ECOLOGICAL AND PHYSIOLOGICAL STUDIES ON THE GREEN ALGA BOTRYOCOCCUS BRAUNII (KÜTZ.)

Thesis presented by

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GENERAL INTRODUCTION

The planktonic freshwater alga Botryococcus braunii (Kütz.) has been of interest since Chodat (1896) drew attention to its unusual form and metabolic activity and since it was established as the causal organism of the Scottish boghead coals (Bertrand, 1893; Renault, 1899) [Appendix 1] and Australian coorognite (Thiessen, 1925). A recent (1965) massive "bloom" of the alga led to Maxwell and Eglinton's paper on the hydrocarbons of this material (1968) and suggested that further knowledge of the metabolic processes of the plant was called for.

The present studies attempt to define conditions under which the alga grows and builds up the wide range of hydrocarbons and fats that are found in the colonies. Actively growing axenic cultures have been obtained which have allowed the use of radioactive techniques in the study of hydrocarbon synthesis.

Two sources of material have been available for the studies: (a) collections from the wild made at Oakmere, Cheshire and Loch Lomond, Dunbartonshire (Material W) and (b) cultures from the Cambridge Culture Collection (Culture No. 207/1B) (Material C).

1. Definition of the Species

The genus Botryococcus with its one species B. braunii was first defined by Kützing in his Species Algarum of 1849 as small, much lobed, irregular, botryoidal colonies enclosed by a delicate hyaline gelatinous membrane, varying in colour between green, dark green and deep red.

W. and G.S. West (1897; 1903) failed to recognise B. braunii in collections from the West of Scotland and named their material Ineffigiata neglecta. They described the colonies as having a central cavity, covered by an outer tough elastic membrane. Expelled cells were thought to be non motile spores. Later the name Ineffigiata neglecta was abandoned. Chodat (1896) gave a detailed account of the structure of B. braunii and assigned it to the Chlorophyceae on account of its green colour and starch production. Pascher (1925) was not convinced of the production of starch and classified it with the Heterokontae. West and Fritsch (1927) agreed with this classification and placed the alga in the Heterocapsales. Blackburn and Temperley (1936) in another detailed description of the plant demonstrated the presence of starch grains in the colonies and assigned the alga to the Chlorophyceae. Engler (1954) was also of the view that the alga was a member of the Chlorophyceae. Belcher and Fogg (1955) showed the presence of chlorophyll b so confirming the alga as a member of the Chlorophyceae.

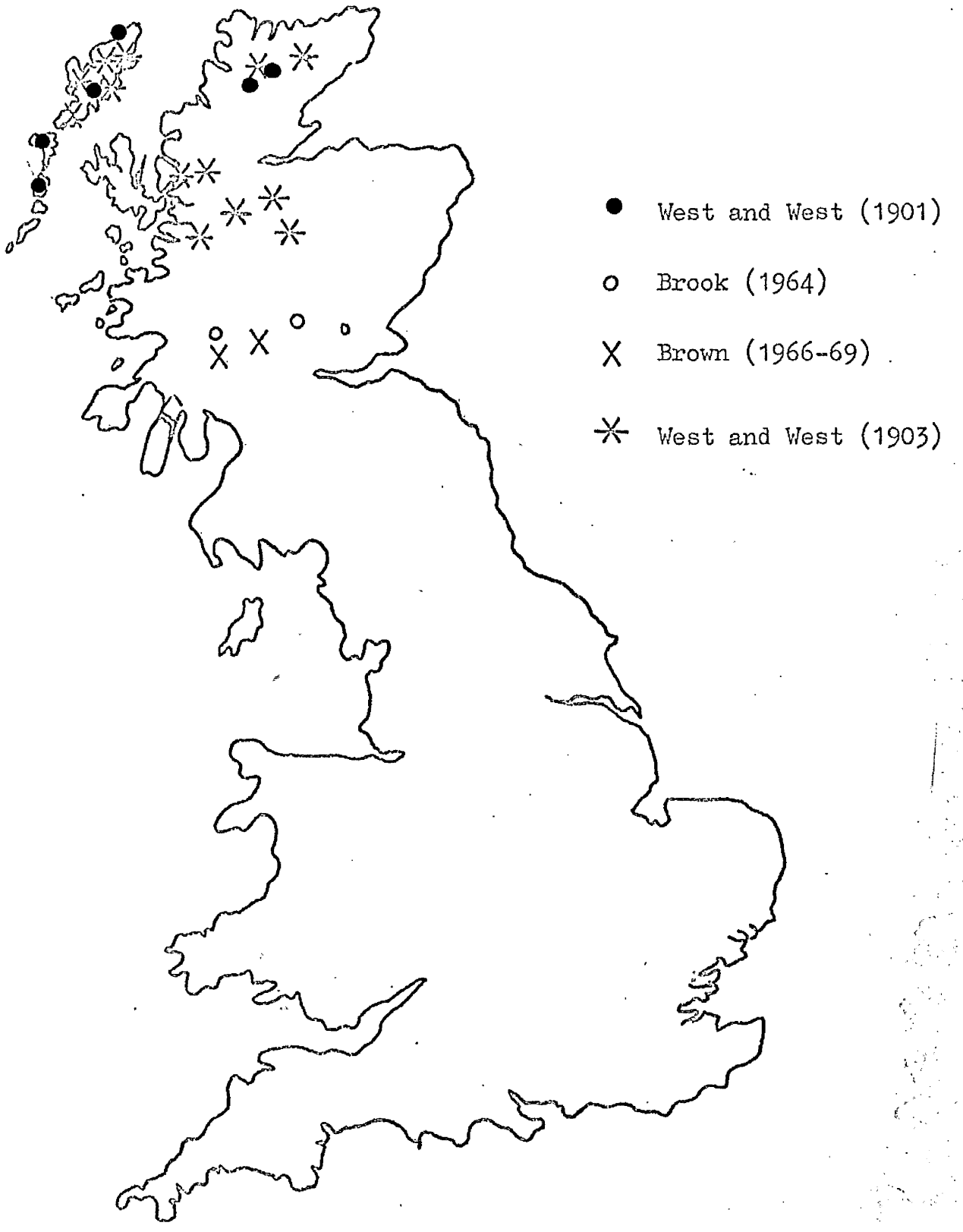


Fig. 1. Records of B. braunii from Scottish sites

Key to Fig. 1.

	Location
⊙ West and West (1901)	
Loch Shin	Sutherland
Loch a Gharbh Bhaid Mhoir	Sutherland
Loch Mor Bhatabhais	Lewis
Loch Laxadale	Harris
Loch nan Eun	North Uist
Loch a Bhursta	Benbecula
✳ West and West (1903)	
Loch Bairness, Loch na Cloiche Sgoilt, Loch na Criche, Loch Gorma	Inverness
Loch Luichart, Loch Rosque	Ross-shire
Loch nan Cuinne, Loch Ruar	Sutherland
Loch Cuthaig, Loch Langa bhat, Loch Roinebhall, Loch an Sgath, Loch Stranabhat	Lewis
Loch Laxadale	Harris
X Brown (1966-69)	
Loch Lomond	Stirlingshire
Lake of Mentieth	Perthshire
Loch Drinishader, Plocrapool Loch	Harris
o Brook (1964)	
Loch Aslaich	
Loch Lomond	Stirlingshire
Loch Ard	Stirlingshire
Loch Leven	Fife

2. Distribution

Botryococcus braunii is cosmopolitan in its distribution being found in both tropical and temperate countries. The original description (Kützing, 1849) was of material found by A. Braun in Lake Neoburgensi, Switzerland and it is common in Europe as far north as Scandinavia and is reported in Africa, Asia, America, Australia and New Zealand. In Australia it gives rise to the peat-stage of the boghead coals, i.e. the "rubbery mats" termed coorognite (Thiessen, 1925). It is occasionally found in salt or brackish waters, e.g. Lake Balkash - a saline lake in Northern Asia (Blackburn and Temperley, 1936), though Belcher (1957) pointed out that this lake is not altogether a saline lake as one end is kept fresh by rivers discharging into it. Lind (1968) reported B. braunii as one of the more common planktonic species for some Kenyan Lakes.

In the British Isles it is widely, but sparsely distributed. Pearsall (1925) records it in a number of the lakes in Westmorland while Lind (1944) and Lind and Galliford (1952) found it to be a dominant alga in Oakmere, Cheshire. A particularly heavy "bloom" was found in November, 1965 (Conway, 1967) and Swale (1968) has reported the presence of the alga in Oakmere during 1966-68.

In Scotland West and West (1897; 1903) reported the occurrence in eight lochs on the mainland, five on Lewis and one on Harris. West and Fritsch (1927) also reported the alga from Harris. Brook (1964) reported it from three of the Scottish Lochs he investigated. Known records for Scottish sites are shown in Figure 1. During 1967 and 1968 two small summer blooms occurred in Loch Lomond and were washed up at Rowardennan

(map ref. 26/37 39 54). On both occasions this material consisted of brown colonies. On the first occasion it was present in plankton samples in about equal concentration with Coelosphaerium, but on the second occasion it proved to be an almost unialgal culture.

Thus, although widely distributed, the slow growth of B. braunii in the field (Swale, 1968) may account for its low density.

Plate 1. Oakmere bloom of B. braunii (1965)

PART I - ISOLATION FROM WILD COLLECTIONS AND DEVELOPMENT OF MATERIAL W. IN CULTURE

Colonies of B. braunii were collected from the Oakmere bloom of 1965 (Plate 1) and cultured in modified Knop's solution (Appendix 2) at a light intensity of 250 foot candles and a temperature of 15°. After about three weeks in culture these colonies gave rise to large green cells which increased in size to about 25μ across and then ceased to develop.

Material was obtained at fortnightly intervals from Oakmere by seventy five yard plankton tows both at the surface of the Mere and at a depth of one metre. As these samples contained very little B. braunii six inch mud cores were taken from a point 1-2 inches in depth and examined. The cores were placed in museum jars two-thirds full of glass distilled water and left overnight. Next day the B. braunii colonies, which were more plentiful in the mud, were removed from the surface of the water with a Pasteur pipette. Colonies were also collected by planktonic tows from Loch Lomond at Rowardennan, Stirlingshire, during June, 1967. In this case the colonies were usually present in about equal proportions with species of Asterionella, Coelosphaerium and Scenedesmus, although during a small summer bloom (1968) B. braunii was present at Rowardennan as an almost unialgal culture. Separation of the Botryococcus from other genera in this case was achieved by differential centrifugation in sucrose solutions of graded concentration

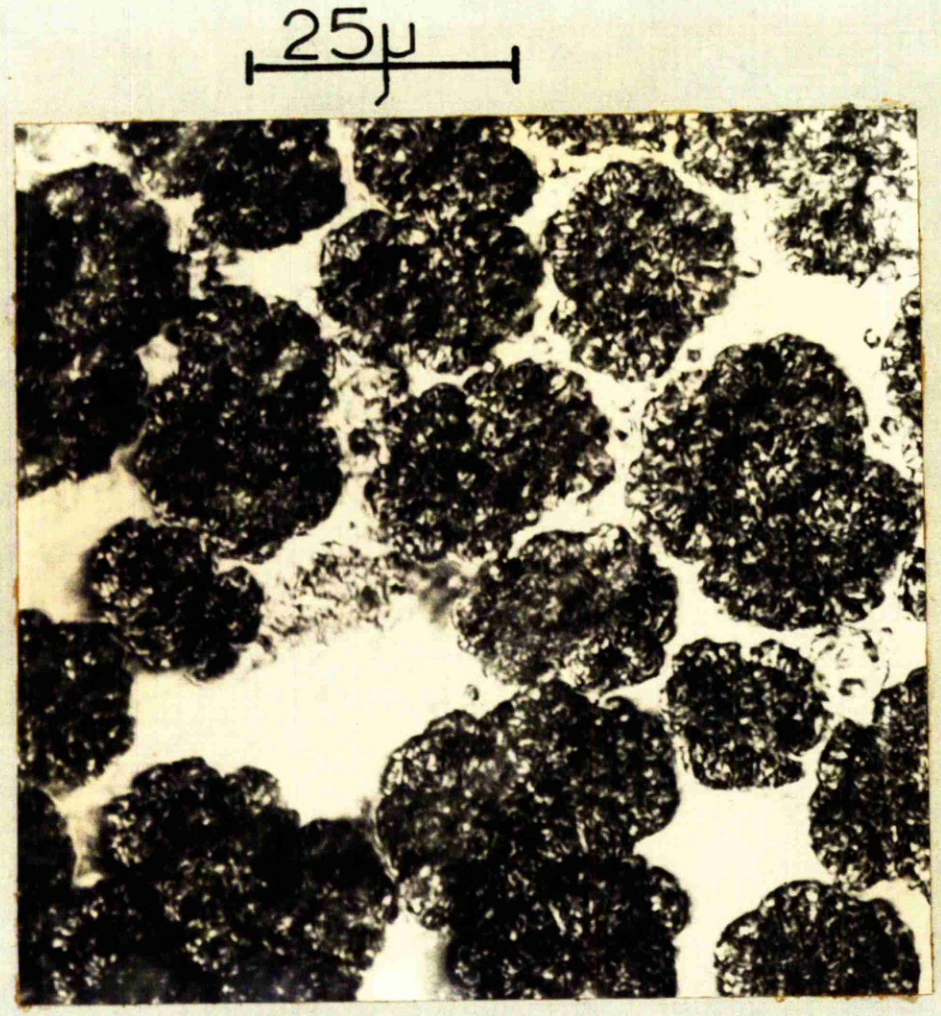


Plate 2. Brown colonies from Oakmere bloom

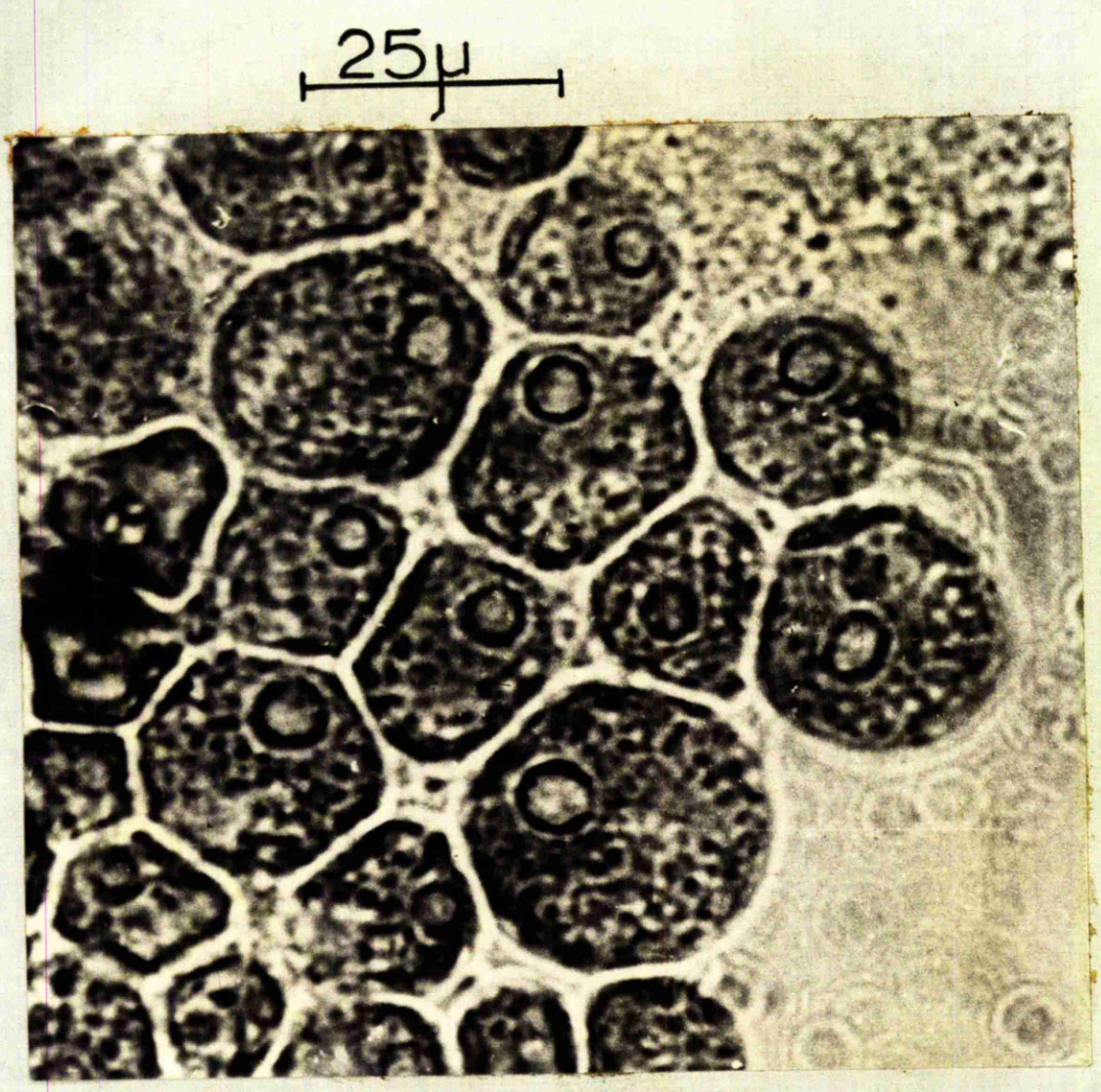


Plate 3. Large green cells forming sheet-like masses

(Appendix 3). Isolated colonies from both Oakmere and Loch Lomond were finally cleaned and prepared for culturing as follows:

A 0.5 ml. aliquot of the colonies was plated out on to 1% Oxoid No.3 Agar prepared in petri dishes. With a binocular microscope and two finely drawn sterile Pasteur pipettes (Pringsheim, 1946) it was possible to remove individual colonies from the agar and to place them in a drop of sterile glass distilled water in a watch glass (Plate 2). After a close microscopic examination to ensure that only B. braunii colonies had been placed in the watch glass the colonies were each moved one at a time through ten similar drops of sterile glass distilled water using a clean sterile Pasteur pipette for each transfer. A final microscopic examination was made of each of the final drops for contaminants after all the transfers had been made. The cleaned colonies were then collected together in tens. Each batch of ten colonies was then used as the inoculum for a 10 ml. tube culture of modified Chu 13 medium, (Appendix 2) and grown at 20° and a light intensity of 250 foot candles from warm white fluorescent tubes for twentyfour hours per day.

When examined after three weeks the colonies in the tube cultures had given rise to large green cells, some of which were stuck together to form sheet-like masses (Plate 3). These cells were about 25μ across and often had a distinct pyrenoid-like body. Cells which were separated from the main mass always seemed to grow a little larger, i.e. to about 30-35μ across. Little obvious morphological change occurred in these large cells even if left in culture for up to twelve weeks. Material isolated from Loch Lomond, in the above manner, behaved similarly giving

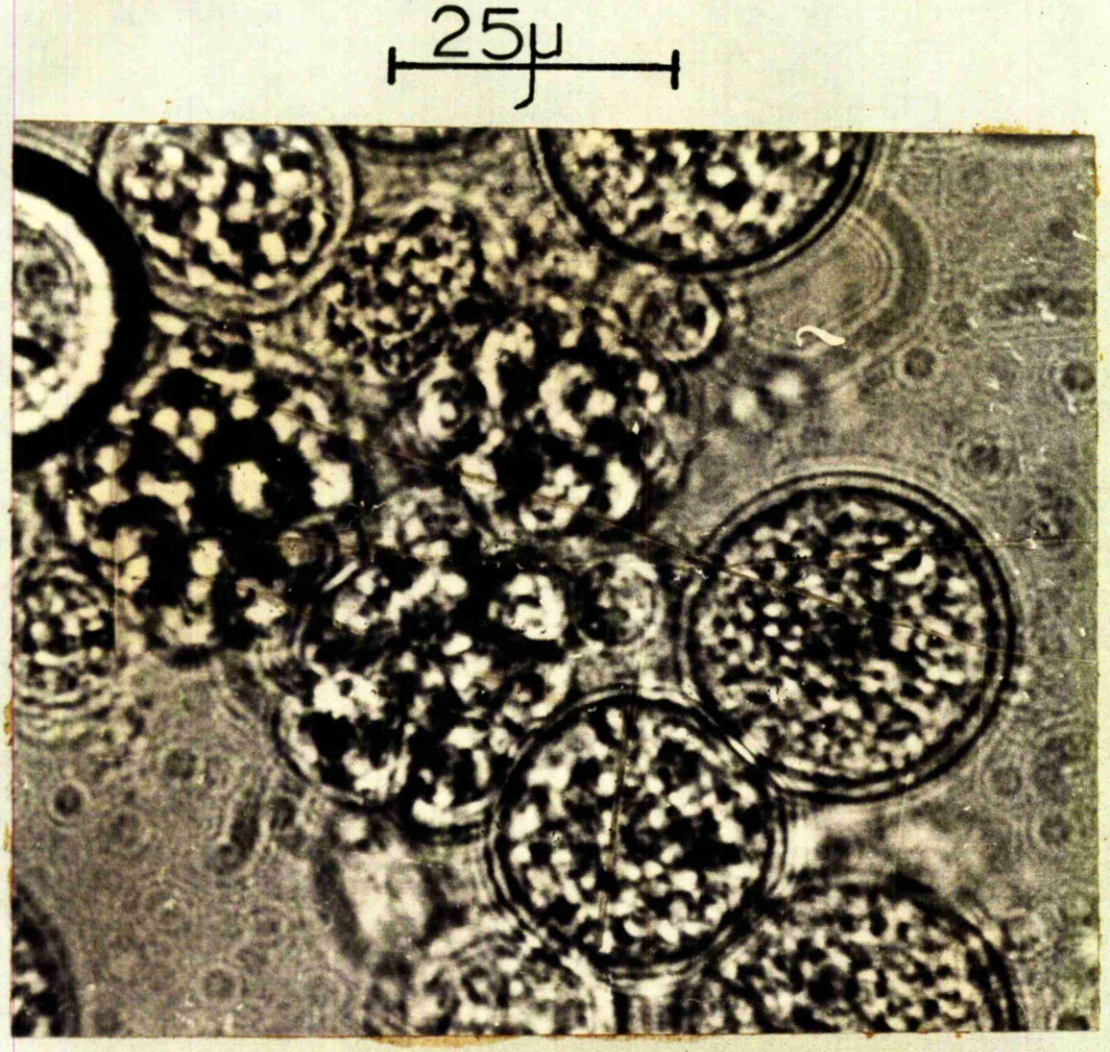
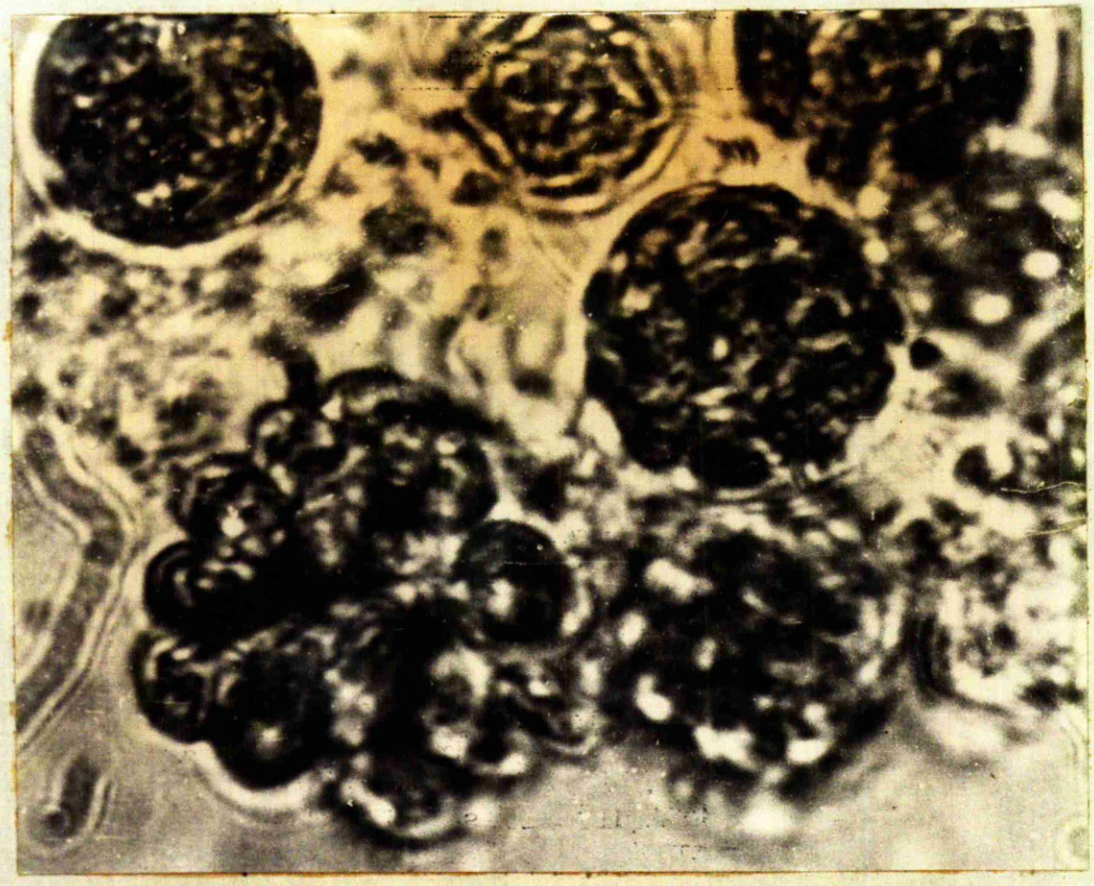


Plate 4. Mulberry-like units produced from Loch Lomond material in culture

rise to large green cells almost identical to those formed by the Oakmere material. The possibility that the large green cells may have been contaminants may be ruled out as on the eight separate occasions that isolations have been made the large green cells have been the constant product of the cultures.

Twice, about two weeks after isolation, large green cells from Loch Lomond material gave rise spontaneously to peculiar tight mulberry-like units in culture (Plate 4). They were smaller than the large green cells (18μ across on average) and were not unlike the wild Botryococcus material in habit except that they were green, and more closely knit. An attempt was made to culture the mulberry habit regularly from the large green cells by the addition of other nutrients to the culture medium. Vitamin B_{12} (at concentrations of 10, 5, 1 p.p.m.) or indole acetic acid (10, 5, 1 p.p.m.) or gibberellic acid A_3 (10, 5, 1 p.p.m.) were added to the nutrient medium in which the colonies were cultured after isolation. The cultures were prepared in duplicate as 10 ml. cultures containing the final concentration of the relevant additive. Controls containing only modified Chu 13 and others containing modified Chu 13 + 0.1 ml. $N/_{10}$ NaOH were included (0.1 ml. of $N/_{10}$ NaOH was used in the indole acetic acid treatments to dissolve the acid). After autoclaving and cooling the media was inoculated with 1 ml. of material isolated from Loch Lomond and since maintained at 15° in modified Chu 13 for a fortnight. After inoculation the cultures were kept at 15° and a continuous light intensity of 250 foot candles and left undisturbed for a period of one month except for a daily shake. They were then examined, but as there were no apparent changes they were left for a further two weeks and re-examined.

25μ



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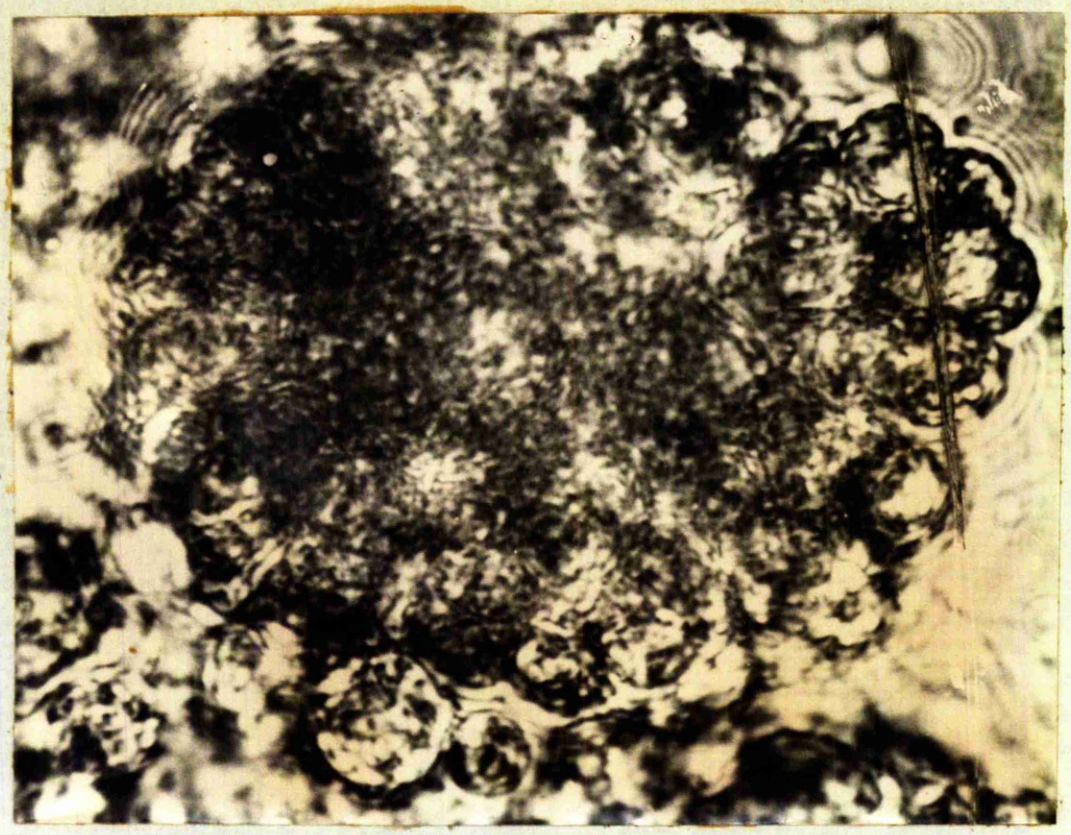


10μ

5a.

Plates 5 and 5a. Mulberry-like units produced in cultures containing vitamin B_{12}.

Culture	Remarks
MC_{13} Vit. B_{12} 1 p.p.m. 5 " 10 "	All contained large green cells as well as well marked "mulberries". The individual cells were larger and fewer per colony than that seen in B. braunii (Plates 5 and 5a)
MC_{13} I.A.A. 1 p.p.m. 5 " 10 "	Sheets of large green cells were formed. In no case were "mulberries" evident. No obvious effect from different I.A.A. concentrations.
MC_{13} GA_3 1 p.p.m. 5 " 10 "	Large green cells formed but not quite as large as those formed in the I.A.A. treatments. No "mulberries" formed.
Controls MC_{13} + 1 ml. H_2O MC_{13} + 0.1 ml. NaOH	Large green cells only, sometimes united in sheets

Table 1.

Results of Culture of large green cells in various media for six weeks

To test if locality affected the behaviour of wild brown colonies in culture a similar experiment was set up using material collected from Oakmere, Cheshire. The vitamin B_{12} treatment was, at each concentration supplied, the only additive to produce the obvious morphological change in the large green cells to produce mulberry-like units.

It was suggested (Droop : personal communication) that the inability

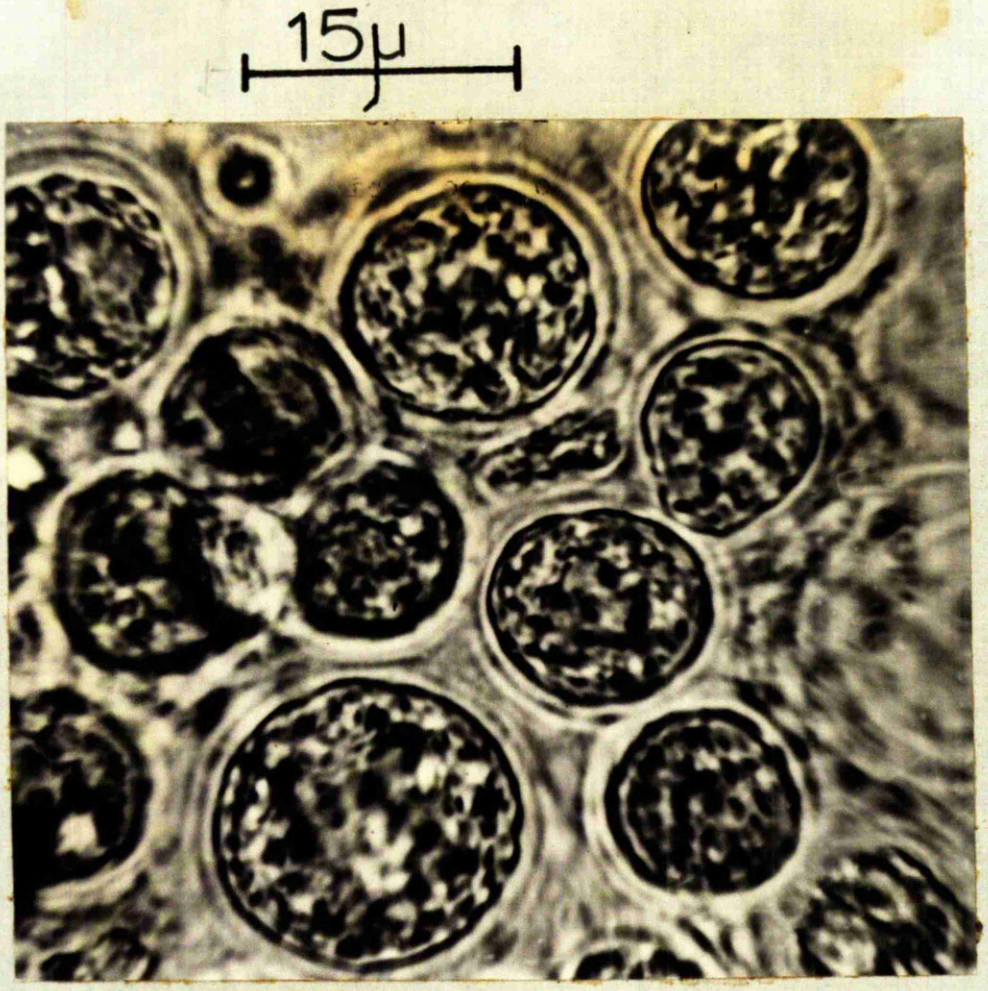


Plate 6. Single cells produced in enriched soil extract medium

of the large green cells to develop further in culture may have been due to the lack of some essential trace elements in the medium, but a culture of such cells grown in sterile enriched soil extract (Appendix 2) for three weeks failed to show any botryococcoid development, and only large numbers of rather smaller single cells could be seen (Plate 6).

The possibility that the modified Chu 13 medium was too concentrated for the unusual form of B. braunii colonies, isolated from the wild, was examined by growing the cultures in 1/10th and 1/100th the normal strength of the medium again with the addition of vitamin B_{12}, indole acetic acid or gibberellic acid. After two weeks in these media the large green cells in all treatments began to turn yellow, and only in the vitamin B_{12} containing cultures was there any sign of morphological change. These cultures contained a few "mulberries", but many fewer than in the undiluted medium. All of these cultures were left in the incubator for twelve weeks in an effort to induce further development of the large green cells by starvation but in all cases, and especially in the case of the vitamin B_{12} cultures all that was observed was that the proportion of individual large green cells to cells held together by mucilage was increased.

The effect of shaking (at 150 r.p.m. in a Gallenkamp orbital incubator) at 20° and a continuous light intensity of 250 foot candles on the morphology of the large green cells has been studied over a three week period. Although the large green cells became extremely numerous in the shaken cultures there was no development towards a colonial habit. The large green cells, from the shaken cultures, were subsequently subcultured into 10 ml. modified Chu 13 tube cultures and placed in a variety of

habitats (Table 2) for a four week period. At the end of this time although the large green cells appeared healthy in all cultures, except those placed in the greenhouse, they showed no obvious morphological changes.

Habitat	Day length (hours)	Light intensity (foot candles)	Effect on Cultures
Constant Temperature Room 10°	12	50	Cultures deep green - no "mulberries"
Constant Temperature Room 10°	12	500	" " "
Incubator 20°	16	1,000	" " "
Incubator 20°	16	250	" " "
Greenhouse 17-20°	Autumn - Winter	Variable	Cultures white and dead

Table 2.

Habitats tested for their effect on the development of the green cells

From the experimental results obtained it appears that brown colonies taken from the wild do not necessarily repeat the colonial

habit in culture. It seems likely that the individual cells escape and behave as autospores whose further development demands some specific and critical conditions not yet determined.

This peculiar behaviour of wild brown colonies, when brought into culture, results in the bulk of the studies on structure, hydrocarbon content and hydrocarbon synthesis being carried out on material from the Cambridge Culture Collection which had the characteristic botryococcoid habit.

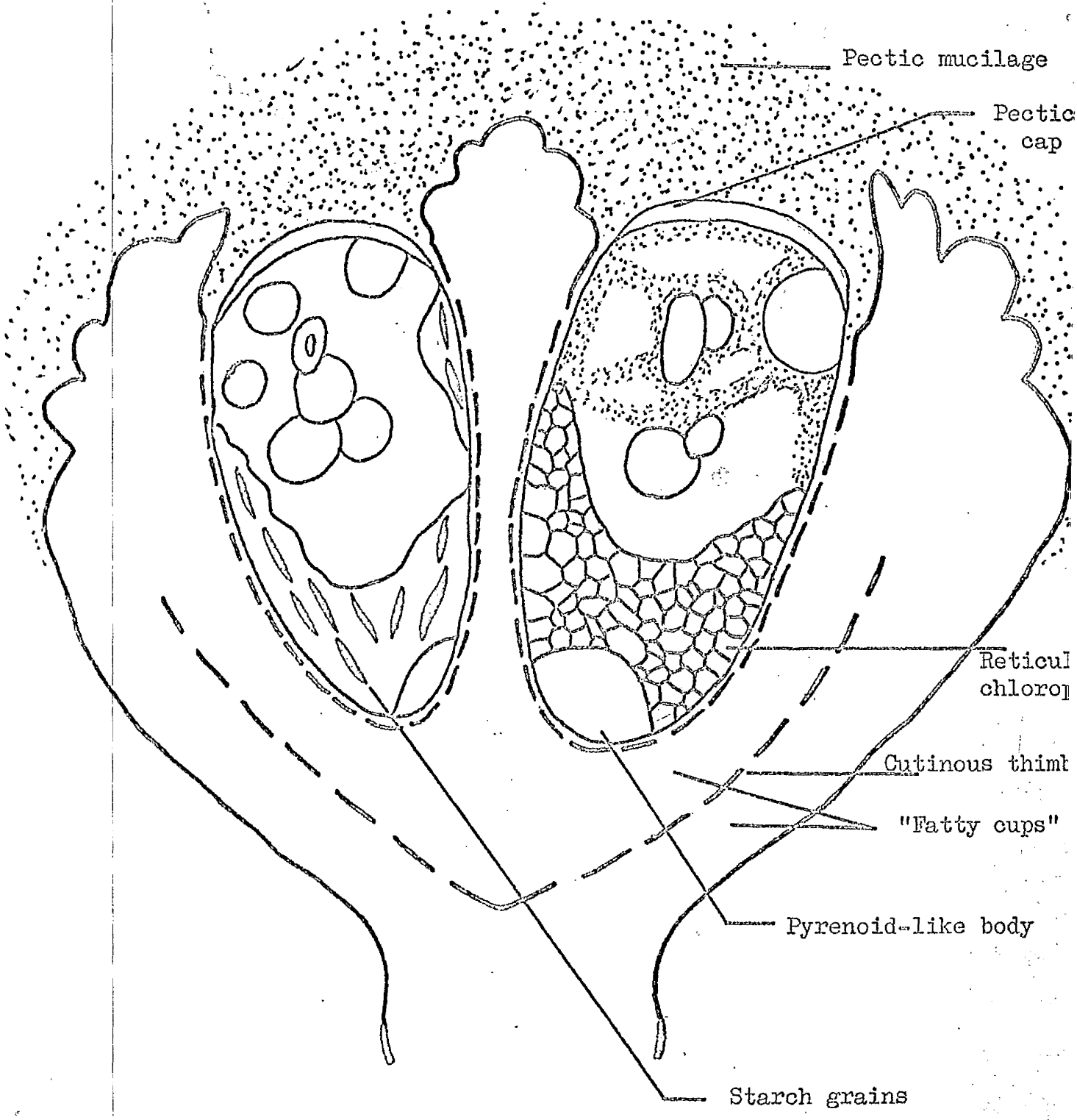


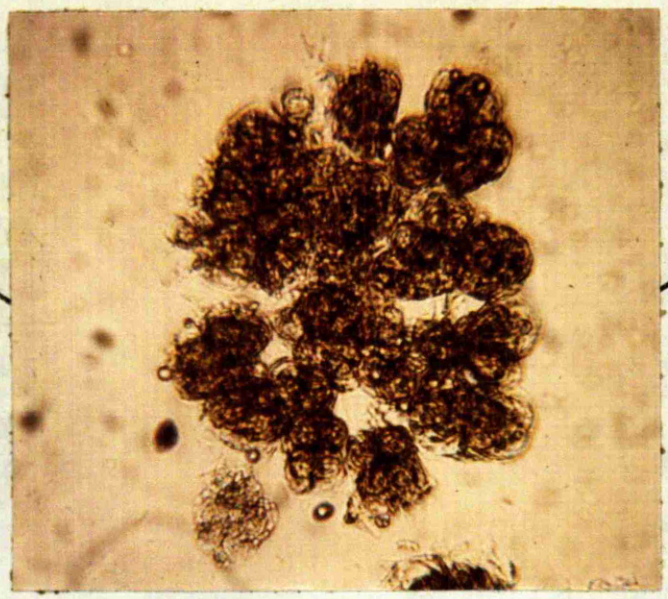
Fig. 2. Diagramatic representation of B. braunii structure (after Blackburn and Temperley, 1936)

25μ

Green active state colonies

Three weeks after subculture into fresh MC_{13} medium

One year in unchanged MC_{13} medium



25μ

Brown resting state colonies

Fig. 3. Life cycle of B. braunii in culture

PART II - FORM AND REPRODUCTION IN BOTRYOCOCCUS

1. Form of the colonies

The structure of the colonies of Botryococcus braunii was first described in detail by Chodat (1896) and this was followed in 1936 by Blackburn and Temperley's very full account of the colonies and individual cells. Under pressure they were able to free individual cells from the embedding mucilage of the colony, and they described the "fatty cups" and the cutinous thimbles in which the protoplasts were embedded (Fig. 2).

