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THE BOTRYOCOCCENES—HYDROCARBONS OF NOVEL STRUCTURE FROM THE ALGA *BOTRYOCOCCUS BRAUNII*, KÜTZING

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Abstract—The hydrocarbon fraction of *Botryococcus braunii*, growing in its natural environment, has been isolated and examined. Surprisingly this fraction was found to comprise 76 per cent of the dry weight of the alga. Furthermore, two unsaturated isomeric hydrocarbons of formula $C_{34}H_{58}$, termed botryococcene and isobotryococcene, constitute the bulk of the hydrocarbons. No saturated hydrocarbons were detected. Approaches to the structural elucidation of the botryococcenes are described and the significance of their occurrence, in terms of the diagenetic fate of *B. braunii*, is discussed. Possible biogenetic routes are considered.

INTRODUCTION

Botryococcus braunii, Kützing is the most widespread of the British *Botryococcus* algae, of which there are four.[1] It is common but not abundant in Britain and occurs throughout the world in a wide variety of climates. In Australia it gives rise to rubbery deposits called Coorongite. Blackburn[2] assigned *B. braunii* to the order Chlorophyceae because of the presence of green chloroplasts and starch in the cells. This finding was later confirmed by Belcher and Fogg[3] who isolated chlorophylls *a* and *b* from the alga. Prior to this *B. braunii* had been assigned to the order Xanthophyceae by some authors and to the Chlorophyceae by others.

The unusual morphology, behaviour and chemical composition have been extensively studied by Blackburn[2] and Belcher.[4] The alga forms approximately spherical colonies which may be attached by threads to form larger compound colonies of varying size, texture and structure. The latter are green or orange, the orange colour being apparent under conditions of nutrient starvation, although in culture they are usually green.[4] Each cell is embedded in a cup of oil and when a cell divides into two daughter cells the latter secrete oil, while remaining inside the cup of the mother cell. Thus the matrix of the colony is built up of the cups of the daughter cells.[2]

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[1] F. E. FRITSCH, *Structure and Reproduction of the Algae*, Vol. 1, Cambridge University Press (1935).
[2] K. B. BLACKBURN and B. N. TEMPERLEY, *Trans. Roy. Soc. Edinburgh* **58**, 841 (1936).
[3] J. H. BELCHER and G. E. FOGG, *New Phytol.* **54**, 81 (1955).
[4] J. H. BELCHER, Ph.D. Thesis, London (1958), and Dr. E. CONWAY, University of Glasgow, personal communication.

B. braunii has also occasioned widespread interest because of its role in the boghead coal controversy. From microscopic examination boghead coals are known to consist largely of minute yellow globules. It was suggested by various authors that the globules were cells in a plant tissue,[5] were composed of bitumen[6] or resin,[7] were globules of oil solidified on an inorganic nucleus[8] or had an algal origin.[9] Finally, Blackburn and Temperley,[2] from a detailed microscopic study of the alga and the boghead coals maintained that the yellow globules were formed from *B. braunii*. Traverse,[10] in an important paper, has surveyed the occurrence of *Botryococcus* in lignites and other Tertiary sediments. He, too, holds the view that certain Paleozoic oil-bearing rocks derive their oil content from this alga and points out that the same genus produces salty sediments in modern brackish and fresh water lakes and very likely is important in the Cenozoic also.

The chemical constitution of the oily colony matrix has been the subject of several investigations. When placed in iodine solution, the alga absorbs so much iodine that it sinks.[2] High iodine values have also been quoted for various fractions of Coorongite[2, 10–13] implying a high degree of unsaturation in the living alga. From these and other results Blackburn thought that the readily-soluble fraction of the alga contained oils and fatty acids, whereas the less soluble fraction probably consisted of more complicated derivatives of fatty acids. Belcher,[4] working with the living alga, found it to contain 7–12 per cent of saponifiable lipid and 16–23 per cent of unsaponifiable lipid: the unsaponifiable lipid was an oil at room temperature but he was unable to obtain it free from ß-carotene. Swain and Gilby[14] examined samples of *B. braunii* floating on Lakes Nicaragua and Manegua in Nicaragua and found them to be composed of more than 90 per cent oily materials.

RESULTS

Figure 1 shows a number of colonies in the *Botryococcus* sample and Fig. 2 represents a single colony under conditions of high magnification, showing the individual cells.

Ultrasonic extraction with acetone of a freeze-dried sample of *Botryococcus* afforded the lipid fraction as a brownish-green oil whose i.r. spectrum had carbon–carbon double bond absorption at 892, 917, 979 and 1002 cm^{-1}, confirming the high degree of unsaturation implied by earlier investigators. However there was no carbonyl absorption, indicating that this extract of the colony matrix was not composed of fatty acids as had been previously believed.[2] This has been confirmed by Douglas *et al.*[15] who have found the fatty acid content of the alga to be very small. Column chromatography on alumina afforded the total hydrocarbon fraction, comprising 76 per cent of the dry weight of the alga. The i.r. spectrum (Fig. 3) of this fraction again had absorption at 892, 917, 980 and 1002 cm^{-1}. Gas–liquid chromatography (Fig. 4) showed the presence of two major components, termed botryococcene and isobotryococcene, which were present in the ratio of about 9:1 respectively.

[5] J. H. BALFOUR, *Trans. Roy. Soc. Edinburgh* **21**, 187 (1854).
[6] J. H. BENNETT, *ibid.* **21**, 173 (1854).
[7] H. R. J. CONACHER, *Trans. Geol. Soc. Glasgow* **16**, 164 (1917).
[8] E. H. CUNNINGHAM-CRAIG, *J. Inst. Petrol.* **2**, 238 (1916).
[9] T. W. E. DAVID, *Proc. Linnean Soc. NSW*, Ser. 2, **4**, 483 (1889).
[10] A. TRAVERSE, *Micropaleontology* **1**, 343 (1955).
[11] M. D. ZALESSKY, *Bull. Comité Géol. Petersbourg* **33**, no. 248, 495 (1914).
[12] M. D. ZALESSKY, *Rev. Gén. Botan.* **38**, 31 (1926).
[13] R. THIESSEN, U.S. Geol. Surv. Prof. Papers, **1321**, 121 (1925).
[14] F. M. SWAIN and J. M. GILBY, *Pubbl. Staz. Zool. Napoli* **33**, suppl. 361 (1964).
[15] A. G. DOUGLAS, K. DOURAGHI-ZADEH and G. EGLINTON, in preparation.

