthonoplastes*, contained poly-unsaturated C_{18} acids.

Botryococcus "*Rubber*"

The rubber (*ca.* 1·5 years old) was saponified and methylated according to the method of Metcalfe *et al.*[38] The total hexane extract was examined by preparative TLC using light petroleum/ether/acetic acid (90:10:1) and a fraction having the retention bracketing methyl stearate and oleate was removed from the TLC plate as a broad band. GLC of the recovered esters was very complex and showed peaks ranging from about C_{11}–C_{30}. Preparative TLC of this crude ester fraction on 10 per cent argentatious silica gel (double development in light petroleum/ether, 95:5) gave four bands, the first three (A, B and C) having retentions similar to methyl stearate, elaidate and oleate respectively; subsequent analytical TLC of each recovered ester fraction showed them to be substantially homogeneous.

A gas–liquid chromatogram of Band A is shown in Fig. 4A. Co-injection of standard *n*-C_{11}–*n*-C_{19} saturated fatty acid methyl esters enhanced the peaks marked 11–19 and the identity of the peaks marked with an asterisk was confirmed by GC–MS.[20] This evidence establishes the presence of saturated fatty acid esters in the range C_{11}–C_{22} and visual inspection of the chromatogram suggests that the homology may extend to about C_{26}.

The gas–liquid chromatogram of Band B is shown in Fig. 4B. The main constituent has the same R_f as methyl elaidate when analysed by TLC (10 per cent argentatious silica gel, development as above) which suggests it contains a *trans*-substituted double bond, double development at $-20°$ with toluene[39] using the same impregnated adsorbent as above gave

* Parker *et al.*[27] have recently reported that for one sample of *Trichodesmium erythaeum* 50 per cent of its total fatty acid content was *n*-decanoic acid. They suggest that *T. erythaeum* may be the source of this acid found in the Gulf of Mexico sea-water by Slowey *et al.*[37]

[28] See, for example: R. E. KALLIO, W. R. FINNERTY, S. WAWZONEK and P. D. KLIMSTRA, in *Symposium on Marine Microbiol.* (edited by C. H. OPPENHEIMER), C. C. Thomas, Springfield, Ill. (1963) and references therein.

[29] T. KANEDA, *J. Biol. Chem.* **238**, 1122 (1963).

[30] M. KATES, in *Advances in Lipid Research* (edited by R. PAOLETTI and D. KRITCHEVSKY) Vol. 2, p. 17, Academic Press, New York (1964).

[31] R. W. HOLTON, H. H. BLECKER and M. ONORE, *Phytochem.* **3**, 595 (1964).

[32] B. W. NICHOLS, R. V. HARRIS and A. T. JAMES, *Biochem. Biophys. Res. Commun.* **20**, 256 (1965).

[33] M. KATES and B. E. VOLCANI, *Biochim. Biophys. Acta* **116**, 264 (1966).

[34] B. W. NICHOLS, *Biochim. Biophys. Acta* **106**, 274 (1965).

[35] H. SCHLENK and J. L. GELLERMAN, *J. Am. Oil Chemists' Soc.* **42**, 504 (1965).

[36] E. KLENK, W. KNIPPRATH, D. EBERHAGEN and H. D. KOOF, *Z. Physiol. Chem.* **334**, 44 (1963).

[37] J. F. SLOWEY, L. M. JEFFREY and D. W. HOOD, *Geochim. Cosmochim. Acta* **26**, 607 (1962).

[38] L. O. METCALFE, A. A. SCHMITZ and J. R. PELKA, *Anal. Chem.* **38**, 514 (1966).

[39] L. J. MORRIS, D. M. WHARRY and E. W. HAMMOND, *J. Chromatogr.* **31**, 69 (1967).

a spot with an R_f of 0·58 compared to methyl elaidate (R_f 0·46). This would accord with a *trans*-mono-unsaturated ester of greater molecular weight than elaidate and visual comparison of the GLC of this fraction (Fig. 4B) with that of the saturated esters (Fig. 4A) suggests that the main constituent is a C_{28} ester.