It has been reported before, Belcher (1957), that the alga may be found in two states: as green colonies when they are thought to be in an actively growing condition and as deep brown colonies when they may be regarded as resting state colonies (Fig. 3). Belcher (1957) found that the orange-brown colouration of the resting state colonies was due to the presence of large quantities of β-carotene which leaked out into the colony matrix. Further, Belcher (1957), showed that variation in β-carotene content may be directly related to light intensity, temperature, ageing of culture medium and to nitrogen and phosphate deficiency. These findings are in line with those for β-carotene in the sub ~~aeriel~~ aerial alga Trentepohlia aurea (Tischer, 1936).

2. Structure of cultured green colonies

(a) With the light microscope

Green colonies (material C) were grown in modified Chu$_{13}$ medium with light intensity of 250 foot candles from fluorescent tubes. When viewed

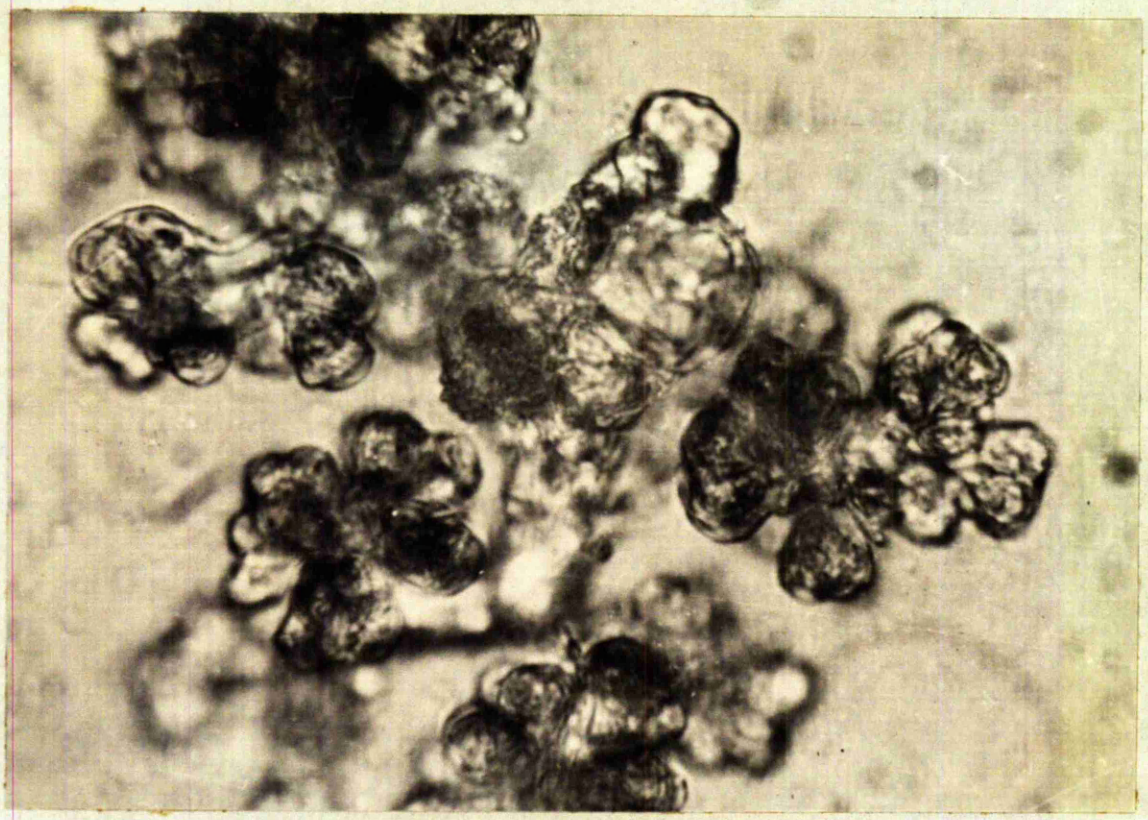


Plate 7. B. braunii colonies in culture

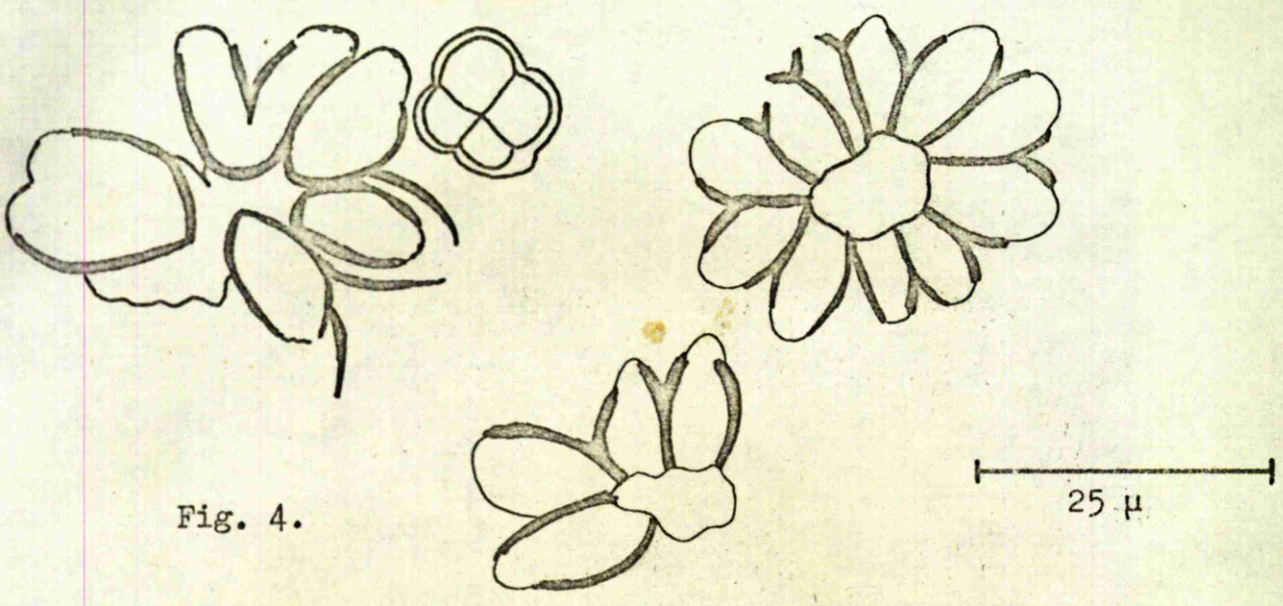


Fig. 4.

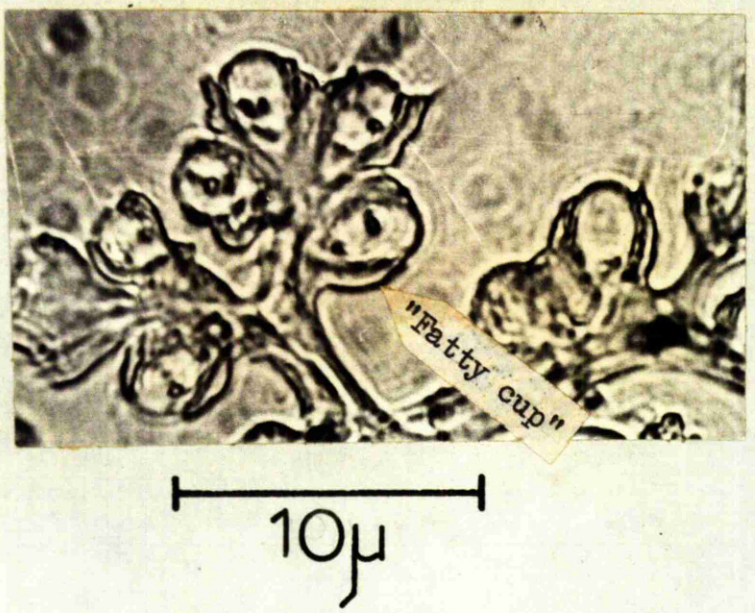


Plate 8.

Colonies showing well marked "fatty cups" (Sudan III stained)

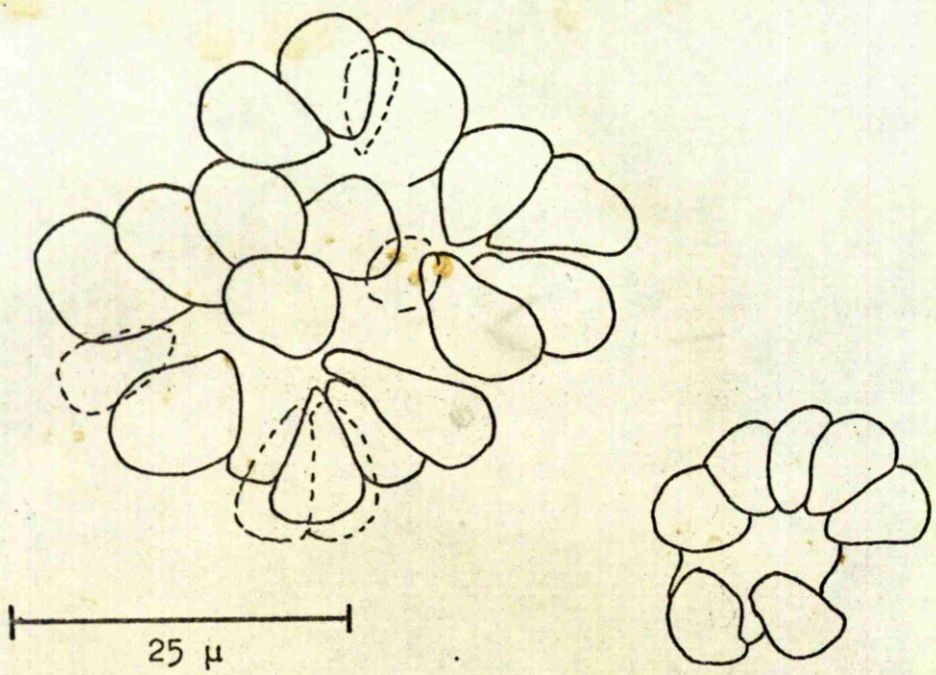


Fig. 5. Colonies stained with iodine and sulphuric acid to show cellulose walls

X 30,000

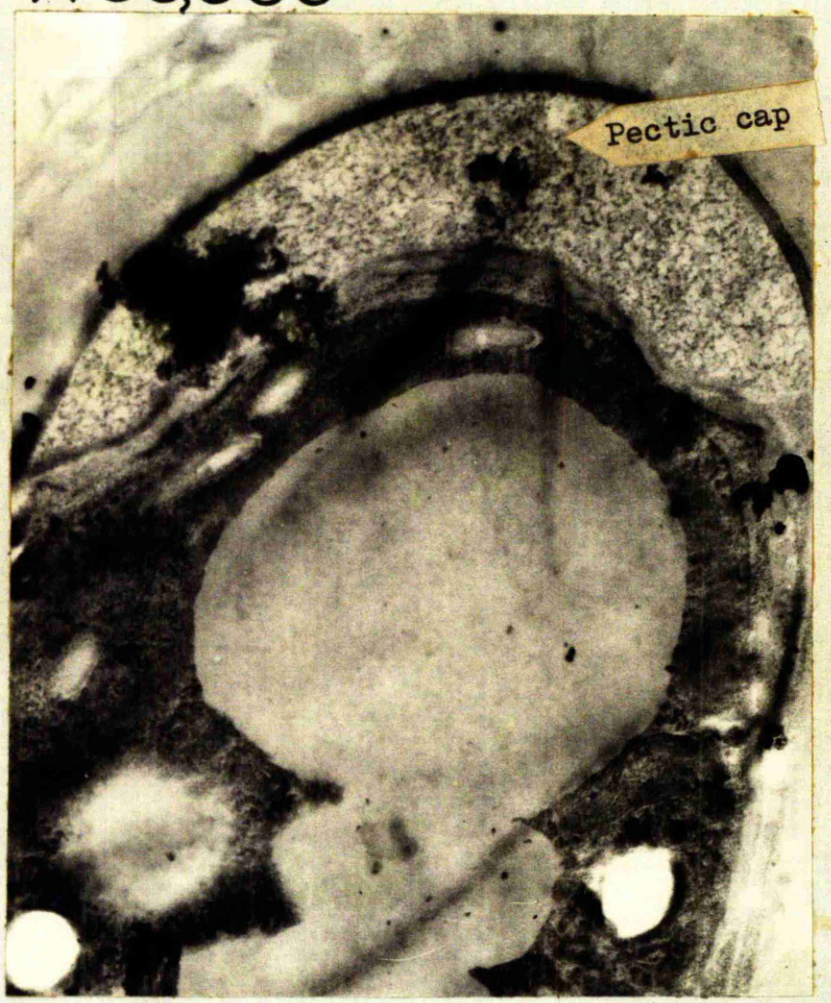


Plate 9. Cell surmounted by pectic cap

(Ruthenium red stained)

X 20,000

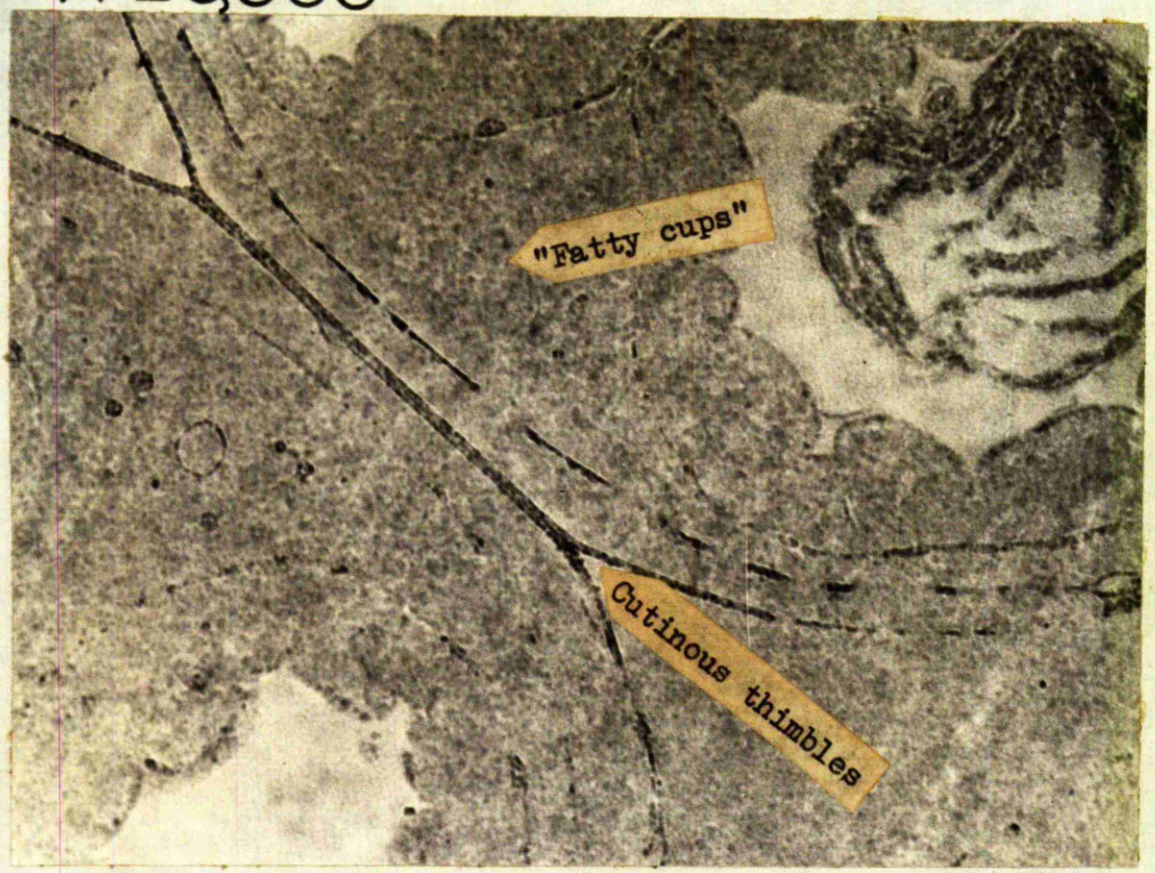


Plate 10. Cutinous thimbles embedded in "fatty cups"

X 80,000

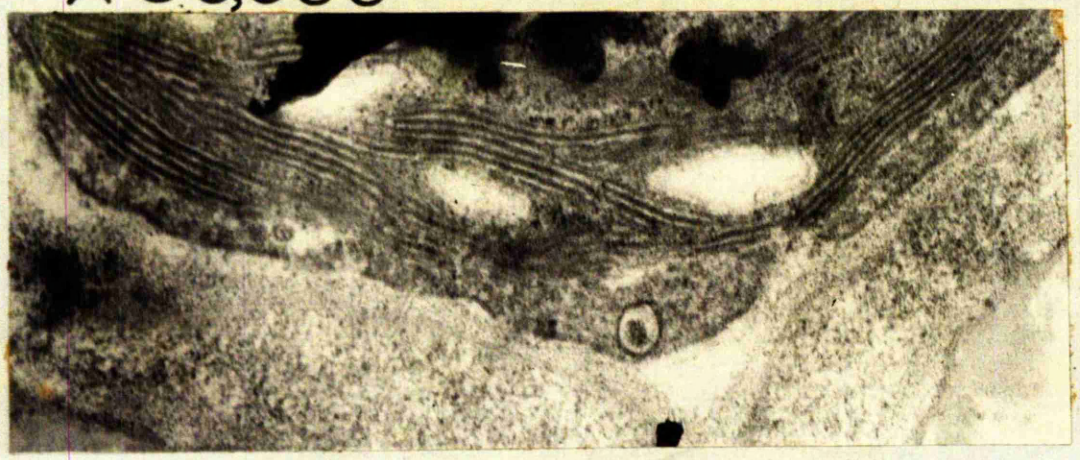


Plate 11. Cell containing starch grains

by light microscopy these showed pear-shaped cells, radially arranged with the broad end outwards in rather loose colonies of varying numbers of cells (Plate 7). With Sudan III, well-marked cups (the "fatty cups" of Blackburn and Temperley, 1936) were clearly visible (Plate 8 and Fig.4). With iodine and strong sulphuric acid each cell in the colony was outlined by a narrow blue line, suggesting the presence of a cellulose wall (Fig.5) and dark areas localised in the chloroplast suggested starch grains. These details of structure are entirely in line with Blackburn and Temperley's (1936) description.

(b) With the electron microscope

More detailed observations with the electron microscope (Appendix 4) showed that each cell bore, on its outer side, a well marked cap bounded both inside and out by its own membrane. This confirms the pectic cap shown by Blackburn and Temperley (1936). The cellulose cell wall entirely surrounds each protoplast and the pectic cap surrounds this as the free outer side of the cell (Plate 9). The relationship of the cutinous thimbles to the fatty cups (Fig. 2) is shown in Plate 10 where, although the protoplasts have been lost during embedding, their position and the cups surrounding them show quite clearly the way in which two daughter cells, each with its cutinous thimble, are themselves embedded in one of the fatty cups. The reticulate chloroplast (suggestive of the form of chloroplast of the Chlorophyceae) show numerous starch grains embedded in the reticulations; in many cases the heavy grains fell out of place during preparation of the sections (Plate 11). The presence of a dark unbounded granular region at the base of the chloroplast suggests the

X 70,000

Plate 12. Pyrenoid-like body at base of cell

X 70,000

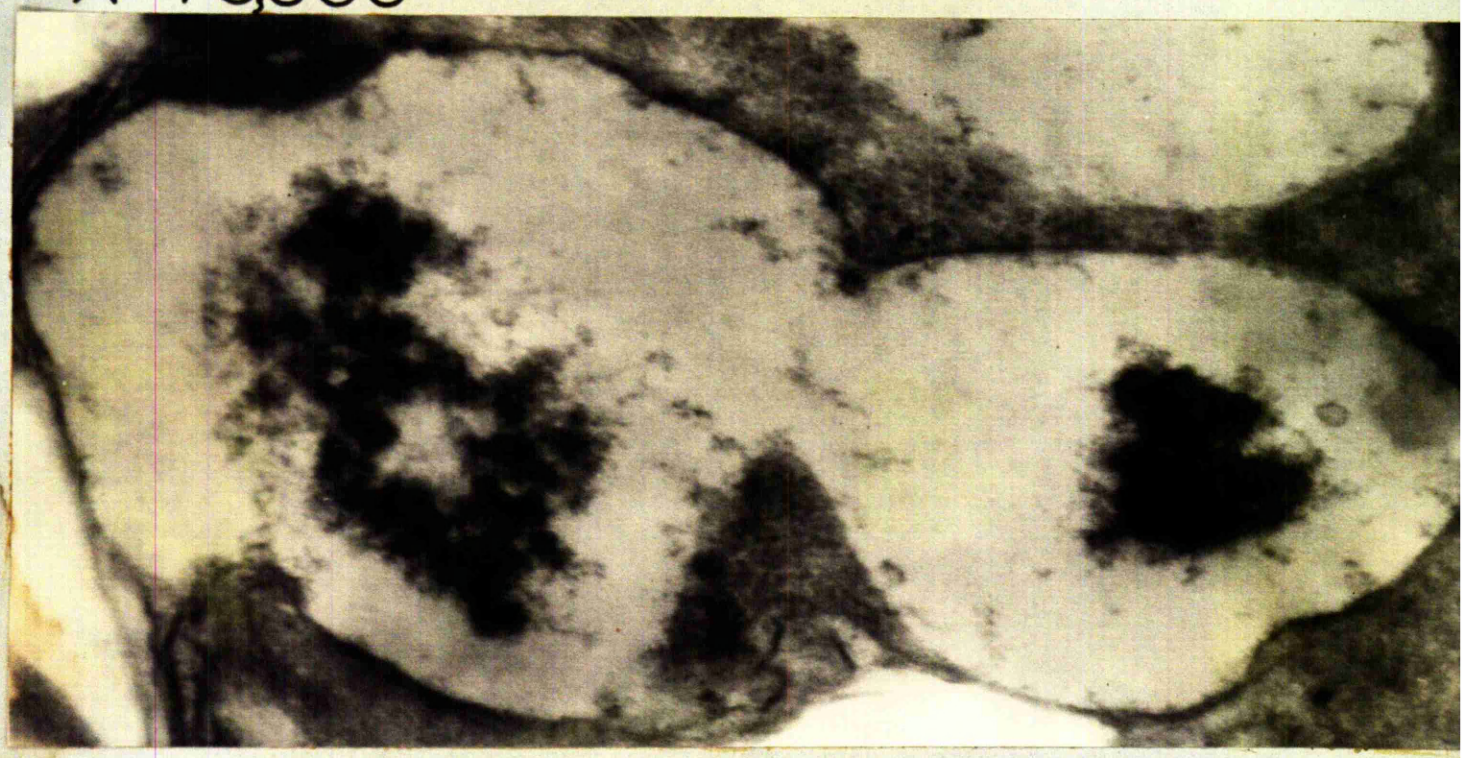


Plate 13. Tannin vesicles containing waste material

X 30,000

Plate 14. Osmophilic droplets escaping from cell.

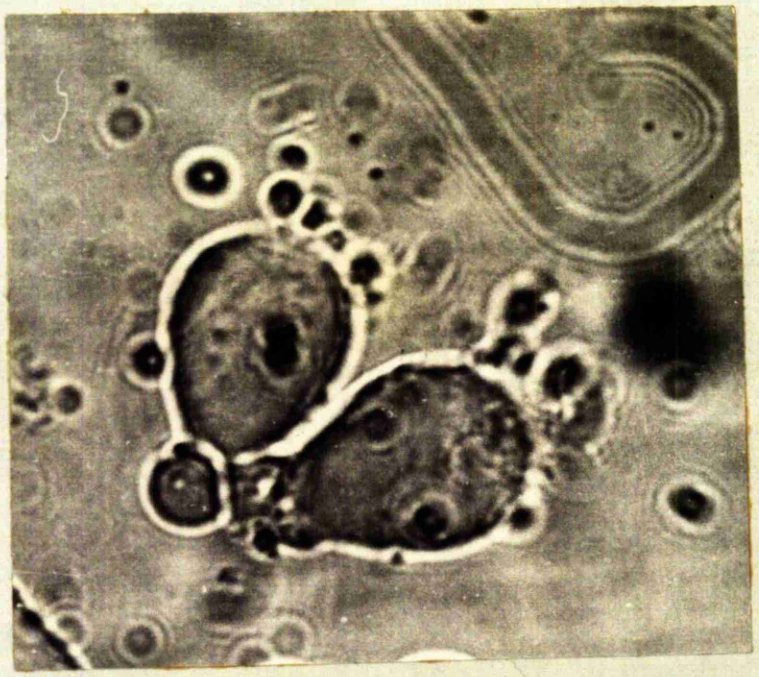


Plate 15. Daughter cells formed from autospore.

pyrenoid-like body reported by Blackburn and Temperley (1936). No starch grains were seen associated with this structure (Plate 12). A number of vacuole-like structures with dark contents also present in the cells (Plate 13) are thought to be tannin vesicles concerned with the disposal of waste materials (Blackburn and Temperley, 1936). As well, numerous globules were seen forming and passing out between the cell wall and thimble (Plate 14). On escaping from the cell they were seen as dark staining osmophilic droplets which are believed to be unsaturated hydrocarbons. Such a metabolite would, no doubt, aid the buoyancy of colonies.

3. Reproduction of B. braunii

(a) Asexual reproduction.

The only definitely known method of propagation in B. braunii is by longitudinal divison of existing cells to form two daughter cells (Chodat, 1896; Pascher, 1925; West and Fritsch, 1927). In this manner colony size and content is increased. According to Blackburn and Temperley (1936) when the colonies contain about twenty to thirty cells, they tend to split into smaller colonies which may or may not be joined by mucilaginous strands. In this way the characteristic groups of colonies are produced. The shedding from mature colonies of whole cells, sometimes surrounded by their "fatty cups" has also been noted and these are taken to be the "autospores" of earlier workers, the term "autospore" being the whole cell protoplast as used by Round (1965). These units divide longitudinally (Plate 15) and by the secretion of mucilaginous substances particularly in the basal region of the cell, colonies are built up with

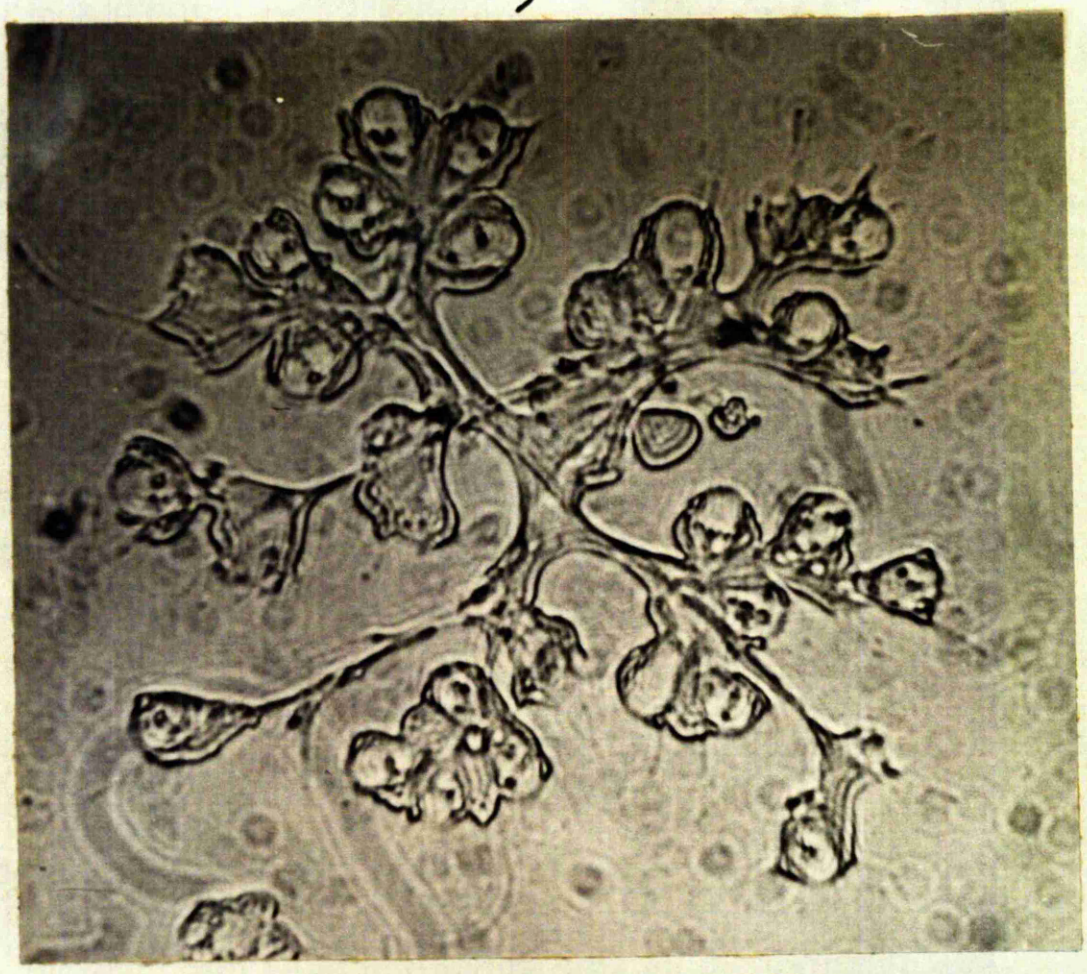


Plate 16. Protoplasts round the periphery of developing colony.

the protoplasts round the periphery of a developing colony (Plate 16).

(b) Sexual reproduction.

A form of sexual reproduction by fusion of motile gametes was reported by Printz (1927). This has been questioned by earlier workers and the present survey has given no indication of any such units.

Colony fragmentation and longitudinal division of autospores freed from mother colonies may therefore be regarded as the modes of reproduction.

4. Speciation in the genus Botryococcus

Kützing (1849) defined the genus Botryococcus as monospecific, naming the form Botryococcus braunii after the Swiss collector A. Braun. With increasing appreciation of botryococcoid forms, other species were recognised, and in 1927 West and Fritsch recorded four species for Britain:-

B. braunii (Kütz.)

B. sudeticus (Lemm.)

B. protuberans (West and West)

B. calcareus (West and West)

The validity of these species has been questioned by a number of authors (e.g. Chodat, 1896; Carlson, 1906). Pascher (1925) had already removed B. sudeticus to the genus Botryosphaera.

Blackburn and Temperley (1936) refer to Botryococcus as a polymorphic form and this is confirmed by the present study. Variation in colony form and size has been marked and, particularly in culture, has been seen to vary with age and local environmental changes. Forms

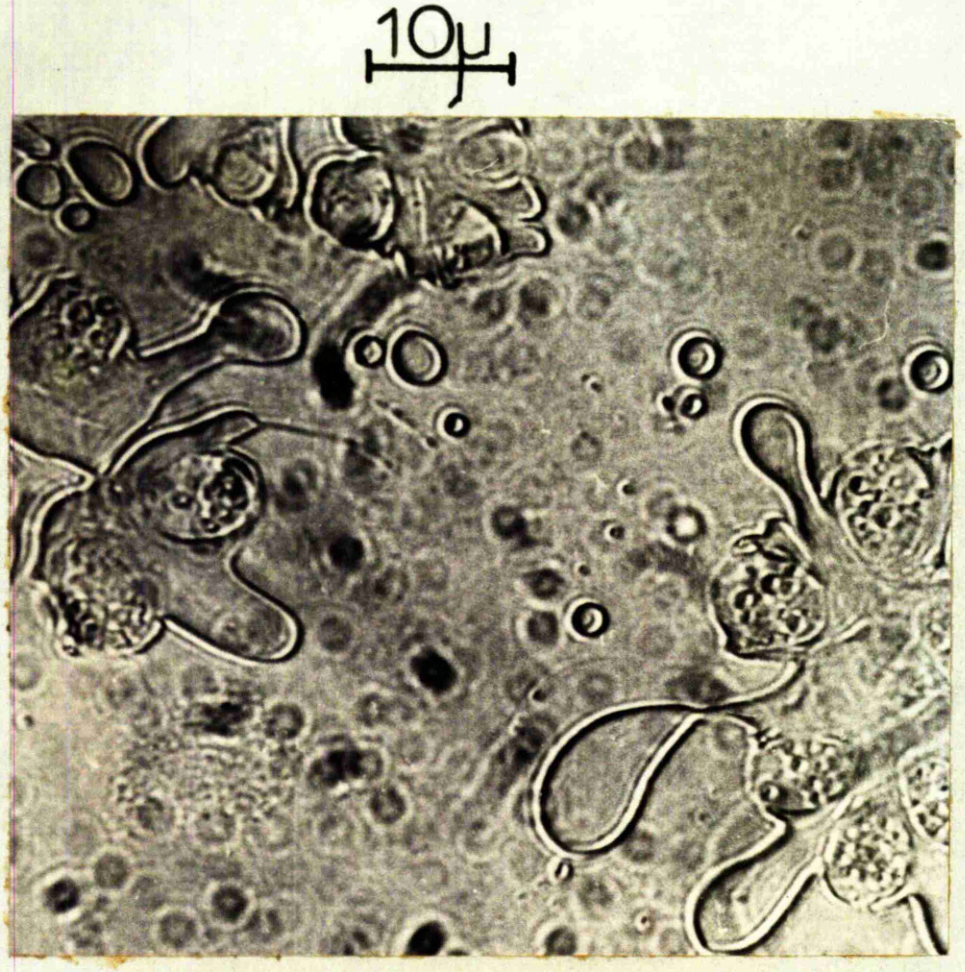
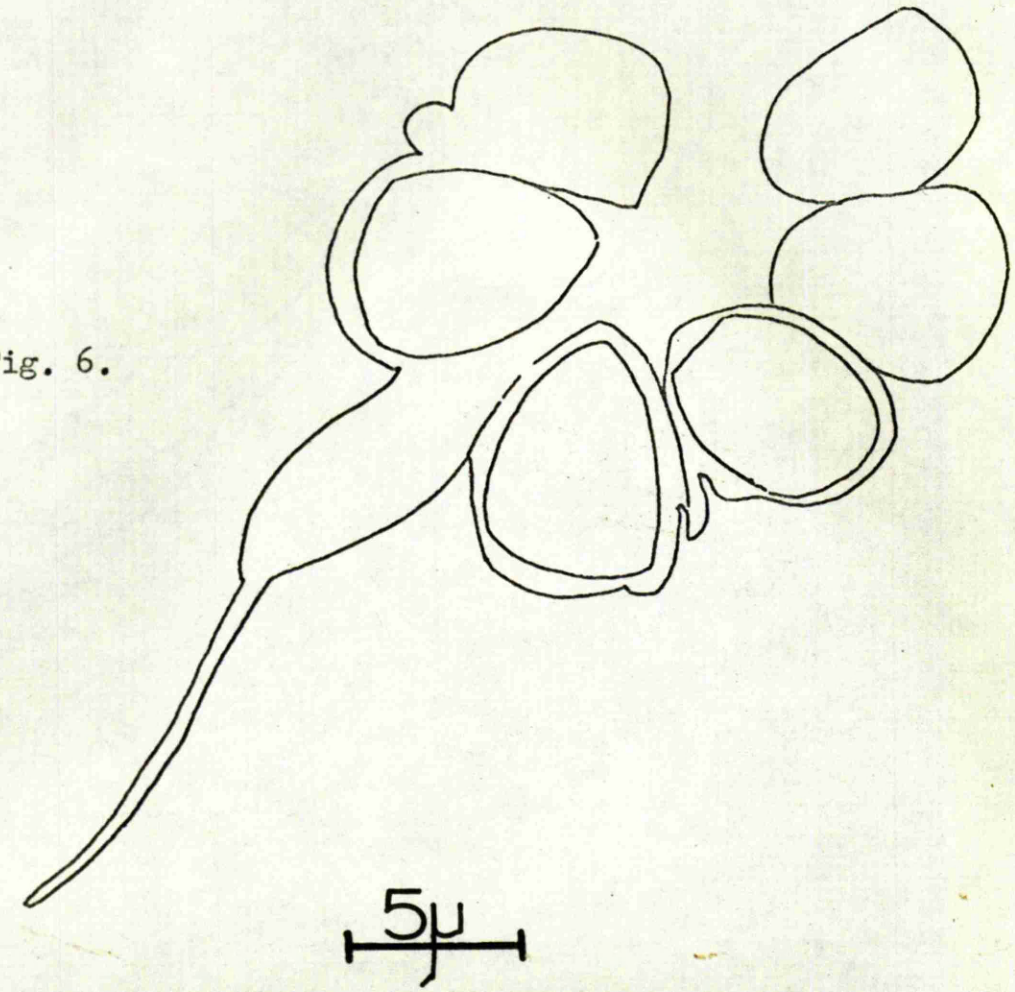


Plate 17. Lipoid bubbles formed by B. braunii.

Fig. 6.



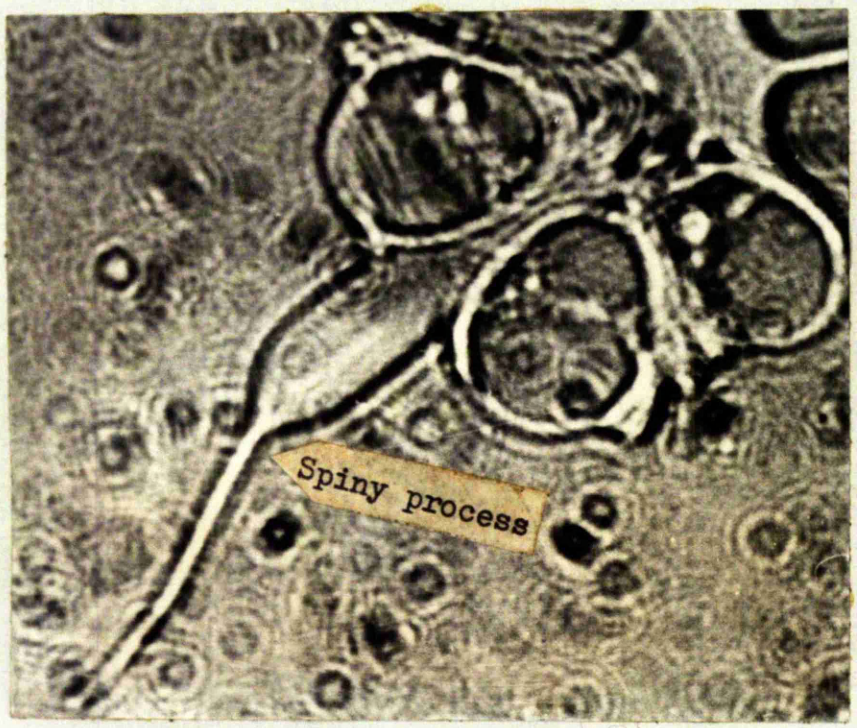


Plate 18. Spiny process formed by B. braunii.

corresponding to the description of B. protuberans and to Smith's var. minor (Smith, 1920) have been repeatedly noted in cultures of our material C (Plate 16) so that there appears to be little evidence for retaining this species, particularly since West's original description (West and West, 1905) was made from preserved specimens from one habitat.

Only one record is known of the species B. calcareus (West and West), a description and drawing again made from preserved specimens. In view of the polymorphic state of the more generally recognised B. braunii the validity of the species B. calcareus may be questioned until further evidence is forthcoming.

The polymorphism of Botryococcus may be emphasised by the fact that West and West (1897; 1903) described a form, with spiny processes protruding from the colonies, which they called Ineffigiata neglecta and which they later recognised as B. braunii and discarded the genus Ineffigiata. Such spiny processes have been frequently seen in material C which had been placed on the microscope stage for a short period. The effect of the heat from the microscope lamp caused the hydrocarbon material surrounding the cells to become less viscous and to flow outwards. On contact with the outer medium this lipoid material became oxidatively polymerised at the outer surface and consequently firmer. As its outward flow was interrupted the more liquid lipoid behind this firm edge tended to round itself off into a bubble. Such bubbles can be seen in Plate 17. These bubbles often broke free, as shown, and left well marked spiny processes behind (Fig. 6, Plate 18).

Thus it appears probable that the polymorphic form Botryococcus braunii is the only representative of the genus known in Britain.

PART III - GROWTH STUDIES ON B. BRAUNII ALREADY IN CULTURE (MATERIAL C)

(1) Production of quick growing cultures

Belcher (1957) reported Botryococcus to be slow growing in culture, and gave a mean doubling time of 5-6 days. In an attempt to produce more rapidly growing, completely uncontaminated cultures which might be used for physiological studies, the present work was based on a unialgal culture from the Cambridge collection [culture No.207/1B - our material C].

Experimental Methods

(a) Method of culture

All cultures, unless otherwise stated, were made up as 95 ml. of medium in 250 ml. Erlenmeyer pyrex widenecked flasks. pH was adjusted to 7.5 with N NaOH before autoclaving. In some experiments the medium was buffered with glycyl-glycine and glycine (S_{66}), or by 2 amino-2-(Hydroxy-methyl) Propane-1:3-Diol [TRIS]. TRIS was thought useful because of its supposed lack of biological activity and because in non-axenic cultures glycyl-glycine and glycine buffering was rendered useless by large scale bacterial development. The TRIS buffer was made up according to Dawson et al. (1959), (Appendix 2).