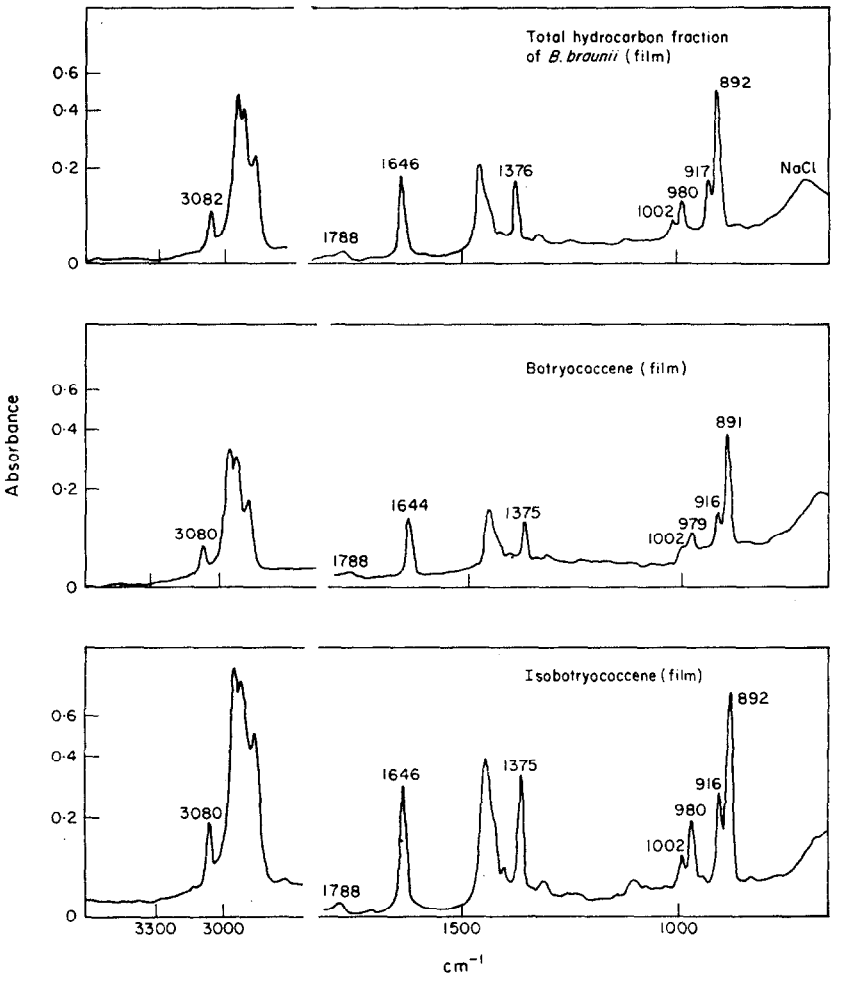


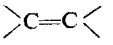
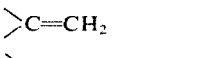
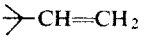
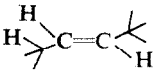
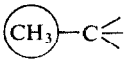
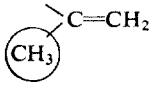
FIG. 3. INFRARED SPECTRA OF *B. Braunii* HYDROCARBON FRACTION AND THE BOTRYOCOCCENES (AS THIN FILMS).

Separation of the two hydrocarbons was achieved by silver ion thin-layer chromatography and a molecular formula of $C_{34}H_{58}$ was assigned to them from their mass spectra (Fig. 5) which show the molecular ions to be at *m/e* 466.

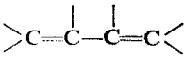
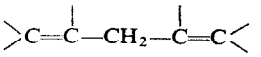
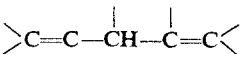
The purity was checked in both cases during combined gas chromatography—mass spectrometry by recording mass spectra at six points on the gas chromatographic peak. There were no differences in the mass spectra recorded for each compound except for the expected small differences in the relative abundances of a few of the ions.

The i.r. spectra of the two hydrocarbons are very alike. The bands at 891 (2); 916 and 1002; and 979 cm^{-1} were assigned to exomethylene, vinyl and *trans* disubstituted carbon–carbon double bond absorptions respectively. Intensity measurements (Table 2) of these bands in the spectrum of botryococcene were compatible with one vinyl double bond, one *trans* disubstituted double bond and four exomethylene double bonds in the hydrocarbon, on comparison with relevant standards. The normal intensity (ϵ_a) for γ (C-H) of a *trans*

TABLE 1. (a) GROUPS PRESENT IN THE STRUCTURE OF BOTRYOCOCCENE

Group	Number	Evidence
	6	i.r., NMR, reduction to botryococcane
	4	i.r., NMR
	1	i.r., NMR, M.S. of botryococcane partial reduction to dihydroiso-botryococcene
	1	i.r., NMR
	5 or 6	NMR
	2	NMR

(b) GROUPS ABSENT IN THE STRUCTURE OF BOTRYOCOCCENE

Group	Evidence
	u.v.
	NMR
	NMR
$-(CH_2)_n-$ [where $n > 4$]	i.r.

double bond is *ca.* 100, indicating that both hydrocarbons have one *trans* double bond (Table 2). The intensities quoted by Cairns *et al.*[16] for the vinyl double bond of rosololactone and deoxyrosololactone are similar to those recorded for both hydrocarbons. The intensity measurement of the exomethylene band of isobotryococcene is compatible with there being one exomethylene bond fewer than in botryococcene. The high frequency of 1002 cm^{-1} for the γ (C-H) absorption of the vinyl double bond in the botryococcenes provides evidence that this group is attached to a fully substituted carbon[17] (see nuclear magnetic resonance data below). The absence of any $-(CH_2)_n-$ absorption around 720 cm^{-1} indicates that both compounds are highly branched. This was confirmed by gas–liquid chromatography since the fully saturated hydrocarbon botryococcane had a retention time of 1·0 relative to n-octacosane. That the double bonds of both hydrocarbons are unconjugated was demonstrated by their u.v. spectra which show end absorption only.

[16] T. CAIRNS, G. EGLINTON, A. I. SCOTT and D. W. YOUNG, *J. Chem. Soc.* (B) 654, (1966).
[17] D. BARNARD, L. BATEMAN, A. J. HARDING, H. P. KOCH, N. SHEPPARD and G. B. B. M. SUTHERLAND, *J. Chem. Soc.* 915 (1950).