Figure 4C shows the gas chromatogram of Band C. Argentatious TLC with both systems described for Band B gave spots with the same R_f as methyl oleate. The peaks in Fig. 4C marked with an asterisk have been confirmed as monoenoic acid esters by GC–MS and co-injection with methyl oleate enhanced the peak so numbered in the chromatogram. This evidence suggests the presence of a series of *cis*-monoeneoic acid esters ranging from C_{16}–C_{22} with the homology perhaps extending to about C_{28}.

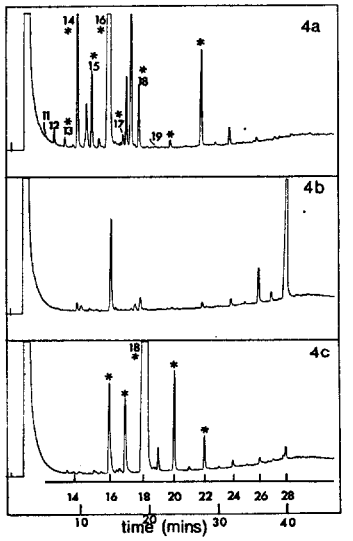

FIG. 4. GAS CHROMATOGRAMS OF FATTY ACIDS (AS METHYL ESTERS) ISOLATED FROM *Botryococcus braunii* "RUBBER".

Conditions: Column 20 ft × 0·040 in. (i.d.) containing 3% OV-1 on 100–120 mesh Gas Chrom Q, temperature programmed from 170–325° at 4°/min, nitrogen 9 ml/min. (A) Saturated esters. (B) *trans*-Unsaturated esters. (C) *cis*-Unsaturated esters.

Concluding Remarks

Our results (Table 1) show that a natural winter bloom of *Botryococcus* contains a small amount of fatty acids in its extractable lipid fraction. The monocarboxylic acids range from about C_{14}–C_{30}, palmitic, oleic and an octacosenoic acid being the major components. Two dienoic C_{18} acids and a dienoic C_{28} acid are present. Other polyenoic acids can only be present in very small amount since none was detected by GC–MS. Acids with even numbers of carbon atoms are more abundant than the acids with odd numbers of carbon atoms. The occurrence of a C_{26} and C_{28} monoenoic acid and a C_{28} dienoic acid is interesting; as far as we are aware no C_{28} dienoic fatty acid has been found in nature and the other two acids are rare. Thus, a $C_{26:1}$ acid has been reported variously in a sponge, a species of lobster, in *Ximenia* seed fat and in brain cerebrosides and a $C_{28:1}$ acid has been reported in a sponge and *Ximenia* seed fat.[40]

[40] T. P. HILDITCH and P. N. WILLIAMS, *The Chemical Constitution of Natural Fats*, Chapman and Hall, London (1964).

A small quantity of α,ω-dicarboxylic acids, ranging from C_{14}–C_{19} with no even/odd preference, have also been characterized in *Botryococcus*. We have previously reported[41] the presence of α,ω-dicarboxylic acids in a Carboniferous sediment, torbanite, which is believed to have been derived from *Botryococcus*; these acids ranged from about C_8–C_{22} with no even/odd preference. Haug *et al.*[42] have recently reported α,ω-dicarboxylic acids in the Green River Shale (Eocene) and it would be interesting to know if any correlation existed between the acids present in this alga-containing shale[43] and the lipid fraction of any contemporary alga of the type present in the shale. Finally, a preliminary examination of the fatty acids present in *Botryococcus* "rubber" (Table 1) has shown that a qualitatively similar distribution exists in both the rubber and the fresh alga. In the former, no attempt was made to examine the lipid extract for dienoic or α,ω-dicarboxylic acids.

EXPERIMENTAL

All solvents were of Analar grade and were distilled through a 30 × 2 cm column packed with glass helices. Light petroleum had b.p. 60–80°, unless otherwise stated. All glassware was cleaned ultrasonically in detergent, rinsed with distilled water, acetone and chloroform and then dried at 80°.

