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HYDROCARBON PRODUCTION IN *ANACYSTIS MONTANA* AND *BOTRYOCOCCUS BRAUNII**

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Abstract—The production of labelled aliphatic hydrocarbons in *Anacystis montana* and *Botryococcus braunii* has been studied using Na_2CO_3 [^{14}C] as a carbon source. The major hydrocarbon produced by *A. montana* is pentadecane (*ca* 93%) accompanied by a pentadecene (*ca* 4%) and other hydrocarbons in the range C_{13}–C_{17}. Long chain (C_{21}–C_{33}) hydrocarbons could not be detected in this organism. The variety of unsaturated hydrocarbons (C_{25}–C_{31}) previously reported in *Botryococcus braunii* is confirmed and contrasts with the synthesis of unsaturated C_{17} hydrocarbons only, in axenic cultures prepared from single cell isolates of this colonial alga.

INTRODUCTION

Aliphatic hydrocarbon production in freshwater and marine algae has been studied extensively [1–6]. In general *Cyanophycophyta* appear to synthesise only *n*- and br-alkanes and *n*-alkenes in a restricted (C_{15}–C_{19}) range [2, 4] whereas some other types produce similar compounds with a wider homologue range [1]. Recent work in this laboratory has failed to demonstrate the presence of hydrocarbons greater than C_{21} in a number of axenically cultured algal species grown in the presence of Na_2CO_3 [^{14}C] and also in a mixed marine zooplankton culture from natural sources utilizing labelled *Phaeodactylum tricornutum* as a food supply [7].

In examinations of a number of microscopic algae, morphologically similar to those found in fossil form in geological sediments, [1, 8–10] *Anacystis montana* and *Botryococcus braunii* were shown to produce long chain aliphatic hydrocarbons (C_{25}–C_{33}). Unfortunately it is not completely clear whether the organisms used in the studies by Gelpi *et al.* [1, 8] were cultured in axenic form or could have been contaminated. This communication reports on hydrocarbon production in (a) axenic cultures of *A. montana*, (b) bacterial contaminated colonial cultures of *B. braunii* and (c) axenic cultures of single cells derived from the latter organism. The study has been facilitated by labelling the algae, using Na_2CO_3 [^{14}C] as the carbon source, thus allowing radioactivity rather than mass to be indicative of biosynthesis by the organism. This system has certain advantages for the detection and determination of micro amounts of hydrocarbon produced by biological material, particularly when large quantities of lipid solvents are used in the work up procedures [7].

RESULTS

The distribution of activity in various fractions from the axenic cultures of *A. montana* and *B. braunii* is shown in Table 1. The values given are averages from a number of growth runs, this being considered justifiable since the scatter associated with each figure is less than 20%. The bulk of the activity was present in the insoluble material that accumulated at the interface of the $CHCl_3$–(MeOH, H_2O) phases and on the silicic acid column after elution with MeOH. The difference in the ratio of activity in the $CHCl_3$ and MeOH, H_2O phases in the two organisms is striking and may reflect a difference in character of the non-lipid material produced by each alga. Typical mass/activity profiles for the GC–RC separations obtained from the hexane eluates of *A. montana* and *B. braunii* are shown in Fig. 1. All *Anacystis montana* samples, including those injected onto the GLC column at high activity loadings, failed to show the presence of activity at the position corresponding to nC_{23}–nC_{33} alkane standards. This finding is in marked contrast to the results obtained from this organism by others [1, 8].

The main peak of activity, ECL 15.0, (Fig. 1a) can be resolved into two components by isothermal elution at lower temperatures on polar and non-polar columns. Thus on Carbowax 20M the major activity peak (ECL 15.0; 93% of the total activity in the hexane fraction) was accompanied by a secondary peak (ECL 15.14; 4% activity). After hydrogenation, GC–RC failed to show the presence of this secondary peak on polar and non-polar columns but quantitation of the activity traces established that it had been incorporated in the main peak (ECL 15.0). This suggested that the major hydrocarbon present was *n*-pentadecane and that the minor peak was C_{15} monoene although co-injection of the sample with authentic 1-pentadecene demonstrated that the minor peak eluted slightly earlier than the standard on polar columns. GC–MS confirmed these identifications but the exact position of the double bond in the monoene could not be established. High level activity injections on polar and non-polar columns showed the presence of active peaks having ECLs of 13.0, 14.0, 16.0 and 17.0 containing 0.2, 1.1, 0.9 and 0.2% of the total activity respectively. It is concluded that these peaks