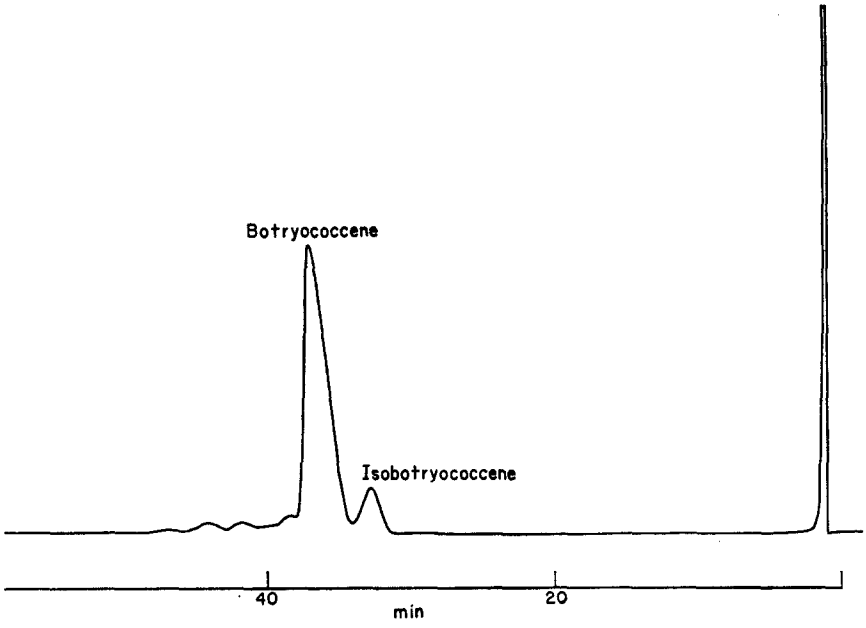


FIG. 4. GAS CHROMATOGRAM OF *B. braunii* HYDROCARBON FRACTION.

TABLE 2. SIGNIFICANT INFRARED BANDS

Compound	Solvent	ν, cm^{-1}	$\Delta\nu_{1/2}$,cm^{-1} [a]	o.d.	ϵ_a
Botryococcene	CS_2	1001	(15)	0·055	60
		977	16	0·098	116
		915	13	0·152	170
		891	8	0·65	757
Isobotryococcene	CS_2	1001	15	0·06	67
		978	17	0·10	112
		916	12	0·15	166
		892	8	0·51	573
Botryococcane	CCl_4	1378	*	0·44	(209)
		1365	*	0·21	(99 sh.)
Squalane	CCl_4	1376	*	0·28	(182)
		1365	*	0·22	(143 sh.)
Dihydrobotryococcene	CS_2	978	12	0·12	127
		890	8	0·63	666
Dihydroisobotryococcene	CS_2	975	11	0·10	151
		888	8	0·35	536
Rosololactone	CCl_4	996	11	—	110
		910	10	—	180
Deoxyrosololactone	CCl_4	999	8	—	90
		913	9	—	170

sh. = shoulder.
Figures in parentheses are approximate.
* Not measured.

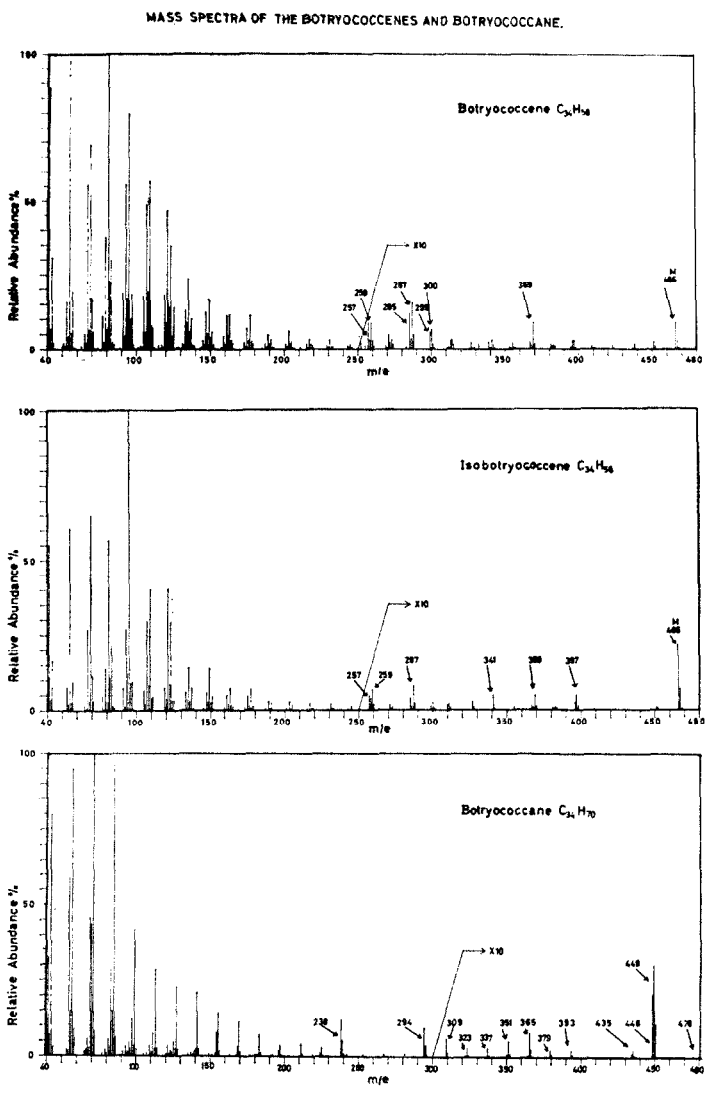
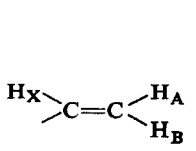


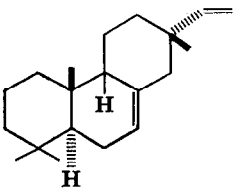
FIG. 5. MASS SPECTRA OF THE BOTRYOCOCCENES AND BOTRYOCOCCANE.

The nuclear magnetic resonance spectra (Fig. 6) of both hydrocarbons show a fairly sharp singlet at 5·4 τ, which was assigned to exomethylene protons and integrated for about eight protons for botryococcene and about six for isobotryococcene. The vinyl group shown to be present by the i.r. evidence was assigned as an ABX (ABC) system (I), corresponding remarkably well with that described by Carman[18] for the hydrocarbon rimuene (II). The two doublets and the singlet in the 5·37–5·1 τ region (rimuene 5·35–5·04 τ) of both spectra were assigned to the AB protons of the vinyl double bond thought to be present in both hydrocarbons. Likewise the singlet, two doublets and singlet in the 4·48–4·2 τ region in the spectrum of botryococcene and 4·46–4·18 τ region in that of isobotryococcene (rimuene 4·4–4·1 τ) were assigned to the X proton of the vinyl double bond. These data suggest that

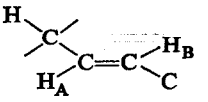
[18] R. M. CARMAN, *Australian J. Chem.* **16**, 1104 (1963).