In order to establish the minimum amount of TRIS buffer needed per 95 ml. culture of medium, to prevent pH fluctuation due to autoclaving, sixteen flasks of modified Chu 13 medium were made up. No TRIS buffer

was added to the first four; 1 ml. to each of the next four; 5 ml. was added to each of the next four; and 10 ml. to each of the last four. pH was then adjusted to 7.5 with N NaOH. The flasks were then bunged and autoclaved at 15 lbs. in.^{2} for 15 mins., allowed to cool and then pH was re-assessed. The lowest concentration of TRIS in the medium which had complete buffering action was 5 ml. Although growth was slower in the media containing TRIS the alga did not seem to be morphologically affected by this concentration.

In all experiments media was sterilized and allowed to stand overnight before inoculation with 5 ml. of a culture of B. braunii which had been maintained at 14° for 3-5 weeks in an incubator continuously lit by "day-light" fluorescent tubes. All cultures were ~~non-aerated and~~ stagnant except for shaking once daily.

All glassware was cleaned before use by standing overnight in a strong solution of 'pyroneg' and rinsed thereafter ten times in tap water and finally three times in glass distilled water. All media examined in the growth studies were made from "Analar" quality chemicals and glass distilled water.

(b) Media

The growth of the alga has been studied in the following media.

1. Modified Chu 10 - MC_{10} Chu (1942)
2. Modified Chu 13 - MC_{13} Chu (1942)
3. Modified Knopp's Medium - K_m Pringsheim (1946)
4. Beijerinck's Medium - B Pringsheim (1946)

5. S_{66} - a ~~light~~ dilute sea water developed by Droop (1961)

6. Enriched soil extract - E_1 - Cambridge Culture Collection, 1966.

Trace elements: Fresh water soil extract was added to the cultures in some experiments. Hoagland's (1933) A-Z solution was added at the rate of 1 ml./litre and 1/2 ml./litre in some experiments. (The constituents and preparation of these media and trace solutions are listed in Appendix 2).

(c) Conditions of daylength and light intensity

In a number of experiments the effect of different light intensities (high light, low light and no light) on the development of the alga was investigated. In all experiments, unless otherwise stated, a 16 hour day-length was employed. Illumination was by alternate daylight and warm white fluorescent tubes giving a total energy of either 2.27×10^{-2} cal/cm^2/min. (high light) or 5.68×10^{-3} cal/cm^2/min (low light) onto the cultures.

(d) Conditions of temperature

Experiments were carried out at 10°, 15° and 20° in constant temperature growth cabinets. The 15° experiments were carried out in a Prestcold constant temperature room, the temperature being recorded by a maximum - minimum thermometer. The cultures were placed on glass trays two inches above the fluorescent light sources and to ensure that the temperature at the glass tray surface was maintained at 15° a thermometer was placed on the tray and read daily.

Experiments at 10° and 20° were carried out in a Saxcil Growth

Environmental Cabinet with a double bank of fluorescent tubes to give high intensity illumination in this cabinet. A limited number of experiments involving shaking of the alga were also carried out at 10^{o} and 20^{o} using a Gallenkamp orbital incubator.

(e) Measurements of growth

Three methods of estimating growth were used:-

(i) Counts by Thoma haemocytometer

Six counts were made at regular intervals on all flasks. By multiplying the average of the six readings by 10^4 the number of colonies per ml. was obtained. A mean doubling time in days was then calculated from the following expression.

$$\frac{\log_{10} No}{\log_{10} 2} = X \qquad \frac{\log_{10} Nf}{\log_{10} 2} = Y$$

$$Y - X = \text{Number of generations}$$

$$\frac{\text{Duration of experiment (days)}}{\text{Number of generations}} = \text{Mean doubling time.}$$

No = Number of colonies/ml. initially

Nf = Number of colonies/ml. finally

(ii) Dry weight measurements

Since dry weight does not increase constantly during growth, such measurements alone cannot be used as a measure of growth. When linked with cell number, an

(ii) Dry weight measurements (contd)

irreversible process, dry weight determinations give a fair estimation of growth.

(iii) Optical density determinations

The optical density of a constant 5 ml. volume of 70% ethanol containing the chlorophylls extracted from the colonies was also measured at 665 mμ. on an S.P.800 spectrophotometer. The height of the absorption peak at this wavelength being taken as a measurement of growth.

(iv) Determinations of the Growth Constant (K) for B. braunii

In a number of experiments in which optimal development was recorded a value for K, the growth constant of the alga was calculated using the following expression. The rate of population increase is a function of cell number

$$\frac{dN}{dt} = f(N)$$

When every cell in the population is maintained under constant environmental conditions the relation becomes

$$\frac{dN}{dt} = KN \qquad \text{(Myers 1964)}$$

integrated and converted to $\log_{10}$:-

$$\log_{10} \frac{N}{No} = K^1 t$$

No = Number of colonies/ml. initially

N = Number of colonies/ml. finally

t = Time interval between No and N

This equation describes a steady state system and is more precisely applicable to unicellular algae e.g. Chlorella, but as colony size is more or less constant it may be applied to B. braunii.

(f) Results of growth experiments with Material C.

(i) Growth in various media at 15°

Measurements of growth were made every three days by haemocytometer counts and mean doubling times were calculated for the total experimental time as well as for the time spent in exponential growth, which was assessed from growth curves.

Medium	Doubling Time (Days)	Total expt. time (days)	Doubling time av. for exponent. phase (days)	Total exponent. growth time (days)
MC_{10}	10.8	27	6.67	17.5
MC_{10} 1/2T	10.7	27	11.40	20.0
MC_{10} T	9.0	27	7.00	21.0
MC_{10} 3% SE	9.6	28	8.50	25.0
MC_{13}	10.4	27	6.70	15.0
MC_{13} 1/2T	10.0	27	6.50	17.5
MC_{13} T	9.0	27	5.60	17.5
MC_{13} 3% SE	9.5	28	7.60	22.5
K_m 1/2T	11.7	25	6.50	14.0
B 1/2T	11.4	25	5.90	13.0
E_1	1.4	20	1.08	15.0
1/2 S_{66} E_1	2.1	20	1.40	13.0
S_{66}	2.1	20	1.80	17.0

Table 3. Mean doubling time of B. braunii in various media under low light intensity at 15°

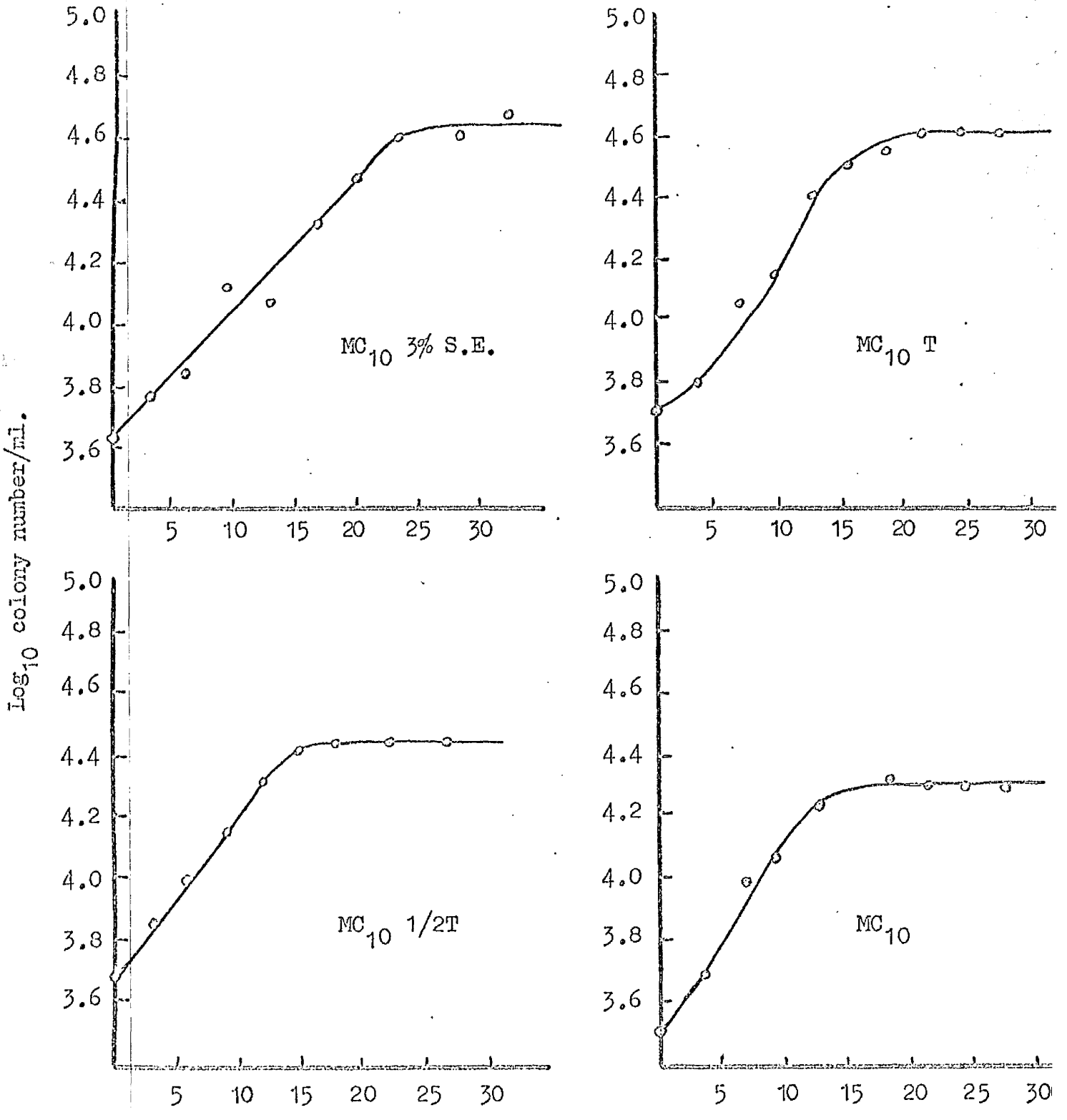


Fig. 7. Growth of B. braunii in MC_{10}

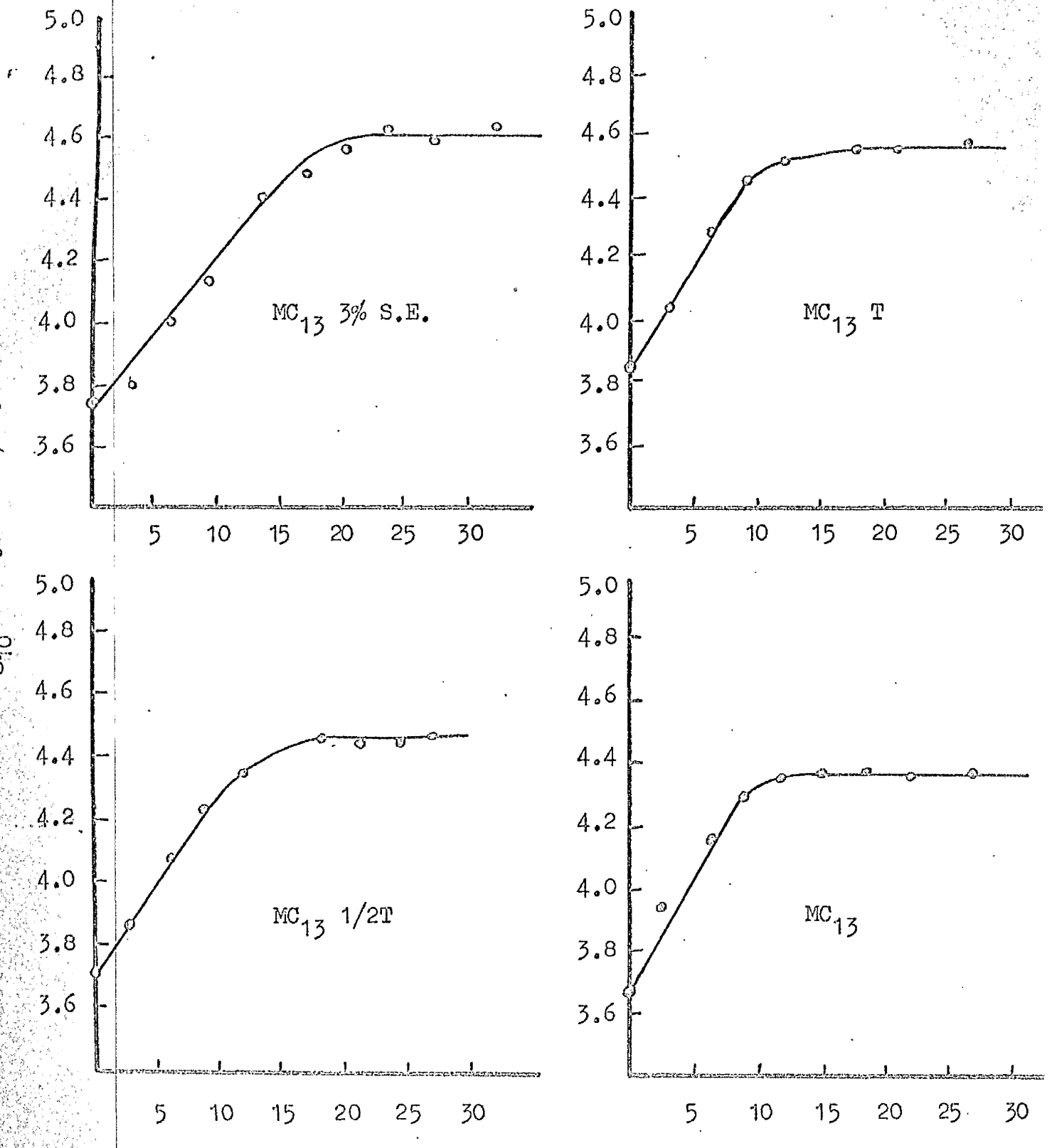


Fig. 8. Growth of B. braunii in MC_{13}

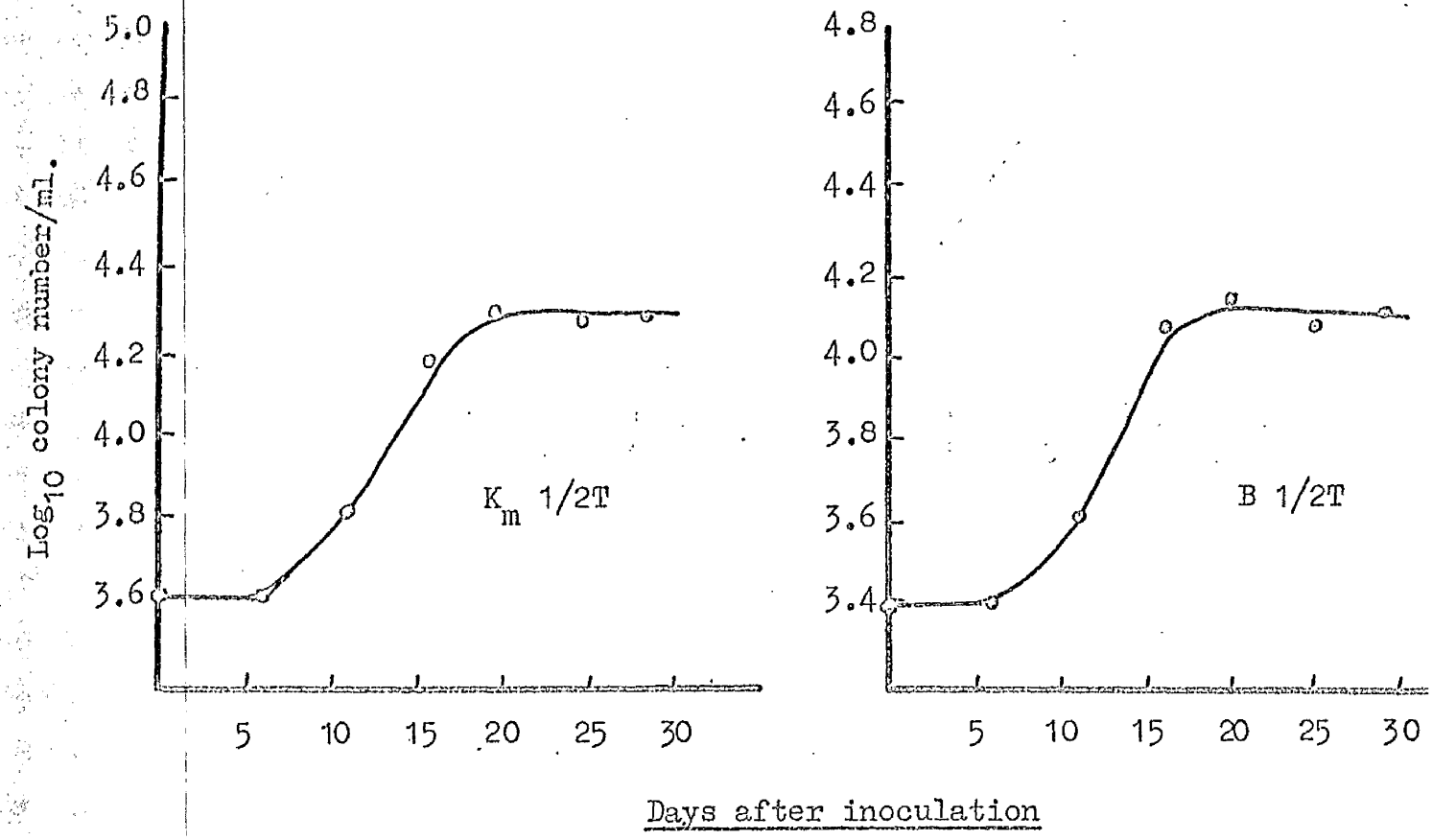


Fig. 9. Growth of B. braunii in K_m 1/2T and B 1/2T

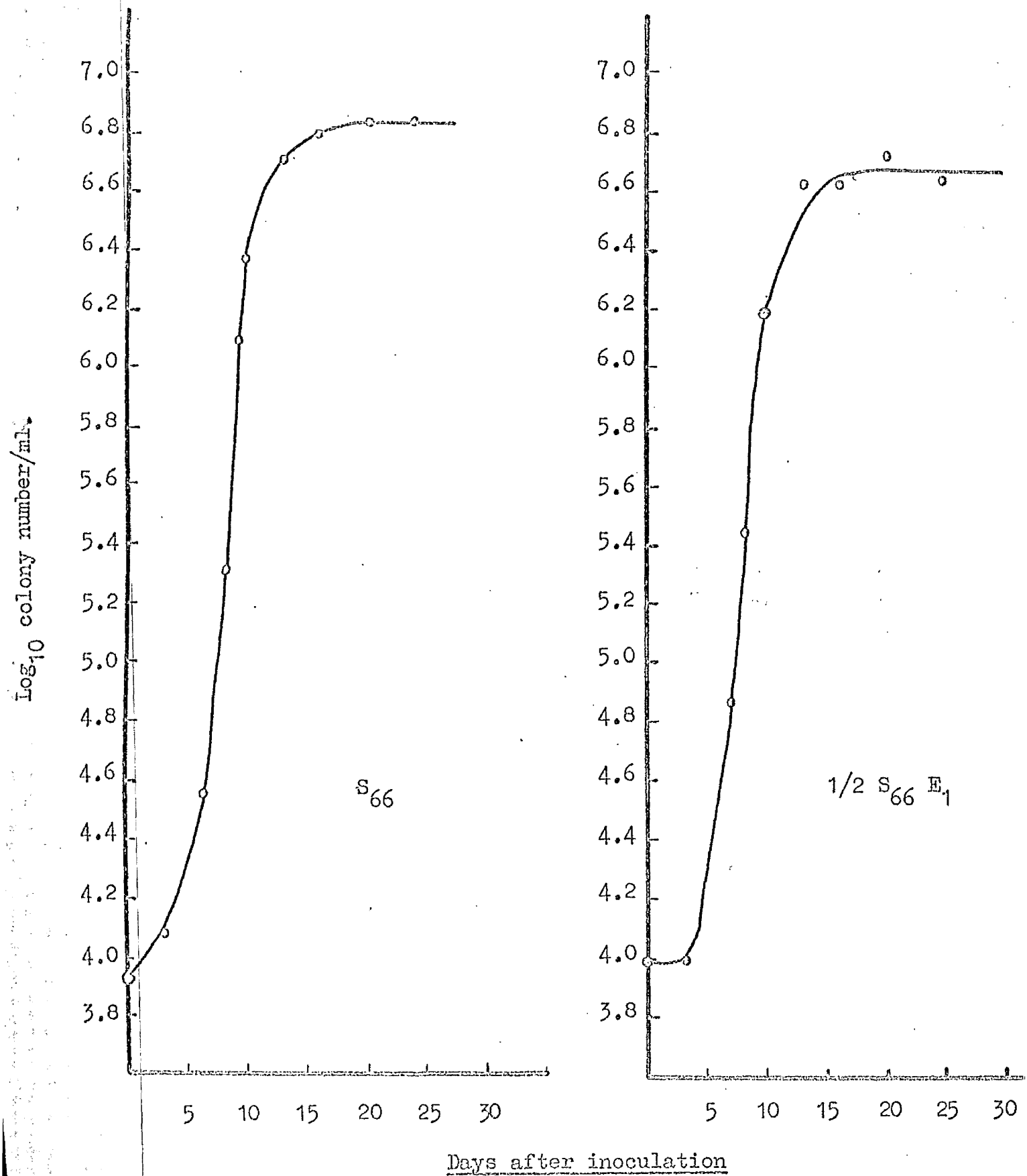


Fig. 10. Growth of B. braunii in S_{66} and 1/2 S_{66} E_1

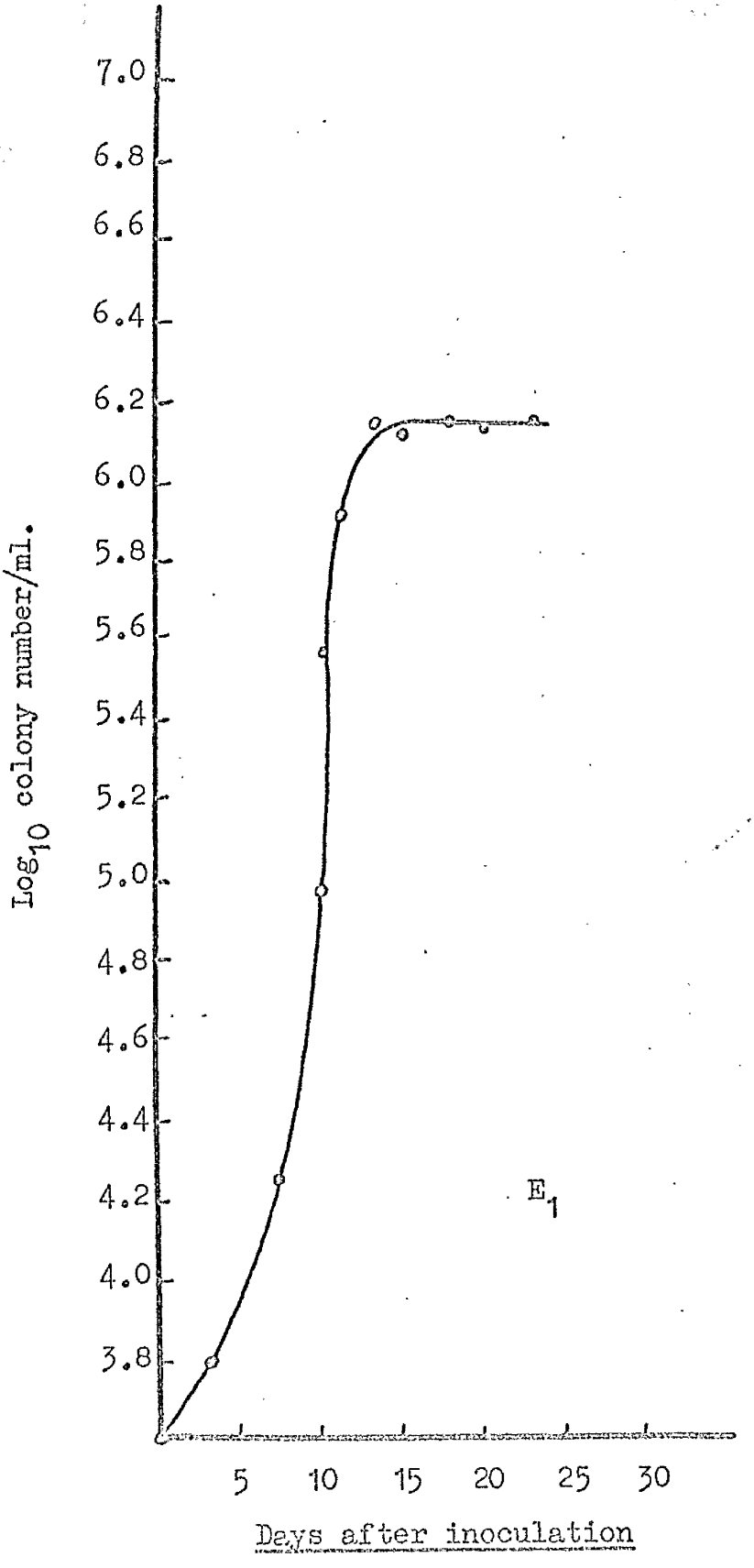


Fig.10a. Growth of B. breunii in E_1

Modified Chu 10 and 13

From the mean experimental period doubling times it is clear that both modified Chu 10 and Chu 13 support very similar growth of B. braunii under the same conditions. In all the growth curves (Figs. 7 and 8) related to these two media there was notably no lag phase and exponential growth was maintained over about 18 days at the longest. Cultures containing no additive (i.e. neither trace solution nor soil extract) grew more slowly than the others, and cultures containing 1 ml. of Hoagland's trace solution per litre of medium grew more rapidly than those with 1/2 ml. of Hoagland's per litre over the total experimental time. The addition of 3% soil extract to both the media resulted in a growth rate intermediate to that obtained by the two concentrations of Hoagland's solutions tested if considered over the total experimental time.

Modified Knop's solution and Beijerinck's medium

In both of these media, which had 1/2 ml. of Hoagland's solution per litre, growth was slower if considered over the total experimental time and the alga showed a well marked lag phase when cultured in them both (Fig. 9).

Enriched soil extract and S_{66}

Growth in these media was much more rapid than in any of the others examined and ceased after 20 days (Figs. 10 and 10a). The rapid growth rate in these media may be ascribed to a number of factors. These media were buffered and so did not suffer the extremes of pH which occur in unbuffered media due to autoclaving. A high pH, even if shortlived, may adversely affect a medium as ferric salts will tend to be precipitated as

insoluble hydroxides and be unavailable to the alga. These media were safeguarded from loss of iron by the presence of a chelating agent (E.D.T.A.) as were the modified Chu media previously mentioned which had an addition of citric acid.

The presence of thiamine and vitamin B_{12} in the S_{66} medium may also be beneficial to B. braunii. The mixture of humic acids present in the soil extract of the E_1 cultures was likely to contain all essential vitamins for the successful development of the alga as well as acting as a buffer and chelating agent.

Different treatments were grown for different lengths of time as shown in Table 3 and, as a result, it was possible for two treatments in different media to have very similar average doubling times for their respective total experimental times. It is also true that cultures grown in different media had different degrees of slope to the exponential portion of their plotted growth curves but showed very similar mean doubling times at the end of the same total experimental time. To be more accurate, and to reflect these growth variations, in different media, which could be masked by an overall figure for mean doubling time calculated from total experimental time, the duration of the exponential phase and the mean doubling time for this period were calculated (Table 3). In the majority of cases the figure for mean doubling time calculated for the total experimental time was satisfactory, and the figure calculated for the mean doubling time during exponential growth only supported it. This latter figure was most useful when comparing the growth of the alga in media in which it showed a pronounced lag phase with those in which it did not. The modified

Chu 13 medium with 3% soil extract would appear from Table 3 to be the third best of the Chu treatments for development of the alga at 15° with a mean doubling time of 9.5 days. If the growth made by the alga during exponential growth only is considered this medium drops to sixth best with a mean doubling time of 7.6 days. It is interesting to note in Table 3 that the slope of the exponential portion of the growth curve (Fig. 6) has placed MC_{10} ahead of MC_{10} T_1 and MC_{10} 3% SE in its ability to support growth of the alga. It appears that a lack of trace solution allows a slightly more rapid development of the alga in this medium, but for a shorter period of time. This was also true in the MC_{13} media where MC_{13} ended exponential growth after fifteen days, whereas MC_{13} $T_{1/2}$ and MC_{13} T_1 continued exponential growth until 17.5 days.

Although the total amount of growth achieved in the cultures with soil extract and those with 1 ml. of Hoagland's trace solution per litre was similar over a 27-28 day period, the cultures with trace solution had a more vertical exponential growth phase than the soil extract containing cultures. Therefore, although the final yield over a 28 day period in these cultures was similar, the manner in which it was achieved was different.

Most rapid growth of the alga was therefore obtained in a complex delicately balanced medium (S_{66}), on the one hand, and in a totally undefined equally complex medium (E_1) on the other hand. Nearly half of the S_{66} cultures became noticeably contaminated by the end of the three week period, no doubt due to the high content of glycyl-glycine (500 mgm/l) and glycine (250 mgm/l) employed as a buffer in this medium. As a result

this medium was discarded for general physiological studies. The E_1 medium was also discarded from general physiological studies because of its undefined nature, but use was made of rapid growth in these two media for culturing the alga in an axenic state.

(ii) Growth under different light intensities at 10°

(i) High light; (ii) Low light; (iii) No light.

Culture		Doubling Time Days	Total exp. Time Days
MC_{13}	H.L.	18.9	21
	L.L.	14.9	21
	N.L.	17.2	21
MC_{13}	H.L.	19.9	42
	L.L.	21.7	42
	N.L.	102.4	42
MC_{13} TRIS	H.L.	50.0	42
	L.L.	62.7	42
	N.L.	-	42
MC_{13}	H.L.	36.7	84
	L.L.	44.7	84
	N.L.	110.5	84
MC_{13}	L.L. Shaker	14.7	42
MC_{13} TRIS	L.L. Shaker	19.8	42

H.L. = high light L.L. = low light N.L. = no light

Table 4.

Growth of B. braunii under different light intensities in the medium MC_{13} at 10°

In contrast to the findings of Belcher (1957) that intensity of light (above 250 foot candles) had little effect on rate of growth, the above results indicate that high light intensity promotes more rapid division

of the colonies than low light, it is noteworthy that there was little difference in dry weight measurements between the different cultures.

(iii) Growth in the presence of TRIS buffer

TRIS buffer appears to have an inhibitory effect on cell division in the alga, as growth in media containing TRIS was always poorer than that in non-TRIS containing controls. In spite of this the dry weight of alga in non-TRIS containing controls after a six week period was always lower than that of the TRIS containing cultures (Table 5).

TRIS Containing Cultures

Dry weight of colonies : 504 mgm.

Weight of hydrocarbon : 0.6 mgm.

Non-TRIS Containing Controls

Dry weight of colonies : 358 mgm.

Weight of hydrocarbon : 5.19 mgm.

Table 5.

As hydrocarbon content proved to be very low in the TRIS containing cultures the greater colony dry weight of these cultures compared with the non-TRIS controls could not be assigned to hydrocarbon production. Neither could it be due to increased fatty acid synthesis in these cultures as shown in Table 6.

	Wt. Sap Lipide	Dry wt. of colonies	% Sap Lipide
TRIS	1.0 mgm	49.08 mgm	2.0
Non-TRIS	3.5 "	46.91 "	7.5

Table 6. Effect of TRIS on Lipide Production

Although TRIS buffer appears to inhibit hydrocarbon and fatty acid synthesis as well as cell division in B. braunii it promotes the increase of colony dry weight.

It is possible that this may simply be a pH effect as the final pH of the control cultures in Table 5 was 9.5 and 9.6 respectively, whereas the final pH of the TRIS containing was only 7.8. This was also the case for the results in Table 6 where the final pH was 8.8 in the controls and 7.7 in the TRIS containing cultures.

Although it is possible that when the alga was in a medium with a high pH the synthesis of fatty acids and hydrocarbons was promoted, and when it was in buffered medium of lower pH that a more 'normal' metabolism was usual although such a system does not account for high hydrocarbon production and subsequent bloom production in the wild. It seems more likely that TRIS buffer has some inhibitory effect on cell division and on lipoid metabolism.

(iv) Growth under different light intensities at 20°

(i) High light; (ii) Low light; (iii) No light.

Culture		Mean doubling time for total exp. period (days)	Duration of exp. (days)
MC_{13}	H.L.	17.1	42
	L.L.	24.6	42
	N.L.	127.3	42
MC_{13} TRIS	H.L.	19.4	42
	L.L.	28.8	42
	N.L.	221.1	42
MC_{13}	H.L.	45.2	84
	L.L.	72.7	84
	N.L.	-	84
MC_{13}	L.L. Shaker	12.5	42
MC_{13} TRIS	L.L. Shaker	14.0	42
MC_{13} 1/10N	H.L.	24.7	42
	L.L.	42.0	42
	N.L.	-	42
MC_{13} O/N	H.L.	127.3	42
	L.L.	127.3	42
	N.L.	-	42

H.L. = high light L.L. = low light N.L. = no light

Table 7.

Growth of B. braunii under different light intensities in the medium MC_{13} at 20°

The results obtained at 20° for six week cultures in MC_{13} were very similar to those obtained at 10°. The TRIS buffered culture results

were better than those obtained at 10° but growth in these cultures was still slower than in the non-TRIS containing controls. Shaking cultures at 20° provided slightly more rapid growth than was obtained by shaking at 10°.

Cultures with 1/10th the normal combined nitrogen supplied in MC_{13} were set up with cultures containing no combined nitrogen. Growth in the MC_{13} 1/10N cultures was markedly slower than in the MC_{13} controls, and in the MC_{13} 0/N growth was almost inhibited altogether. Any growth made in the latter cultures was probably due to combined nitrogen already in the colonies when they were inoculated into the MC_{13} 0/N medium.

(g) Estimation of a Growth Constant for B. braunii

As growth, measured over the total experimental period, was most rapid in $MC_{10}T_1$, $MC_{13}T_1$ and in S_{66} of all the treatments examined an estimation of the growth constant of the alga in these media and under these growth conditions was calculated (Table 8).

The optimum value of K established for B. braunii under these conditions was 0.60 over a two day period in S_{66}. In comparison, Myers (1964) reported a K maximum of 1.96 for Chlorella pyrenoidosa. As shown in Table 8 the value of K varies little under the conditions of the three media used in these experiments.

S_{66}		$MC_{10}T_1$		$MC_{13}T_1$	
Time (days)	Value of K	Time (days)	Value of K	Time (days)	Value of K
0 - 2	0.55	0 - 2	0.52	0 - 2	0.52
2 - 4	0.51	2 - 4	0.52	2 - 4	0.52
4 - 6	0.55	4 - 6	0.52	4 - 6	0.52
6 - 8	0.58	6 - 8	0.52	6 - 8	0.52
8 - 10	0.60	8 - 10	0.52	8 - 10	0.51
10 - 12	0.52	10 - 12	0.52	10 - 12	0.50
12 - 14	0.51	12 - 14	0.52	12 - 14	0.50
14 - 16	0.51	14 - 16	0.51	14 - 16	0.50
16 - 18	0.50	16 - 18	0.51	16 - 18	0.50
18 - 20	0.50	18 - 20	0.50	18 - 20	0.50
20 - 22	0.50	20 - 22	0.50	20 - 22	0.50
22 - 24	0.50	22 - 24	0.50	22 - 24	0.50

Table 8.

Estimation of K for B. braunii grown in the media $MC_{10}T_1$, $MC_{13}T_1$ and S_{66} at 15° and low light intensity.

2. Production of an Axenic Culture

The culture obtained from the Cambridge culture collection although unialgal was not axenic. When an 0.5 ml. aliquot of colonies were plated out onto 1% nutrient agar and incubated at 25° for 48 hours subsequent microscopic examination of the agar surface showed the presence of a yeast and two bacteria of the genus Vibrio as well as the B. braunii colonies.

A number of techniques for the production of axenic algal cultures have been evolved over the years. Pringsheim (1946) washed algae in sterile drops of medium to remove bacteria. Zobell and Long (1938) experimented with heat, ultra violet irradiation and various chemical bactericidal compounds, of which acriflavine proved to be bacteriostatic in concentrations that did not greatly interfere with the growth of diatoms. Gerloff, Fitzgerald and Skoog (1950) used ultra violet irradiation to obtain bacteria free cultures of blue green algae. The use of antibiotics in the production of axenic cultures was first reported by Fish (1950), who pre-treated algae in 5,000 units/ml. of penicillin then sub-cultured into a peptone broth in which the concentration of pencillin was decreased to 500 units/ml. This prevented the growth of bacteria for up to seven days. Spenser (1952) elaborated on this work by using penicillin and streptomycin to purify Nitzchia closterium forma minutissima. Subsequently Droop (1967) has produced a method by which various mixes of antibiotics may be used to purify algal cultures.

To obtain bacteria free cultures of B. braunii the following methods were tried.

(a) Washing Methods

Pringsheim's (1946) method of repeated washing in sterile medium proved to be impractical in a non motile colony protected by a heavy mucilaginous sheath in which bacteria can lodge; but an extension of this method was tried by washing with sterile medium and then plating out on agar containing 0.01% potassium tellurite, a bacteriostatic agent (Ducker and Willoughby, 1964). The colonies were washed in ten successive sterile batches of S_{66} medium and then plated out on 1% Oxoid No.3 agar, made up with S_{66}, and containing 0.01% potassium tellurite. After a three week period there was no obvious growth. Colonies were carefully picked from the agar, in a sterile inoculating room, by means of a sterile platinum loop and then inoculated into fresh S_{66} liquid medium. After a three week period at low light intensity an 0.1 aliquot was removed from each of the cultures, after spinning down, by means of sterile pasteur pipettes and inoculated onto a sterility test medium of casein (50 mgm./100 ml.) and glucose (1 gm./100 ml.). After incubation at 25° for 48 hours all petri dishes of sterility test medium showed bacterial development indicating that the culture was infected. This method was therefore no more successful than washing alone.

(b) Ultraviolet irradiation of colonies

Ultra-violet irradiation of the colonies was carried out with a Camlab U.V. apparatus type (TL-900) using a wavelength of 254 mμ.

The colonies to be sterilized were spread evenly in 0.5 ml. of medium on 1% Oxoid agar in petri dishes and then placed beneath the U.V. source at a distance of three inches from the lamp. Treatment by U.V.

irradiation was initially for 1, 5, 10 and 15 seconds. Colonies from each treatment were then removed by sterile pasteur pipettes, inoculated onto petri dishes of 1% nutrient agar and incubated at 25° for 48 hours. The remaining colonies in each treatment were inoculated into marked test tubes, each containing 6 ml. of sterile S_{66} medium. These cultures were grown at 25° and a light intensity of 250 foot candles until the results of the sterility tests were known. After 48 hours the sterility test plates were all seen to be heavily contaminated, and so all cultures were discarded. The exposure to U.V. irradiation was increased to 20, 25, 30, 35 and 40 seconds before subculturing into S_{66} and carrying out sterility tests as before. Again all the test dishes were contaminated and the cultures were discarded, after 48 hours.

In an effort to obtain axenic cultures by this method exposures to U.V. radiation were greatly increased as shown in Table 9.

Exposure time (secs)	No. of bacterial colonies in 0.1 ml.	Percentage Kill
0	250	0
45	180	28.0
60	186	25.6
75	156	37.6
90	80	68.0
120	38	84.8
150	85	66.0
180	70	72.0
240	7	97.2
300	1	99.6 *
360	1	99.6 *

* After two weeks in culture alga lost colour and died.

Table 9. Ultraviolet irradiation of colonies.

After U.V. treatment 0.1 ml. of culture supernatant was withdrawn as usual and placed in separate marked petri dishes containing 1% nutrient agar. The petri dishes were incubated in duplicate at 25° for 48 hours and the number of bacterial colonies which grew in each petri dish were noted as well as the relevant exposure time to the U.V. radiation. The remaining colonies in each treatment were inoculated into S_{66} and cultured as before. Although this method came very close to producing an axenic culture after five minutes irradiation it proved useless as the colonies so treated died about two weeks later.

To test if the medium was at fault the experiment was repeated with colonies inoculated into S_{66} medium which contained an extract of the Vibrio bacteria (5 ml. medium : 1 ml. extract) after U.V. treatment. This extract was prepared by removing 100 bacterial colonies from nutrient agar and autoclaving in 5 ml. of S_{66} for twenty minutes at 15 lb. in.^{2}. This failed to promote the development of the colonies. In the light of later work with antibiotics this experiment was repeated, the colonies being inoculated into E_1 after treatment, but in all cases they died. It was concluded that the long exposure to U.V. radiation, as well as killing almost all the bacteria had killed all the algal colonies.

(c) Antibiotic Treatments

The method used in this treatment was essentially that of Droop (1967). The antibiotic mixes (Appendix 5) were stored at 7° until immediately before use. It is important to note that although antibiotics are easily applied there is no way of knowing if the cells exposed to antibiotics have become damaged or otherwise altered. This

latter point is exceedingly important as often it is reasonably easy to kill the bacteria but almost impossible (in some cases) to keep the alga alive subsequently.