I.r. spectra were recorded in CCl_4 on a Perkin-Elmer 257 instrument. Gas chromatographic conditions are shown in the figure legends. The combined gas chromatograph–mass spectrometer (L.K.B. 9000) was fitted with a 7 ft × $\frac{1}{8}$ in. column containing 2% JXR on 100–120 mesh Gas Chrom Z; helium flow rate was 30 ml/min, scan times were 4–5 sec, ionization potential was 70 eV.

The alga *Botryococcus* was received live as an oily rust-coloured aqueous suspension which was stored in polythene containers at −20°. The sample was collected by Mr. J. Osborne, warden of Rostherne N.W.R., from the surface of Oakmere (Cheshire) on 5 November 1965. The authenticity and purity of the alga was confirmed by Dr. J. W. G. Lund (Freshwater Biological Association, Windermere) and Dr. E. Conway (Botany Department, Glasgow University).

Micro-hydrogenation

A micro-hydrogenation vessel was devised and tested using heptadec-1-ene as a standard olefin. A solution of heptadec-1-ene (400 μg) in ethyl acetate (0·5 ml) was placed in the hydrogenation vessel together with palladium-charcoal (10 per cent, 30 mg). The apparatus was swept with hydrogen for about 2 min, the outlet and the inlet taps were closed and the tube was partially immersed in an ultrasonic tank for about 30 min. The mixture was passed through a short column (2·0 cm × 4 mm) of neutral alumina and the clear eluate was concentrated to near dryness. The i.r. spectrum and the GLC of the recovered oil showed the absence of the starting material. TLC on silver nitrate–silica gel gave one spot with R_f 0·93 (*n*-heptadecane as standard had R_f 0·93).

Extraction of Fatty Acids from Botryococcus *and Preparation of Methyl Esters*

A suspension (250 ml) of the alga was centrifuged and the wet residue was dried azeotropically with benzene/methanol (1:2, 3 × 150 ml) and finally with benzene only; it was then vacuum dried (at 55°). The dried alga (31·8 g) was saponified with a mixture of methanol (100 ml), KOH (5·6 g), water (5 ml) and benzene (10 ml) by boiling under reflux for 3·5 hr. This mixture was subsequently diluted with water (25 ml), thoroughly washed with *n*-hexane and evaporated under reduced pressure; acidification to pH 1 followed by continuous extraction with ether (100 hr) gave an oil whose i.r. spectrum showed carboxylic acid absorption (3000 and 1710 cm^{-1}) together with bands due to botryococcene. An ethereal solution of this oil was extracted with dilute KOH and the latter then thoroughly washed with *n*-hexane, acidified and extracted with *n*-hexane to give a crude acid fraction. This fraction of acids was methylated with methanol/benzene/sulphuric acid (20:10:1) to give 4·5 mg of crude esters (equivalent to 0·014 per cent based on 31·8 g of dried alga).

The prominent bands in the i.r. spectrum correlated with those reported for long-chain fatty acid esters, i.e. 2940 and 2860 (ν_{CH} for CH_2 and CH_3); 1745 ($\nu_{C=O}$); 1200 and 1170 cm^{-1} (ν_{C-O} for esters). However, bands at 890, 915, 1650 and 3020 cm^{-1} suggested that small quantities of botryococcene were still present.

[41] A. G. DOUGLAS, K. DOURAGHI-ZADEH, G. EGLINTON and J. R. MAWELL, in *Advances in Organic Geochemistry* 1966 (edited by G. D. HOBSON and G. C. SPEERS), Pergamon Press, Oxford, in press.
[42] P. HAUG, H. K. SCHNOES and A. L. BURLINGAME, *Science* **158**, 772 (1967).
[43] N. DE NEVERS, *Scient. Amer.* **214**, 21 (1966).

Preparative TLC of Fatty Acid Esters from Botryococcus

The methyl esters were chromatographed on silica gel G containing 10% silver nitrate (200 × 200 × 0·5 mm) using *n*-hexane/ether (95:5); the bands were removed after visualization with 2′,7′-dichlorofluorescein and the esters were recovered by elution with ether. The R_f values of Bands 1–5 were 0·45, 0·40, 0·30, 0·18 and 0·09 respectively; comparative R_f values were methyl hexacosanoate (0·45), methyl stearate (0·40), methyl oleate (0·30), methyl linoleate and dimethyl brassylate (0·09).