(I)



(II)



(III)

in both hydrocarbons the vinyl group is attached to a fully substituted carbon atom as in rimuene. In both spectra the *trans* disubstituted double bond protons were assigned as an AB quartet ($J_{AB} = 16$ c/s) with the A part further split by an allylic proton as in (III). Irradiation of the saturated CH, CH_2 region by sweeping the region of absorption between 7·6 and 8·2 τ caused the A part of the AB system to collapse to a doublet as expected.

The NMR spectra of these two hydrocarbons differ only slightly. The spectrum of botryococcene shows no absorption between 7·5 and 7·7 τ whereas that of its isomer has a broad allylic absorption at 7·55 τ integrating for one or two protons. Also the spectrum of botryococcene has a singlet at 8·38 τ whereas that of isobotryococcene has a narrow doublet at the same position. Integration of these signals is compatible with two methyl groups on double bonds in each hydrocarbon. The saturated C-methyl region in both spectra integrates for five or six methyl groups.

Hydrogenation of botryococcene and isobotryococcene afforded the same hydrocarbon botryococcane. In the mass spectrum (Fig. 5) the ion of highest mass is at *m/e* 476. Acceptance of this as the molecular weight implies one unreduced double bond or one ring in the molecule. However, the spectrum is typical of a saturated acyclic hydrocarbon, having all of the important fragment peaks in the C_nH_{2n+1} series. In particular, loss of C_2H_3 from *m/e* 476 to give *m/e* 449 seems very unlikely. A more logical explanation is that the highly branched botryococcane molecule exhibits no parent molecular ion in its mass spectrum and that *m/e* 476 is due to a trace of unsaturated impurity. Similar occurrences have previously been reported.[19] Increasing the unsaturation in a given hydrocarbon molecule increases the relative intensity of the parent molecular ion in its mass spectrum.[20] Thus it is very likely that *m/e* 466 in the spectra of the highly unsaturated botryococcene and isobotryococcene does in fact represent the molecular ion. The botryococcenes therefore have the formula $C_{34}H_{58}$ and botryococcane is $C_{34}H_{70}$. This latter formula corresponds to a molecular weight of 478 and this was supported by osmometric measurements. On the basis of this molecular weight and formula a satisfactory and self-consistent explanation of most of the characteristic peaks in the botryococcane spectrum can be given.

On electron impact saturated hydrocarbons fragment preferentially at the branching points. The positive charge remains on the more highly substituted carbon atom and elimination of the longest carbon chain attached to the branching point is favoured. Further, the positive ions thus formed tend to lose a hydrogen atom so that such fragmentations give rise to doublets at C_nH_{2n+1} and C_nH_{2n}. The absence of an $(M\text{-}15)^+$ ion in the mass spectrum of botryococcane is not surprising although there are twelve or thirteen methyl groups present (see below). The methyl radical is the least stable of the alkyl radicals and will not be eliminated readily if other fragmentations are facile. The ions at *m/e* 449 and 448 are probably due to loss of the ethyl group formed by hydrogenation of the vinyl group of botryococcene

[19] K. BIEMANN, *Mass Spectrometry*, p. 81, McGraw-Hill, New York (1962).
[20] K. BIEMANN, *Mass Spectrometry*, p. 51, McGraw-Hill, New York (1962).

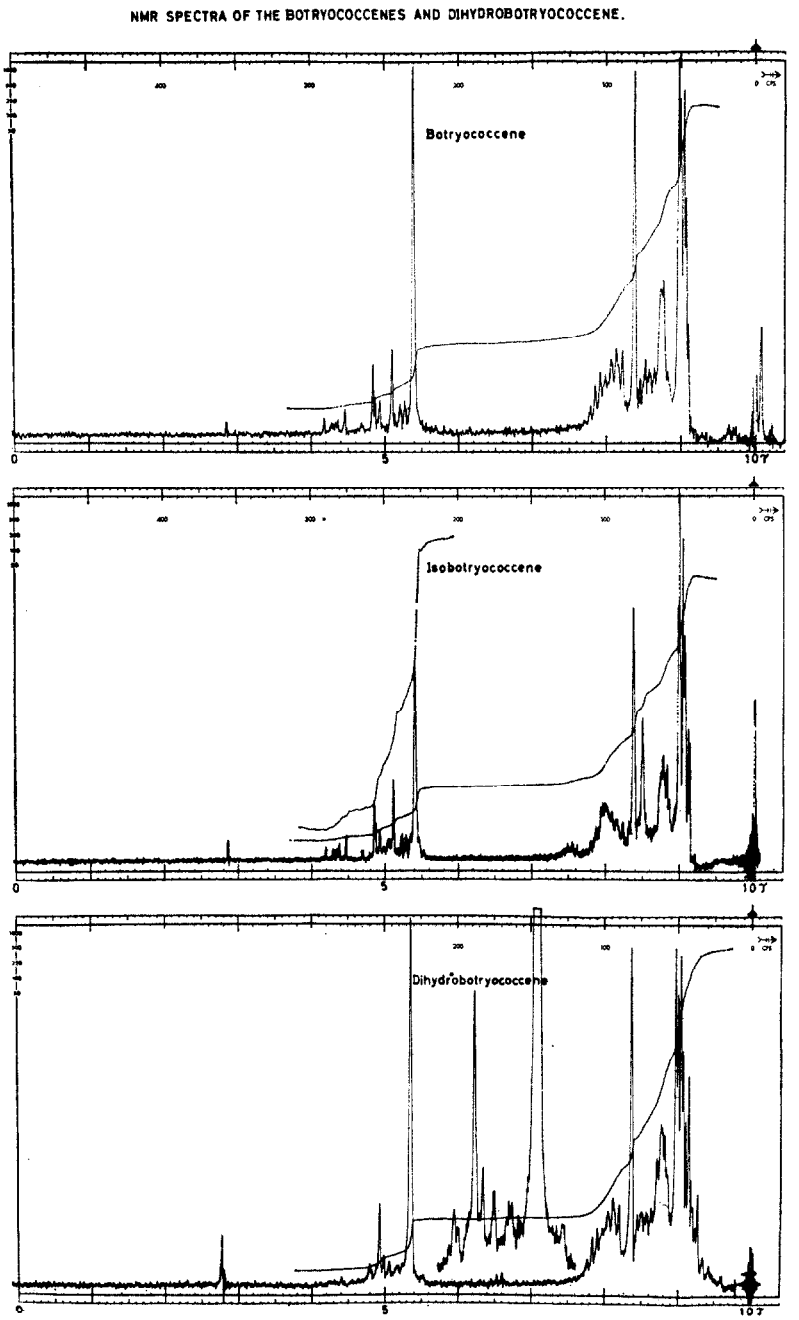
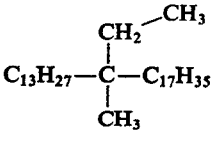
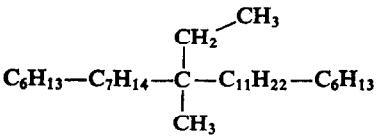


