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HYDROCARBON CONTENT AND ITS RELATIONSHIP TO PHYSIOLOGICAL STATE IN THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

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Abstract—Three physiological states, characterized by their hydrocarbon content are described for the green alga *Botryococcus braunii*: (1) Green active state colonies containing a complex mixture of hydrocarbons of the general formula C_nH_{2n-2} and C_nH_{2n-4}, (2) Brown resting state colonies containing a high concentration of a nearly pure (90 per cent) hydrocarbon botryococcene, (3) Large green cells showing very little synthesis of hydrocarbons.

INTRODUCTION

IT HAS been suggested that the green colonial alga *Botryococcus braunii* (Kütz) may be implicated in the formation of hydrocarbons found to occur in oil shales of the tertiary period.[1] As a result the hydrocarbon content of this alga and of the blue green alga *Anacystis montana* have been the subject of study.[1,2] In the case of the so called "golden brown" alga *B. braunii* it was found that six hydrocarbons of the general formula C_nH_{2n-2} and one of the formula C_nH_{2n-4} could be demonstrated using combined gas chromatography/mass spectrometry (GC-MS). The alga *B. braunii* is known to exist in several physiological states and, working with the resting state material a very high percentage of hydrocarbon per dry weight of colonies has been noted. This was shown to consist very largely of two isomeric hydrocarbons—botryococcene and isobotryococcene (in 9:1 ratio).[2]

In view of the interest in this alga as a source of hydrocarbons in geological deposits, comparative studies including light and electron microscopy and chemical analysis have been made for the various physiological states of development. It has been found that the nature of the hydrocarbon fraction may be correlated with the stage of development, and it is the object of this paper to discuss these findings.

RESULTS AND DISCUSSION

Hydrocarbons from *Botryococcus braunii* were isolated by acetone extraction of colonies, from which the aqueous phase of the culture medium had been removed by rotary evaporation. The extract was subject to chromatography on alumina and the hydrocarbon fraction analysed by gas–liquid chromatography (GLC) on SE-30 and OV-17. Molecular weights of

[1] E. GELPI, J. ORO, H. J. SCHNEIDER and E. O. BENNETT, *Science* **161**, 700 (1968).
[2] J. R. MAXWELL, Ph.D. Thesis, University of Glasgow (1967).

some of the components of these mixtures were confirmed using GC-MS. The resultant GLC data for the hydrocarbons observed is listed in Table 1.

The data listed in Table 1 (obtained from green exponential colonies) suggested the presence of three homologous series of hydrocarbons in which double bond isomerism and polyunsaturation may occur. Analysis by GC-MS using an OV-1 column demonstrated the general formula C_nH_{2n-2} for the A series of compounds and n was shown to be 27, 29 and 31 respectively for compounds 4, 6 and 9. This result is in agreement with that reported previously[1] for this alga when the presence of six hydrocarbons [$n=23, 25, 27, 28, 29, 31$] of this general formula had been demonstrated. In our experiments no trace of the hydrocarbon $C_{28}H_{54}$ could be seen on either SE-30 or OV-17. The B series was found to have the general formula C_nH_{2n-4} from GC-MS data and that for peak 8 $n=29$. No GC-MS data for the C series was obtained, but the GLC data suggested the possibility of isomerism with the B series.

TABLE 1. GLC DATA FOR HYDROCARBONS IN *Botryococcus braunii*

Hydrocarbon	Series	Retention index SE-30	Retention index OV-17	M.W.	Relative composition (percentage)
1	A	2305	2295	—*	0·3
2	A	2500	2495	—	1·0
3	B	—	2605	—	0·2
4	A	2705	2705	376	7·2
5	B	2765	2820	—	0·9
6	A	2905	2915	404	32·6
7	C	2945	2995	—	4·8
8	B	2965	3015	402	23·0
9	A	3100	3120	432	25·1
10	C	3135	3195	—	1·9
11	B	3155	3225	430	3·0
Botryococcene		2790	2800	466	—
Isobotryococcene		—	—	466	—