Table 1. Percentage of whole cell activity in fractions from algae

Organism	Chloroform*	Methanol Water*	Hexane†	Benzene†	Methanol†
A. montana	15.37	2.14	0.98	0.61	7.62
B. braunii	12.38	14.01	0.25	0.67	5.60

* Phases from Bligh and Dyer [19] extraction.
† Eluates from silicic acid column fractionation.

arise from C_{13}, C_{14}, C_{16} and C_{17} *n*-alkanes. Two additional components each containing *ca* 0.3% activity were also noted. From their GLC behaviour, before and after hydrogenation, on a variety of stationary phases they have been tentatively identified as C_{17} alkene and C_{16} alkane.

The colonial alga *B. braunii* as supplied produced unsaturated C_{25}–C_{31} hydrocarbons in addition to a relatively small amount of unsaturated C_{17} material (Fig. 1b). Detailed examination of the hexane eluates from this organism by GC–MS showed that it contained long chain material identical to that obtained from the green active state colonies of this organism [9, 10] with the exception of C_{25} monoene. Squalene [1] and botryococcene [9] reported to be present in *B. braunii* could not be detected by GLC or TLC in the hexane or C_6H_6 eluates. The unsaturated C_{17} material was resolved on polar columns into two components having identical RI to those possessed by the two main components of the single cell isolate cultures (see below) but insufficient quantities were available for GC–MS analysis. Hydrocarbons produced by axenic single cell isolates differ markedly from those synthesized by the alga when grown in bacterial contaminated colonial form. Long chain (C_{23}–C_{33}) material could not be detected in axenic culture even at high activity loadings. GC–RC profiles of the hexane eluates show a single region of activity only on non-polar phases (ECL 16.6) which is resolved on DEGS columns into three peaks (ECLs 17.15, 17.52 and 18.0) containing 19.2%, 77.7% and 3.1% of the total activity respectively. Hydrogenation led to the appearance of a single peak (ECL 17.0) on polar and non-polar phases suggesting that all three compounds are unsaturated C_{17} hydrocarbons. Confirmation of the MW of the two major components was obtained by using DEGS columns. The peak of ECL 17.15 gave a MS entirely consistent with a monoene with a M^+ at *m/e* 238 ($C_{17}H_{34}$), while the peak of ECL 17.52 displayed a M^+ at *m/e* 236 ($C_{17}H_{32}$) and had a fragmentation pattern consistent with either an alkadiene or alkyne. Further work is needed to unequivocally establish the structure of this compound. While the MS evidence clearly identifies these two peaks as unsaturated C_{17} hydrocarbons insufficient material was available for characterization of the minor peak (ECL 18.0). Coinjection with authentic 1-heptadecene indicated that the monoene (ECL 17.15) elutes slightly earlier than this standard from polar columns, behaviour which is comparable to that observed for the C_{15} monoene found in *A. montana*.

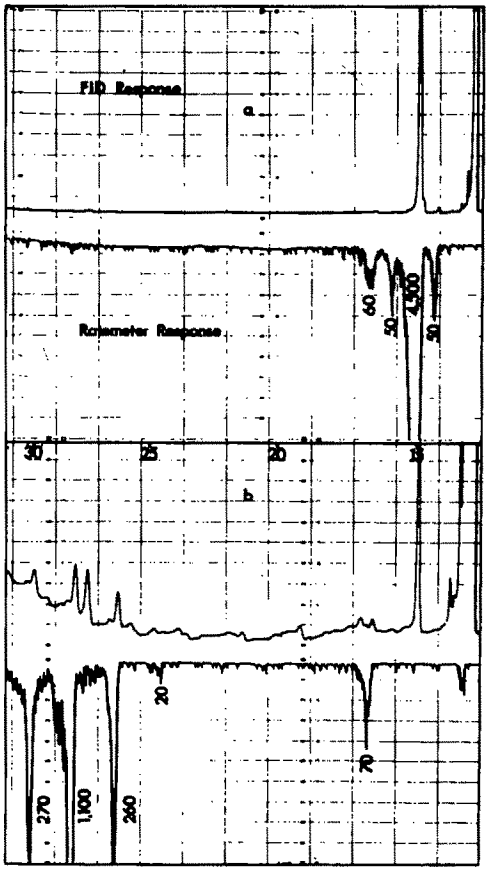

Fig. 1. GC–RC mass/activity profiles of the hexane fractions of (a) axenic cultures of *Anacystis montana* and (b) impure cultures as supplied of *Botryococcus braunii* on SP 2100 temperature programmed from 150–300° at 4°/min, carrier gas argon at 50 ml/min. The counts associated with each peak and the elution position of *n* alkane standards are displayed.