Micro-hydrogenation of Fatty Acid Esters from Botryococcus

A portion (1·5 mg) of the total ester fraction described below in ethyl acetate (1 ml) was hydrogenated with palladium-charcoal (30 mg). The i.r. spectrum of the product was characteristic of saturated methyl ester. Analytical TLC with *n*-pentatriacontane and a mixture of C_{10}–C_{22} methyl esters as reference materials showed the presence of a hydrocarbon with (R_f 0·9), probably perhydrobotryococcene, and monocarboxylic acid methyl esters (R_f 0·42–0·46).

Preparative TLC of Hydrogenated Fatty Acid Esters

Preparative TLC of these esters on argentatious silica gel afforded three main bands. Band 1 had an i.r. spectrum similar to that of botryococcane (perhydrobotryococcene)[18] and the gas chromatogram had, in addition to a number of small peaks, one large peak with the same retention time as botryococcane. Band 2 had an i.r. spectrum characteristic of a long-chain methyl ester [o.d. $\nu_{C=O}=0{\cdot}20$, o.d. $\nu_{C=O}$/o.d. $\nu_{CH(2860)}=0{\cdot}67$; this ratio is $\sim 1{\cdot}0$ for methyl stearate]. Methyl esters ranging from C_{14}–C_{30} are evident from the gas chromatogram (Fig. 1B); those identified from their mass spectra are listed in Table 1. Band 3 had i.r. bands at 1740, 1620, 1280 and 1070 cm^{-1}. The C—H stretching region (2850–2980 cm^{-1}) showed a high ratio of CH_3 to CH_2 and o.d. $\nu_{C=O}$/o.d. $\nu_{CH}=2{\cdot}0$. The GLC was complex and no components have as yet been identified.

Preparation of Fatty Acid Esters from Botryococcus "*Rubber*"

Botryococcus "rubber" (1·5 g) was cut into small pieces and boiled under reflux with 0·5 N methanolic NaOH (50 ml) for 1·5 hr. Excess BF_3/methanol was added and the mixture was boiled for 15 min, cooled, distilled to remove methanol (*ca.* 75 ml) and extracted with *n*-hexane to give an oil (240 mg).

Preparative TLC of Esters from Botryococcus "*Rubber*"

Preparative TLC on silica gel G (light petroleum/ether/acetic acid, 90:10:1) gave a large number of bands. A broad band with R_f 0·42–0·60 (methyl stearate 0·58, methyl oleate 0·54) was removed and extracted with ether to give a very small amount of the crude methyl esters. This fraction had a very complex GLC pattern. It was separated into three main fractions by preparative TLC on argentatious silica gel; a diffuse band extending from the origin to the bottom (third) band was not examined further. Analytical TLC of three recovered fractions on argentatious silica gel gave discrete spots with the same R_f values as methyl stearate, elaidate and oleate respectively.

TLC at −20° *of Esters from* Botryococcus "*Rubber*"

Fractions 2 and 3 from above were chromatographed on 10% argentatious silica gel at −20° by double development with toluene. Fraction 2 had R_f 0·58 (methyl elaidate R_f 0·46) and fraction 3 had R_f 0·26 (methyl oleate R_f 0·26).

Acknowledgements—We are indebted to S.R.C. for provision of the LKB 9000 gas chromatograph–mass spectrometer (grant B/SR/2398 to Drs. Eglinton and C. J. W. Brooks), N.E.R.C. (A.G.D.) and N.A.S.A. (NsG 101-61, K.D-Z) for financial support, Mrs. F. Lawrie and Miss N. Robertson for the i.r. measurements, Mr. J. Osborne for collecting *Botryococcus*, Drs. J. W. G. Lund and E. Conway for helpful advice, particularly in relation to the authenticity and purity of the sample of the alga, Misses J. Malcolm and H. Humphrys for gas chromatography–mass spectrometry recordings, and to Dr. A. McCormick for assistance in mass spectral interpretation.