* Denotes not recorded

Whilst the location of double bonds in these compounds is not known for certain, the i.r. spectrum of the mixture showed absorption at 990 cm^{-1}, 910 cm^{-1} and 1643 cm^{-1} consistent with the presence of a vinyl group [CH_2=CH—R] in at least some of the components of the mixture. Mass spectra of these components (Fig. 1) suggested that they were homologous series of molecules with little or no branching along the length of the chain. The predominance of even mass number fragments, particularly in the high molecular weight range, in the A series suggested the loss of a small fragment by rearrangement, followed by successive fragmentation along the chain. Alternatively, should the two double bonds be located :$\alpha\omega$, then double cleavage α to the double bond might produce a predominance of ions of even mass number. The absence of ions in the region m/e 200–230 is partly due to masking of expected ions (at m/e 208 and 222) by background derived from column bleed. For the B series there appears to be a predominance of ions of odd mass number, usually three mass units less than for the A series.

The compounds isolated from the brown resting state colonies were shown by GLC, GC-MS, i.r. and NMR spectroscopy to be the previously characterized hydrocarbons botryococcene and isobotryococcene.[2, 3]

Three physiological states of the alga *B. braunii* are readily discernable. The first of these consists of green active state colonies [obtained from the Cambridge Culture Collection] (Fig. 2a) characterized by three homologous series of hydrocarbons. These were found to account for up to 17 per cent of the dry weight of the colonies. The A series of hydrocarbons (Table 1) consisted chiefly of three compounds: 4, 6 and 9 and the B series principally of compound 8. Series C accounted for no more than 6 per cent of the total in most cases.

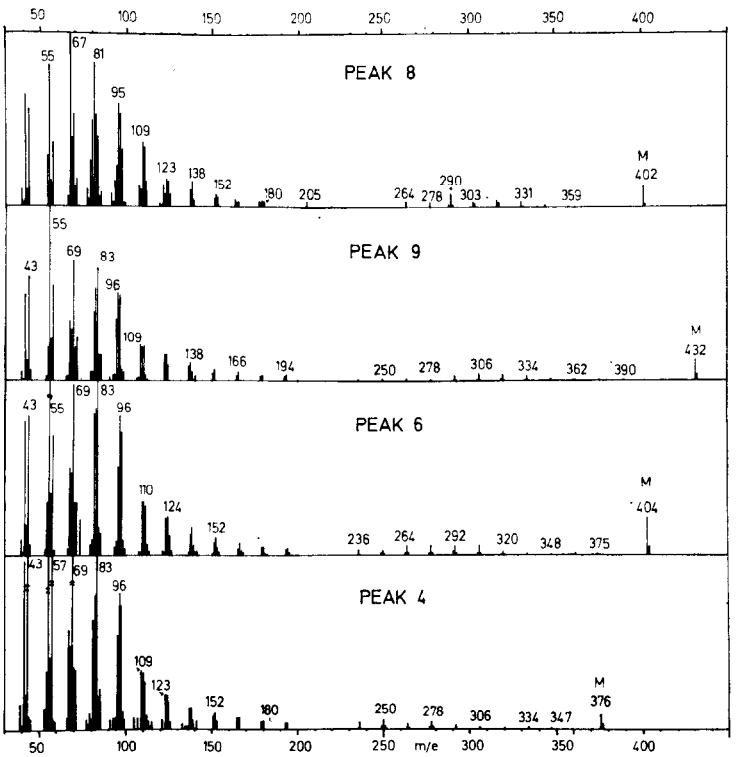

FIG. 1. MASS SPECTRA FOR COMPOUNDS CORRESPONDING TO PEAKS 4, 6, 8 AND 9 (TABLE 1).

The second consists of brown resting state colonies (Fig. 2b) and are characterized by almost unilateral development of the hydrocarbon botryococcene. These colonies are derived from green active state colonies and have turned brown and entered the resting phase of growth, if considered in terms of a growth curve. The hydrocarbon content of such colonies may be up to 86 per cent of their dry weight. When colonies of this physiological type were inoculated into fresh medium, the botryococcene was found to have largely disappeared within 1 week and the characteristic hydrocarbons of green, or exponential colonies, had been formed.