From the considerable number of cultures of *B. braunii* examined, both axenic and non-axenic, we have observed in only one case for each culture type the presence of radiolabelled hydrocarbons additional to those already noted. These hydrocarbons had ECLs of 19.24, 19.47 and 19.70 on DEGS columns and gave after hydrogenation a single active peak which on a variety of GLC phases corresponded with phytane (2,6,10,14-tetramethylhexadecane). It is of interest that these unsaturated hydrocarbons all display ECLs greater than their saturated counterpart phytane on non-polar (APL) as well as polar columns, an unusual feature which is similar to the behaviour of isoprenoid C_{19} di- and tri-olefins relative to pristane (2,6,10,14-tetramethylpentadecane) [11]. These unsaturated hydrocarbons are probably identical to some of the phytol derived phytadienes present in zooplankton [12].

DISCUSSION

The distribution of activity in the various fractions from the algae can be compared with previously published values obtained by gravimetric methods. Thus the activities in the $CHCl_3$ extractable lipid fall within the upper range of figures quoted for a variety of algal species [13]. Activity in the hexane fraction from the axenic single cell isolates of *B. braunii* indicates a hydrocarbon content (1%) which is very much less than that obtained (17%) from green active state colonies of this alga, but the organism has been shown to produce widely different amounts of hydrocarbon depending upon its physiological state [9]. We have previously noted [7] that hydrocarbon artefacts are readily introduced into similar analyses and this can lead to erroneously high values being obtained.

The aliphatic hydrocarbon pattern of *A. montana* noted by Gelpi *et al.* [1, 8] differs substantially from that displayed by other prokaryotic cyanophycean algae [1]. The pattern obtained in the present work, with no detectable long chain hydrocarbon, is similar to that obtained in a study of a large number of blue–green algae [4]. Although the culture in which long chain hydrocarbon was noted was stated to be bacteria free [8] a later communication from the same group [1], incorporating results from this organism, indicates that bacterial contamination was monitored by microscopic examination only. It is possible that this relatively insensitive technique failed to detect contaminating organisms; the point is of some importance since certain bacteria have also been shown to produce long chain hydrocarbons [14]. Other factors which might explain the different findings are related to the possibilities that either different strains of the organism or culturing in different media may produce different hydrocarbon patterns. The culture we obtained was composed mainly of single and diploid cells with little evidence of colony formation. Microscopic examination of axenic cultures showed the cells to be morphologically similar to those in the impure culture and we conclude that changes were not introduced as the result of the isolation process.

The non-axenic culture of *B. braunii* as supplied and after culturing was similar to that depicted by Brown *et al.* [9] and appeared to consist mainly of colonies of green cells growing in a 'mulberry' habit. Axenic single cell isolates from this culture when grown closely resembled in size, shape and colour the individual cells of the colony but the characteristic 'mulberry' growth habit had been lost and the cells were discrete. It has been shown that this alga exists in different physiological states [9] and we believe on the basis of the above evidence that our preparation is composed of the individual cells of the green active state colonies and is quite distinct from the large green cells [9] which develop from resting state colonies of this alga. The presence of identical unsaturated C_{17} hydrocarbons in both impure colonial and single cell axenic cultures lends support to the view outlined above and suggests that the isolation technique did not alter the metabolism of the isolated cell.