FIG. 6. NUCLEAR MAGNETIC RESONANCE SPECTRA OF THE BOTRYOCOCCENES AND DIHYDROBOTRYOCOCCENE.

and isobotryococcene. These ions are very intense, providing further evidence that the vinyl group of botryococcene and isobotryococcene is attached to a tetrasubstituted carbon atom. Likewise the ions at *m/e* 295 and 294 (M-$C_{13}H_{27}$) and 239 and 238 (M-$C_{17}H_{35}$) are very much more intense than their neighbours. On this basis IV is a possible schematic structure for botryococcane.

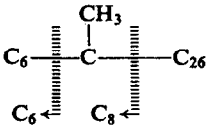


(IV)

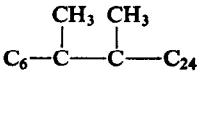


(V)

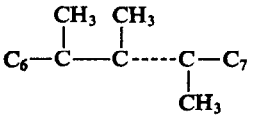
The ion at 435 (M-C_3H_7) is very small in intensity but this does not exclude isopropyl groups from the structure of botryococcane since the mass spectrum of squalane also shows a negligible loss of a C_3 fragment. There is no observable loss of a C_4 or C_5 fragment in the spectrum of botryococcane, so it is unlikely that there is a branch situated on the fifth or sixth carbon atoms from either end of the hydrocarbon chain. This indicates that there is at least a six carbon chain before branching occurs, as in V. In V a methyl branch on C_7 would give an $(M\text{-}C_6)^+$ secondary ion at *m/e* 393 on electron impact. Such a fragmentation would also give an (M-C_8) primary ion as in VI.



(VI)

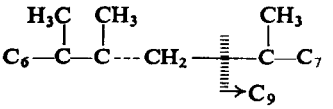


(VII)



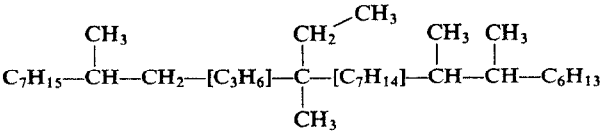
(VIII)

However, in the mass spectrum of botryococcane the $(M\text{-}C_8H_{17})^+$ ion is much more intense than its neighbours. It therefore seems more likely that this ion is a secondary ion as would be expected from VII. This partial structure explains the presence of the large $(M\text{-}C_8H_{17})^+$ ion since it would produce a secondary carbonium ion as well as a secondary radical on electron impact. Structure VII also explains the $(M\text{-}C_6H_{13})^+$ ion but not the $(M\text{-}C_7H_{15})^+$ ion. Botryococcane therefore probably has another chain of seven carbon atoms attached to the branching point, as in VIII. The ion at *m/e* 351 (M-C_9) is slightly more intense than expected and may be a primary ion arising from fission at a branching point as in IX.

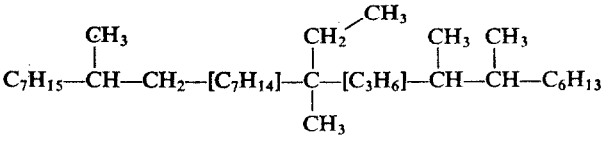


(IX)

From the mass spectral evidence presented above (X) and (XI) are possible partial structures for botryococcane.



(X)



(XI)

The i.r. spectrum of botryococcane shows no double bond absorption and under conditions of high resolution the methyl bending region shows absorption at 1386, 1378 and 1366 cm^{-1} (Fig. 7). The same region for squalane is also shown for comparison. In squalane the

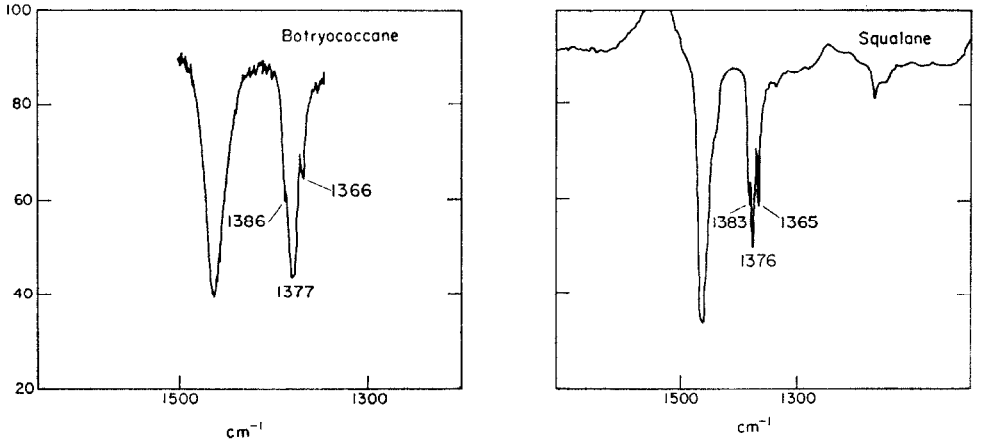
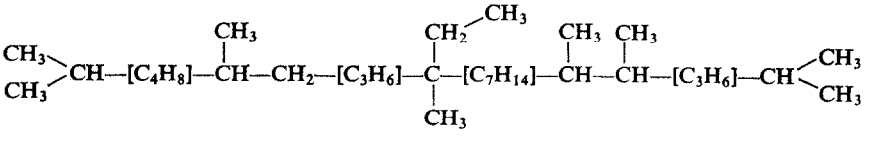


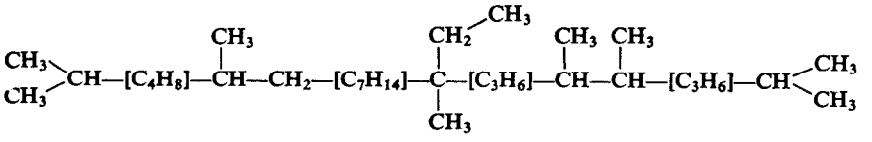
FIG. 7. INFRARED SPECTRA OF BOTRYOCOCCANE AND SQUALANE IN THE δ(C-H)REGION(CCl_4).

bands at 1386, and 1365 cm^{-1} arise from the four geminal methyls of the two isopropyl groups. For squalane the 1365 cm^{-1} band has ϵ_a *ca.* 143 (Table 2) whereas the 1366 cm^{-1} band in the spectrum of botryococcane has ϵ_a *ca.* 99, so it is probable that botryococcane has one or two gem dimethyl groups. It appears that the latter situation is the more likely since botryococcene appears from NMR evidence to have two methyl groups attached to exomethylene double bonds.