In the third state (Fig. 2c), the characteristic "mulberry" habit of the *Botryococcus* colony has been lost and large green cells have developed. These may be made to revert to

[3] J. R. MAXWELL, A. G. DOUGLAS, G. EGLINTON and A. MCCORMICK, *Phytochem.* 7, 2157 (1968).

the more normal habit of the alga only with great difficulty. This state, characterized by very poor hydrocarbon development, arose when brown resting state colonies (collected from Oakmere, Cheshire and Loch Lomond, Stirlingshire) were brought into unialgal culture in modified Chu 13 medium[4] and grown under constant conditions of temperature, day length and light intensity.

In order to facilitate the study of the biosynthesis of the various hydrocarbons it was considered necessary to produce an axenic culture[5] of the alga containing the relevant hydrocarbons, i.e. the three series from the green state and botryococcene from the brown state.

The effect upon hydrocarbon synthesis of varying the culture conditions was investigated. In particular the effects of variation in light intensity, combined nitrogen and duration of culturing were studied and the results are indicated in Table 2. Using the normal nitrogen

TABLE 2. PERCENTAGES OF EACH HYDROCARBON FROM CULTURES OF *B. braunii*

Culture conditions		Peak number (Table 1)								
		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
O.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
Typical 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.

content in the medium, the characteristic hydrocarbon development of green state colonies was noted during the first 6 weeks after inoculation. However, after 12 weeks about one in every three cultures contained botryococcene in addition (identified by GLC). Production of this intermediate state was rather haphazard and the botryococcene was found to disappear after inoculation into a fresh medium. However by culturing in one-tenth the normal combined nitrogen supply for a period of 6 weeks, using high light intensity at 20° for a 16-hr day, the intermediate state could be produced routinely. It is worth noting that, when *B. braunii* was cultured in darkness or with one-tenth the combined nitrogen supply, relatively more C_{31} hydrocarbon was produced than under other culturing conditions although light appears essential for the synthesis of botryococcene.

Whilst it may be seen from the foregoing that three physiologically distinct states of the alga *B. braunii* may be recognized directly by light microscopy and by hydrocarbon content,

[4] S. P. CHU, *J. Ecology* **30**, 284 (1942).
[5] M. R. DROOP, *Br. Phycological Bull.* **3**, 295 (1967).