Six parallel (6 ml.) tube cultures were prepared in each case for each antibiotic treatment. The cultures being as heavy as possible but still vigorous. The antibiotic mixes were prepared as 12 ml. mixtures, 6 ml. allowing for wastage. Six ml. of the mixture was sterilized by passing through a sterile Millipore filter system containing a sterile 25 mm. Millipore "filter-paper" (HAWP 02500 H.A. 0.45μ) into one of the cultures. This culture then contained 12 ml. (i.e. 6 ml. of medium and 6 ml. of antibiotic mixture) and the original antibiotic concentration had been halved. After thorough mixing (by whirlimixer) half of this 12 ml. was decanted, over a flame, into a clean sterile stoppered test tube. One of the two tubes was set aside marked 1. The other was mixed, over a flame, with another of the 6 ml. cultures. After mixing and placing half in each tube one was again set aside and marked 2. This process was continued until there were seven tubes each containing the alga and decreasing concentration of antibiotics. By this method a serial dilution of antibiotics was obtained - each tube containing half the antibiotic concentration of the previous one. One drop of a sterility test medium (50 mgm. casein + 1 gm. glucose/100 ml. glass distilled water) was always added to each tube to stimulate bacterial division as antibiotics attack dividing bacteria. The cultures were then illuminated by fluorescent tubes (light intensity 250 foot candles) at 25°. After 24 hours the cultures were removed. One drop of medium was withdrawn from each tube by means of a

sterile pipette and inoculated onto nutrient agar in petri dishes, which were then marked to indicate which culture they represented. After incubation of these plates at 25° for 48 hours there were no bacterial contaminants visible on any of them. The cultures were then spun down at 1,500 r.p.m./5 min. and the antibiotic mixture decanted. The colonies were then resuspended in 6 ml. of sterile glass distilled water and subcultured into duplicate marked 100 ml. flasks each containing 30 ml. of MC_{13} medium. These cultures were grown in a light intensity of 250 foot candles from fluorescent tubes at 25° for a three week period. Although these cultures were bacteria free they did not survive long. They all died within six weeks of the antibiotic treatment. It was concluded that either the vigorous antibiotic treatment or else the subsequent conditions of environment and/or medium had an adverse effect on the colonies.

The experiment was repeated using antibiotic mixes VI and VII. After 24 hours exposure to the antibiotics the cultures treated with antibiotic mix VI were subcultured from the original MC_{13} used in the treatment into MC_{10}, MC_{13} and Beijerinck's medium, all as 10 ml. tube cultures. Those treated with antibiotic mix VII were subcultured into MC_{10}, MC_{13} and modified Knop's solution as 10 ml. tube cultures. Only one of these subcultures - from the mix VI treatment - which had been subcultured into MC_{10} survived and was bacteria free after a three week period. It had been prepared from the third tube in the dilution series of antibiotics. It was then subcultured into MC_{10} and MC_{13}, both enriched with 3% soil extract and grown at 25° at the usual light intensity

but it died within a month.

From these results it became obvious that the original treatment needed modification in its application to B. braunii. It was possible that the alga died for a number of reasons, of which three seemed most likely to repay study:-

(i) That the alga had been placed in medium too concentrated in mineral nutrients after the bacterial flora was removed.

(ii) That a certain concentration of antibiotics had been carried over into the subcultures and was having a progressive effect.

(iii) That the culture medium lacked an essential growth factor formerly supplied to the alga by its bacterial flora.

To test the importance of (i) the alga was subcultured, after antibiotic treatment, into S_{66}, 1/10 S_{66}, 1/100 S_{66}, 1/10 MC_{13} and 1/10 MC_{10} media in an attempt to obtain successful axenic cultures. Although the 1/10 S_{66} subcultures lasted almost a month after treatment none of the cultures were successful, so it appeared that the concentration of the media used previously for subculturing was not too concentrated and that some other factor was preventing the alga's development in axenic culture.

To investigate whether a certain concentration of antibiotics had been carried over into the subcultures (ii) and/or whether there may have been an adhering coat of antibiotics on the colony surface the following modifications were made to the treatment.

The antibiotic treatment period was reduced to 1, 3 and 5 hours, and after removal of a 0.1 ml. aliquot of the culture supernatants for sterility tests the remainder was decanted and the colonies resuspended in sterile

glass distilled water, three times over, before subculturing into a variety of media. Although the period of exposure to antibiotics was much reduced it was noted that the results from sterility tests were very similar to those obtained from 24 hour exposure to antibiotics (Table 10).

Dilutions	1	2	3	4	5	6
1 hour treatment	-	-	+	-	+	+
3 hour treatment	-	-	-	+	+	+
5 hour treatment	-	-	+	-	+	+

- = no bacteria + = bacteria

Table 10.

1, 3 and 5 hour treatments with antibiotic mix IV

From this table of results the following cultures were assumed safe to subculture.

Antibiotic Mix

IV 1 hour treatment dilutions 2 + 4 i.e. 1_2 and 1_4

IV 3 hour treatment dilutions 2 + 3 i.e. 3_2 and 3_3

IV 5 hour treatment dilutions 2 + 4 i.e. 5_2 and 5_4

These cultures were subcultured into S_{66} (5 ml.) supplemented with 1 ml. of a bacterial extract prepared by autoclaving 100 bacterial colonies (grown on nutrient agar) in 5 ml. of S_{66} for 20 minutes at 15 lb. $in.^2$. After two weeks all cultures were alive but growth was extremely slow, but by the end of one month all the cultures had died. The shortened

exposure time killed bacteria successfully but in spite of rinsing in sterile glass distilled water before subculturing and in spite of the addition of a bacterial extract to the subcultures they still died.

The possibility that the media used for the growth of B. braunii, after antibiotic treatment, lacked some essential growth factor for the development of the alga in its axenic state (iii) was investigated in the following manner.

A three hour antibiotic treatment in mix IV was carried out on three separate series of cultures (IV_1, IV_2 and IV_3). After treatment, and preparation of the usual sterility test plates, each of the cultures was washed three times in sterile glass distilled water, then taken up in 6 ml. of sterile glass distilled water and subcultured into each of the following media -

A : S_{66} + vit. B_1, vit. B_2, vit. B_6 + vit. B_{12} All 100µg./l.

B : S_{66} + GA_3 (1 p.p.m.) + folic acid (0.5 p.p.m.).

C : S_{66} + inositol (0.1 p.p.m.) + nicotinic acid (0.1 p.p.m.).

D : S_{66} + 5 mgm./l. coconut milk + 5 mgm./l. yeast extract.

F : S_{66} + casein (10 p.p.m.) + Bactopeptone (10 p.p.m.).

G : S_{66} + Pectin (0.1%) + Asparagine (10 p.p.m.).

H : S_{66} + Ascorbic acid (10 p.p.m.) + glutamic acid (10 p.p.m.).

I : S_{66} + coconut milk 5 mgm./l. ⟶ 0.5%.

E_1 - Enriched soil extract.

Sterility tests were performed three weeks after subculture. Results are indicated in Table 11. Bacteria-free cultures which were alive and healthy were left to grow. By the end of another three week period only the E_1 cultures remained alive. They were retested and

	IV_1 DILUTIONS						IV_2 DILUTIONS						IV_3 DILUTIONS					
Additives	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
S_{66} + A	-	-	-	-	+	+	-	-	-	+	+	+	-	-	-	-	-	+
B	-	-	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	+
C	-	-	-	-	+	+	-	-	-	-	-	D	-	D	-	-	-	-
D	-	+	+	+	+	+	-	D	+	+	+	+	D	D	+	+	+	+
F	-	+	-	+	-	D	D	-	+	-	+	D	D	-	-	-	-	+
G	+	+	D	+	+	+	D	+	+	+	+	D	D	D	+	D	+	+
H	D	-	-	D	-	D	D	D	D	-	-	D	D	-	-	D	-	D
I	-	-	-	-	-	D	D	-	-	D	+	D	D	D	D	D	-	D
E_1	-	-	-	-	-	-	-	-	-	-	-	-	D	-	+	-	-	-

D = dead; - = bacteria free + alive; + = contaminated

Table 11.

Growth in various media after antibiotic treatment.

proved bacteria-free and have since been maintained in this axenic state. Later it was noted that about 6-8 weeks after treatment colonies were able to grow in MC_{13} in the axenic state. The initial period in E_1 has always been found necessary before subculturing into the defined medium.

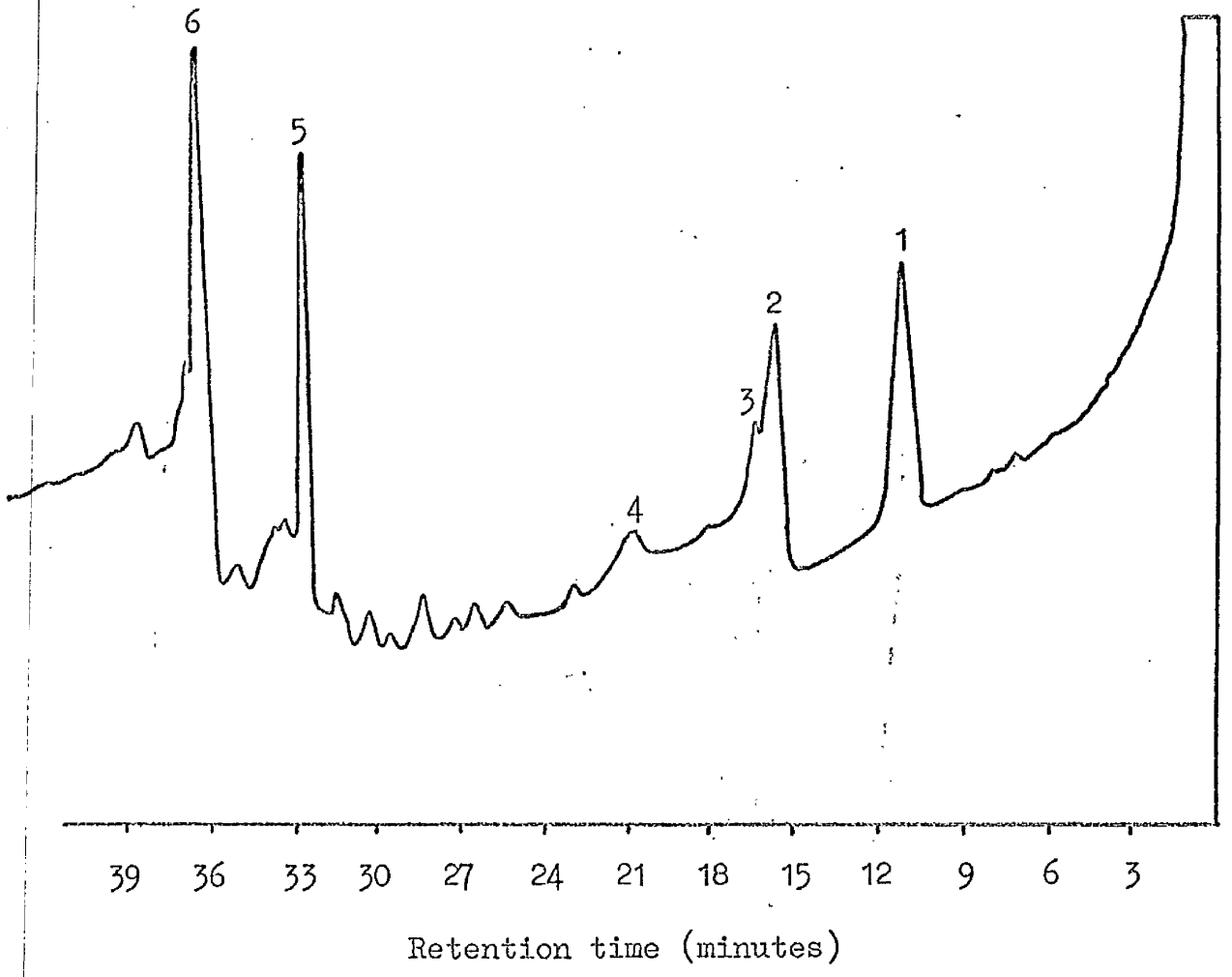


Fig. 11. GLC trace of fatty acids from B. braunii.

PART IV. LIPIDS OF BOTRYOCOCCUS (MATERIAL C)

Introduction

When B. braunii was stained with Sudan III "fatty cups" became visible surrounding each protoplast, and as a result a study of the fatty acid content of the alga was undertaken.

The colonies were cultured in MC_{13} under constant environmental conditions and then extracted in the following manner.

The colonies were oven dried on weighed filter paper, weighed and then ether extracted in a Soxhlet apparatus for seven hours. The solvent was then removed by rotary evaporation and the residue was treated with about a dozen sodium hydroxide pellets, 25 ml. of water and 0.5 ml. of ethyl alcohol. After shaking gently the flask was boiled for three hours under reflux and then allowed to cool after which the contents were diluted 1:1 with water. Extraction with ether (2 x 1/3 volume) yielded the unsaponifiable lipoid. The sodium hydroxide/water layer was then acidified with 6N hydrochloric acid and extracted with ether (2 x 1/3 volume). Both ether extracts were then treated with anhydrous sodium sulphate to remove water.

Gas chromatographic examination

Gas chromatography was carried out on the methyl esters of the fatty acids. They were prepared from the fatty acids by reaction with diazomethane (CH_2N_2) and when temperature programmed on 5% SE-30, a dimethylpolysiloxane, coated on Gas Chrom P, between 150° and 300° at 3° a minute they produced a trace as shown in Figure 11. By comparative GLC of

known fatty acid esters it was shown that each of these peaks represented a certain fatty acid with two well marked maxima at C_{16-18} and C_{24-26} i.e. the major components of the fatty acid mixture contained in the colonies consisted of palmitic acid ($C_{15}H_{31}COOH$: peak 1), stearic acid ($C_{17}H_{35}COOH$: peak 3), oleic acid ($CH_3[CH_2]_7CH=CH-[CH_2]_7COOH$: peak 2) and acids with 24 and 26 carbon length chains having an unknown number of double bonds (peaks 5,6). To examine fatty acid production under different environmental conditions experiments were conducted in duplicate in MC_{13} and MC_{13} containing TRIS buffer at 10 and 20° to study the effect of different media and environment on fatty acid production in the alga (Table 12).

Media	10° % Sap. lipids	20° % Sap. lipids
MC_{13}	7.5	2
MC_{13} TRIS	2.0	3
MC_{13} 1/10N	-	5
MC_{13} 0/N	-	27

MC_{13} 1/10N - Modified Chu $_{13}$ with 1/10 normal combined nitrogen

MC_{13} 0/N - Modified Chu $_{13}$ with no combined nitrogen

Table 12.

Fatty acid content of colonies in various media

Although there was a wide fluctuation in the fatty acid content of the colonies under different treatments, expressed as a percentage of colony dry weight, there was no change in the constituent fatty acids in any of the treatments.

Since similar depth of colour was noted with Sudan III in all cultures and as the basis of Sudan III staining is its reaction with unsaturated carbon atoms (-C=C-) and as the fatty acids recorded were mostly fully saturated ones, it seems unlikely that the action of the stain could be the result of the fatty acid content. Such a suggestion appears to invalidate the hypothesis of Blackburn and Temperley (1936) that the cups surrounding the cells of Botryococcus are composed of true fats. The possibility that these cups might be composed of hydrocarbons called for further investigation, since such molecules, if present in an unsaturated state, would give rise to the observed colour reaction with Sudan III.

1. Extraction of hydrocarbons from colonies

Hydrocarbons were extracted from green, actively growing, colonies of material C cultured in MC_{13} under constant environmental conditions of 15° and a light intensity of 1,000 foot candles (16 hour day) in the following way.

The colonies were vacuum dried, using a rotary evaporator, weighed, and then extracted with 'Analar' acetone at 4° for three days. After evaporation of the acetone the residue was dissolved in 10 ml. of re-distilled petroleum ether ($40-60^{\circ}$ B.P.). The hydrocarbons were separated in a glass chromatography column (1.5 x 15 cm.) provided with a sintered glass filter disc and filled to a depth of two centimetres with neutral alumina. The hydrocarbons were eluted from the column using redistilled petroleum ether. This hydrocarbon fraction was weighed and stored, ready for gas chromatography, in diethyl ether under nitrogen in

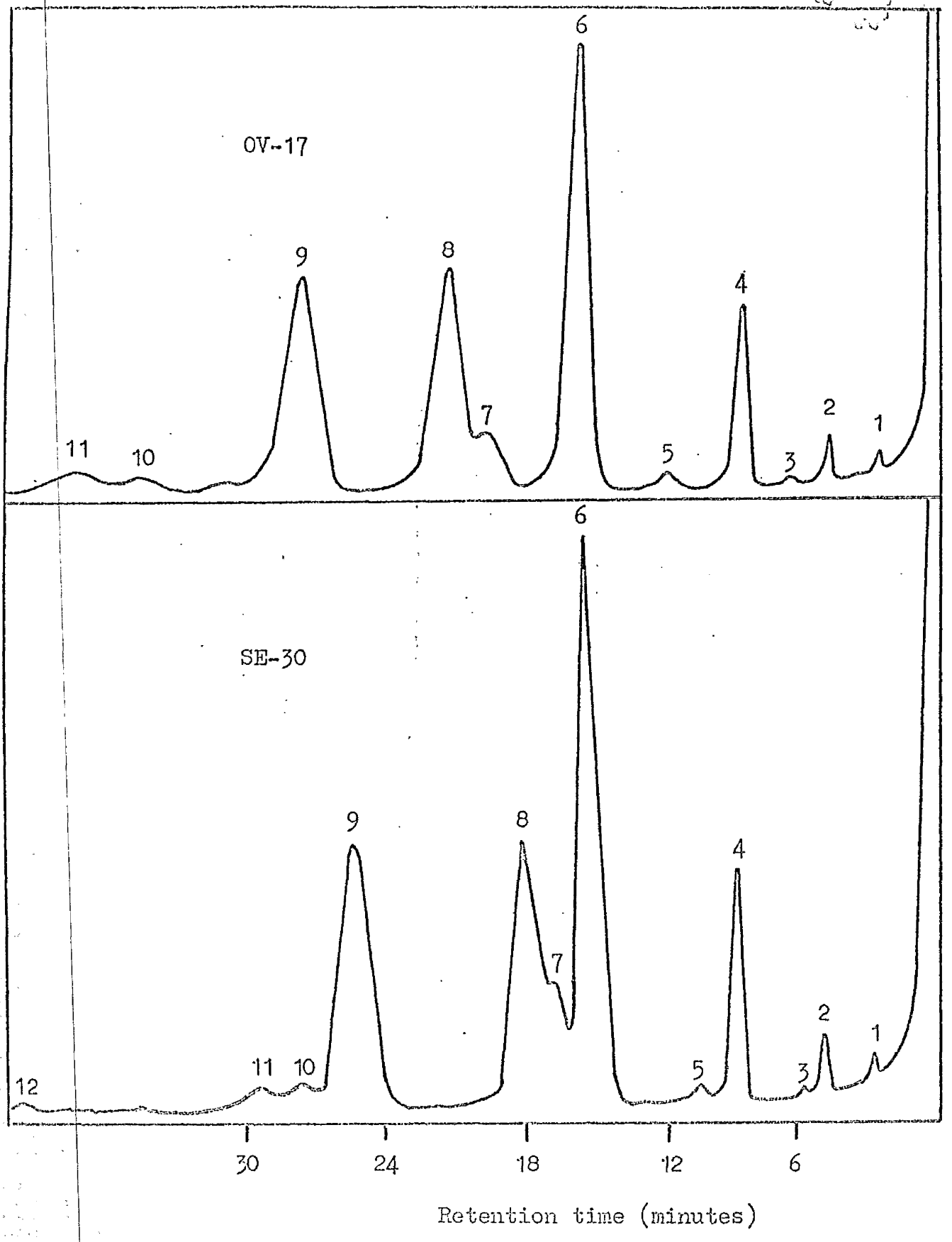


Fig. 12. GLC trace of hydrocarbons from B. braunii

a deep freeze to prevent polymerisation.

2. Analysis by gas liquid chromatography (GLC)

The hydrocarbons were dissolved in diethyl ether (1 ml.) and chromatographed on a Pye series 104 gas chromatograph. A nine foot glass column packed with 3% OV-17, a phenyl-methylpolysiloxane coated on 100-120 mesh Gas Chrom Q, was used routinely and on some occasions a column packed with 5% SE-30, a dimethylpolysiloxane coated on Gas Chrom P was also used. Operating conditions were 244° with a nitrogen carrier gas flow rate of approximately 40 ml./min. The n-alkane octacosane [$C_{28}H_{58}$] was used as a routine check for operating parameters. Retention data were determined relative to this and as retention indices. (Ambrose and Ambrose, 1961) (Ettre, 1964).

3. Analysis by gas chromatography/mass spectrometry (GC/MS)

An L.K.B. 9000 combined gas chromatograph/mass spectrometer was used with a ten foot OV-1 column, similar in properties to SE-30, with operating conditions as previously described.

4. Chromatographic results

The hydrocarbon fraction produced chromatographic traces as shown in Figure 12 which shows that there were three well marked homologous series of hydrocarbons, as indicated by the retention data listed in Table 13:-

Hydrocarbon	Series	Retention index		M.W.
		SE-30	OV-17	
1	A	2305	2295	- *
2	A	2500	2495	-
3	B	-	2605	-
4	A	2705	2705	376
5	B	2765	2820	-
6	A	2905	2915	404
7	C	2945	2995	-
8	B	2965	3015	402
9	A	3100	3120	432
10	C	3135	3195	-
11	B	3155	3225	430
Botryococcene		2790	2800	466
Isobotryococcene		-	-	466

* Not recorded

Table 13. GLC data for hydrocarbons in B. braunii

These results bear a marked similarity to these reported recently by Gelpi et al. (1968) for the alga.

(a) An A series of six members (peaks 1, 2, 4, 6, 9 and 12)

(b) A B series of four members (peaks 3, 5, 8 and 11)

(c) A C series of two members (peaks 7 and 10), which, from the GLC data are probably isomeric with the B series.

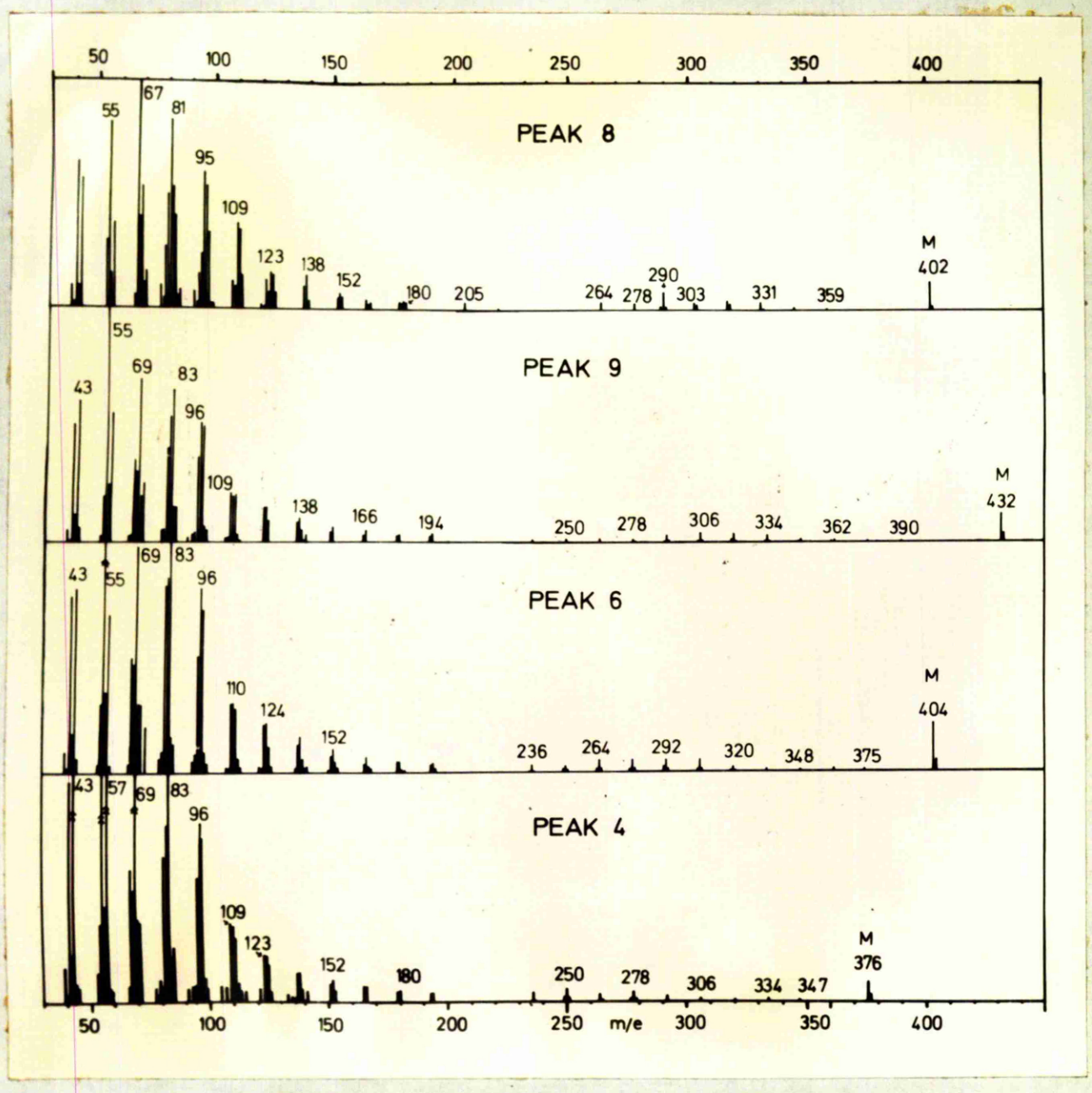


Fig. 13. Mass spectra of peaks 4, 6, 8 and 9.

The percentage that each hydrocarbon represented of the total hydrocarbon fraction was assessed by measuring the area under each peak and expressing it as a percentage of the total peak area (Ambrose and Ambrose, 1961). Retention indices of the components of the samples were determined by analysing the samples and a mixture of suitable normal alkanes (in this case $nC_{28}H_{58}$, $nC_{30}H_{62}$, $nC_{32}H_{66}$) and retention times determined (expressed as distances on the chromatographic chart). The retention distances of the n-alkanes were plotted on the logarithmic axis of semi-logarith paper against the arbitary numbers of 2,800, 3,000 and 3,200 respectively, which are taken (by definition) to be the retention indices assigned to $nC_{28}H_{58}$, $nC_{30}H_{62}$ and $nC_{32}H_{66}$. The retention indices of the members of the sample were then calculated from this scale by interpretation of retention distances and reading off the corresponding point on the retention index scale (Ettre, 1964 : Kovat's Method).

As peaks 4, 6, 8 and 9 were the dominant peaks of the hydrocarbon mixture they were studied further.

5. Mass spectra of peaks 4, 6, 8 and 9. (Fig. 13)

The compounds were shown by mass spectrometry (Table 14) to have the following molecular weights.

Peak	Molecular wt.		
4	376	C_nH_{2n-2}	n = 27
6	404	C_nH_{2n-2}	n = 29
8	402	C_nH_{2n-4}	n = 29
9	432	C_nH_{2n-2}	n = 31

Table 14.

Compounds represented by peaks 4, 6 and 9 are hydrocarbons of the

general formula C_nH_{2n-2} each having two "double bond equivalents" and differing from the next member by 28 mass units, i.e. a C_2H_4 fragment. They are therefore all members of one series, which may be designated the A series.

A double bond equivalent may be defined as a functional group which reduces the number of hydrogen atoms by two from the predicted number : C_nH_{2n+2}. A C=C double bond, a ring structure such as a cyclohexane or a carbonyl group [C=O] etc. are examples of "double bond equivalents".

After calculation of their retention indices these hydrocarbons were designated $2^{\Delta}C_{2705}$ (peak 4), $2^{\Delta}C_{2915}$ (peak 6) and $2^{\Delta}C_{3120}$ (peak 9). The 2^{Δ} indicating that each hydrocarbon contained two double bond equivalents.

Peak 8 ($3^{\Delta}C_{3015}$) is a member of a B series having the general formula C_nH_{2n-4}, i.e. each member of the series had three double bond equivalents per hydrocarbon. A C series (peaks 7 and 10) was also present which from the GLC data appeared to be isomeric with the B series. Thus these green active colonies produced three homologous series of aliphatic hydrocarbons A, B and C which are thought to be straight chain, or at the most slightly branched. There was no evidence of aromatic structure in these hydrocarbons from their mass spectra.

As these findings on hydrocarbon content and structure, for green active colonies, were in total contrast to the findings on hydrocarbon content and structure for brown resting colonies (material W) and for coorognite, the peat-stage equivalent of B. braunii, a study of the structure of the hydrocarbons of the green material was undertaken. The mass spectral evidence pointed to the hydrocarbons being straight chain, or almost so, and therefore this study resolved itself into a search for the sites of the

presumed double bonds in the molecules. For convenience, subsequent discussion will assume a straight chain diolefin structure but it should be stressed that branched chain and/or cyclic structures are not rigourously excluded.

6. Hydrocarbon content of other simple green algae in culture

Since the hydrocarbon content, and the hydrocarbon series of Botryococcus braunii is regarded as unusual for simple green algae, comparisons with other forms regarded as being taxonomically related were made by analysing the amount and nature of the hydrocarbons present under similar cultural conditions. The algae tested were:-

	Cambridge Culture No.
Chlorococcum multinucleum	213/1A
Chlorococcum macrostigmatum	213/9
Pediastrum duplex	LB261/2
Pandorina morum	60/1C

A 5% inoculum of each of these were grown in duplicate and in parallel series with Botryococcus in MC_{13} medium at 15° with a 16 hour day of low light (250 foot candles) intensity, and analysed after six weeks and after twelve weeks. Table 15 confirmed that the amount and composition of the hydrocarbon present in Botryococcus was markedly different from the other species examined. Thus the hydrocarbon synthesis by Botryococcus may be a metabolic process of considerable significance and one which warrants further investigation and appreciation.

Species	Hydrocarbon as % of dry wt.
Chlorococcum multinucleum	2.90
Chlorococcum macrostigmatium	0.01
Pediastrum duplex	2.10
Pandorina morum	2.88
Botryococcus braunii	15.61

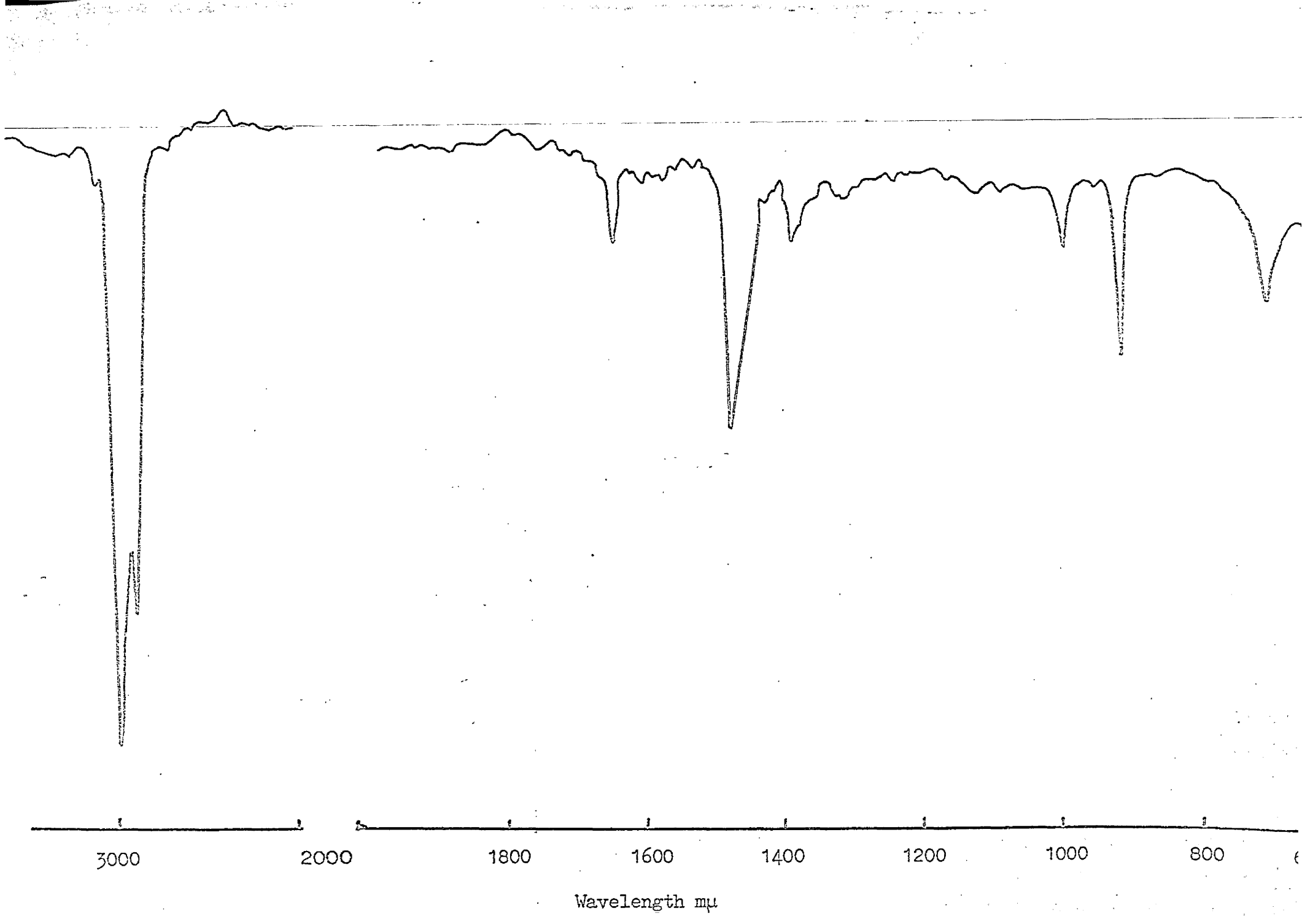
Table 15.

Percentage Hydrocarbon in Cultures after twelve weeks

Retention Indices	Pandorina morum	Chlorococcum multinucleum	Chlorococcum macrostignatium	Pediastrum duplex	Botryococcus braunii
2275	-	8.7	1.7	1.3	-
2295	-	-	-	-	0.07
2395	-	8.7	1.3	1.8	-
2450	-	-	10.2	6.3	-
2500	0.3	17.4	0.9	6.4	0.20
2605	-	8.7	0.9	1.3	0.04
2705	12.5	8.7	8.5	10.3	16.00
2820	Trace	34.8	3.4	2.1	0.19
2915	61.1	Trace	45.5	50.3	71.60
2995	-	13.0	6.4	-	1.10
3015	Trace	-	-	-	5.10
3055	Trace	-	-	-	-
3120	-	-	-	-	5.60
3195	-	-	-	-	0.40
3225	26.1	-	21.3	20.2	0.10
m hydrocarbon/100 mgm lture dry wt.	2.87	2.94	0.01	2.11	15.61

Table 15a.

Retention indices and percentages of individual hydrocarbons present in cultures after twelve weeks


3000
2000
1800
1600
1400
1200
1000
800
Wavelength mμ

7. Structure of the main A series hydrocarbons : $2^{\Delta}C_{2705}$-, $2^{\Delta}C_{2915}$- and $2^{\Delta}C_{3120}$-

Infra red spectroscopic analysis of the total hydrocarbon mixture from green active colonies was carried out using a Unicam S.P. 200 spectrophotometer (Fig. 14) and showed absorption at 995 cm^{-1}, 910 cm^{-1} and 1,640 cm^{-1} consistent with the presence of a vinyl group ($R-CH=CH_2$) in at least some components of the mixture. This leaves one other double bond to be accounted for in the A series, assuming that the vinyl absorption is related to this series.

To study the main A series hydrocarbons adequately it was necessary to separate them from the B and C series which also occurred in the crude mixture extracted from the colonies. The mixture was applied as a narrow zone on to thin layer (0.25 mm.) silver nitrate impregnated silica gel plates (Morris, 1966), (Zabkiewicz et al., 1968) which were then developed using redistilled petroleum ether (40-60 B.P.). After drying, material on the plates was located by spraying with dichlorofluorescein and scanning with a U.V. lamp [350 mμ] when zones enhanced the fluorescence of the dye. The double bonds in the hydrocarbons complex with the silver nitrate to form π-complexes [i.e. the π-electrons of the olefinic linkage are involved] as shown:

$$R_1-CH=CH-R_2-CH=CH-R_3 + AgNO_3$$

$$\downarrow$$

$$R_1-CH=CH-R_2-CH=CH-R_3$$

$$\downarrow \qquad\qquad \downarrow$$

$$Ag^+ \qquad\qquad Ag^+$$

The members of the B and C series with their three double bonds per hydro-

carbon tend to complex more with the silver than do the members of the A series which have only two double bonds per hydrocarbon. The zone of the separated A series was then scraped from the plate. Recovery of the hydrocarbons from the silica gel was effected by placing the absorbent in small chromatography tubes and then eluting with diethyl ether. After collection and evaporation of the ether the hydrocarbons were dissolved in diethyl ether (1 ml.) for examination by GLC.

(a) Location of double bonds in long chains

(i) Cleavage of ethylenic linkages.

Subbaram (1964) devised a method by which ethylenic linkages were subjected to oxidative cleavage. The resulting free carboxyl groups were esterified with diazomethane and analysed by GLC. In relation to an A series hydrocarbon this would result in:

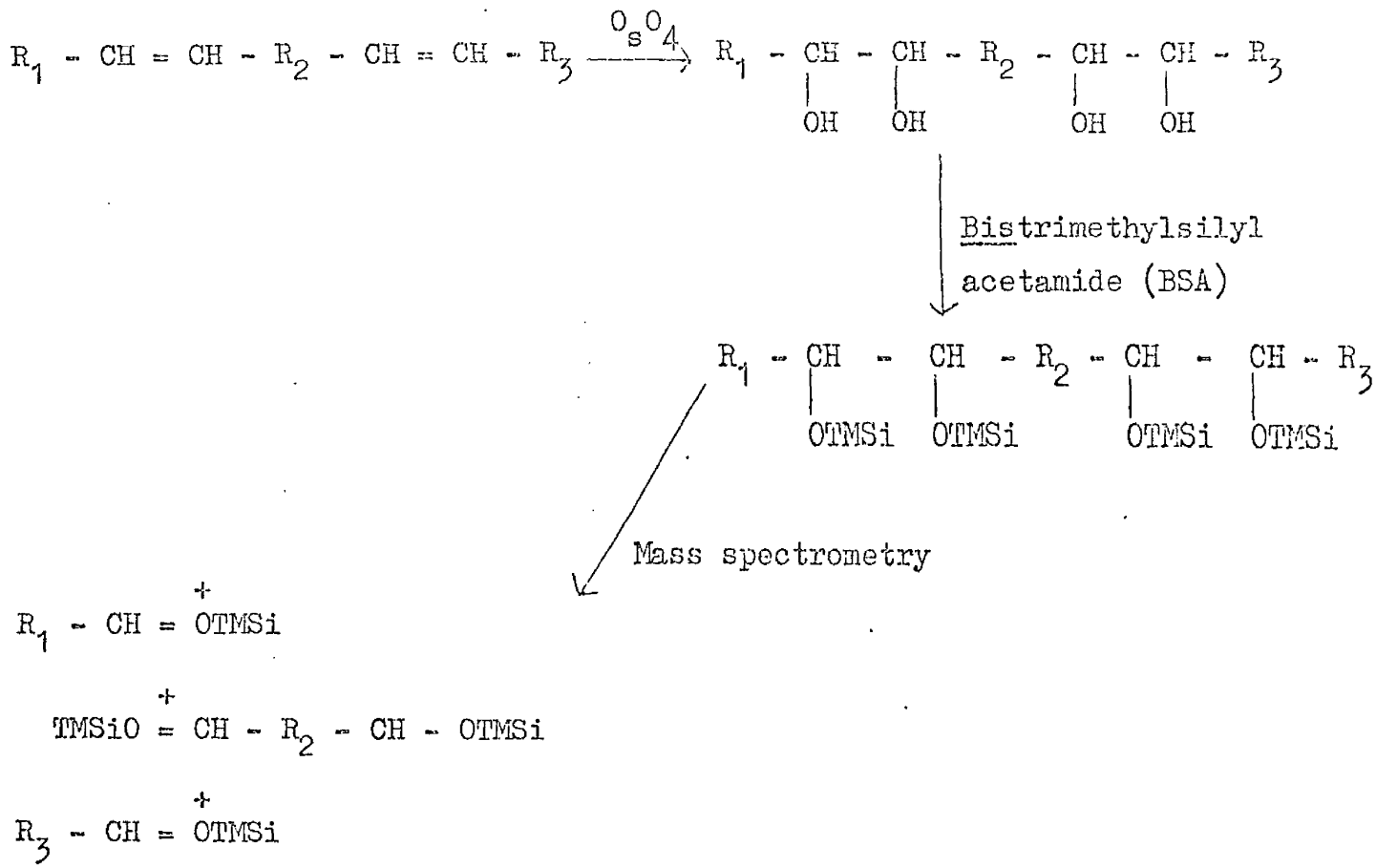
$$R_1\text{-}CH{=}CH\text{-}R_2\text{-}CH{=}CH\text{-}R_3$$

$$\downarrow \quad (1)\ KMnO_4/KIO_4 \quad (2)\ CH_2N_2$$

$$R_1\text{-}COOMe + MeOOC\text{-}R_2\text{-}COOMe + R_3COOMe$$

These products might then be identified by combined GC/MS and the sites of the double bonds determined.

The method of Eglinton and Hunneman (1968) involved the preparation of water insoluble diols which were subsequently converted to trimethylsilyl (TMSi) ethers. The products of this treatment were identified by GC/MS. A knowledge of the structure of these fragments would allow one to determine the double bond sites in molecules (Appendix 6).



$R_1 - CH = \overset{+}{O}TMSi$

$TMSiO = \overset{+}{C}H - R_2 - CH - OTMSi$

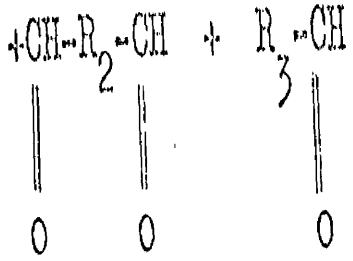
$R_3 - CH = \overset{+}{O}TMSi$

On the two occasions that the above method was used it proved inconclusive, possibly due to the high molecular weight of the products.