Possible partial structures for botryococcane are therefore as follows (XII and XIII), if there is indeed a gem dimethyl group present at each end of the chain.



(XII)



(XIII)

The i.r. spectrum of botryococcane (Fig. 7) also shows this hydrocarbon to be more highly branched than squalane since the ratio of the integrated area of the δCH_2, CH_3 band to the area of the δCH_3 bands is 2:1 for squalane compared with 1·57:1 for botryococcane. The very high degree of branching is also shown by the nuclear magnetic resonance spectrum of botryococcane (Fig. 8); indeed the methyl region integrates for twelve or thirteen methyl

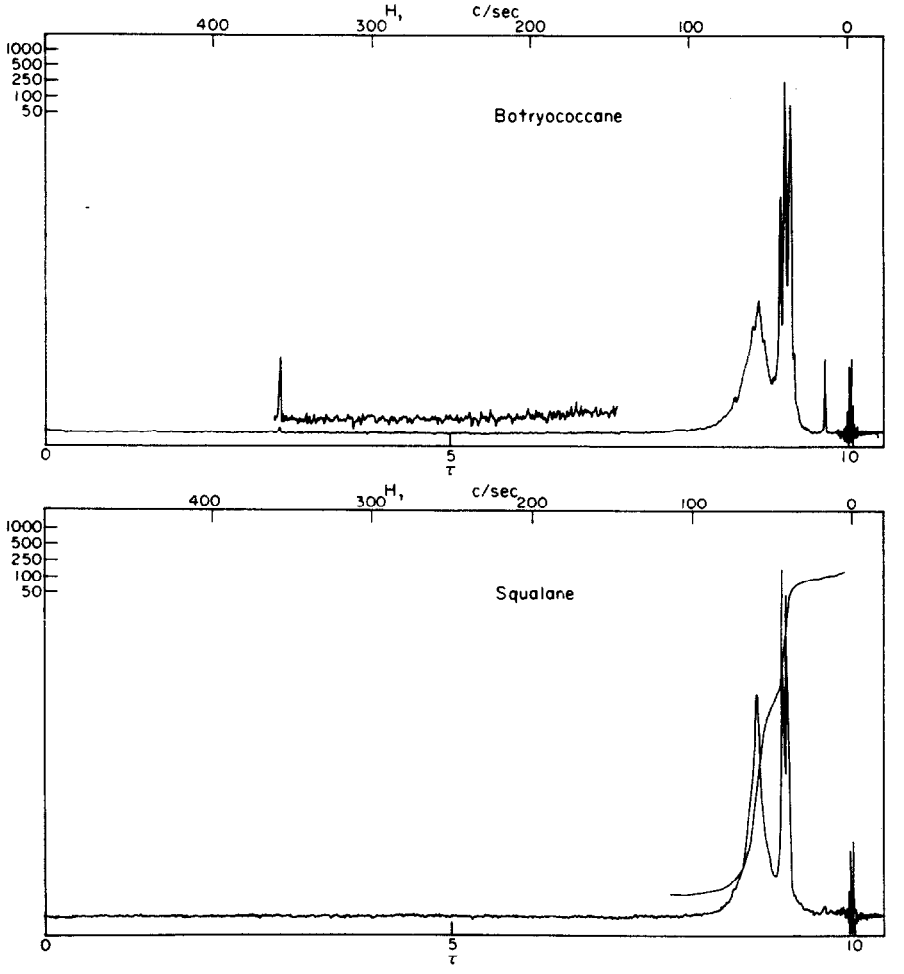


FIG. 8. NUCLEAR MAGNETIC RESONANCE SPECTRA OF BOTRYOCOCCANE AND SQUALANE.

groups and the above possible partial structures when expanded will need to take this into account. The NMR spectrum of squalane is included in Fig. 8 for comparison, to show the greater proportion of methyl groups to methylene and methine groups on botryococcane. These data suggest that botryococcene does not have a simple isoprenoid skeleton.

Attempts to partially hydrogenate the hydrocarbon fraction of *B. braunii* with palladium on charcoal were unsatisfactory since isomerization of the double bonds appeared to be taking place in addition to the hydrogenation. The intention was to see if *trans* mono-olefins derived from botryococcene and isobotryococcene could be obtained by thin-layer chromatographic separation of the products. However gas–liquid chromatography of the fractions with *trans* disubstituted absorption in the i.r. showed that they consist of complicated mixtures of products. Partial reduction of the hydrocarbon fraction with P-2 nickel boride,[21] which inhibits isomerization, was more successful. Preparative scale silver ion thin-layer chromatography allowed the isolation of two partially reduced products, viz. dihydrobotryococcene and dihydroisobotryococcene, whose infrared spectra showed the disappearance of the vinyl double bond in each case. This was confirmed by the mass spectra which showed the parent ions to be at *m/e* 468 in each case. Furthermore, the disappearance of the vinyl double bond was shown by the nuclear magnetic resonance spectrum of the dihydrobotryococcene (Fig. 6). These data confirm the presence of the vinyl double bond in botryococcene and isobotryococcene since P-2 nickel boride only reduces such bonds. Table 1 summarizes both the groups thought to be present and those known to be absent in the structure of botryococcene.

DISCUSSION

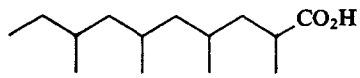
There is no doubt that *Botryococcus braunii* gives rise to Coorongite since the transformation has been observed,[22, 23] and the morphological evidence[2] indicates that it also gives rise to the algal coals. In fact the algal sediment Torbanite (Carboniferous, 300×10^6 years) is almost entirely composed of minute yellow globules morphologically resembling colonies of *B. braunii*.