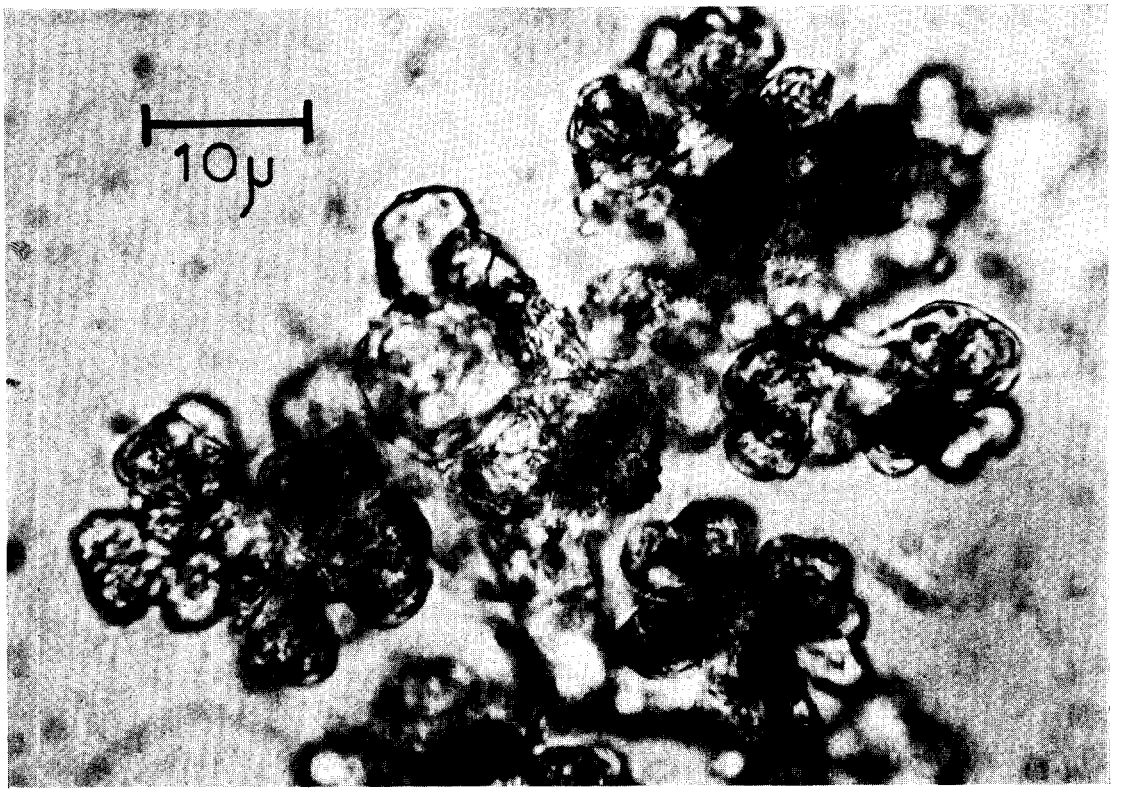

FIG. 2a. LIGHT MICROGRAPH OF GREEN ACTIVE STATE *B. braunii* COLONIES.

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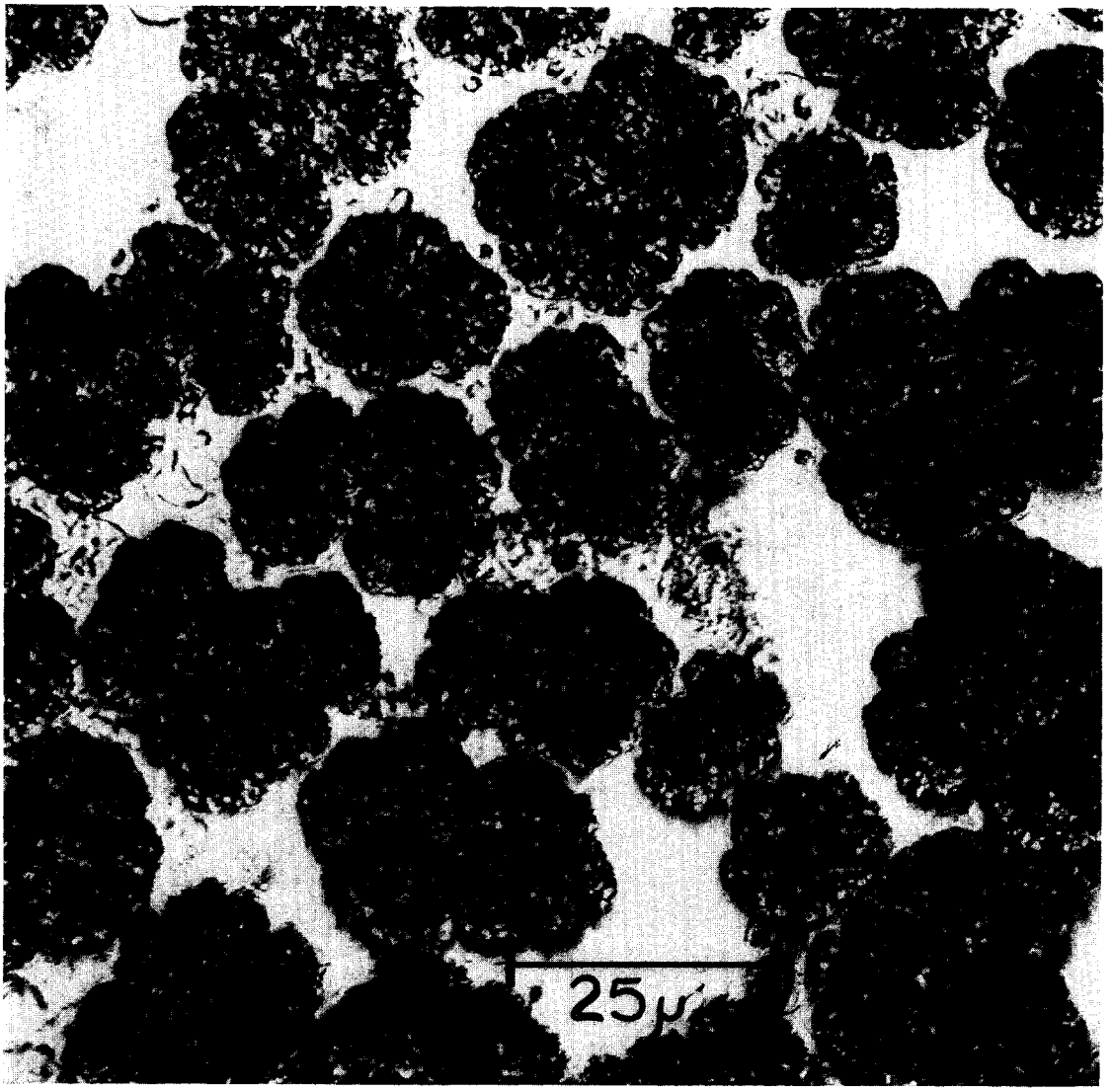