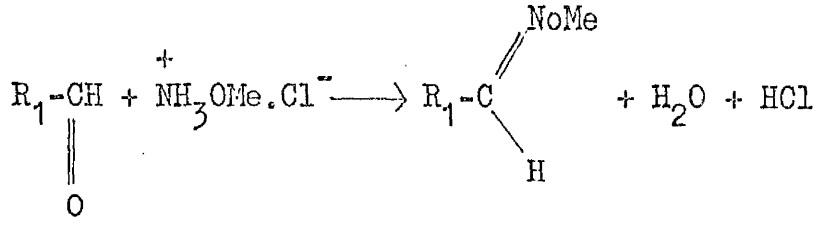
(ii) Preparation of aldehydes

The TMSi groups were removed from 10 mgm. of the above diol mixture, in a small phial, by hydrolysis. They were then reacted with 0.4 ml. of 95% ethanol and 0.007 gm. of potassium periodate (in 0.3 ml. of N H_2SO_4 - this reagent is commonly used for cleavage of 1,2 diols (Fieser and Fieser, 1967). After ten minutes the colourless solution was cooled to 15°, water was added to dissolve the precipitated potassium sulphate. The mixture was extracted with ether and the ether solution was then washed with water to remove periodate. This was expected to yield aldehydes (formed from the hydrocarbons) on evaporation.

OH OH OH OH



This material was treated with methoxyamine hydrochloride ($HCl.NH_2OMe$) in an attempt to form O-methyloxime derivatives for GLC:-



The resultant mixture was analysed by GLC on SE-30 by linear programming of the temperatures from 100-250° at 3°/minute, and with a final period of twenty minutes at 250°. Temperature programming was carried out in case any of the fragments from these hydrocarbons were extremely small (e.g. formaldehyde, acetaldehyde etc.) and would have otherwise been lost in the solvent front under the more usual operating temperature of 244°.

This method also proved inconclusive. A check to ensure that there was a residue present proved that there was very little, possibly due to mechanical losses in an elaborate working procedure. These methods were discarded as unlikely to determine the double bond sites in the hydrocarbons. This was not wholly due to the methods used to form the diols and aldehydes, but nore specifically to the method of separation on $AgNo_3$

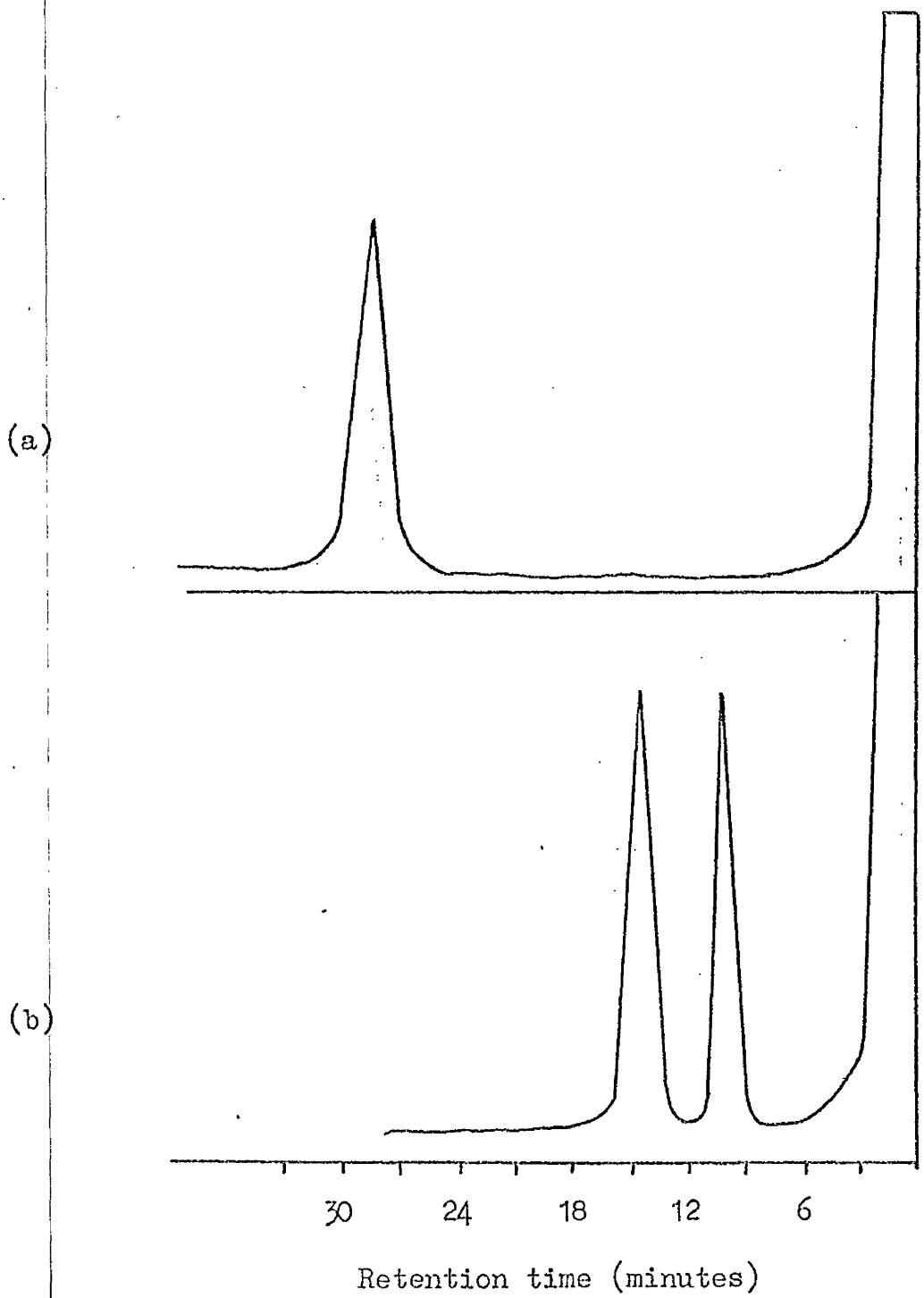


Fig. 15. GLC trace of (a) methylester of elaidic acid and (b) products obtained after ozonolysis

impregnated silica gel plates. As the A series differs only in having one double bond less than the B and C series, separation was not always complete, especially when using a larger scale, and the need to take only a narrow zone from the TLC plate was rather wasteful, and the hydrocarbon fraction was only available in limited quantities.

(iii) Preparation of ozonides

Colonies grown in MC_{13} with 1, 5 or 10 p.p.m. of actidione for a six week period produced the A series of hydrocarbons almost exclusively. This method of obtaining these hydrocarbons was used in the ozonolysis experiments in contrast to the TLC separation employed in the previous experiments.

A rapid, simple procedure for locating the position of double bonds in as little as 1 μg. of an organic compound is now possible with the Supelco micro-ozonizer and a gas chromatograph (Appendix 7, Method I). The design of the ozonizer and the procedure for testing were based on the work of Beroza and Bierl (1966 and 1967). In this method the position of unsaturation was determined by ozonization followed by reductive cleavage of the ozonides and GLC of the ozonolysis fragments.

Initially, as a test of this method, a sample of elaidic acid (the isomer of oleic acid) was ozonized. After ozonolysis the sample was re-chromatographed. Two peaks were produced indicating cleavage of the original elaidic acid at the ethylenic linkage (Fig. 15a + b).

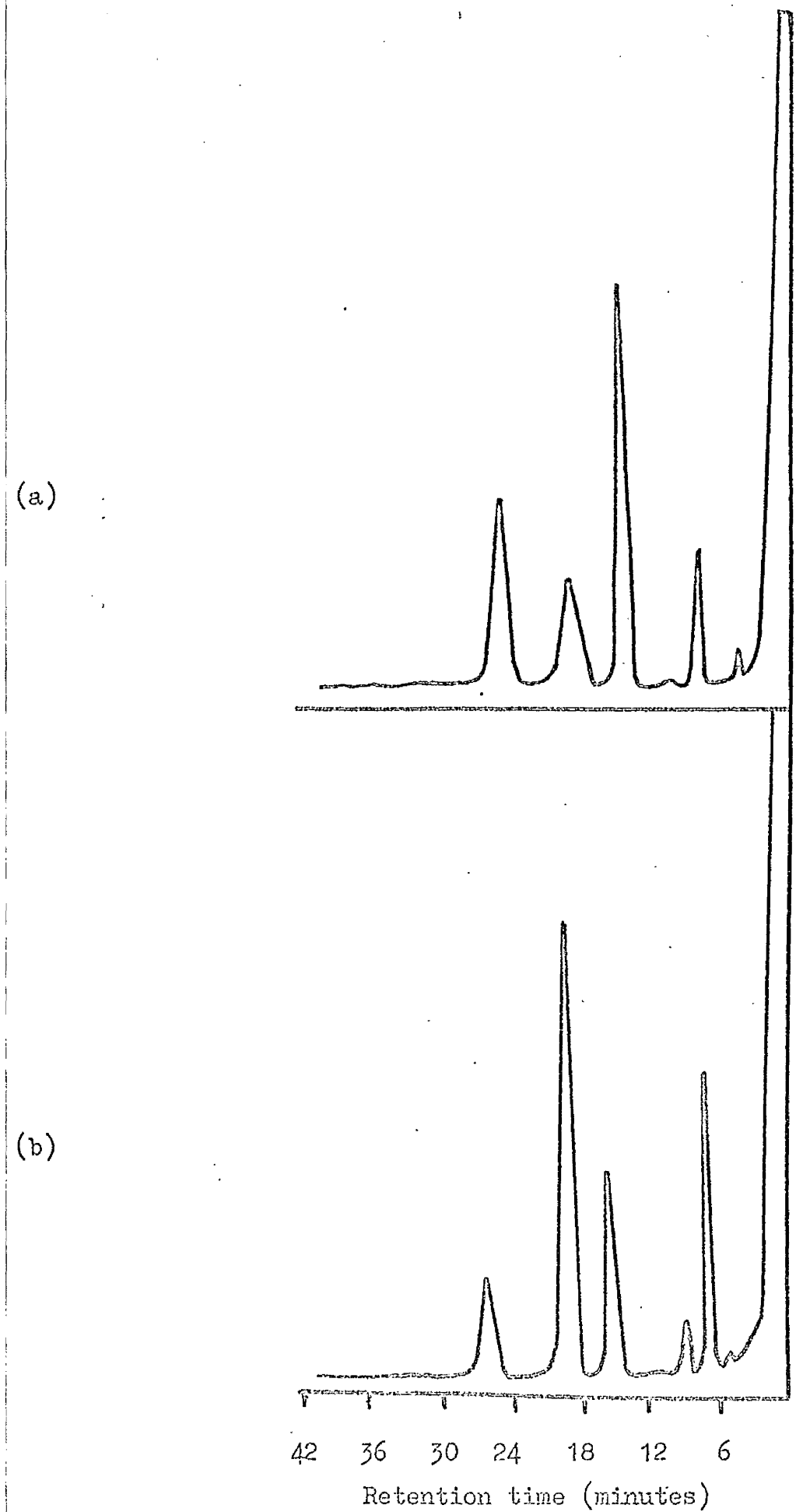
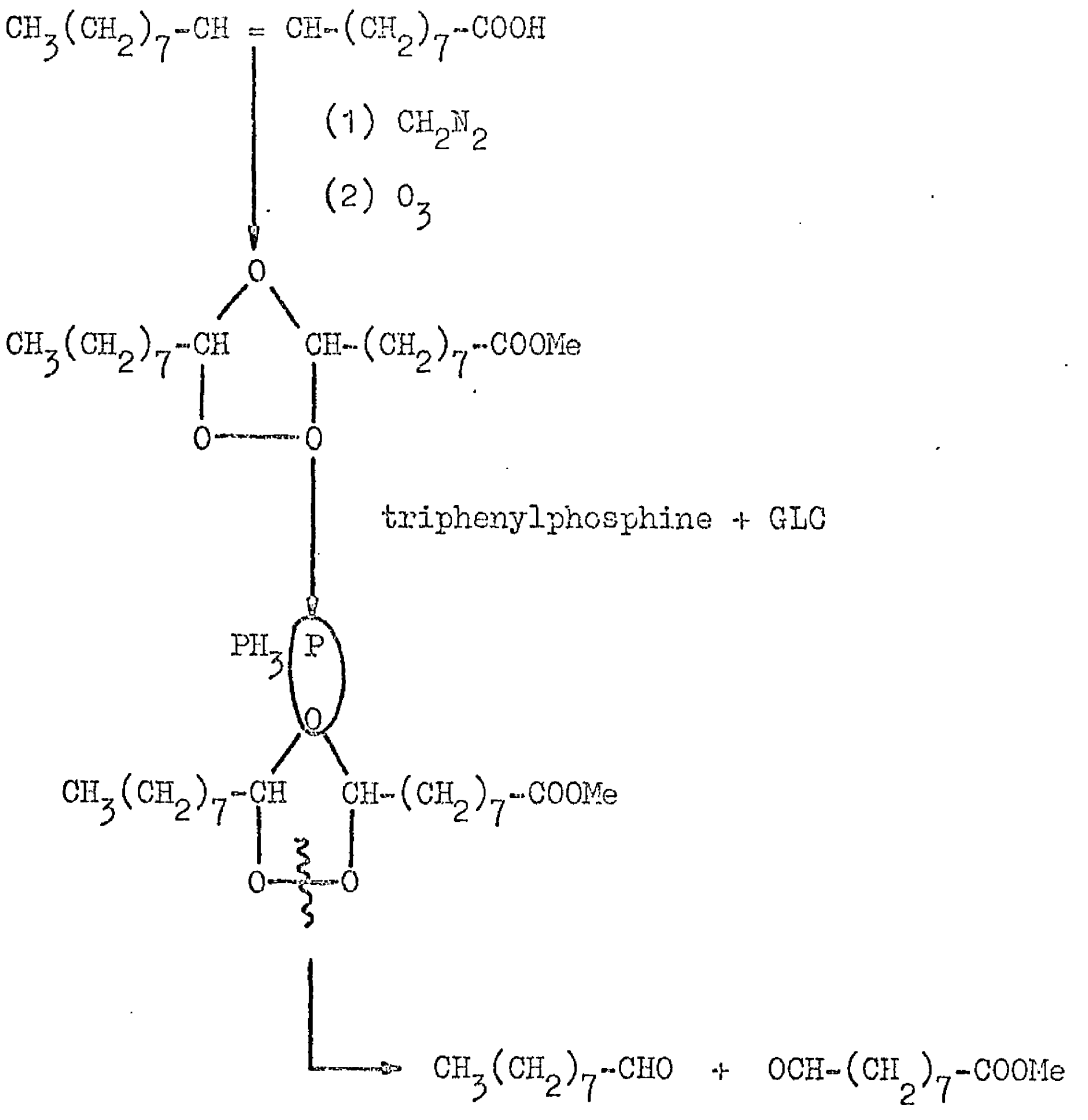


Fig. 16. GLC trace of (a) hydrocarbons from B. braunii and (b) products obtained after ozonolysis



Thus ozonolysis of the A series of hydrocarbons and subsequent GLC produced a trace with two extra peaks together with the three main components of the hydrocarbon mixture (Fig. 16a + b).

(b) Mass spectra of extra peaks produced by ozonolysis

Mass spectrometry indicated that the two extra compounds produced by ozonolysis were triphenylphosphine and triphenylphosphine oxide. The first of these compounds was added to decompose any ozonides formed in the reaction and the second compound is the expected oxidation product formed from triphenylphosphine. The presence of these compounds in GLC

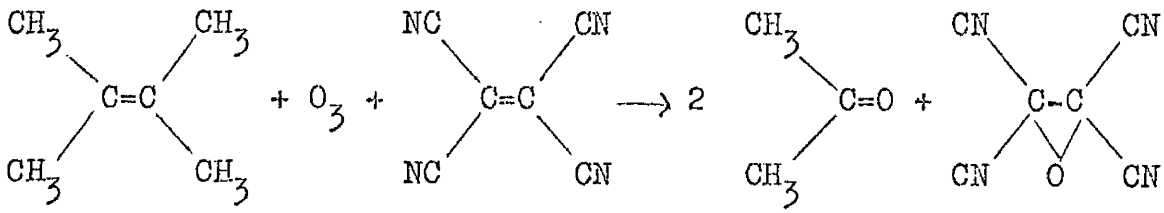
Aldehydes				O-methyloximes			
Peak	SE-30 Retention Index	Peak	OV-17 Retention Index	Peak	SE-30 Retention Index	Peak	OV-17 Retention Index
1	1910	1	1910	1	1910	1	1580
2	1960	2	2000	2	2030	2	1725
3 (A)	2030	3	2105	3 (A)	2130	3	1930
4	2120	4 (A)	2135	4	2200	4	2025
5 (B)	2225	5	2200	5	2225	5	2120
6	2320	6	2225	6 (B)	2325	6 (A)	2230
7	2355	7	2300	7	2420	7	2310
8 (C)	2420	8 (B)	2340	8	2470	8	2330
9	2475	9	2400	9 (C)	2520	9 (B)	2435
10	2685	10	-	10	2565	10	2530
11	-	11	2500	11	-	11	2555
12	-	12 (C)	2545	12	-	12 (C)	2630
13	-	13	2630	13	-	13	2690
14	-	14 (H)	2705	14	-	14 (H)	2710
15	-	15	2765	15	-	15	2730
16	-	16 (H)	2900	16	-	16	-
17	-	17	-	17	-	17 (H)	2905
18	-	18	-	18	-	18	2930

A, B and C are the three peaks common to both fractions.
H denotes a hydrocarbon of the A series.

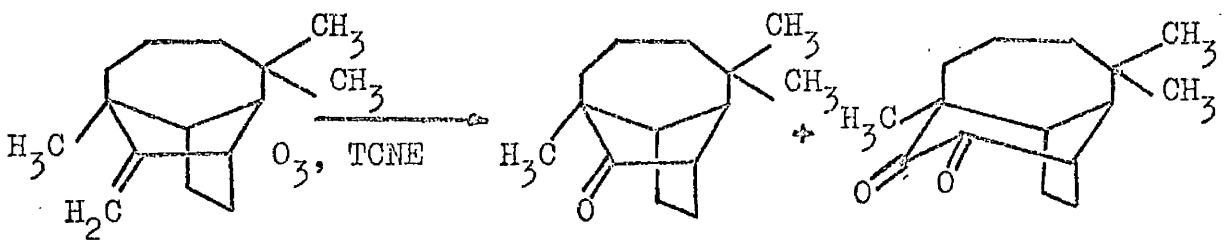
Table 16.

Retention indices for products of ozonolysis

traces, using this method, had not been previously mentioned (Pelick and Supina, 1968) and was therefore not expected. The use of triphenylphosphine was clearly precluded and tetracyanoethylene (TCNE) was used to decompose ozonides (Appendix 7, Method II). This compound was found to be stable to ozone and also to decompose ozonides to the corresponding carbonyl compound and tetracyanoethylene oxide:-



Using method II Munavalli and Ourisson (1964) noted that longifolene was converted to the keto compound whereas "normal" ozonolysis produced only the epoxide, an abnormal product:-



Ozonolysis by method II for up to thirty minutes, at room temperature, resulted in a number of products. Three of these products (A, B and C, Table 16) were dominant and constant, and with the majority of the other products, were examined by GC/MS. O-methyloxime derivatives of these compounds were also prepared and examined by GC/MS. Retention data for the aldehydes and their O-methyloxime derivatives are detailed in Table 16.

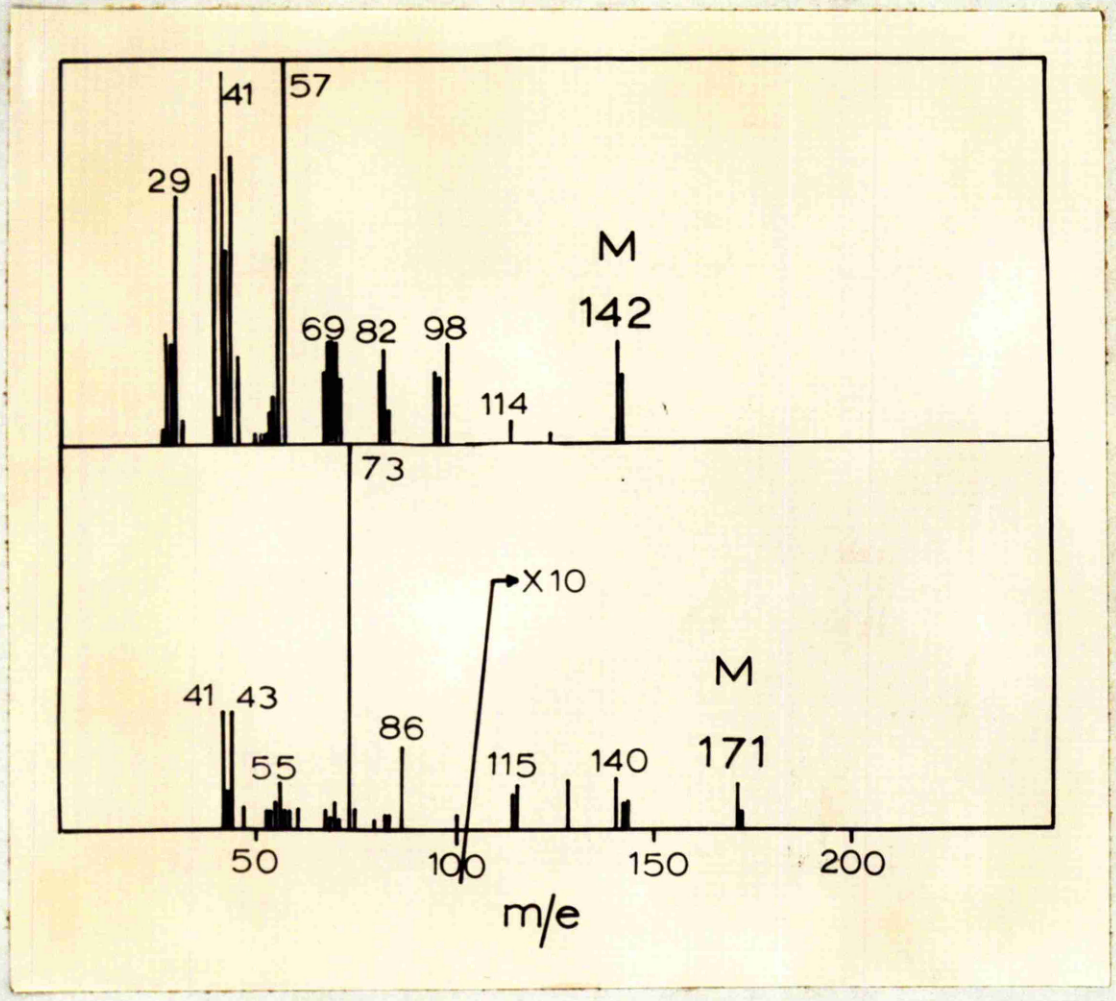


Fig. 17. Mass spectra of compounds with molecular weights of 142 and 171.

The general formula for an A series hydrocarbon may be written:-

$$R_1 - CH = CH - R_2 - CH = CH - R_3 \qquad [1]$$

From infra red spectroscopy of a mixture of the hydrocarbons it has been shown that $R_1 = H$ (i.e. vinyl absorption and therefore the general formula [1] now becomes:-

$$CH_2 = CH - R_2 - CH = CH - R_3 \qquad [2]$$

On complete ozonolysis of an A series hydrocarbon of formula [2] three products would be formed:-

$$CH_2O + OCH - R_2 - CHO + OCH - R_3 \qquad [3]$$
$$\text{(i)} \qquad \text{(ii)} \qquad \text{(iii)}$$

It is also possible that only mono-ozonolysis may occur in which case either of the following two sets of products would result.

$$CH_2O + OCH - R_2 - CH = CH - R_3 \qquad [4]$$

or

$$CH_2 = CH - R_2 - CHO + OCH - R_3 \qquad [5]$$

Such mono-aldehydes [4 and 5] would have a general formula of $C_nH_{2n-2}O$.

Examination by GC/MS of the aldehydes produced by ozonolysis indicated one with a molecular weight of 142 (Fig. 17), i.e. $CH_3-(CH_2)_7-CHO$. On the assumption that such a compound is an aldehyde it should be possible to prepare its O-methyloxime derivative which would add 29 mass units to its molecular weight, i.e. there should be a product with a molecular weight of 171 in the O-methyloxime fraction. Further examination of the O-methyloxime derivatives of the aldehydes showed such a compound to be present (Fig. 17). No related compounds differing in molecular weight

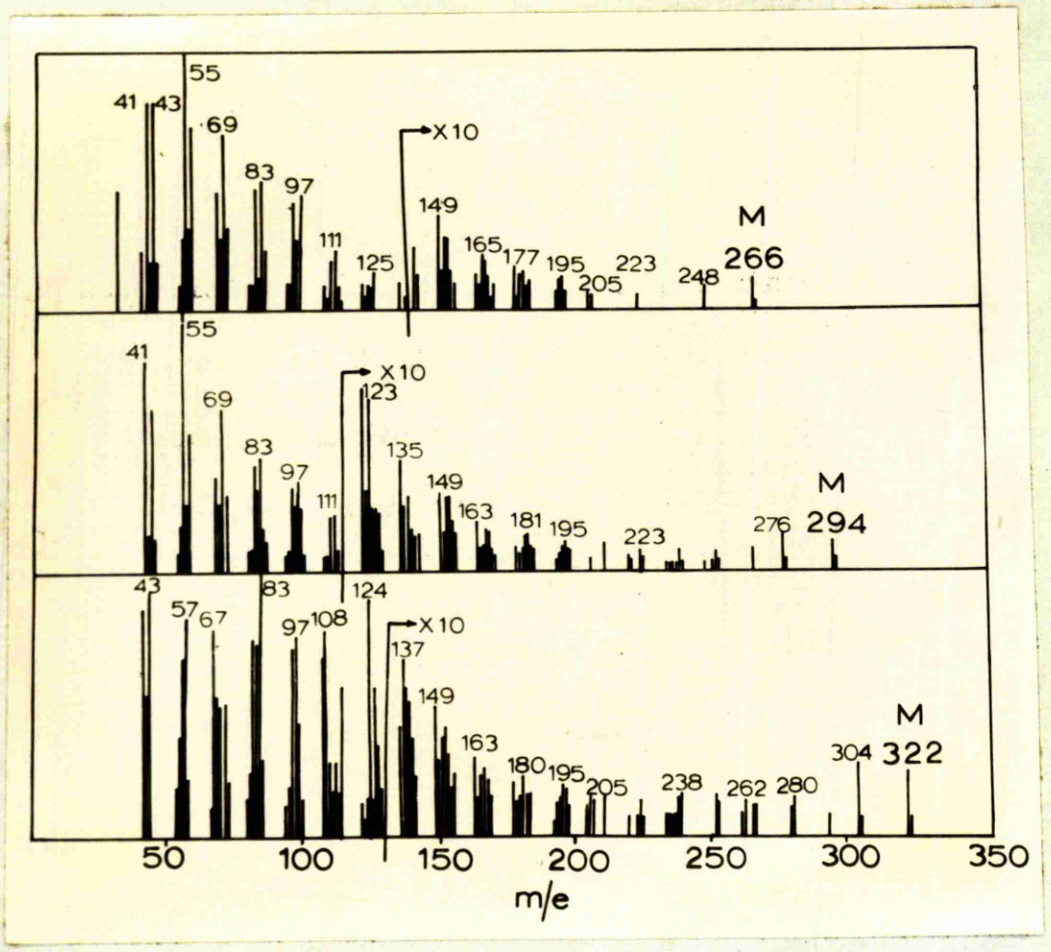


Fig. 18. Mass spectra of compounds with molecular weights of 266, 294 and 322.

by 28 mass units from these two compounds could be detected thus this aldehyde appears to be a unique structure common to all three major components of the hydrocarbon mixture. When this information was incorporated into the general formula for the A series hydrocarbons equation [5] became:-

$$CH_2 = CH - R_2 - CHO + OCH - (CH_2)_7 - CH_3$$

$$\text{i.e. } R_3 = (CH_2)_7 - CH_3$$

The general formula for the mono-aldehyde containing - R_2 - is $C_nH_{2n-2}O$ and it may be noted from GC/MS that three products were present after the ozonolysis differing from each other by increases of 28 mass units in their molecular weights. These were 266, 294 and 322 ($C_nH_{2n-2}O$ where n = 18, 20 and 22) (Fig. 18).

The production of corresponding O-methyloxime derivatives with molecular weights of 295, 323 and 351 was noted and this may be taken to indicate the presence of single carbonyl groups in each of the corresponding presumed aldehydes (i.e. M.W. 266, 294 and 322).

These molecular weights would give the mono-aldehyde the following formulae.

266		$C_{18}H_{34}O$
294	$C_nH_{2n-2}O$	$C_{20}H_{38}O$
322		$C_{22}H_{42}O$

Substituting these values into equation [5] determines the chain-length of R_2.

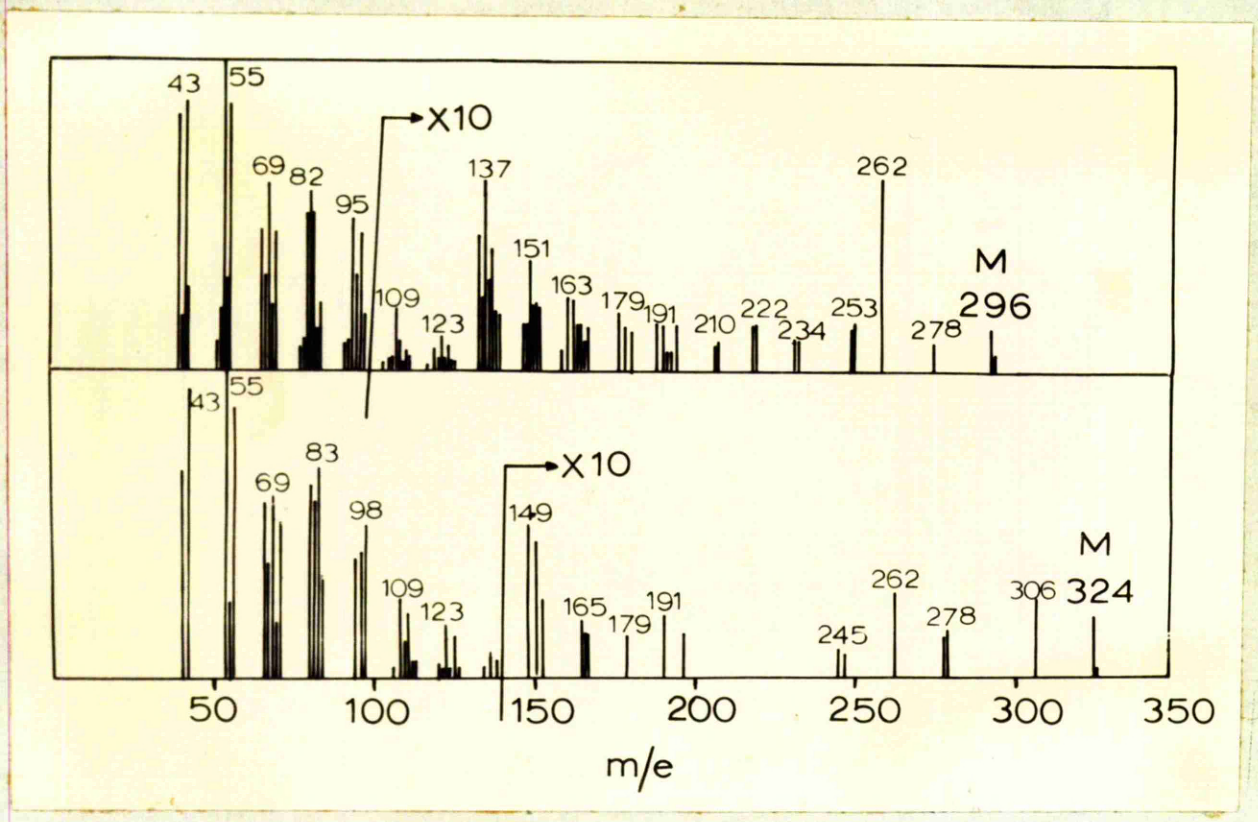


Fig. 19. Mass spectra of compounds with molecular weights of 296 and 324.

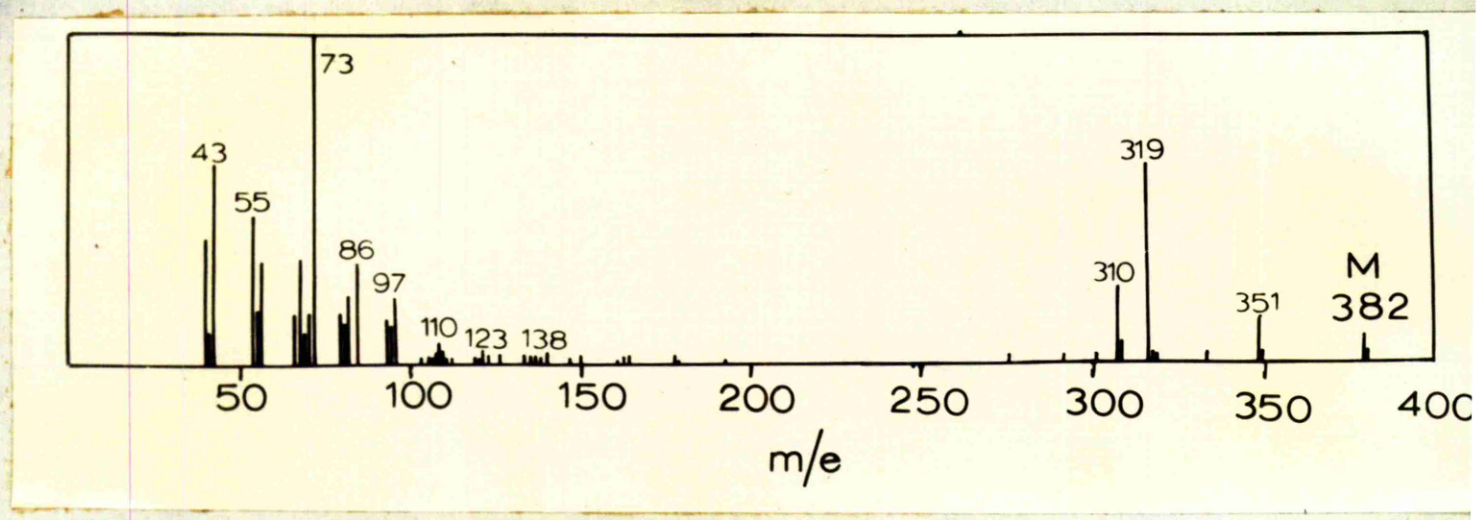


Fig. 20. Mass spectrum of the compound with molecular weight of 382.

Molecular Weight		
266	$- CH_2 = CH - (CH_2)_{15} - CHO$	i.e. $R_2 = (CH_2)_{15}$
294	$- CH_2 = CH - (CH_2)_{17} - CHO$	i.e. $R_2 = (CH_2)_{17}$
322	$- CH_2 = CH - (CH_2)_{19} - CHO$	i.e. $R_2 = (CH_2)_{19}$

Further ozonolysis of these structures may be expected to produce the following results.

$CH_2O + OCH - (CH_2)_{15} - CHO$	dialdehyde molecular weight 268
$CH_2O + OCH - (CH_2)_{17} - CHO$	" " " 296
$CH_2O + OCH - (CH_2)_{19} - CHO$	" " " 324

Two of these products (296 and 324) have been found by GC/MS from the aldehyde fraction (Fig. 19) thus indicating that these structures for the A series hydrocarbons are essentially correct. As further evidence, these dialdehydes would, on preparation of their O-methyloxime derivatives, be expected to give products with molecular weights of:-

326	i.e. 268 + 58
354	i.e. 296 + 58
382	i.e. 324 + 58

One of these products (M.W.382) has been found and identified by GC/MS (Fig. 20) and some evidence obtained for a second compound (M.W.354).

From this it appears that the structures of the three main A series hydrocarbons are:-

$C_{27}H_{52}$: $CH_2 = CH - (CH_2)_{15} - CH = CH - (CH_2)_7 - CH_3$

$C_{29}H_{56}$: $CH_2 = CH - (CH_2)_{17} - CH = CH - (CH_2)_7 - CH_3$

$C_{31}H_{60}$: $CH_2 = CH - (CH_2)_{19} - CH = CH - (CH_2)_7 - CH_3$

The occurrence in green algae of a hydrocarbon having a terminal double bond (1-hexacosene $C_{26}H_{52}$) has been reported for an unnamed species of Scenedesmus and of Chlorella (Iwata et al. 1961, 1963). The second double bond in the A series of B. braunii occurs at the position expected in relation to the double bond of oleic acid, if the terminal methyl group of the hydrocarbon is taken to correspond to the methyl group of the fatty acid chain.

These oxidation products have not yet been unambiguously confirmed as aldehydes. Oxidation to the corresponding carboxylic acids, attempted by Dr. B. A. Knights, using zinc permanganate, produced an unsatisfactory result and further confirmation is being sought.

The mass spectra of these compounds are complicated by the presence of the vinyl group at the end of the chain; however it seems probable that the molecules have essentially linear structures since there are no obvious breaks in cracking patterns such as might possibly be associated with a branch in the chain.

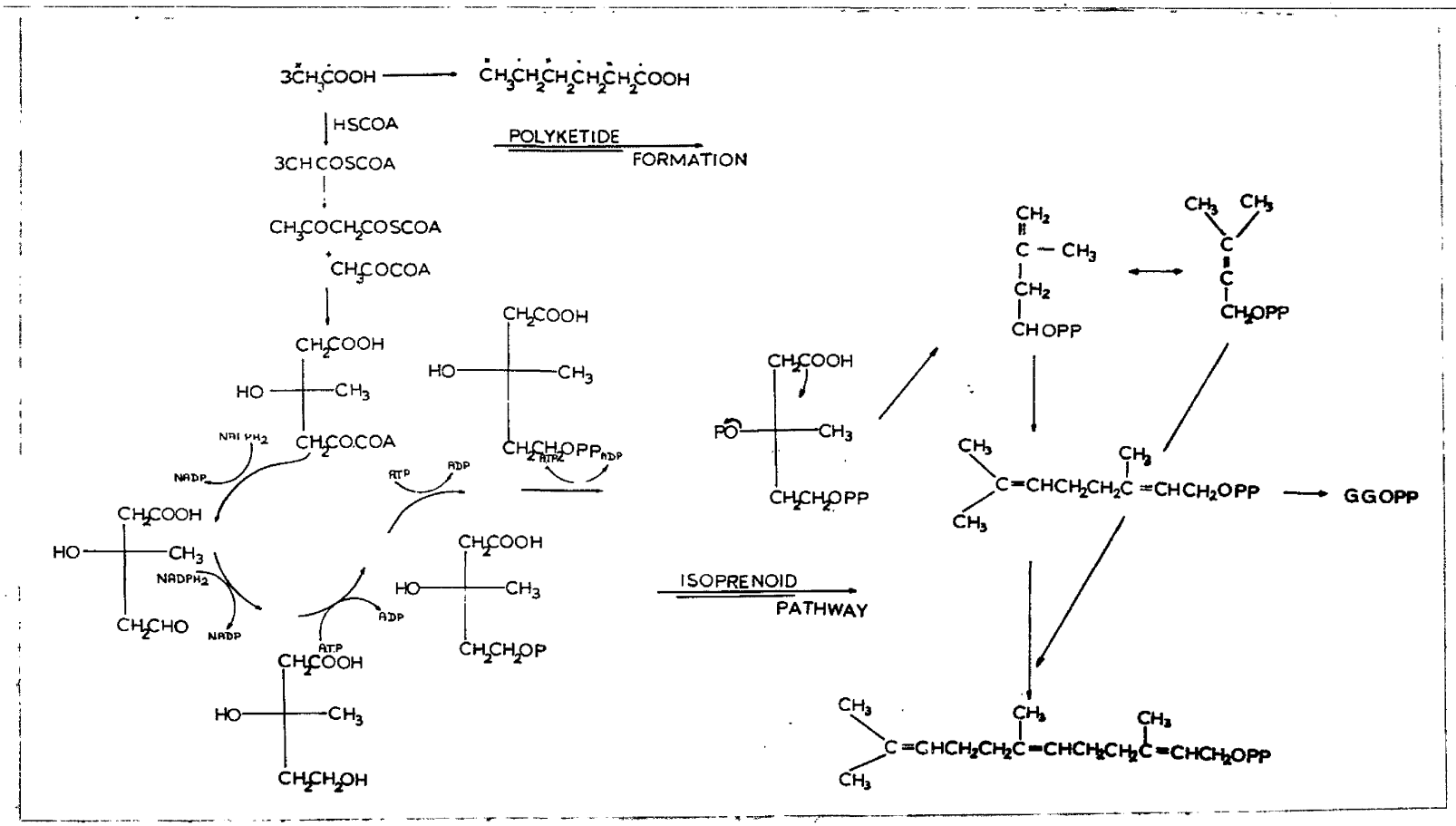


Fig. 21. Possible pathways for hydrocarbon synthesis

8. Hydrocarbon synthesis in culture

Two biosynthetic pathways are known which could lead to the production of a long chain hydrocarbon, viz. the Isoprenoid pathway and the Polyketide pathway (Bu'lock, 1965), (Fig. 21).

In the polyketide pathway, which is basically the joining together of two carbon units to form a long chain, there are a number of possible starter units including acetyl CoA, malonyl CoA, propionyl CoA [although this latter could lead to the formation of hydrocarbons of odd carbon number]; it is more likely that decarboxylation of a fatty acid having an even number of carbon atoms occurs (Kates, 1966)]. The possibility that the acetate pathway may be implicated in hydrocarbon synthesis in the cultured strain of Botryococcus is made all the more probable from the knowledge (GC/MS) that each of the hydrocarbons, in each of the series, differs in formula from the next by C_2H_4, i.e. by a two carbon fragment.

The Isoprenoid pathway of synthesis of hydrocarbons which is essentially the formation of long branched chains by the uniting of five carbon isopentenoid fragments (Bu'lock, 1965) does not appear readily to afford a suitable carbon skeleton for the formation of the hydrocarbons isolated from Botryococcus (material C).