It was this resemblance, first brought to our attention by Professor T. N. George and Dr. W. D. I. Rolfe of the Geology Department at Glasgow University, which led to the present study.

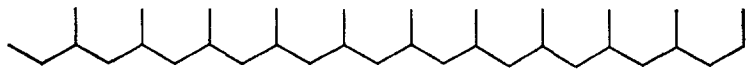
The finding that the colony matrix of the living alga almost entirely comprises two highly unsaturated hydrocarbons of high molecular weight accounts for the observed formation of Coorongite and presumably Torbanite. Polymerization of the botryococcenes, perhaps as a result of bacterial action, sunlight, heat or atmospheric oxidation would result in the formation of Coorongite. This appears likely since the botryococcenes were found to polymerize readily on standing. Also a small sample of *B. braunii* became rubbery in texture after exposure to the atmosphere at room temperature for 48 hr. The botryococcenes could still be recovered from the exposed *B. braunii*, but in much smaller quantities. This provides further evidence that polymerization of the botryococcenes is intimately connected with the formation of Coorongite.

[21] H. C. BROWN and C. A. BROWN, *J. Am. Chem. Soc.* **85**, 1005 (1963).
[22] A. C. BROUGHTON, personal communication to H. R. J. CONACHER, *Oil Shale and Cannel Coal*, Institute of Petroleum (1938).
[23] E. DE HAUTPICK, *Bull. Soc. Géol. France*, Ser. **4**, 26 (1926).

Two biosynthetic pathways are known which could lead to a highly branched long chain hydrocarbon, viz. the isoprenoid pathway and the polyketide pathway. In the polyketide pathway there are a number of possible starter units including acetyl CoA, propionyl CoA, isobutyryl CoA and α-methylbutyryl CoA. Highly branched systems are found where one of the extenders is methylmalonyl CoA, an example being 2,4,6,8-tetra-methyldecanoic acid (XIII) isolated from the preen gland of the mute swan.[24]



(XIII)

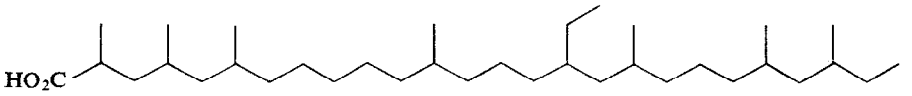


(XIV)

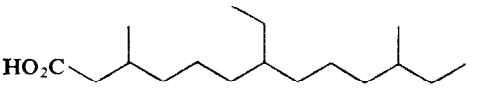
It is easy to envisage a highly branched C_{34} hydrocarbon (XIV) which could be synthesized by the simple polyketide pathway using acetyl CoA as starter and methylmalonyl CoA as extender. This structure has the requisite number of methyl groups but obviously does not satisfy the other evidence obtained for the structures of the botryococcenes. Kates[25] has reviewed the methods whereby unsaturation may be introduced into a polyketide. Normally unsaturation is introduced after synthesis of a saturated structure, but in the botryococcenes it may be difficult for the alga to introduce the variety and number of double bonds found.

The isoprenoid pathway does not readily afford a suitable skeleton but there is no difficulty in accommodating the variety and number of double bonds since examples of these types of bonds are well known in the terpene and steroid series. Additional methyl substitution in isoprenoid biogenesis is normally the result of methylation with methionine. The same process of alkylation can also occur with non-isoprenoid structures. It is known that in plant sterols the isoprenoid side chain can be methylated[26] and there appears to be no reason why methylation should not occur with an acyclic isoprenoid thereby resulting in a more highly branched structure. If the botryococcenes are indeed alkylated isoprenoids then alkylation with a C_2 unit might provide the vinyl group, as an alternative to its being derived from a pre-existing isoprenoid unit. There seems little point in further discussing possible biogenetic pathways while the structures still require complete elucidation. However, several natural products with unusually branched carbon skeletons have been reported recently, for instance monensic acid[27] and the juvenile hormone;[28] the carbon skeletons of these two compounds are illustrated diagramatically in (XV) and (XVI) respectively. A novel six carbon unit, based on homomevalonic acid (C_7), has been suggested[29] as a possible source of the ethyl groups in (XVI): such a unit would parallel the C_3, C_4 etc. acyl starter units now known to act in the same way as the long-known C_2 acyl starter in polyketide biogenesis.

[24] G. Ödham, *Ark. Kem.* **23**, 431 (1965).
[25] M. Kates, *Ann. Rev. Microbiol.* **20**, 13 (1966).
[26] L. J. Goad and T. W. Goodwin, *Biochem. J.* **99**, 735 (1966).
[27] A. Agtarap, J. W. Chamberlin, M. Pinkerton and L. Steinrauf, *J. Am. Chem. Soc.* **89**, 5737 (1967).
[28] H. Röller, K. H. Dahm, C. C. Sweeley and B. M. Trost, *Angew. Chem.* **79**, 190 (1967).
[29] C. Sweeley, personal communication.



(XV)



(XVI)

In addition there remains the problem of why this unique alga, at a certain stage of its life cycle, synthesizes an extensive colony matrix comprising these highly unsaturated hydrocarbons. Numerous possibilities come to mind: *inter alia*, provision of a food store, a flotation sack and a protection device during difficult environmental conditions.

Again, evolutionary selection may have arisen as a result of the hydrocarbons proving distasteful or toxic to foraging zooplankton, or preventing parasitism by fungi.[30] Laboratory culture of the alga generally produces a growth which has much less oil evident in the colonies: such cultures are green.

Work is proceeding on the structural elucidation of the botryococcenes.

EXPERIMENTAL

Botryococcus Braunii Kützing

The sample used in the present study was collected from an extensive orange bloom floating on the surface of Oakmere, Cheshire, England (map reference SJ (33) 575677, 1-inch sheet no. 109).

Thin Layer Chromatography

Silica gel (Kieselgel G) impregnated with 10 per cent by weight of silver nitrate[31] was used as absorbent. The plates were activated at 120° for approximately 1 hour and stored in a desiccator. Preparative plates were always cleaned by development in ethyl acetate prior to activation. Detection was achieved by spraying with 50 per cent polyphosphoric acid followed by charring at 200°. Preparative plates were sprayed with a 0·2 per cent solution of 2′,7′-dichlorofluorescein in ethanol and viewed under an u.v. lamp (254 nm).