FIG. 2b. LIGHT MICROGRAPH OF BROWN RESTING STATE *B. braunii* COLONIES.

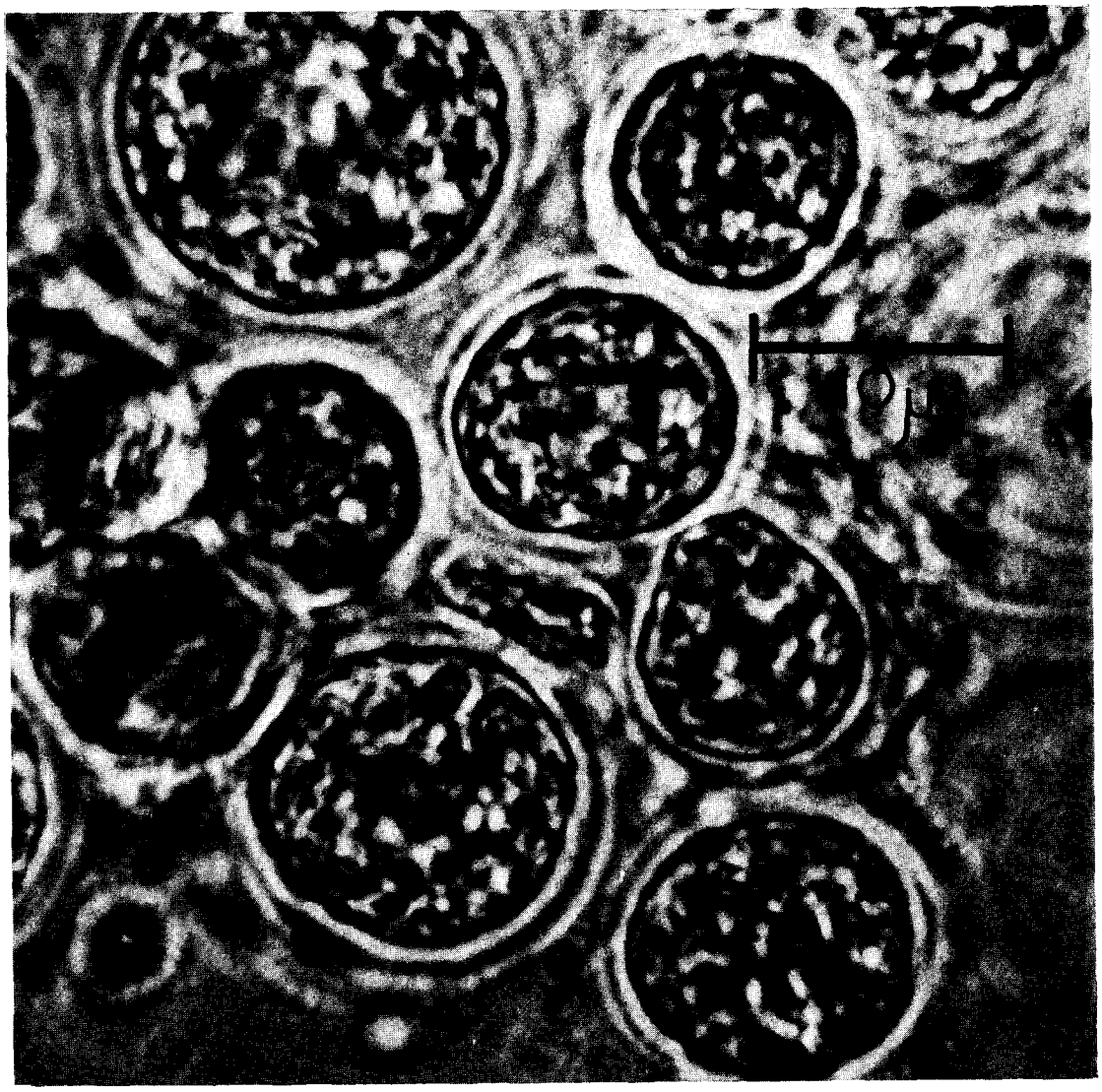

FIG. 2c. LIGHT MICROGRAPH OF "LARGE GREEN CELLS" DEVELOPED FROM RESTING STATE COLONIES OF *B. braunii.*

further work is needed in an attempt to assess fully the inter-relationship of these three states. The variation in relative percentage of hydrocarbons with age in the green state suggests that the biosynthesis and, especially interconversion of members of the A, B and C series, may be in an active state, dependent to some extent upon the culturing conditions. Whilst these compounds may have a common origin, the highly branched nature of botryococcene[2,3] suggests a different mode of biosynthesis for this hydrocarbon. It is hoped to obtain evidence for this in the future using the axenic culture.

EXPERIMENTAL

Extraction of Hydrocarbons and Subsequent Analysis

Colonies of the alga were dried in a rotary evaporator and extracted with Analar acetone at 4° for 3 days. After evaporation of the acetone, the residue was taken up in redistilled petroleum ether (b.p. 40–60°), chromatographed on neutral alumina and the hydrocarbon fraction was then dissolved in 1 ml of diethyl ether for GLC.

GLC. Was as described previously[6] using 3% OV-17 and 5% SE-30 stationary phases. Operating conditions were 244°, and carrier gas nitrogen flow rate of 40–50 ml/min. Retention times in min for hydrocarbons were $n C_{28}H_{58}$:11·4, $n C_{30}H_{62}$:19·5, $n C_{32}H_{66}$: 33·0 on both columns and $n C_{28}H_{58}$ was used as a routine standard.

GC-MS. An LKB 9000 combined gas chromatograph-mass spectrometer was used with a 10 ft OV-1 column, with operating conditions as previously described.[7]