The importance of these two major pathways (Fig. 21) in hydrocarbon synthesis in green active colonies has been initially studied using radioactively labelled precursors:-

Sodium [1-^{14}C]-acetate or [1-^{14}C]- mevalonic acid lactone.

Belcher (1957) has already shown that Botryococcus can utilise acetate,

and acetate is known as a precursor of isoprenoid molecules as is mevalonic acid. The known highly branched structure of botryococcene isolated from the brown material (Maxwell, 1967) makes it possible to envisage mevalonic acid as an intermediate compound in its synthesis. Uptake of either of the two labelled precursors by green colonies might therefore indicate if either plays a part in the synthesis of the hydrocarbon in the green state. Further, an intermediate growth state, containing botryococcene as well as the aliphatic hydrocarbons of the green colonies, can be postulated and experiments were set up to produce such a growth state in the hope of being able to study the biosynthesis of all hydrocarbons in one culture.

(a) Production of an intermediate growth state

Green colonies cultured for three or six week periods in MC_{13} at 10^{o} in high light (1,000 foot candles), low light (250 foot candles) or in the absence of light developed the normal A, B and C series of hydrocarbons with the A series the dominant one. If, however, these cultures were left intact and only extracted after twelve weeks in about one out of every three cultures another hydrocarbon was produced in addition to the normal A, B and C series (Table 17). From GLC data this compound has been identified as botryococcene. Thus by allowing cultures to age to a certain extent botryococcene was produced as well as the homologous A, B and C series in the cultures. By supplying cultures with one tenth the normal combined nitrogen supply in MC_{13} and culturing them for six weeks in a light intensity of 1,000 foot candles (16 hour day) and at a temperature of 20^{o} this intermediate state was produced regularly with botryococcene as well as the A, B and C series of hydrocarbons.

Culture conditions		Peak number								
		1	2	3	4	6	7	8	9	B*
3 weeks	H.L.	T	-	-	12.3	59.0	-	-	28.5	-
	L.L.	-	-	-	9.5	61.0	-	-	29.2	-
	N.L.	-	-	-	5.5	52.5	-	-	42.0	-
6 weeks	H.L.	-	T	-	6.0	37.0	1.0	7.0	49.0	-
	L.L.	-	-	-	4.0	62.0	T	2.5	31.0	-
	N.L.	-	-	-	-	39.0	-	-	61.0	-
1/10th N_2	H.L.	T	1.5	-	5.0	20.0	-	22.5	47.0	4.3
0.N_2	H.L.	-	1.3	-	13.1	66.0	-	-	19.2	-
12 weeks	H.L.	1.0	2.0	-	7.0	41.5	3.0	4.0	39.5	2.0
	L.L.	T	T	-	7.0	38.5	2.5	2.0	47.5	1.5
10 week old inoculum		3.8	5.5	4.0	16.5	51.0	2.0	-	15.0	2.0

* Botryococcene

T trace detected

\- not observed

H.L. denotes high light; L.L. low light; N.L. no light

Table 17.

Percentages of each hydrocarbon from cultures of B. braunii

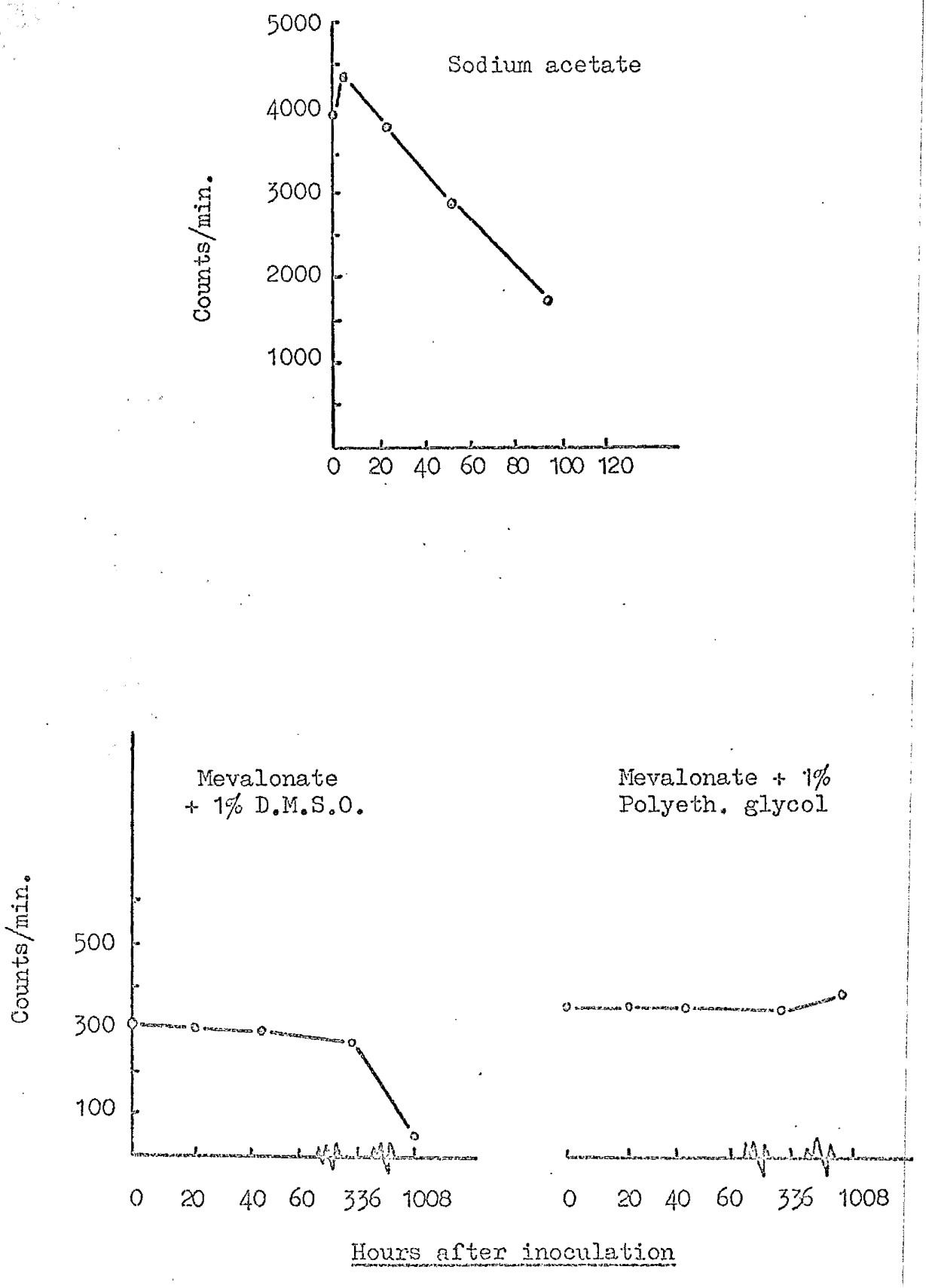


Fig. 22. Level of radioactivity in 0.1 ml. of culture supernatant

(b) Uptake of sodium [1-^{14}C]-acetate

Six ml. cultures of the intermediate culture state were set up in graduated centrifuge tubes with MC_{13} medium containing a tenth the normal combined nitrogen supply, and radioactively labelled sodium acetate (5 p.p.m.) with 4,000 counts per minute (c.p.m.) in 0.1 ml. of the culture supernatant, measured on an I.D.L. counter apparatus. The cultures were kept in a constant temperature incubator at 15° with light intensity of 1,000 foot candles. They were removed every hour initially, and spun down at 2,000 r.p.m./5 min. Two 0.1 ml. aliquots were withdrawn from each of the duplicates. These were then placed on 2.5 cm. planchettes, which had been standing in methanol to reduce surface tension forces, evaporated to dryness, by the heat from a lamp, and then counted. In this way the uptake of labelled sodium acetate by the alga was followed. After 96 hours there was a marked decrease in the activity of the culture supernatants indicating uptake by the alga (Fig. 22). The colonies were then removed and washed thoroughly before extracting in acetone for three days. A 0.1 ml. aliquot of this acetone extract proved to be labelled, and when the hydrocarbons were separated by column chromatography they were also found to be labelled. To ensure that no fatty acids present in the colonies had inadvertantly been included with the hydrocarbon fraction 1 ml. of normal sodium hydroxide was added to the hydrocarbon fraction contained in 1 ml. of diethyl ether. After shaking a 0.1 ml. aliquot of the sodium hydroxide was withdrawn, evaporated to dryness, as before, and counted, but proved to have no activity. The sodium hydroxide would have extracted, as sodium salts, any fatty acids present,

and if they had been labelled this would have been demonstrated.

To ensure that the "hydrocarbon extract" which was labelled, was indeed hydrocarbon, it was subjected to TLC on silica gel plates against a known unlabelled hydrocarbon sample. Spraying with ceric ammonium sulphate indicated that both samples travelled the same distance on the chromatographic plate in the same time (i.e. they had the same R_f values). Autoradiographs were prepared from the plates and indicated that the hydrocarbons extracted from the colonies were labelled. A final test to ensure that this labelled material was hydrocarbon was carried out by scraping the labelled material from an unsprayed zone on the plate and gas chromatographing an ether extract of it. This produced the normal GLC trace associated with green colonies.

Although this experiment indicated that labelled acetate was utilised in hydrocarbon synthesis it did not indicate the pathway of biosynthesis as acetate is a starting material for both pathways.

(c) Uptake of [1-^{14}C]-mevalonic acid lactone

A similar experiment to the previous one was set up with mevalonic acid lactone and its uptake by the colonies was measured by decrease in activity in the culture supernatant. There was no uptake of the mevalonic acid over a two week period. As this may have been due to the large size of the mevalonic acid molecule, cultures were set up as before but with the addition of 1% dimethyl sulphoxide (DMSO), and others were set up with 1% polyethylene glycol, as well as the labelled mevalonic acid lactone. It was thought that these additives, from their action on membrane structure, would facilitate the uptake of the mevalonate. After

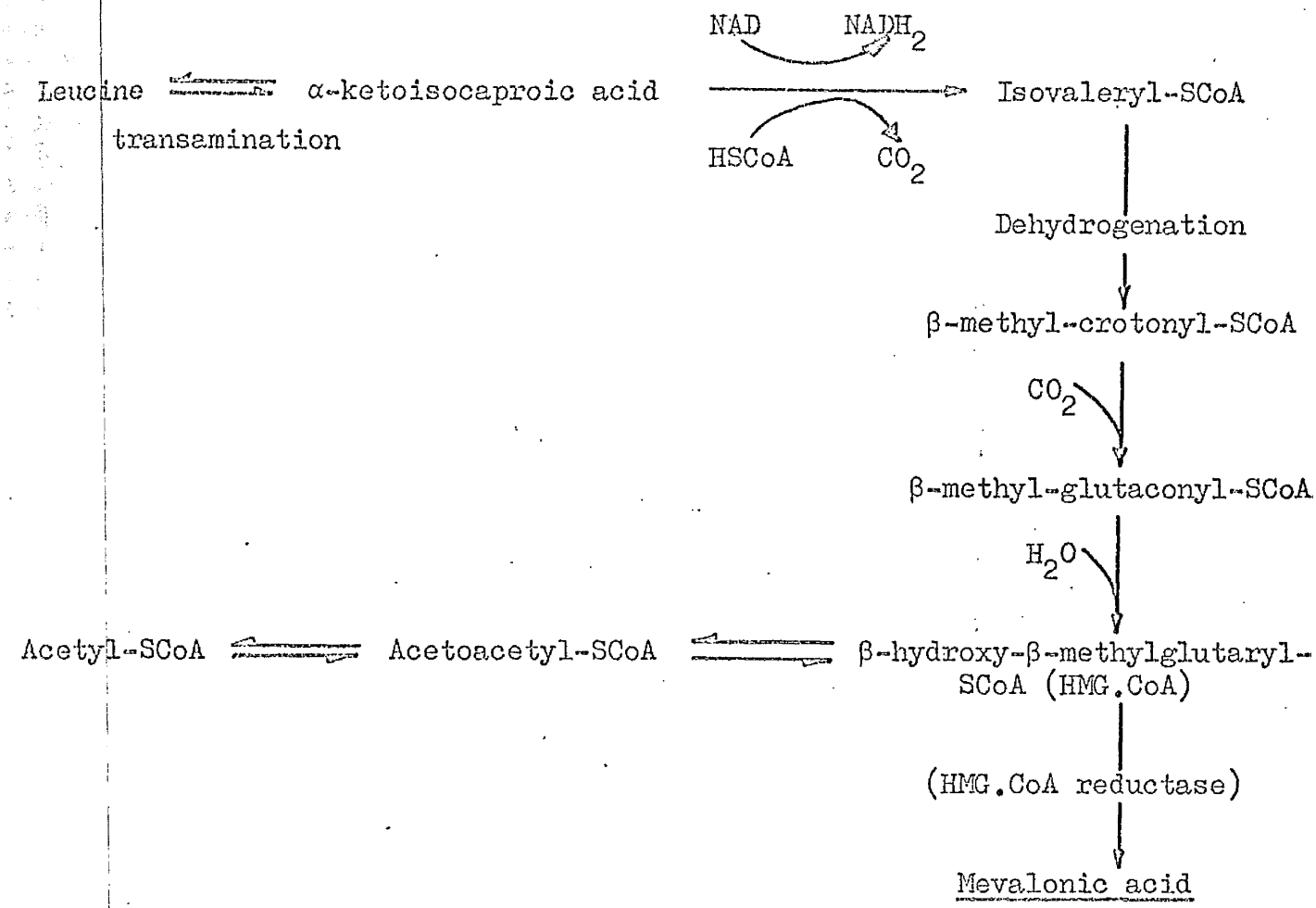


Fig. 23. Scheme for synthesis of mevalonic acid from leucine or acetyl SCoA via 3-hydroxy-3-methyl-glutaryl-SCoA (after Burnett, 1968)

a six week period in the incubator the 1% DMSO cultures showed a considerable decrease in the supernatant's activity whereas the 1% polyethylene glycol containing cultures did not (Fig. 22). The 1% DMSO containing cultures were then washed as previously described and extracted in acetone. Although this acetone extract was radioactive the hydrocarbons subsequently extracted from it were not. This would seem to indicate that although mevalonic acid may be used in such synthetic pathways as sterol production (hence its occurrence in the acetone extract) it is not important in hydrocarbon synthesis in these colonies.

As the exposure to the labelled acid had been of long duration it was necessary to verify this result in some way involving a much shorter exposure to a labelled substrate. Burnett (1968) has described how leucine may be metabolised to mevalonic acid via 3-hydroxy-3-methylglutaryl-SCoA (Fig. 23). Labelled leucine was therefore added to cultures, in an effort to overcome the problems of uptake associated with the large size of the mevalonic acid molecules.

(d) Uptake of [1-^{14}C]-Leucine

A similar experiment to the previous two, except that a total darkness treatment was included, was set up with labelled leucine and uptake into the colonies was followed. After 96 hours the supernatant radioactively had decreased to almost half its original activity and the colonies were removed and extracted. In both light and dark treatments the acetone extract was labelled but the hydrocarbons were not. A second experiment in both the light and the dark was set up with labelled leucine. This time the colonies were extracted (as before) after twelve hours, and

the fatty acid and hydrocarbon fractions were both examined for radioactivity, but in neither case were they labelled.

These experiment on hydrocarbon formation in B. braunii indicate synthesis by the Polyketide Pathway. This is in agreement with what is known of their structure as each member in any of the three series of hydrocarbons differs from the next by a two carbon fragment. The synthesis of botryococcene, the hydrocarbon of brown resting colonies, is somewhat of an enigma. Its synthesis could still be by either the Polyketide or Isoprenoid Pathways. In these experiments botryococcene usually accounted for about 4% of the hydrocarbon fraction and if such a small quantity were labelled in say the mevalonic acid lactone or leucine experiments it is just possible that the I.D.L. counter apparatus (which has about 4% efficiency) would not record its activity. To make a thorough investigation of the synthesis of botryococcene it would probably be necessary to employ a radio gas chromatograph.

9. Fluctuations in hydrocarbon content

(a) Fluctuations in culture

To examine variation in hydrocarbon content in relation to media, temperature and day length, cultures were grown for varying lengths of time at 10° or at 20° in MC_{13} or MC_{13} tris buffered medium for a 16 hour day in a high or a low light environment or in complete darkness. The hydrocarbons were extracted, as previously described, and hydrocarbon content determined in the following manner.

(i) by weight of hydrocarbon

(ii) by gas chromatography

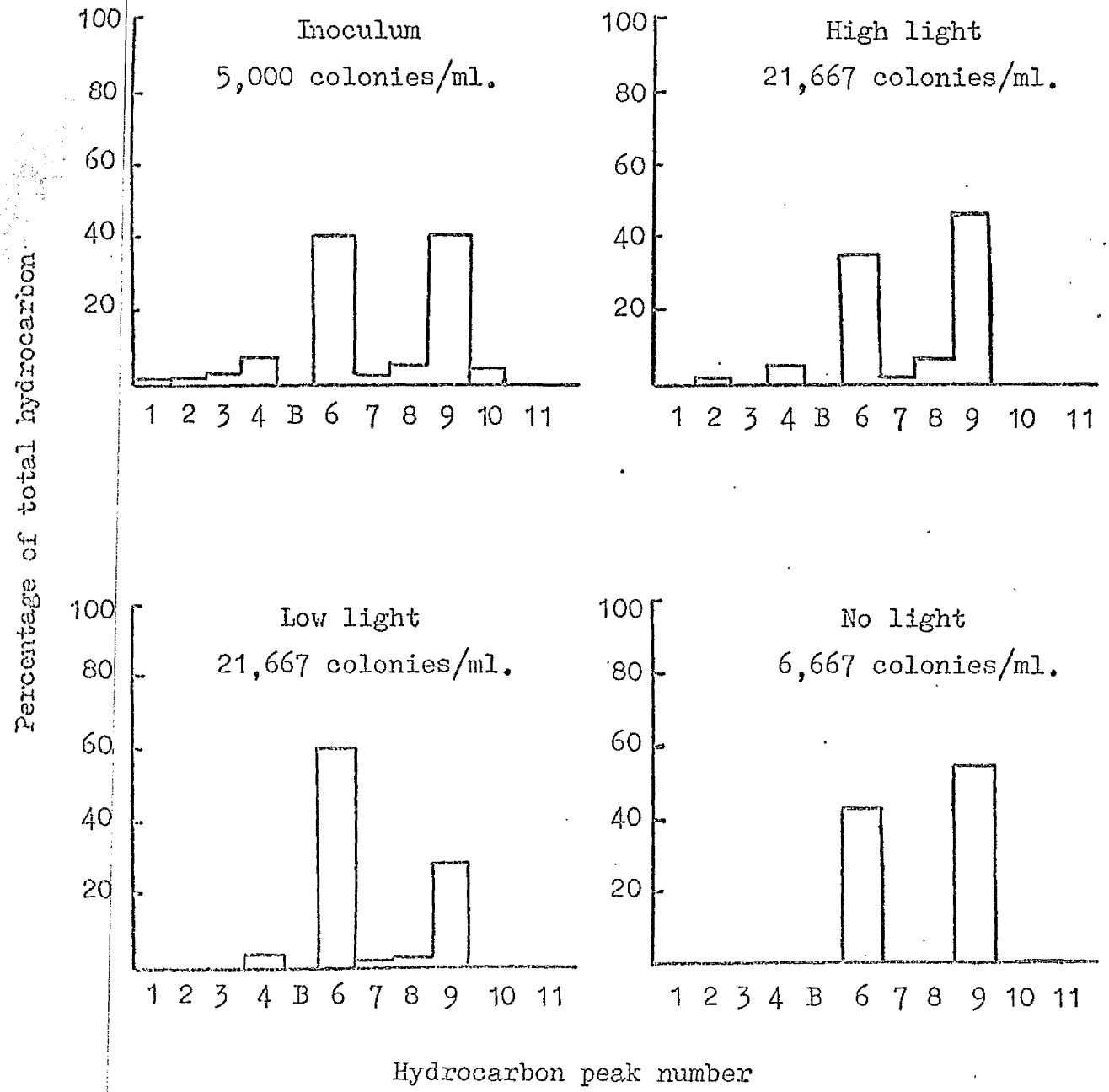


Fig. 24. Hydrocarbon content of colonies after six weeks in MC_{13} at 10°

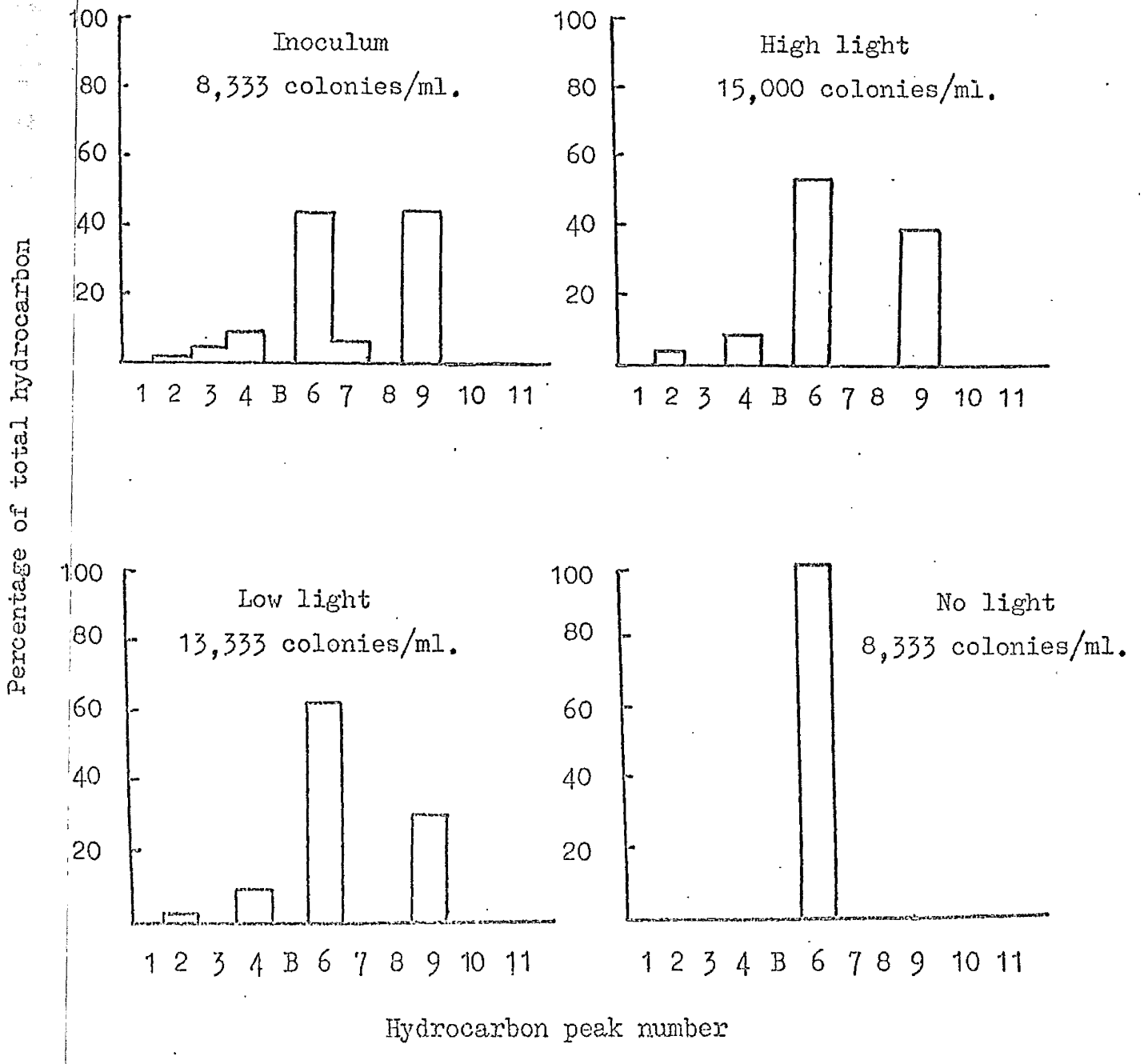


Fig. 25. Hydrocarbon content of colonies after six weeks in MC_{13} TRIS at 10°

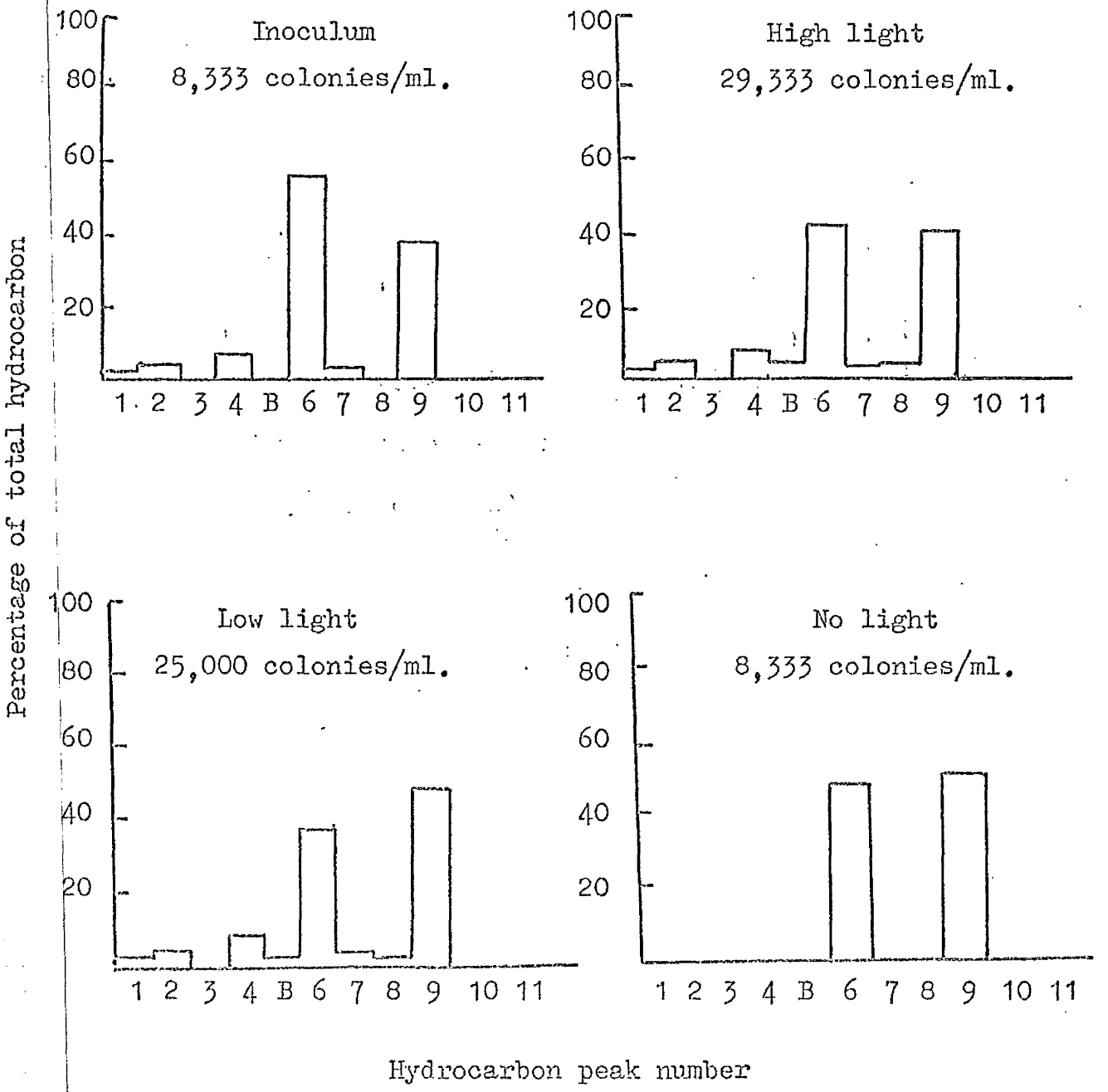


Fig. 26. Hydrocarbon content of colonies after twelve weeks in MC_{13} at 10°

the hydrocarbon $nC_{28}H_{58}$ being included so that retention ratios could be calculated for each hydrocarbon. The area under each peak was then determined (base X altitude) and expressed as a percentage of the total hydrocarbon content (Ambrose and Ambrose, 1961) (Figs. 24 to 27). Additionally the number of colonies at the start and at the end of each experiment was counted. After six weeks in MC_{13} medium at 10° Fig. 24 shows that the greatest colony count per ml. was obtained in the high and low light cultures and that these high light cultures had the greatest weight of hydrocarbon. In all light treatments there was a lessening of the number of hydrocarbons present. This was especially evident in the dark treatment where only the two main members of the A series of hydrocarbons were detected by GLC ($2^{\Delta}C_{2910}$, peak 6 and $2^{\Delta}C_{3120}$, peak 9).

Variation was also evident in MC_{13} cultures buffered with TRIS buffer (Fig. 25) where the dark treatment contained only the hydrocarbon $2^{\Delta}C_{2910}$ (0.2 mgm). On the other hand a twelve week experiment in MC_{13} produced a somewhat different result (Fig. 26). In this case the range of hydrocarbons present increased in both the high and low light treatments compared with the initial inoculum. The cultures grown in the dark in this case gave very similar results to the dark cultures of the six week experiment in that only members of the dominant A series were present.

It appears, from these results, that over a six or twelve week culture period in high or low light the A series remains the dominant series of hydrocarbons formed, and that the weight of hydrocarbon formed is greater after twelve weeks reflecting the greater colony numbers in these cultures.

Although TRIS buffer inhibited cell division and reduced the weight of hydrocarbon present (see growth studies) the A series of hydrocarbons were still dominant in these cultures. A low light intensity experiment shaken for a six week period produced essentially the same result as the twelve week treatment in that there was an increase in the number of hydrocarbons present at the end of the experiment. A similar series of experiments conducted at 20° showed the same trends with the six week high and low light treatments producing a narrower range of hydrocarbons than the inoculum and the twelve week high and low light treatments producing a wider range.

(b) Effect of different nitrogen concentrations on hydrocarbon production

Cultures were grown at 20° for six weeks in MC_{13} medium containing 1/10th the normal combined nitrogen supply and others in MC_{13} with no combined nitrogen. The 1/10th nitrogen cultures produced less weight of hydrocarbon (7.1 mgm. in the high light treatment, and 9.8 mgm. in the low light treatment) than produced by control cultures containing the normal combined nitrogen supply associated with MC_{13} (high light treatment - 13.1 mgm.; low light treatment - 13.8 mgm.). After six weeks similar cultures containing no combined nitrogen contained very little hydrocarbon (high light treatment - 1.5 mgm.; low light treatment - 1.60 mgm.). In the 1/10th combined nitrogen cultures there was the usual wide range of hydrocarbons associated with the green colonies, but in addition, especially in the high light treatment, there was the hydrocarbon botryococcene - the dominant hydrocarbon in brown wild colonies. The cultures with no combined nitrogen contained a narrower range of hydrocarbons, namely the main

members of the A series $2^{\Delta}C_{2710}$, $2^{\Delta}C_{2915}$, $2^{\Delta}C_{3120}$.

In conclusion it appears that:

(i) high light (1,000 foot candles) promotes greater cell division in the alga over low light (250 foot candles) in the six and twelve week experiments but that the final weight of hydrocarbon produced is higher in the low light cultures,

(ii) cultures maintained in darkness utilise the hydrocarbons contained in the initial inoculum and undergo little or no cell division,

(iii) cultures with 1/10th the normal combined nitrogen supply contain botryococcene as well as the normal A, B and C series hydrocarbons associated with green colonies, and

(iv) culturing in a medium with no combined nitrogen led to the utilisation of the hydrocarbons that existed in the inoculum as did culturing in the dark.

(c) Effect of lessened U.V. radiation on hydrocarbon production

As six week old cultures at 10^{o} have been shown to have a narrower range of hydrocarbons than similar cultures after twelve weeks it was thought possible that the other hydrocarbons present after twelve weeks, but which were not present after six weeks, may have arisen by reduction of the existing A series members and/or by polymerisation due to the action of U.V. radiation from the fluorescent tubes producing the illumination. Possibly some or all of the A series members may have been actively synthesised and some or all of the others may have been produced by polymerisation. To test the effect that fluorescent lighting

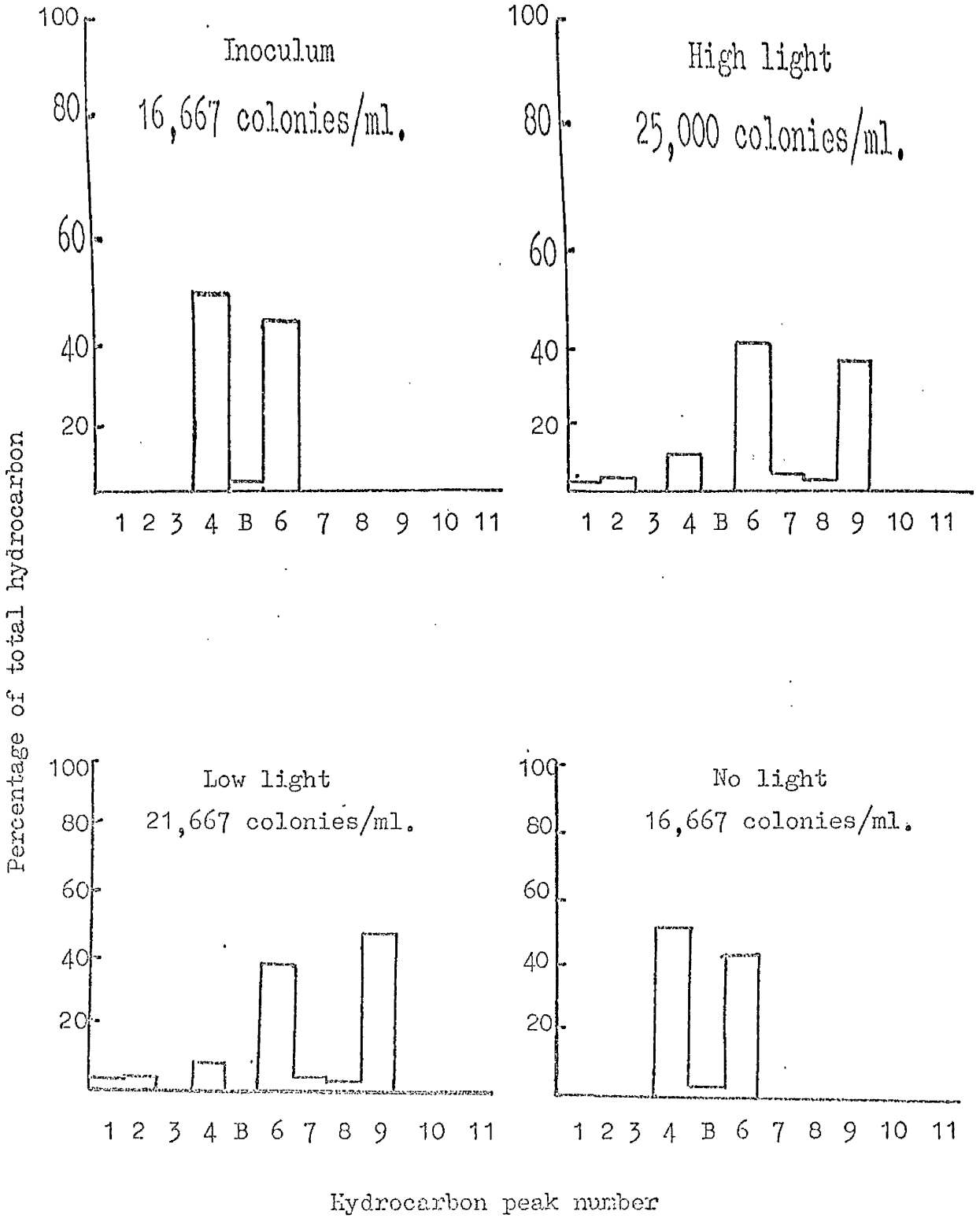


Fig. 27, Hydrocarbon content of colonies after four weeks in $^{14}C_{13}$ at 10°

might have on hydrocarbon synthesis a series of cultures were grown under tungsten illumination of 250 foot candles and subsequently extracted and examined by GLC.

These cultures produced a similar amount of hydrocarbon by weight (9.3%) as control cultures grown under illumination from fluorescent tubes (8.5%) and the hydrocarbon traces from both treatments were identical. Therefore it would not appear that any of the hydrocarbons are produced as a result of polymerisation due to U.V. radiation from the fluorescent tubes.

(d) Utilisation of hydrocarbons

In all cases the hydrocarbons contained in the inoculum for dark treatment cultures gradually disappeared during the treatment, although colony numbers never increased during these treatments. It appears that when the alga is denied the opportunity to photosynthesise it has the ability to break down its own hydrocarbons and to utilise them as a source of energy. This was well marked in twelve week experiments in which some of the cultures were extracted and analysed every fortnight (Fig. 27). This inoculum contained two A series hydrocarbons and a little botryococcene initially and after two weeks each culture from all treatments still contained only these hydrocarbons. After four weeks both the high and low light treatments had developed the normal wide range of hydrocarbons, but the colonies maintained in darkness contained only the two A series hydrocarbons and the botryococcene that they had contained initially. These gradually decreased until at the end of six weeks they were no longer discernible by GLC. Therefore it appears that the colonies utilised the hydrocarbons as a source of energy in the absence of light for photosynthesis.

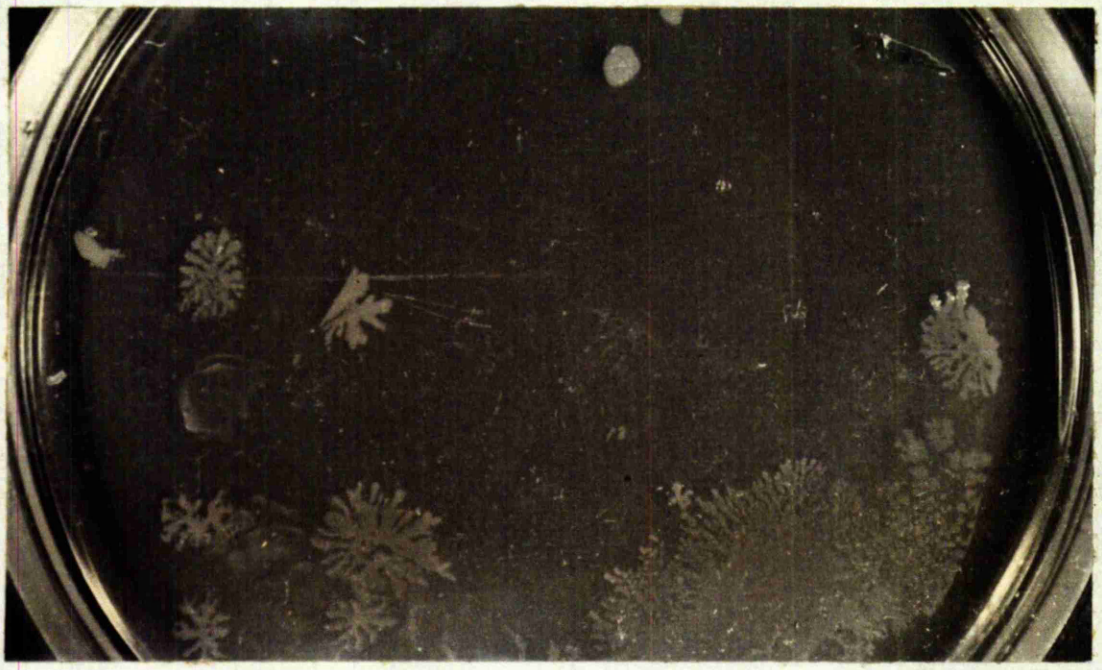


Plate 19. Motile bacteria isolated from B. braunii

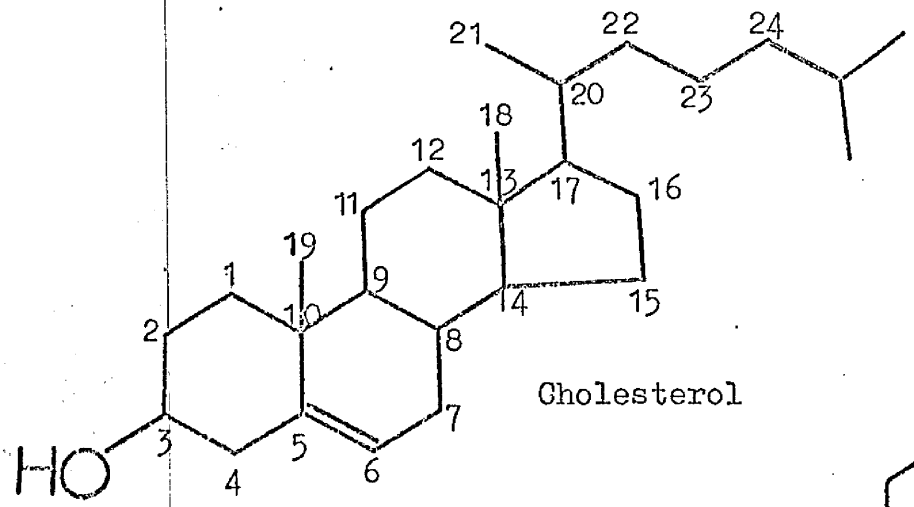
10. Hydrocarbon content of bacteria associated with Botryococcus

To determine if natural contaminants play a part in the hydrocarbon content of colonies Botryococcus (material C) was spread over nutrient agar in petri dishes and incubated at 25° for 48 hours. Samples from these plates were then inoculated into nutrient broth (Appendix 8) and incubated at 25° overnight. Duplicate samples from the nutrient broth cultures were replated onto nutrient agar in the following dilutions 1×10^4; 1×10^5; 1×10^6; 1×10^7. These cultures were incubated for 48 hours at 25° and from the dilution 1×10^7 two freshwater bacteria of the genus Vibrio which showed slight motility (Plate 19) were isolated. As these bacteria grew very slowly on minimal medium agar (Appendix 8) they were inoculated into minimal liquid medium and incubated at 25° for ten days. After rotary evaporation to remove the medium the hydrocarbons were extracted and examined by GLC.