Gas–Liquid Chromatography

Gas–liquid chromatograms were obtained on a Perkin-Elmer Model F-11 equipped with a flame ionization detector. Retention times were measured on a 10 ft × 1/16 in. stainless steel column operating at 240° and a flow rate of 25 ml/min unless otherwise stated. The column was packed with Chromosorb G (100–120 mesh), coated with 2 per cent seven-ring *m*-polyphenyl ether.

Infrared Absorption Spectroscopy

I.r. spectra were recorded on a Unicam SP 100 double beam spectrophotometer, equipped with an SP 130 sodium chloride prism-grating double monochromator and operated under vacuum conditions (accuracy ± 1 cm^{-1}).

Nuclear Magnetic Resonance Spectroscopy

NMR spectra were recorded on a Varian HA 100 (100 megacycles) spectrophotometer.

Mass Spectrometry

Mass spectra were recorded on an AEI MS9 double focusing mass spectrometer.

Combined Gas Chromatography-Mass Spectrometry

The instrument used was the LKB 9000 gas chromatograph-mass spectrometer equipped with a 10 ft × 0·12 in. i.d. glass column packed with 1 per cent SE-30 on Gas Chrom P. The carrier gas was helium with a flow rate of 30 ml/min. The scanning time used was 4 sec.

[30] Dr. JOHN WEST, personal communication.

[31] L. J. MORRIS, in *New Biochemical Separations* (edited by A. T. JAMES and L. J. MORRIS), pp. 295–319, Van Nostrand, London (1964).

Extraction of the Lipid Fraction

An aliquot (800 ml) of the greeny-orange algal suspension was freeze dried (12 hr) and the resulting orange powder (12 g) was extracted (30 min) in an ultrasonic tank with acetone (Analar grade, 400 ml). The suspension was centrifuged at 3000 rev/min (20 min) and the yellow extract was decanted off. The extraction was repeated five times, whereupon the last extract was colourless. Evaporation of the combined extracts afforded a brownish-green oil (10 g, 83 per cent of the dry wt. of *Botryococcus braunii*).

Isolation of the Total Hydrocarbon Fraction and the Botryococcenes

The combined extracts were dissolved in the minimum volume of *n*-hexane and chromatographed on neutral alumina (70 g). Elution with *n*-hexane (800 ml) gave the hydrocarbon fraction as a colourless oil (9·14 g, 76 per cent of the dry wt. of *B. braunii*) on evaporation. An aliquot of the hydrocarbon fraction (51·5 mg) afforded botryococcene (45 mg) and isobotryococcene (4·3 mg), after preparative silver ion thin-layer chromatography (benzene developer). The botryococcene was shown to be 96 per cent pure by gas–liquid chromatography ($t_R = 0{\cdot}81$ relative to *n*-octacosane). GLC (Pye Argon chromatograph) on 4 ft × 3/8 in. glass columns packed with Gas Chrom P, 100–120 mesh, coated with 1 per cent CHDMS/2 per cent PVP, 1 per cent DC710 and 1 per cent polymer Z respectively, failed to reveal any further separation. The isobotryococcene was shown to be 98 per cent pure by gas liquid chromatography ($t_R = 0{\cdot}76$ relative to *n*-octacosane). Once again the gas chromatographic conditions used above failed to reveal any further separation.

Subsequent examination on capillary columns did not further segregate the hydrocarbons but it is still possible that they are mixtures of stereoisomers or closely related positional isomers.

Hydrogenation of Botryococcene to Botryococcane

Botryococcene (75 mg) was hydrogenated with stirring for 4 hr in ethyl acetate (20 ml) in the presence of 10 per cent Pd on C (10 mg) until no more H_2 was absorbed. Removal of the catalyst, evaporation of the solvent, and preparative silver ion TLC gave the fully saturated hydrocarbon, botryococcane (60 mg, $R_f = 1{\cdot}0$ relative to *n*-octacosane, $t_R = 1{\cdot}0$ relative to *n*-octacosane).

Reduction of the Botryococcenes by the Method of Brown and Brown[21]*—Dihydrobotryococcene and Dihydroisobotryococcene*

Nickel acetate (0·62 g, 2·5 m. mole) was dissolved in benzene-free ethanol (20 ml, 95 per cent) in the hydrogenation flask. The system was flushed out with H_2 and $NaBH_4$ (2·5 ml of a 1·0 M solution) in ethanol was added. An aliquot (200 mg) of the hydrocarbon fraction of *B. braunii* was slowly added via a side arm and the hydrogenation was allowed to proceed (3 hr) at atmospheric pressure. The solution was evaporated to dryness and the residue rinsed with *n*-hexane. The resulting solution was passed through a small column of neutral alumina (5 g). Evaporation of the solvent and azeotroping with benzene gave the crude product as a colourless oil (170 mg) whose i.r. spectrum was very similar to that of the starting material. Preparative thin-layer chromatography (benzene developer) afforded four fractions which were then investigated. The fractions were shown to be: (*a*) dihydroisobotryococcene (9 mg, $t_R = 0{\cdot}82$ relative to *n*-octacosane), (*b*) dihydrobotryococcene (55 mg, $t_R = 0{\cdot}79$ relative to *n*-octacosane), (*c*) unchanged botryococcene, (*d*) unchanged isobotryococcene.

Acknowledgements—The authors wish to thank Mr. J. Osborne, warden of Rostherne N.N.R. for collecting the algal samples. The authenticity and purity of the samples were vouched for by Dr. J. W. G. Lund of the Freshwater Biological Association, Windermere and Dr. E. Conway of the Botany Department, Glasgow University. Dr. Lund also kindly arranged for the collection of the *B. braunii*. Photomicrographs were made by Dr. J. S. Gillespie of the Physiology Department, Glasgow University. We also wish to thank the Science Research Council for a Research Fellowship (A. McC.), and a maintenance grant (J. R. M.), and the Natural Environment Research Council for a Research Associateship (A.G.D.) and general support (GR/3/655). The support of the National Aeronautics and Space Administration is also gratefully acknowledged (NASA grant NsG 101-61, Subtask C). The gas chromatograph-mass spectrometer was purchased with a generous grant (B/SR/2398) from the Science Research Council to Drs. G. Eglinton and C. J. W. Brooks.

We thank Mrs. F. Lawrie for the i.r. measurements, Dr. W. McCrindle and Mr. James Galt for the NMR data and helpful suggestions relating to them, and Misses Helen Humphreys and Janice Malcolm for assistance with the mass spectral measurements. We also gratefully acknowledge helpful advice from Dr. C. A. Brown in respect of the hydrogenation experiments and Professor T. N. George, F.R.S., and Dr. W. D. I. Rolfe for their suggestions and keen interest.