Culture of Colonies

All experiments were carried out under 16 hr illumination per 24 hr using alternate day light and warm white fluorescent tubes giving a total energy of either $2{\cdot}271 \times 10^{-2}$ cal/cm²/min (High light) or $5{\cdot}68 \times 10^{-3}$ cal/cm²/min (Low light) on to cultures. All cultures were "stagnant" i.e. non aerated and shaken once daily. Culture medium—modified Chu 13 double strength: chelated with citric acid: $2C_{13}$ [Fe $M \times 10^{-5} \times 3{\cdot}5$] (Citrate).[4] KNO_3 (0·10 g), K_2HPO_4 (0·02 g), $MgSO_4.7H_2O$ (0·05 g), $CaCl_2.6H_2O$ (0·04 g), Fe citrate (0·01 g), and citric acid (0·10 g) made up to 1 l. with glass distilled water. pH adjusted to 7·5 before autoclaving at 15 lb/in² for 15 min.

Isolation from Wild

Plankton samples were taken by plankton tows at 1 m depth from Loch Lomond (Stirlingshire) and Oakmere (Cheshire). The material was spread on 1% oxoid agar plates and single colonies were removed using two Singer Micro Manipulators. These colonies were washed three times in sterile medium, checked microscopically for contaminants and, if clean, inoculated into 10 ml $2C_{13}$ [Fe $M \times 10^{-5} \times 3{\cdot}5$] (citrate) and grown up at 20° under low light for experimentation.

Green active state colonies used throughout this work were obtained from the Cambridge culture collection, Culture No. LB 807/1 Droop 1950 Maddingley Brick Pits, England.

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[6] D. S. INGRAM, B. A. KNIGHTS, I. J. MCEVOY and Miss P. MCKAY, *Phytochem.* 7, 1241 (1968).
[7] B. A. KNIGHTS, *J. Gas Chromatog.* 5, 273 (1967).