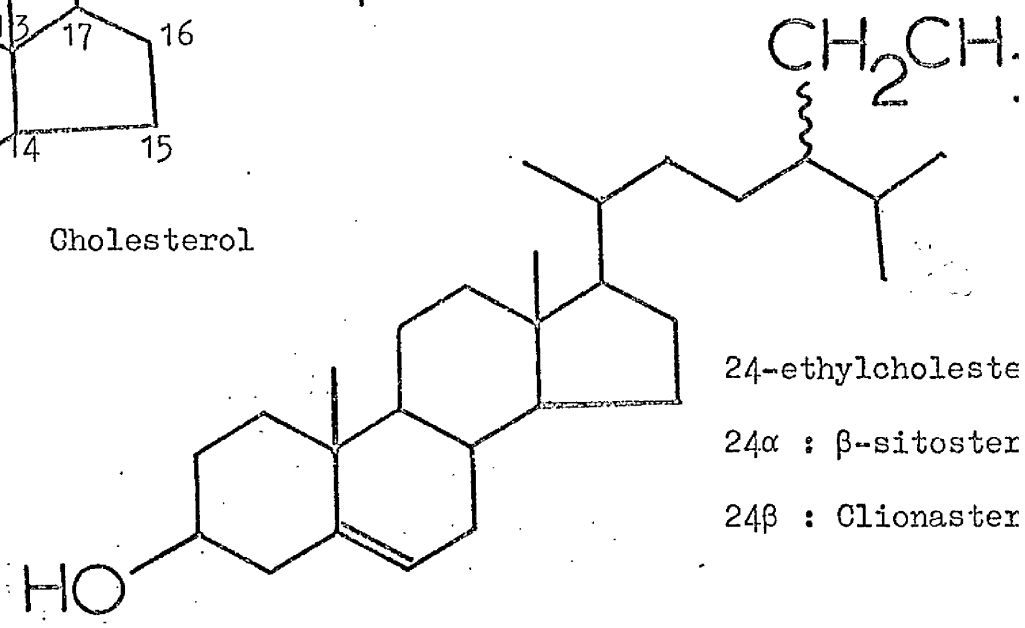
Both species of bacteria were found to have a very low hydrocarbon content, less than 1% of their dry weight.

Since the same range of hydrocarbons have been extracted from axenic and bacterially contaminated cultures of Botryococcus, and since the bacteria show such little hydrocarbon development and are therefore unlikely to accelerate the rate of hydrocarbon production it is reasonable to assume that the alga alone is responsible for the high hydrocarbon production. The intricate colonial form may be an acceptable habitat for bacteria and there is some evidence that they can utilise hydrocarbons in their metabolism (Norris, 1968).

STEROLS



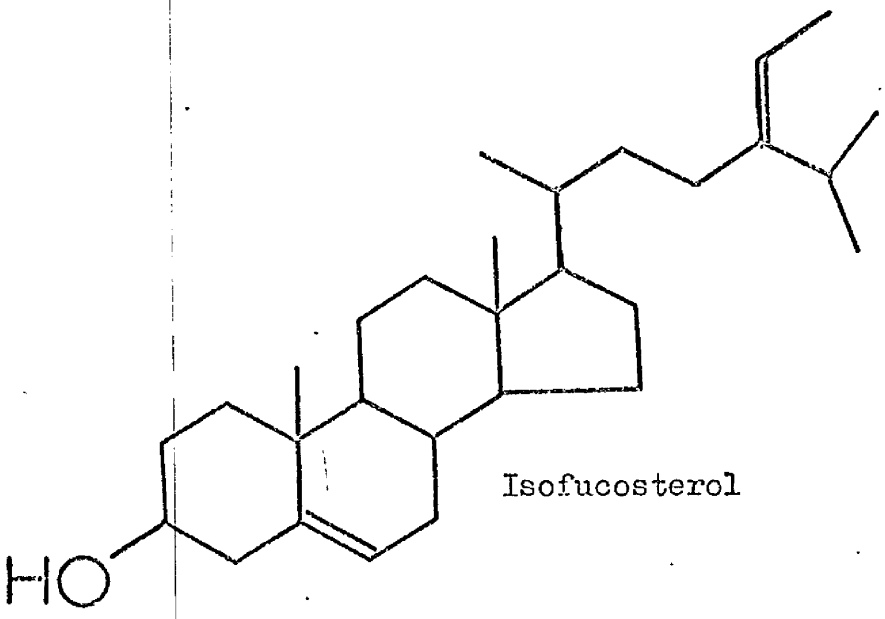
Cholesterol



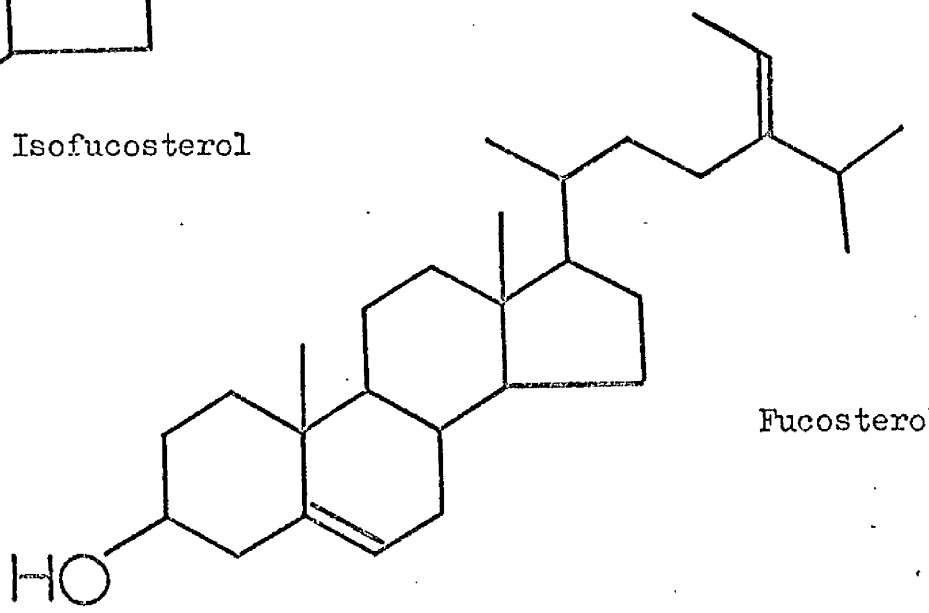
24-ethylcholeste

24α : β-sitoster

24β : Clionaster



Isofucosterol



Fucosterol

11. Sterols in Botryococcus

Willstätter and Page (1914) were first to record that the algae contained sterols when they noted the existence of a new phytosterol in the brown alga Fucus vesiculosus. Montignie (1935) also recorded the presence of steroid material in certain algae. The first detailed investigation of algal sterols was carried out by Heilbron, Phipers and Wright (1934) who isolated, from both F. vesiculosus and Pelvetia canaliculata a doubly unsaturated sterol of the formula $C_{29}H_{48}O$ to which the name fucosterol was assigned. An investigation by Carter, Heilbron and Lythgoe (1940) of seven of the algal classes showed that the Chlorophyceae were characterised by the production of the sitosterol mixture common to higher plants.

More recent investigations (Patterson and Krauss, 1965; Ikekawa et al., 1968; Gibbons et al., 1968) on the Chlorophyceae indicated that the situation was more complex than originally thought. Thus, Patterson and Krauss (1965) demonstrated that in species of Chlorella sterols similar to those of higher plants (e.g. stigmasterol and α-spinasterol) had opposite stereochemistry at C_{24} while Ikekawa (1968) recorded cholesterol as the major sterol of Ulva petusa and of Chaetomorpha crassa where it occurred together with an isomer of 24-ethylcholesterol; and in Enteromorpha intestinalis and Ulva lactuca Gibbons et al. (1968) identified 28-isofucosterol, the isomer of the typical sterol of the Phaeophyta while Bergmann and Feeney (1950) demonstrated the presence of chondrillasterol in Scenedesmus obliquus.

Tsuda (1957, 1958, 1958, 1960) has shown the presence of cholesterol,

24-methylenecholesterol and 22-dehydrocholesterol in the Rhodophyta while, from studies on Rhodymenia palmata and Porphyra purpureum Gibbons et al. (1967) concluded that only cholesterol or closely related C_{27} sterols were present.

In an endeavour to determine if the sterols of Botryococcus braunii were the same as those known in any other group, material C was extracted as follows.

Dried colonies were extracted for seven hours using ether in a Soxhlet apparatus, and the solvent was then removed by rotary evaporation. After treatment by boiling under reflux for three hours with sodium hydroxide and 2% ethanol in water the mixture was allowed to cool before diluting 1:1 with more water; this solution was then extracted with ether (2 x 1/3 volume) and the ether extract was washed with water (2 x 1/3 volume). The ether extract was then treated with anhydrous sodium sulphate to remove traces of water and evaporated to dryness to yield the unsaponifiable lipoid.

Some of this material was chromatographed on thin layer silica gel plates and developed in 20% ethyl acetate in redistilled petroleum ether. The plates were then sprayed with ceric ammonium sulphate heated to 120° for a few minutes and examined. The hydrocarbon content of this extract was obvious at the solvent front and another spot of R_f, about 0.5, was also noticeable. As this may have been sterols the remainder of the unsaponifiable lipoid was dissolved in 1-2 ml. of hot ethanol, an equal volume of a hot solution of 2% digitonin in 90% ethanol was added and the mixture left to stand overnight. Precipitation of sterol digitonides

Peak 3 : Cholesterol

Peak 6 : 24-ethylcholesterol

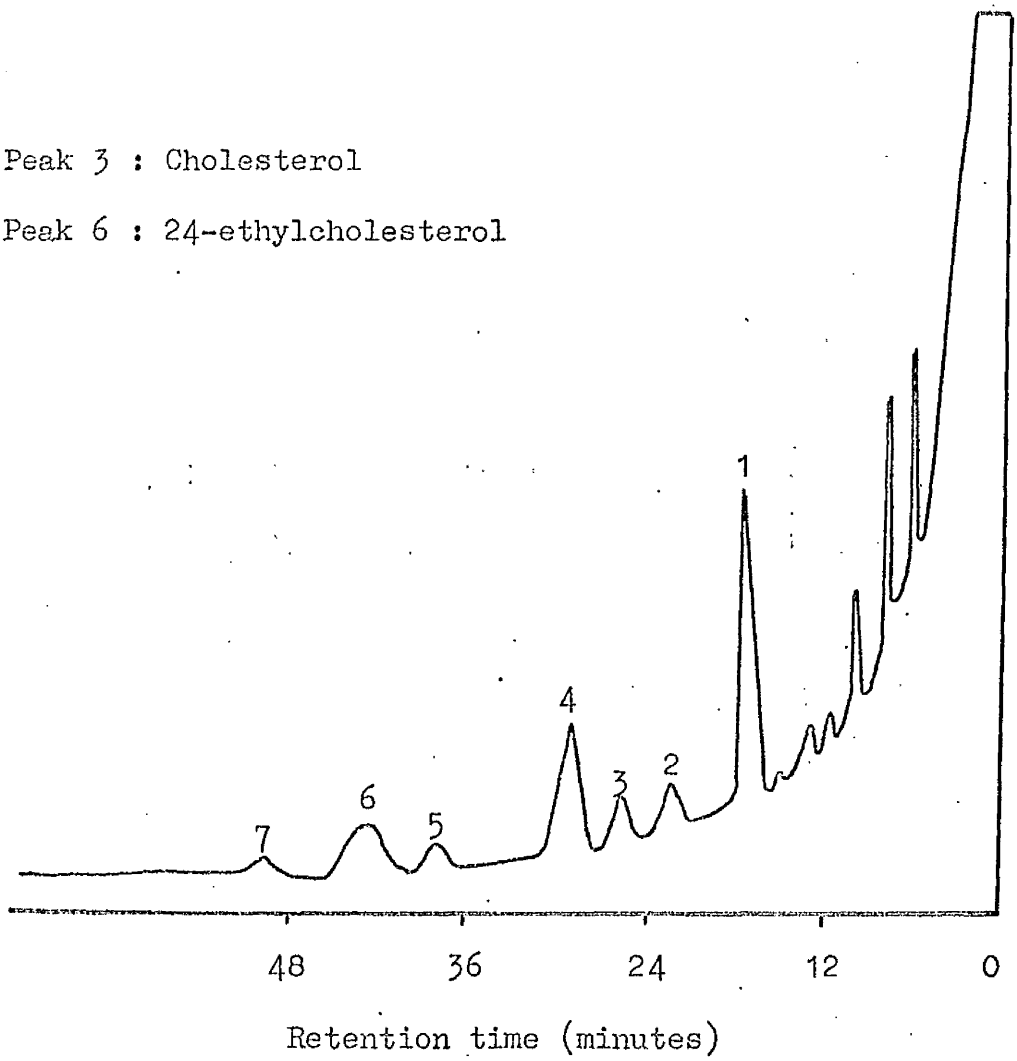


Fig. 28. GLC trace of sterols from B. braunii

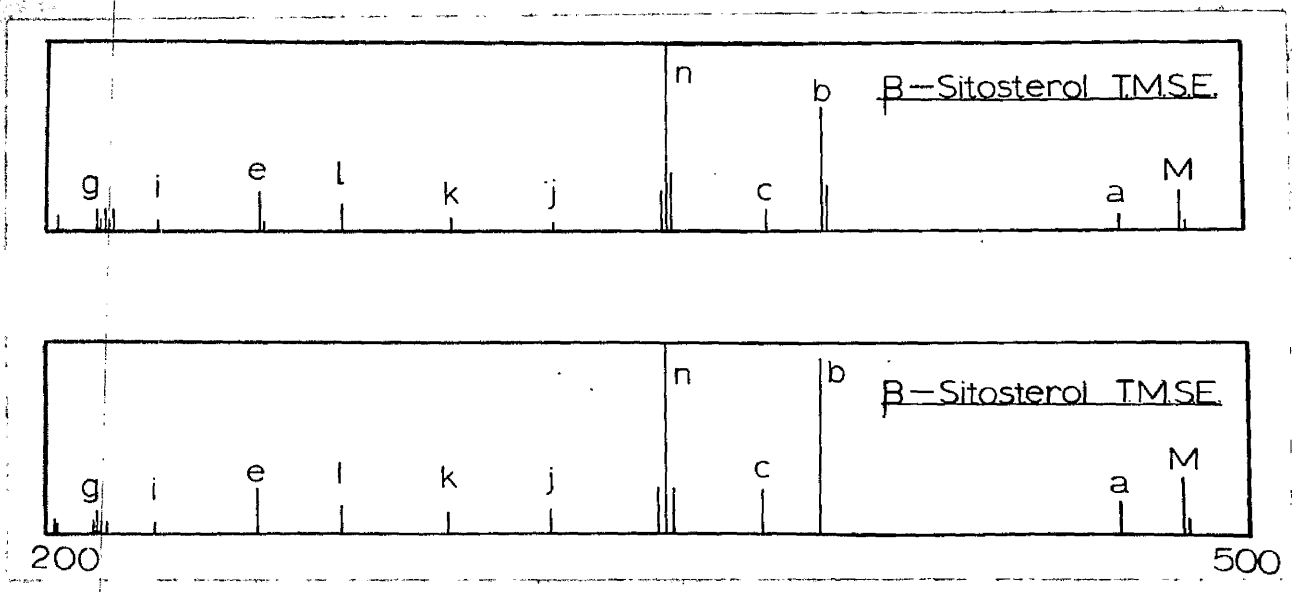


Fig. 29. Mass spectra of β-sitosterol obtained from (a) Avena sativa L. (Knights, 1967); (b) B. braunii

was completed by adding 25 ml. of ether which also precipitated excess digitonin. The solution was centrifuged (2,000 g/10 mins.) and decanted; solid material was then resuspended in ether and recentrifuged. In this way the digitonides were left in the precipitate. To liberate the sterols the digitonin and digitonides were dissolved in 1 ml. of pyridine by warming to about 100° for 2-10 minutes. Ether was then added to precipitate digitonin. Centrifugation and decantation yielded the sterol containing solution. The sterols were obtained by evaporation followed by trituration of the residue with ether to remove traces of unprecipitated digitonin (Fieser and Fieser, 1959). The basis of this method is the reaction of sterols containing a 3β-hydroxyl group to form an insoluble 1:1 complex with digitonin and was first reported by Windaus (1909).

GLC of the sterols as trimethylsilyl ethers (TMSE) was performed on 3% OV-17 at 256° and indicated the presence of several compounds, two of which appeared to be 24-ethylcholesterol (β-sitosterol or its isomer) and cholesterol (Fig. 28, peaks 3 and 6 respectively). The presence of 24-ethylcholesterol was confirmed by GC/MS analysis when a spectrum was produced (Fig. 29) identical to that of β-sitosterol isolated from Avena sativa (Knights, 1967). There was insufficient cholesterol present for GC/MS analysis and its presence was only confirmed by chromatographing a known cholesterol sample under identical conditions and comparing their retention times.

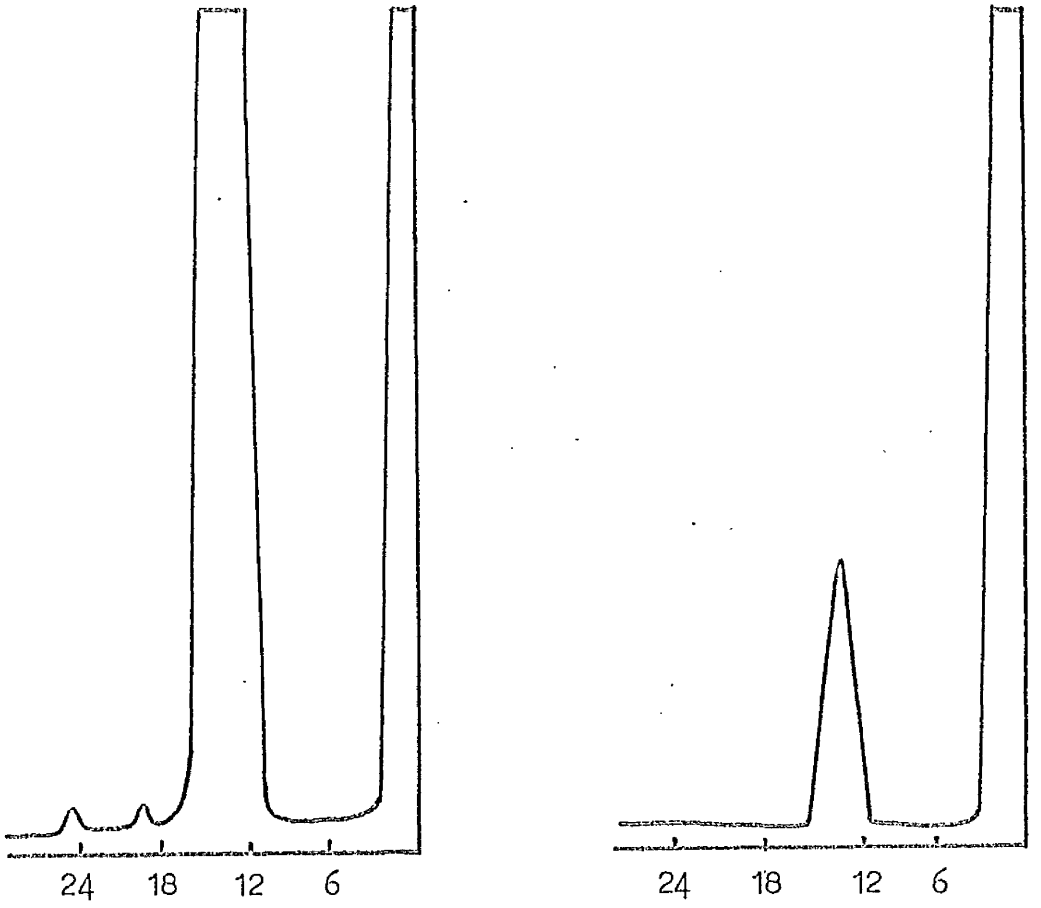
Several other compounds can be demonstrated by GLC of the sterol fraction of B. braunii but their identity is uncertain. However, it can

be stated that neither fucosterol or 28-isofucosterol (Gibbons *et al.*, 1968) were present in detectable quantities. There was no sign of ergosterol as found in *Chlorella ellipsoida* by Otsuka (1963) but quantities of isomers of 24-methyl- and 24-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol (Peaks 4 and 5) may be present.

Thus, in its sterol pattern, *Botryococcus braunii* shows closer relation to *Chaetomorpha crassa* and appears to have little affinity with *Scenedesmus obliquus*, a form to which it is usually allied taxonomically. This supports the contention that there seems to be no fixed taxonomic relationship with respect to sterols in green algae.

Plate 20. Colonies from Oakmere bloom of 1965

(a)



(b)

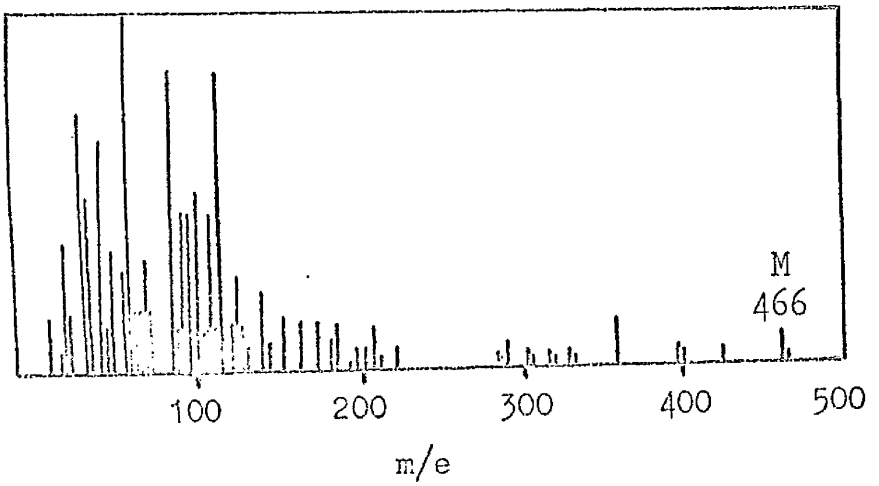


Fig. 30(a) and (b). GLC trace and mass spectrum of Botryococcene

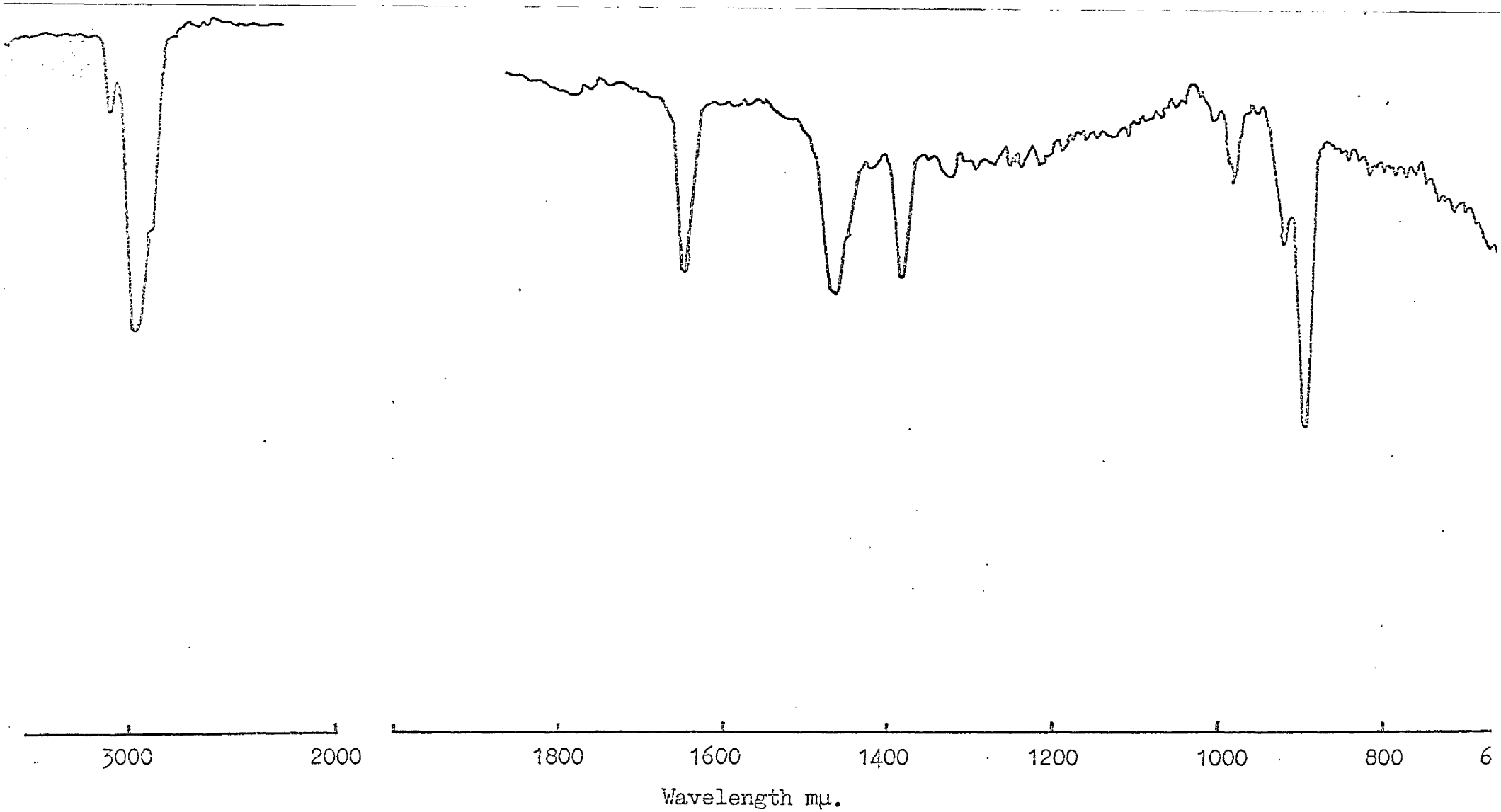


Fig. 31. Infra-red absorption spectrum of Botryococcene

PART V. STUDIES ON THE HYDROCARBONS OF BROWN RESTING COLONIES OF B. BRAUNII FROM THE OAKMERE BLOOM OF 1965 (MATERIAL W.) AND OF AUSTRALIAN COOROGNITE

Introduction

Brown resting colonies from the Oakmere bloom of 1965 (Conway, 1967) were collected and examined microscopically. They were golden brown in colour and had a rubbery appearance. The colour was due to carotenes diffusing out from the cells into the surrounding colony matrix, (Plate 20).

Although the main part of this study is on the hydrocarbon structure, formation and content, of green active colonies from the Cambridge Culture Collection, brown resting colonies from Oakmere have been extracted and the hydrocarbons examined by the previously described method (Part III). Between 70 and 80% of the dry weight of the alga was found to be composed of hydrocarbons and when analysed by GLC (3% OV-17) the chromatogram was dominated by a single peak. From mass spectrometry this compound was shown to have a molecular weight of 466 (Fig. 30a + b). Maxwell (1968) has called this acyclic polyunsaturated hydrocarbon botryococcene and has noted that it occurs in the colonies in the ratio of about 9:1 with its isomer iso-botryococcene.

Examination of the hydrocarbon botryococcene was made by infra-red spectroscopy (i.r. spec.) and nuclear magnetic spectroscopy (n.m.r.). Infra-red spectroscopy of botryococcene (liquid film) showed strong absorption at 890 $cm.^{-1}$ due to the presence of four exomethylene double bonds, and absorption at 910 and 1,000 $cm.^{-1}$ consistent with the presence of a vinyl group [$R\text{-}CH = CH_2$] (Fig. 31). Absorption at 980 $cm.^{-1}$ showed a *trans* disubstituted bond. Nuclear magnetic resonance spectroscopy

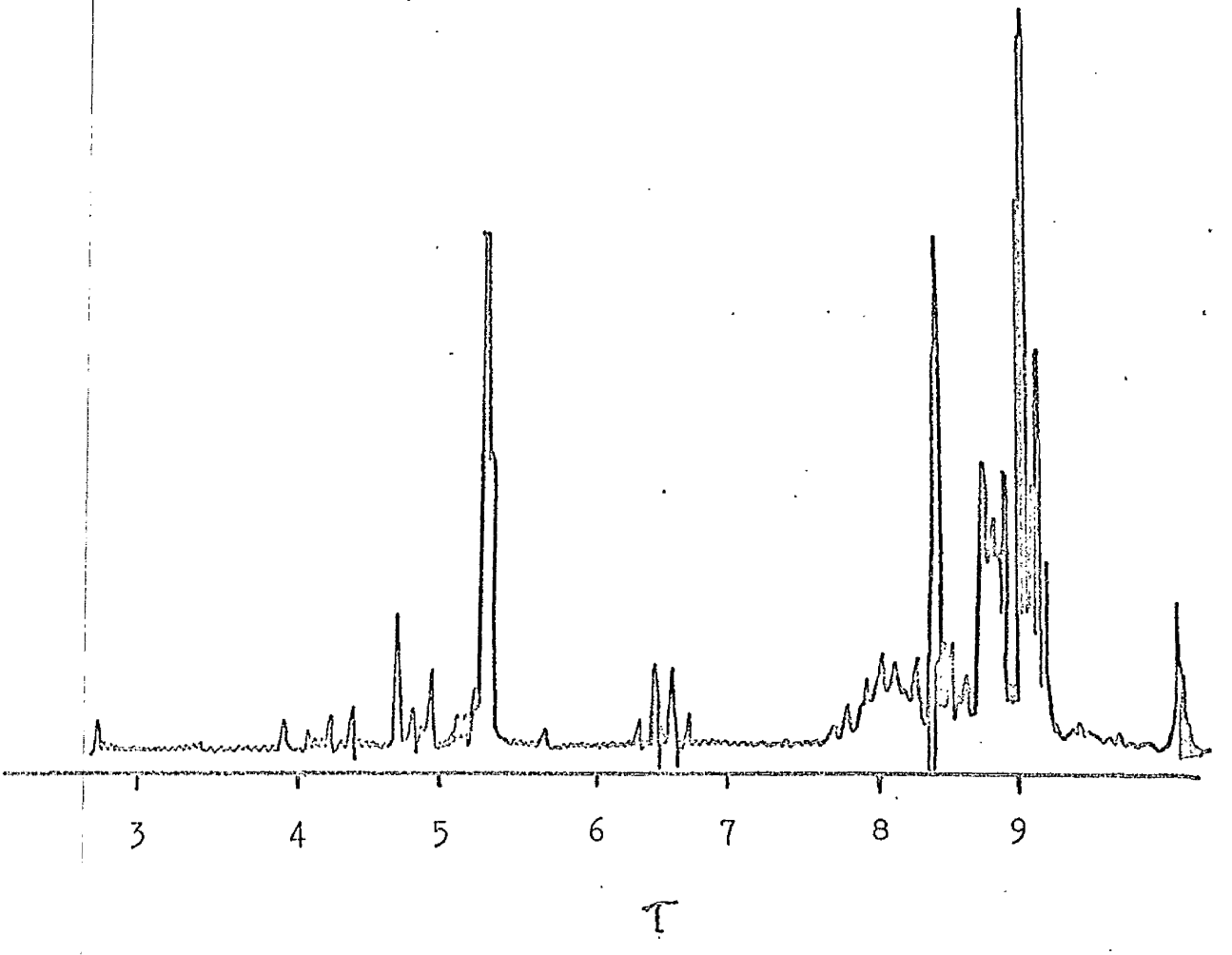
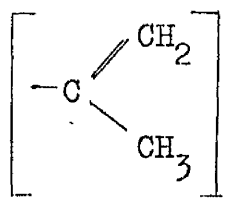
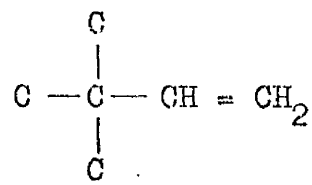


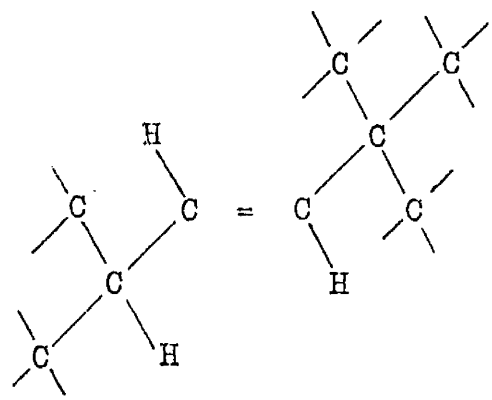
Fig. 32. Nuclear magnetic resonance spectrum of Botryococcene

carried out on botryococcene at 60 m/c on a Perkin Elmer instrument with $CDCl_3$ solvent and tetramethylsilane $[Si(CH_3)_4]$ internal standard gave results as shown in Fig. 32. These were very similar to results obtained by Maxwell (1968) except for impurities which gave a quartet at 6.54 τ and a doublet centred at 8.75 τ. This contaminant may have been due to iso-botryococcene the isomer of botryococcene. A detailed analysis of the n.m.r. spectrum of botryococcene has been described by Maxwell and has been taken to indicate the presence of four exomethylene double bonds, a vinyl group attached to a quaternary carbon and a trans double bond substituted by a quaternary carbon and a tertiary carbon. The presence of two isopropenyl groups  together with several methyl groups was also indicated.

Vinyl group attached to quaternary carbon



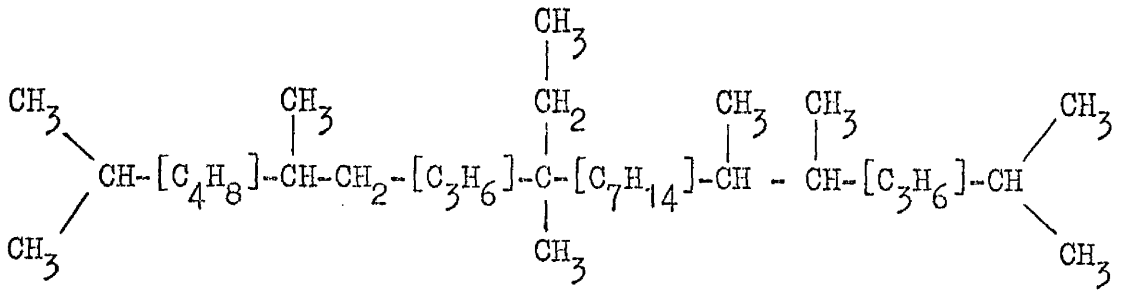
Trans double bond substituted by a quaternary carbon and a tertiary carbon



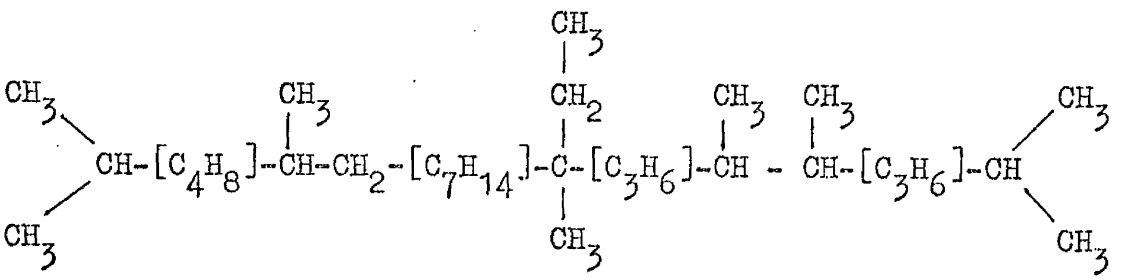
There was no carbonyl absorption (i.r.), indicating that the extract contained little or no fatty acids or other carbonyl containing substances, as would have been expected from the method of extraction. It had previously been thought that the "fatty cups" of these colonies were composed

of fats (Blackburn and Temperley, 1936) but from this evidence and the results of Douglas et al. (1969), who found the fatty acid content of the colonies to be very small, this does not seem to be the case.

Maxwell (1968) prepared botryococcane by hydrogenation of botryococcene and from n.m.r. and i.r. spectroscopy as well as GC/MS has concluded that it has either of the two following partial structures.



or



1. Hydrocarbon content of 'Green mulberries' grown from Material (W) in culture.

It was brown resting colonies of this type, from Oakmere, and from a small summer bloom on Loch Lomond, which when isolated and cultured gave rise to the peculiar large green cells which could only be made to revert to the characteristic 'mulberry' habit of Botryococcus with great difficulty. Once the 'mulberries' were obtained they were cultured in MC_{13} with 5 p.p.m. vit. B_{12} for six weeks at 20° and a light intensity

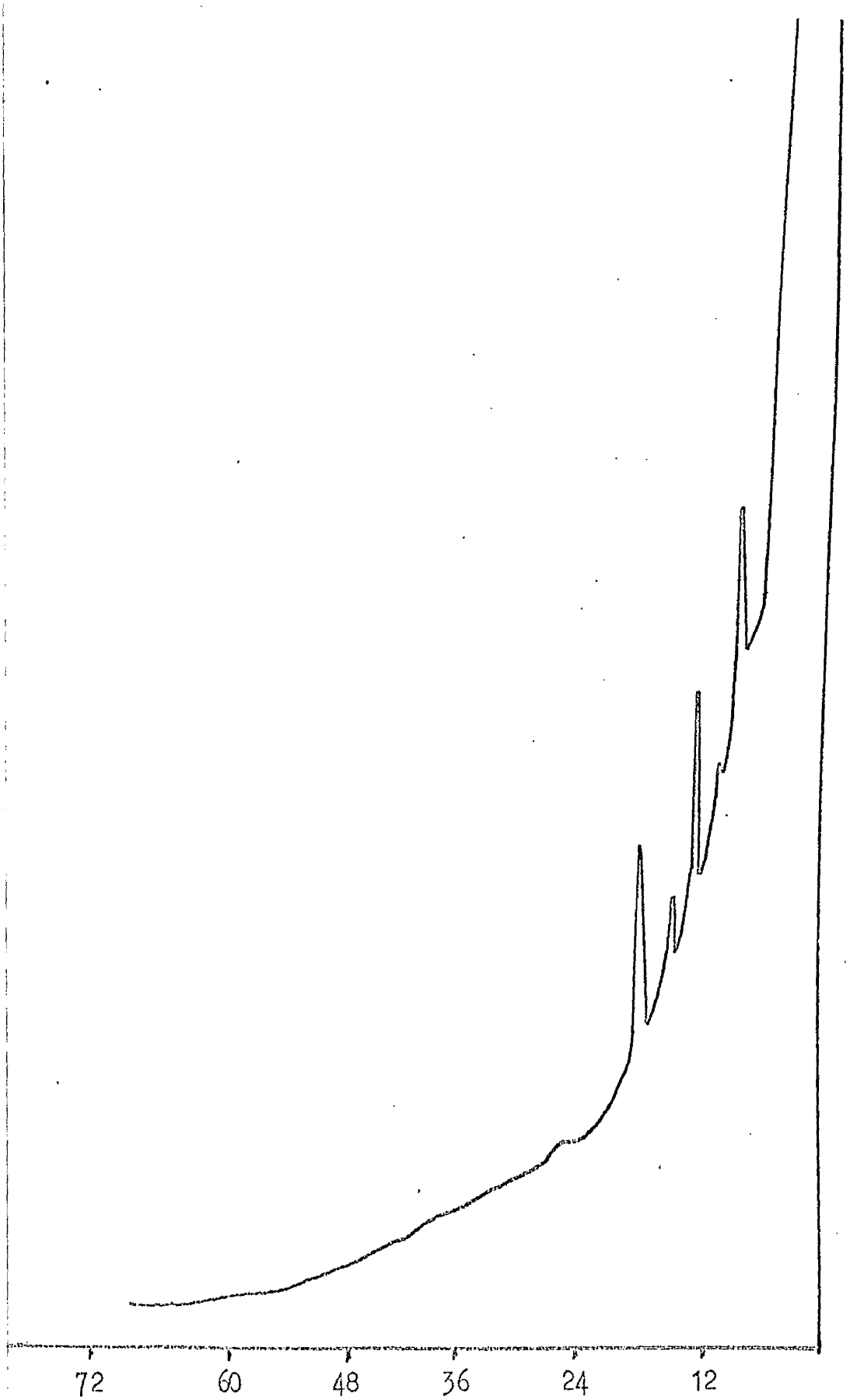


Fig. 33. GLC trace of hydrocarbons obtained from "mulberries" formed from brown wild material.

of 250 foot candles before hydrocarbon extraction and examination by GLC. The 'mulberries' were very poor in hydrocarbons (less than 1% of the algal dry weight). Gas chromatography demonstrated that only very little of this hydrocarbon consisted of the characteristic hydrocarbons obtained from the green active colonies of the Cambridge culture, Fig. 33, Table 18.

Retention distance (mm)		Retention ratio with $nC_{28}H_{58}$
11	(A)	0.23
15		0.32
20	(A)	0.43
26		0.55
31		0.66
35	(A)	0.75

Retention distance of $nC_{28}H_{58}$ = 47 mm.

(A) denotes member of the A series of hydrocarbons found in Cambridge culture material.

Table 18.

Retention data for the hydrocarbons isolated from the 'mulberries' formed from brown resting wild type material.

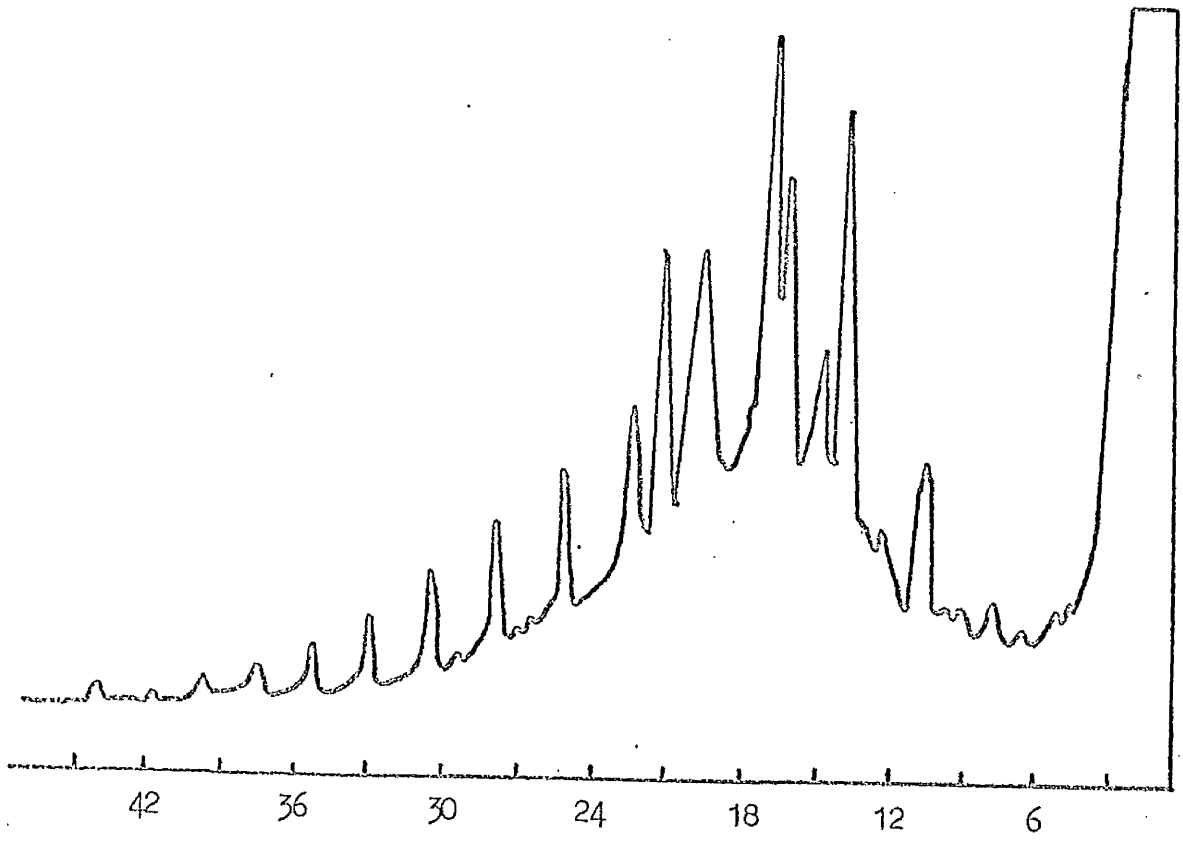


Fig. 34. GLC trace of hydrocarbons from coorognite

2. Study of Coorognite

Coorognite, the peat-stage equivalent of B. braunii (Thiessen, 1925) has been analysed from specimens given by Professor G.E. Fogg, Westfield College, London. This was a brown rubbery material, about 1/4" thick, which had been obtained from the deposits in the Coorong district of Australia.

This material was extracted, after grinding in a pestle and mortar with liquid nitrogen, in the usual manner and produced a hydrocarbon fraction of 0.3% of the dry weight of the coorognite. When gas chromatographed it was apparent that the hydrocarbons were all of lower molecular weight than that of botryococcene the hydrocarbon from brown resting B. braunii colonies (Fig. 34). The lower molecular weight of these hydrocarbons and their pattern of distribution suggests that they may be produced as a result of chlorophyll decomposition. [Oró, Nooner and Zlatkis (1965), Han et al. (1968)].

After elution of these hydrocarbons the chromatographic column was flushed with diethyl ether and a further fraction was collected which accounted for about 10% of the initial dry weight of the coorognite. This extract was examined by infra-red spectroscopy and by ultra violet spectroscopy to obtain some insight into its structure.

(a) Properties of this diethyl ether eluate from coorognite

When chromatographed on thin layer silica gel chromatographic plates, in 20% ethyl acetate in redistilled petroleum ether, this compound appeared non-polar running almost with the solvent front. One mgm. in

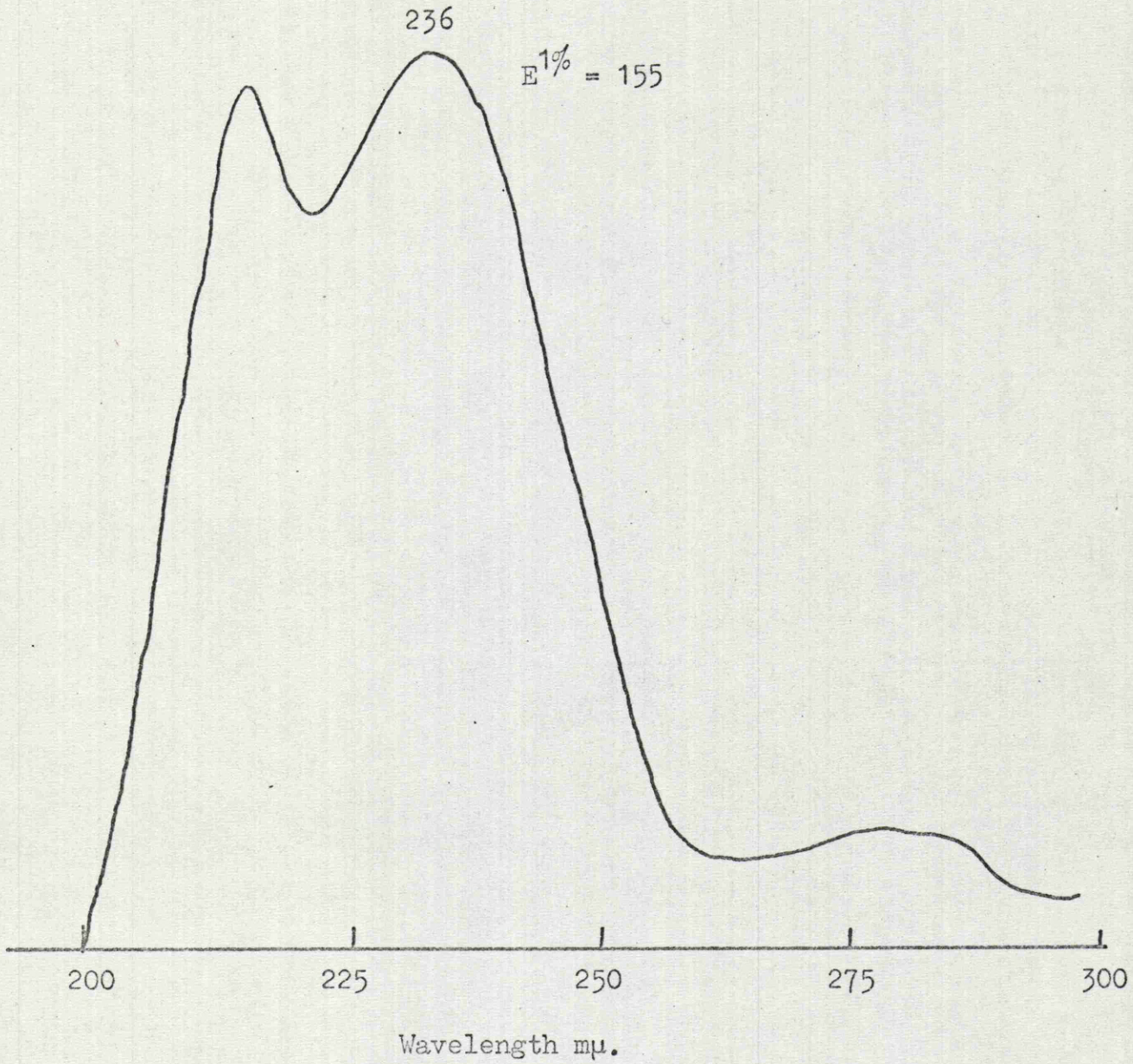


Fig. 35. U.V. absorption spectrum of the diethyl ether eluate from coorognite.

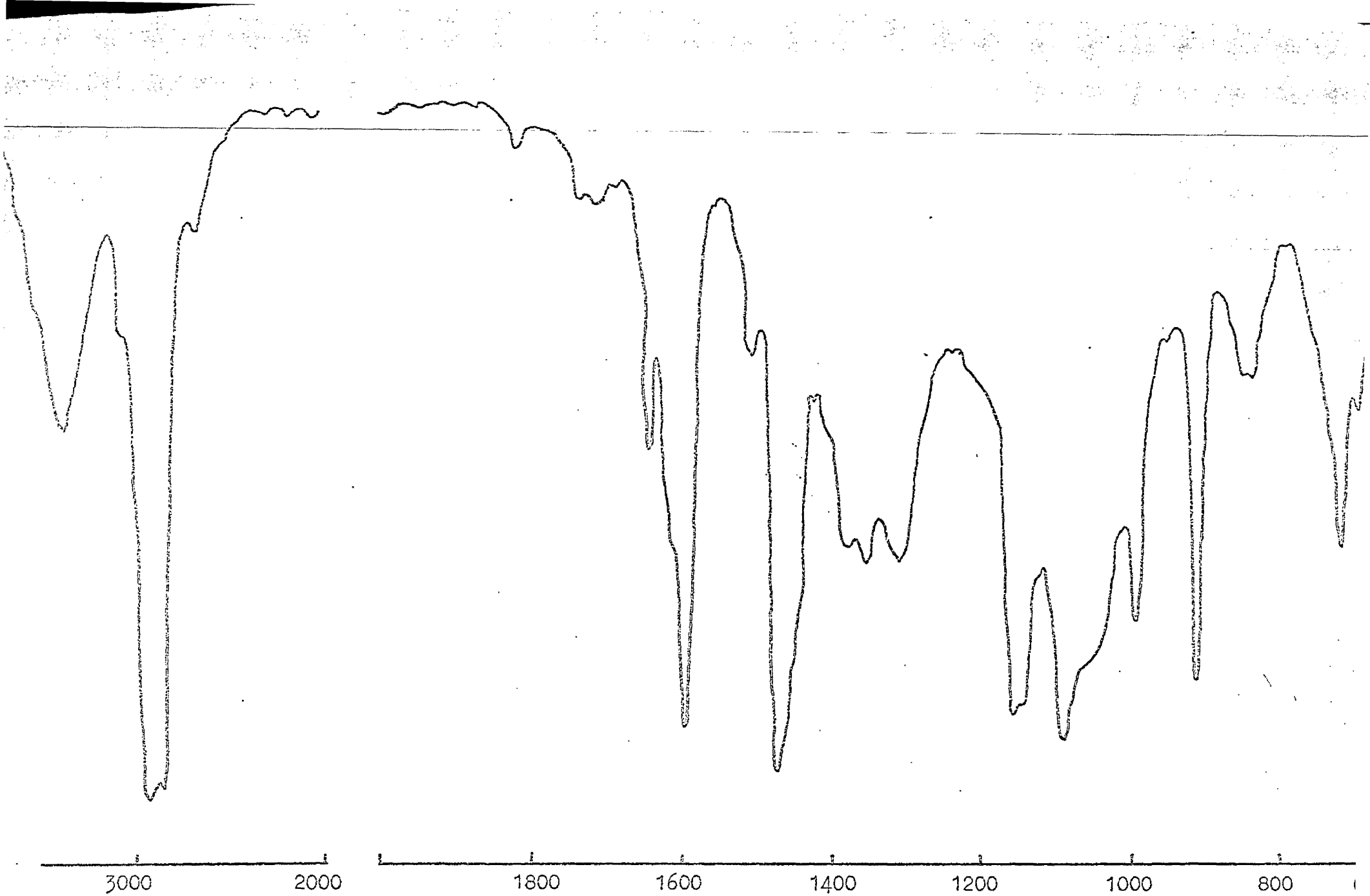
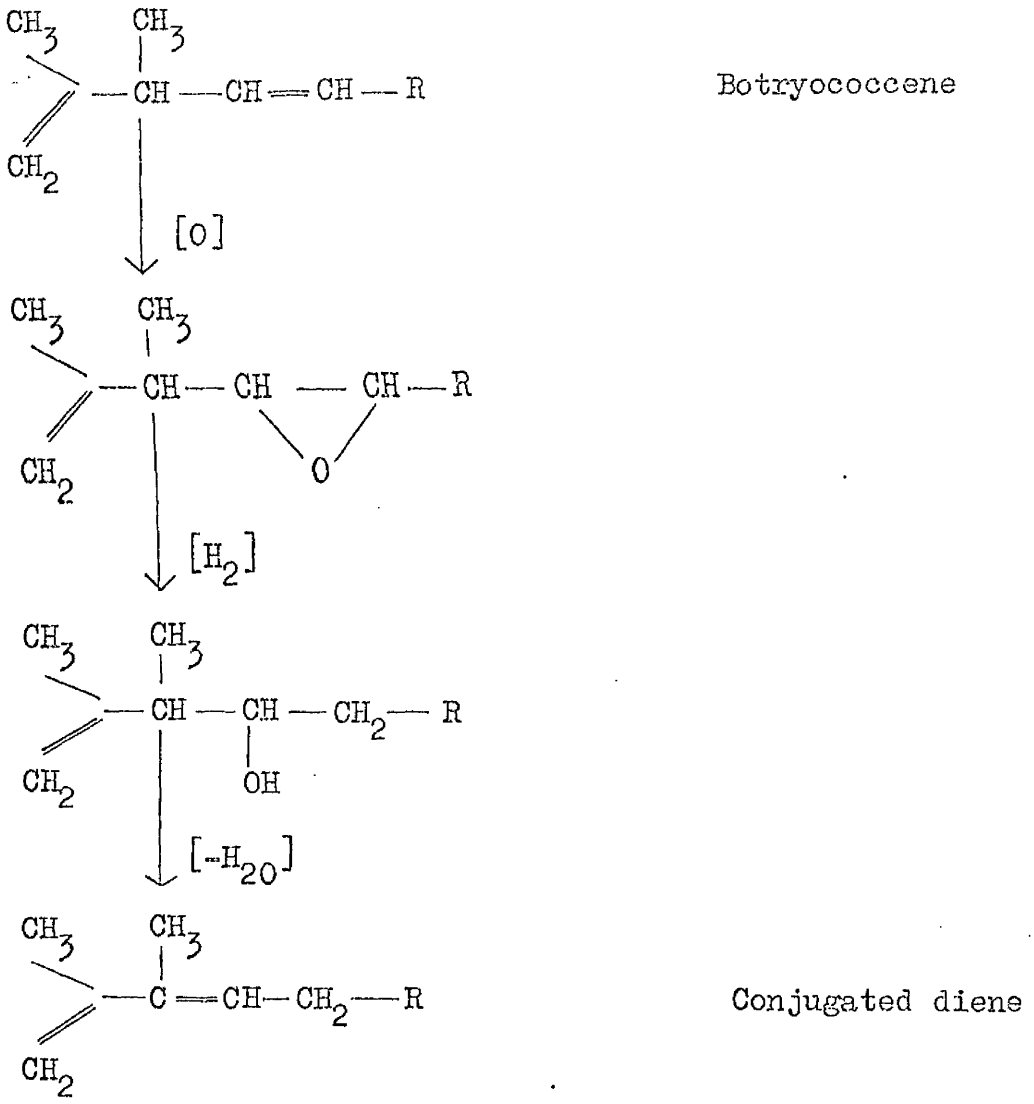


Fig. 36. Infra-red absorption spectrum of diethyl ether eluate from coorognite.

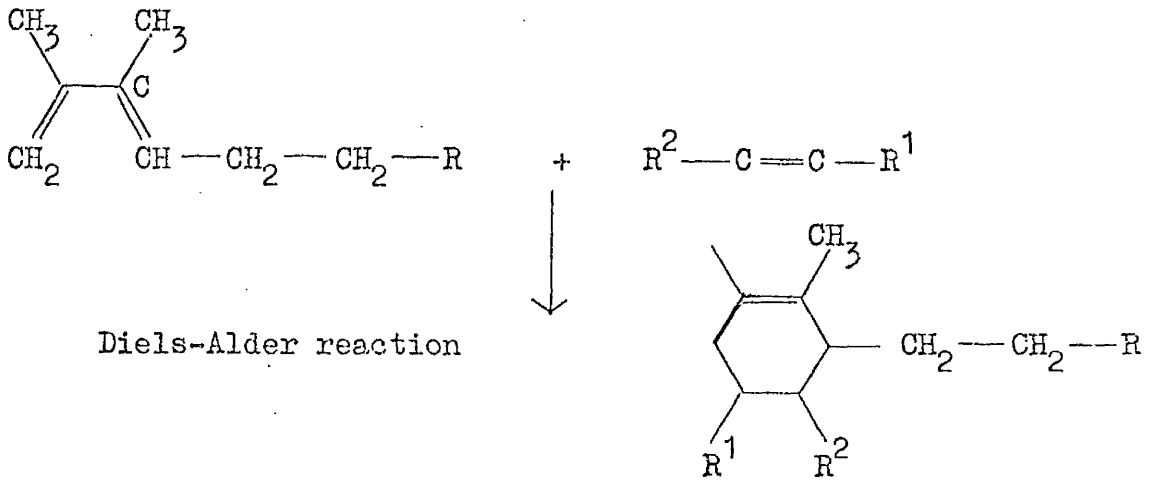
10 ml. hexane gave a U.V. spec with absorption at 233 mμ indicative of a possible aromatic structure (Fig. 35.) or a conjugate diene or polyene. Infra-red spectroscopy (liquid film) on the other hand (Fig. 36) showed vinyl absorption at 1,000 cm^{-1}, 910 cm^{-1} and 1,640 cm^{-1}. The absorption at 1,600 cm^{-1} could be indicative of an aromatic structure or of a conjugated double carbon bond. The latter seems more likely due to the absence of absorption due to aromatic hydrogens in the region 650 - 1,000 cm^{-1}. There was no ketone (C=O) absorption. Summarising the i.r. evidence it may be said that this compound is probably a conjugated diene (or polyene) with extensive C-H absorption, some OH absorption and some C-O absorption but no C=O absorption. This material could not be successfully analysed by GLC under normal operating conditions, suggesting partial polymerisation from a compound such as botryococcene.

(b) Possible formation of the diene

As coorognite is formed from brown resting colonies of B. braunii [Broughton (1938), Hautpick (1926)] it is interesting to speculate how the conjugated diene (or polyene) of coorognite can be formed from botryococcene, the hydrocarbon of brown resting colonies, by exposure to the atmosphere. A simple possible scheme for the production of a conjugated diene from botryococcene is detailed below.



It is possible that some such system is responsible for the production of this conjugated diene (or polyene) isolated from coorognite and polymerisation reactions may be envisaged as a consequence of this, e.g.



and possible free radical polymerisation of intermediates.

DISCUSSION

Outstanding among the characteristic features of Botryococcus braunii are the complex form of the colony, the distinctive green and brown states in which the alga is found at different times in the same habitat and the large amounts of hydrocarbon which may be present at any stage in the maturation. Although the alga appears to fit into the usual pattern of photosynthesis in the Chlorophyta, with the presence of chlorophyll a and b and the end product of starch, the main product of photosynthesis appears to be hydrocarbon. Because of their degree of unsaturation, these hydrocarbons are usually seen as liquid droplets inside the cells in cultures (Plate 14). These droplets are capable of passing out through the cell wall, and as they do so, they become oxidatively polymerised. Since extraction of lipid (page 43) has shown fats to be present only in very small amounts it is probable that the much larger amounts of hydrocarbon present give rise to the so called "fatty cups" of Blackburn and Temperley (1936). These form a structural unit which, by its resistance to decay, gives the alga its characteristic form and a structure which can survive long periods of time (Thiessen, 1925). The cups which give marked protection to the cells, undoubtedly isolate the protoplasts from the external environment, and Belcher (1957) estimated that almost 90% of each cell is so protected. Such specialised form of the Botryococcus colonies together with the massive amounts of surrounding mucilage may account, not only for the polymorphism of the genus, but also for the slow growth in the field (Swale, 1968) and for the fact that although widely distributed, the alga is seldom found in anything but sparse amounts. This is evident from

earlier records (West and West, 1901; Brook, 1964) and is noticeably true from numerous plankton hauls made in Loch Lomond, Stirlingshire and Oakmere, Cheshire over the past three years. Nevertheless, records exist (Conway, 1967) of temporary massive growths which give rise to "bloom" conditions of the plant in its golden brown form: no accounts are known of "blooms" in the green state. This may be accounted for by the variation in hydrocarbon content between the two states. Fogg (1965) suggested that the massive production of lipoid he detected in brown colonies would lower their specific gravity and render them buoyant. Maxwell (1967) recognised the hydrocarbon botryococcene in brown colonies and pointed out that this accounted for some 80% of the dry weight of the colonies; an amount sufficient to account for their buoyancy. On the other hand green colonies are now known (Brown, Knights and Conway, 1969) to contain not more than 20-30% hydrocarbon, and these hydrocarbons differ quantitatively and qualitatively from botryococcene. Since it has been found possible (page 64) to induce the development of botryococcene by growing colonies in MC_{13} medium containing 1/10th of the usual combined nitrogen supply it may be suggested that depletion of combined nitrogen in natural freshwater leads to high botryococcene production which lowers the specific gravity of the colonies and allows them to float.

Further, Belcher and Fogg (1955) found that in media with low combined nitrogen formation of β-carotene was markedly increased and this would account for the development of golden-brown colour in the colonies at the same time as the increase in the lipoid content.

The formation of such large amounts of hydrocarbon call for further

consideration of its synthesis. Earlier workers (Chibnall and Piper, 1934) postulated that in microorganisms hydrocarbon synthesis was linked with fatty acid decarboxylation, and this theory was supported by Kates (1966). Kolattukudy (1967) demonstrated a similar relationship in higher plants (Brassica oleraceae) where palmitic acid became the substrate for an elongation-decarboxylation enzyme complex which elongated the palmitic acid to a C_{30} acid. Subsequent decarboxylation of this unit released the C_{29} hydrocarbon. Later, however, Oró et al. (1967) found it difficult to accept the hypothesis of hydrocarbon biosynthesis by simple fatty acid decarboxylation since in bacteria and simple algae from both freshwater and marine environments there was incomplete correlation between fatty acids and hydrocarbons present.

In tests on culture of Botryococcus (Material C) the GLC trace showed the presence of fatty acids of chain length C_{24} and C_{26} and the hydrocarbon trace demonstrated the presence of hydrocarbons of chain length C_{23} and C_{25}. These support the theory of hydrocarbon synthesis by fatty acid decarboxylation. But of the main A series hydrocarbons ($2^{\Delta}C_{2705}$, $2^{\Delta}C_{2915}$ and $2^{\Delta}C_{3120}$) there were no detectable amounts of corresponding fatty acids C_{28}, C_{30} and C_{32}. This discrepancy may be accounted for by the rapid decarboxylation of the higher chain length fatty acids to form the great amounts of the main A series hydrocarbons known to be present (page 46).

Finally the determination of such large amounts of hydrocarbon in Botryococcus in the living state raises questions of its presence or absence in shales and boghead coals where Botryococcus has been cited as

the causal organism. Analyses have not been attempted but earlier work with the Green River shale of the U.S.A., a possible algal deposit, has shown the presence of hydrocarbons of high molecular weight (nC_{20} - nC_{33}) particularly of nC_{27}, nC_{28} and nC_{31}. Such findings were supported by the work of Han et al. (1968) on an algal ooze in Florida U.S.A. These latter workers also reported that populations of simple algae such as species of Nostoc, Anacystis, Spirogyra and Chlorella showed only hydrocarbons in the range nC_{15} - nC_{20} with nC_{17} dominant. This suggests that in the formation of such deposits algae able to synthesise longer chain length hydrocarbons, such as have been found in Botryococcus, may be implicated.

Appendix 1.

B. braunii and its relationship to the Boghead Coals.

Bituminous shales (Thiessen, 1925) of the types that give rise to boghead coals, torbanite, oil shales, tasmanite, cannel coals and cannel slates have long been known to be of plant origin, and Ralph (1865) was probably the first to suggest that the organic constituent of tasmanite (a form of coal) was algal in origin. In this he was incorrect as the organic constituent is now known to be spores of higher plants; but David (1890) working on Australian kerosine shale recorded the presence of spherical bodies, which he thought might be of some fresh-water alga. Bertrand (1893) and Renault (1899) working on a series of shales of European, Australian and American origin, reported the presence of large numbers of "yellow bodies" particularly in boghead coals (so called after a deposit found near the Boghead Estate, Bathgate, Scotland) sometimes called torbanite (after a deposit found at Torbane Hill, also near Bathgate). The generic name of Pila was established for these "yellow bodies". Similar deposits from the southern hemisphere were named Reinschia (Renault, 1899) and were thought to be of different form. Bertrand (1930) examined the "yellow bodies" in greater detail and concluded that Pila was, in fact, B. braunii although he made no decision about Reinschia. It is now usually accepted (Travers, 1955) that both Pila and Reinschia are Botryococcus braunii, the slight differences in form and extent of wall formation being due to fossilization under differing climatic conditions.

Conacher (1917) has stated that the "yellow bodies" were a constant feature of oil shales and in boghead coal they were so closely packed together that there was little matrix between. In this state, a close grained deep brown or black "coal" is formed, which is strong, rubbery and difficult to break. Such coals are, according to Anderson and MacGregor (1938) and Conacher (1917), comparatively rare and give more gas than cannel coal.

Controversy on the nature of the "yellow bodies" was marked through the latter part of the Nineteenth Century and the early part of the Twentieth Century, particularly since most authors recognised them as algal in origin but failed to link them with any living algal form. Working with coorognite (a rubber-like deposit) from Southern Australia, Thiessen (1925) came to the conclusion that coorognite was the peat-stage equivalent of the boghead coal of the northern hemisphere, and he named the organic constituent Eleophyton coorongiana, an oil producing alga. His description and plates fit well with the form of Botryococcus braunii, though he himself believed it to be an unknown alga. It was Zalessky (1926), working on balkashite (a paraffin-rich earth in Russian Lakes) who recognised the "yellow bodies" as consisting of vast numbers of an alga capable of secreting oil. From Chodat's (1896) description of B. braunii he believed it to be the causal organism. Zalessky believed that deposits on the shores of the lake were formed by vast numbers of algal colonies drying in situ, and becoming impregnated with silica, but that if the algal deposit (which was resistant to decay) was formed at the bottom of the lake the algal colonies would not coalesce but would remain

separated in a matrix of organic mud, which on consolidation would resemble boghead coal.

Naumann (1921), Bradley (1924) and Blackburn and Temperley (1936) re-examined boghead coals and came to the conclusion that although "yellow bodies" differed somewhat in the coal from different areas, they were all forms of the polymorphic alga B. braunii and that different specific names were not justifiable. Fremy and Dangeard (1938) working on Swedish and French deposits agreed with this and Travers (1955) in his review of the literature of lignites and tertiary deposits agreed that the causal organism of the oil found there was B. braunii.

Appreciation of the oil-forming potentialities of this alga, gave rise to geochemical interest in the nature of its oil. Maxwell (1967) estimated that 76% of the dry weight of brown colonies of B. braunii, collected from Oakmere, was composed of hydrocarbon and that almost all of that was an acyclic polyunsaturated hydrocarbon (botryococcene) and its isomer (isobotryococcene). [See Part V].

Appendix 2.

Media tested in growth studies on B. braunii.

Modified Chu 13 (MC_{13}) (Chu, 1942)

KNO_3	0.1 gm.
K_2HPO_4	0.02 gm.
$MgSO_4.7H_2O$	0.05 gm.
$CaCl_2.6H_2O$	0.04 gm.
Fe citrate ($M \times 10^{-5} \times 3.5$)	0.0093 gm.
Citric acid	0.1 gm.
Glass distilled water to	1 litre

pH adjusted to 7.5 before autoclaving.

Modified Chu 10 (MC_{10}) (Chu, 1942)

$Ca(NO_3)_2$	0.08 gm.
K_2HPO_4	0.02 gm.
$MgSO_4.7H_2O$	0.05 gm.
Na_2CO_3	0.04 gm.
Na_2SiO_3	0.05 gm.
Fe citrate ($M \times 10^{-5} \times 3.5$)	0.0093 gm.
Citric acid	0.1 gm.
Glass distilled water to	1 litre

pH adjusted to 7.5 before autoclaving.

Beijerinck's Medium (B) (Pringsheim, 1946)

NH_4NO_3	1 gm.
K_2HPO_4	0.2 g.m
$MgSO_4.7H_2O$	0.1 gm.
Fe citrate	0.0093 gm.
Citric acid	0.1 gm.
Glass distilled water to	1 litre

pH adjusted to 7.5 before autoclaving.

Knop's Medium (Modified after Chu, 1942) (K_m) (Pringsheim, 1946)

KNO_3	1 gm.
$Ca(NO_3)_2$	0.1 gm.
K_2HPO_4	0.2 gm.
$MgSO_4.7H_2O$	0.1 gm.
Fe citrate	0.0093 gm.
Citric acid	0.1 gm.
Glass distilled water to	1 litre

pH adjusted to 7.5 before autoclaving.

S_{66} Medium (Droop, 1961)

NaCl	300 mgm.
$MgCl_2.6H_2O$	40 mgm.
KCl	8 mgm.
$CaSO_4.2H_2O$	10 mgm.
Glycylglycine	500 mgm.
Glycine	250 mgm.
KNO_3	100 mgm.
K_2HPO_4	10 mgm.
$VitB_{12}$	100 mμ gm.
Thiamine	100 μ gm.
$EDTANa_2$	50 mgm.
Trace solution:	
Fe	500 μ gm.
Mn	50 μ gm.
Zn	5.0 μ gm.
Cu	5.0 μ gm.
Co	500 mμ gm.
Mo	500 mμ gm.
Glass distilled water to	1 litre

pH adjusted to 8 before autoclaving.

Hoagland's A-Z Trace Solution (Hoagland and Snyder, 1933)

LiCl	0.5 gm.
$CuSO_4.5H_2O$	1.0 gm.
$ZnSO_4$	1.0 gm.
H_3BO_4	11.0 gm.
$Al_2(SO_4)_3$	1.0 gm.
$SnCl_4.H_2O$	0.5 gm.
$MnCl_2.6H_2O$	7.0 gm.
$Co(NO_3)_2.6H_2O$	1.0 gm.
TiO_2	1.0 gm.
KI	0.5 gm.
KBr	0.5 gm.
$NiSO_4.6H_2O$	1.0 gm.

Made up to 18 litres with glass distilled water and applied at 1 ml. or less per litre of culture medium. [1 ml. per litre : T, 1/2 ml. per litre : 1/2T]

Soil Extract (S.E.)

1 litre of tap water

1 Kg. of garden soil

Autoclaved at 20 lbs. $in.^2$/30 minutes.

After standing for one week, the supernatant was decanted and autoclaved at 20 lbs. $in.^2$/30 minutes before use.

Trace stock solution for S_{66} (Droop, 1961)

	Added to 100 ml. g. dist. water	Conc./100 ml.
$CuSO_4.5H_2O$	2 mgm.	Cu : 0.5 mgm.
$COCl_2$	111.1 mgm.	CO : 50 "
Fe citrate	237.5 mgm.	Fe : 50 "
$MnCl_2$	11.46 mgm.	Mn : 5 "
Na molybdate	119 mgm.	MO : 50 "
$ZNSO_4$	1.25 mgm.	Zn : 0.5 "

1 ml. of this solution was added to 999 ml. of the S_{66} to give the relevant concentrations of trace elements mentioned.

E_1 Medium (Cambridge Culture Collection of Algae and Protozoa 2nd edit., 1966)

Soil extract	50 mgm.
KNO_3	200 mgm.
K_2HPO_4	20 mgm.
$MgSO_4.7H_2O$	20 mgm.
Glass distilled water to	1 litre

pH adjusted to 7.5 before autoclaving.

TRIS Buffer (Dawson et al., 1959)

pH 23°	pH 37°	0.2M TRIS	0.1 NHCl	
9.10	8.95	25 ml.	5.0 ml.	Dilute to 100 ml. with glass distilled water.
8.92	8.78	25 "	7.5 "	
8.14	8.00	25 "	25.0 "	
8.05	7.90	25 "	27.5 "	
7.96	7.82	25 "	30.0 "	
7.20	7.05	25 "	45.0 "	

TRIS M.W. = 121.14; ∴ 0.2M solution contains 24.23 gm./l.

Analar grade chemicals were used in the preparation of all media.

Appendix 3.

Differential centrifugation for the isolation of B. braunii colonies.

25 ml. of graded sucrose solutions were made up from 5% - 25%.

10 ml. of each solution was added to two 100 ml. centrifugation tubes starting with the most concentrated and ending with the weakest. 5 ml. of the non unialgal culture of B. braunii was then added to each tube and they were then spun at 35,000 r.p.m./60 minutes. After centrifugation the B. braunii colonies were isolated from the rest of the starting material and could be removed with a fine Pasteur pipette and washed.

Appendix 4.

Preparation of B. braunii for Electron Microscopy

(a) Preparation of Sörensen phosphate buffer.

80 ml. of $^{M}/_{15}$ Na_2HPO_4 was combined with 20 ml. of $^{M}/_{15}$ KH_2PO_4. 2 ml. of this solution was then added to 8 ml. of MC_{13} medium, (see Appendix 2) and pH adjusted to 7.38 before adding 1 ml. of a 25% gluteraldehyde solution.

(b) Fixation and Embedding. Method I.

1. The colonies were fixed for 4 hours at 4° in 2.1/2% gluteraldehyde.
2. Washed in buffered medium (2 x 1 hour) and left overnight in medium (at 4°).
3. Fixed for 4 hours at 4° with 1% osmic acid made up in buffered medium.
4. Washed in buffered medium (2 x 1 hour at 4°).
5. Dehydrated through alcohols:-

30% (1/4 hr. at 4°) 70% (1/2 hr. ⟶ O/N) 90% (1/2 hr.) 100% (2 x 1 hr.) warming to room temperature. Alcohol removed by washing in propylene oxide (2 x 15 minutes) and then embedded in the following manner.

propylene oxide	:	araldite G + accelerator	
3	:	1	for 4 hours
1	:	1	overnight (O/N)
1	:	3	4-8 hours

(b) 5. (contd)

They were then soaked in araldite G + accelerator for 24 hours before hardening at 30° (1 day), 50° (1 day) and 60° (1 day).

Araldite G is prepared from Resin Cy212 (35 parts) and Hardener DDSA (40 parts).

Sections were then cut on an LKB ultrotome (type 4801A), mounted on copper grids and examined at various magnifications in an A.E.I. EM.6B electron microscope with an accelerating voltage of 60 K.V.

Method II.

Addition of Ruthenium red for studies on cell pectic cap.

Colonies fixed in gluteraldehyde as before but with the addition of 500 ppm. ruthenium red. Ruthenium red was included in the washing and in the osmic fixation at the same concentration. Subsequent treatment of the colonies was as before.

Appendix 5

Antibiotic mixtures used to purify B. braunii (Droop, 1967).

Amounts are quoted in µg. per ml. as prepared for the six tube procedure described.

	Mixtures			
	IV	V	VI	VII
Benzyl penicillin sulphate .	5,000	8,000	8,000	8,000
Streptomycin sulphate	800	1,600	2,000	1,600
Chloramphenicol	400	200	8	80
Neomycin	-	-	-	400
Actidione	800	-	-	-
Total	7,000	9,800	10,008	10,080

Appendix 6.

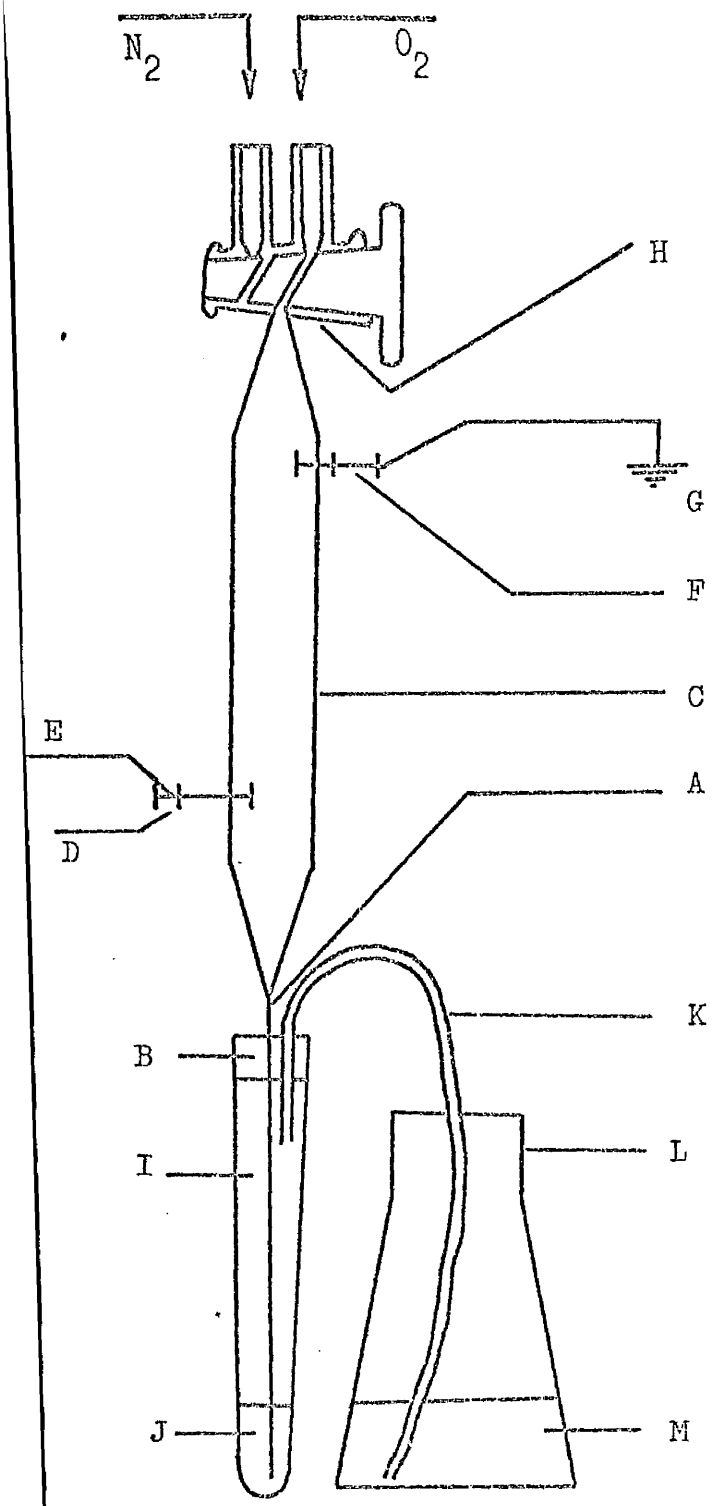
Preparation of Diols (Eglinton and Hunneman, 1968)

11 mgm. of the A series hydrocarbon mixture was dissolved in 3 ml. of pyridine and ether (1:8). 50 mgm. of osmic acid in 1 ml. of ether was then added and the solution was allowed to stand for an hour and a half before adding 6 ml. of Na_2SO_3 suspension (1.5 ml. of 16% aqueous Na_2SO_3 [16 gm./50 ml.] plus 160 ml. of methanol). It was then stirred magnetically for one hour and spun down at 2,000 r.p.m./15 min. Supernatant was then decanted and evaporated and the substrate was taken up in 2 ml. of ethyl acetate for analysis.

Appendix 7.

Ozonolysis of the A series hydrocarbons

Supelco Micro-ozoniser



A = Needle stock tubing

B = Silicone rubber septum

C = Glass tubing

D = Gold plated electrode

G = Earth

H = 3-way stopcock

K = Teflon tubing

L = 10 ml. Erlenmeyer flask

M = Indicating solution

Cold Bath not shown.

OZONOLYSIS

Method I.

A 25 μg. sample of the hydrocarbons in 100 μl. of redistilled carbon disulphide was placed in tube I. Ten ml./min. of oxygen was passed into the solution cooled at ca. -70° in an acetone dry ice bath. The cord at electrode D was clipped to the Tesla coil to generate ozone. When the blue colour of excess ozone was seen in the indicating solution M in the flask L the high voltage was turned off at the Tesla coil. The indicating solution was made up of 5% potassium iodide in 5% sulphuric acid with added starch.

Ozone generation usually required between 1-1.1/2 min. Stopcock H was then turned to purge the solution in I with nitrogen for 30 secs. The cold bath was removed and tube I was slipped off the needle stock tubing, the silicon septum B removed and about 1 mgm. of powdered triphenyl phosphine dropped into the solution. The tube was immediately stoppered and swirled to dissolve the powder. When the temperature of the solution reached ambient a 3 μl. aliquot was injected into a Pye Series 104 gas chromatograph with a 9 ft. 3% OV-17 column operated at 244° and a nitrogen carrier gas flow rate of 40-50 ml./min.

Method II

The reaction was repeated at room temperature and tetracyanoethylene (TCNE) was substituted for triphenyl phosphine.

Appendix 8.

Media for culture of bacteria.

Nutrient Broth

Lab lemco (beef extract)	10 gm.
Bactopeptone	10 gm.
Sodium chloride	5 gm.
Glass distilled water to	1 litre

pH adjusted to 7.0 - 7.2 before autoclaving

Minimal medium (Starr, 1946)

Glucose	0.5 gm. (added separately)
KH_2PO_4	0.1 gm.
NH_4Cl	0.2 gm.
$MgSO_4$	0.02 gm.
Trace stock solution:	
$B(H_3BO_3)$	0.5 µg.
$Ca(CaCO_3)$	10.0 µg.
$Cu(CuSO_4.5H_2O)$	1.0 µg.
$Fe(FeSO_4[NH_4]_2SO_4.6H_2O)$	10.0 µg.
$Mn(MnSO_4.H_2O)$	1.0 µg.
$Mo(MoO_3)$	1.0 µg.
$Zn(ZnSO_4.7H_2O)$	5.0 µg.
Glass distilled water to	100 ml.

pH adjusted to 6.8 before autoclaving

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