The markedly different hydrocarbon patterns obtained from the contaminated and axenic single cell cultures of *B. braunii* indicated that such differences might be due to the presence of contaminating organisms. Addition of bacteria, isolated on agar plates from the impure culture, to growing cultures of the axenic single cell isolates caused clumping of the alga within 24 hr; such clumps however did not show the characteristic 'mulberry' habit. Hydrocarbon profiles from these mixed cultures were identical to those obtained from the axenic isolates and suggest that the contaminating bacteria do not affect hydrocarbon production. A similar finding can be inferred from other results where essentially identical hydrocarbons were produced in cultures rendered axenic by antibiotic treatment [9] and in other cultures not specified as being so treated [10]. Differences in the hydrocarbon patterns obtained in this work and in that of others [1, 8–10] might be explained by postulating that certain parts of the colony matrix other than the green cell synthesise the long chain material found in this organism. Details of the unusual morphology of this alga [15] and of its hydrocarbon production when growing in its natural environment [16] have been published. It should be pointed out however that examination of the organism as supplied, by light and scanning electron microscopy, showed the presence of a variety of bacteria and at least two other organisms which have been tentatively identified as motile protozoa. These organisms which may survive antibiotic treatment could be responsible, at least in part, for the production of long chain hydrocarbon. We are unable to offer an explanation for the presence of what are considered to be phytadienes in one axenic and one impure culture and have been unable to reproduce this finding thus far in either culture grown under different conditions of temperature and light intensity. It is unlikely that the compounds are artefacts produced by catalytic dehydration and isomerization of phytol on the chromatographic adsorbent [11] since all such fractionations were performed on water deactivated silicic acid.

Previous work from this laboratory has shown [7] that pure algal cultures synthesize a limited number of aliphatic hydrocarbons in the C_{14}–C_{21} region. This is in contrast to the analyses of planktonic material from natural sources which show the presence of long chain (C_{25}–C_{33}) material [17]. From these results it was inferred that such long chain hydrocarbon was of exogeneous origin. The present results from the impure culture. *B. braunii* indicate that this view may have to be modified.

EXPERIMENTAL

Organisms and cultural conditions. Unialgal cultures of *A. montana* and *B. braunii* were obtained from the Cambridge Culture Collection, numbers 1404/1 and 807/1 respectively, both cultures as provided being heavily contaminated. Single cell, bacteria free, isolates were prepared from *A. montana* by micromanipulation techniques using a modification of the agar block method of ref. [18]. Difficulty was experienced in obtaining pure cultures of *B. braunii* but axenic single cell isolates were obtained by the above technique after the alga had been given a preliminary exposure for 10 min to 1% PhOH. This treatment presumably allows the release of single cells from the colony matrix. Isolates of both organisms were grown in light in 3 ml aliquots of the modified Chu No 10 medium [3] except that $NaHCO_3$ was omitted and the final pH brought to 7.9 before autoclaving. Algal cultures to be labelled, both axenic and contaminated by bacteria, were grown in the above medium in 150 batches within 1 l. flasks plugged with cotton wool. Cultures were exposed to a constant light intensity of *ca* 1000 lx at 25° and the initial inoculum allowed to multiply for 5 days before addition of Na_2CO_3 [^{14}C]. Usually 2 ml sterile soln containing 75 μCi (sp. act. 59.3 mCi/mmol) was added and the cells harvested by centrifugation after a further 7 days. Where appropriate, sterility tests to confirm the absence of bacteria

were carried out by inoculation on nutrient agar plates, microscopic examination being performed concurrently in order to check that morphology of the cells had remained constant during growth.

Extraction of hydrocarbons. Moist cells were extracted with hot $CHCl_3$–MeOH (2:1) using a modification of the technique in ref. [19]. The $CHCl_3$ layer was taken to dryness under red. pres. in a rotary evaporator and the residue dissolved in hexane. Fractionation of this lipid extract was carried out on deactivated (5% H_2O) silicic acid columns (50 × 12 mm) prewashed with hexane. Elution from the column with hexane (50 ml) gave the aliphatic hydrocarbon fraction, similar volumes of C_6H_6 and MeOH yielding the aromatic and fatty acid material respectively. Fractions were reduced to a small vol. by rotary evaporation and held under N_2 prior to chromatography.

Chromatography. GLC of the hexane eluates was performed on a GC–RC system in which 90% of the total flow passed to a furnace tube in which radioactive material was converted to CO_2 [^{14}C], the remainder being directed to the FID. Full details of this system appear elsewhere [7]. Columns used for separation of hydrocarbons (1.5 m × 4 mm) were packed with either APL, DEGS, Carbowax 20M, SP2100 or SP2330, Ar being used as carrier gas. The ECL of separated peaks was determined by the usual method and peak areas calculated by triangulation or by summing the counts associated with each peak. GC–MS was carried out on packed columns in a system coupled via a Biemann separator. TLC on Si gel plates was followed by scanning activity with a radiochromatogram scanning system.

Other details. Hydrogenation of material was performed in a micro scale apparatus using a Pd–C catalyst. Activity in soln was measured using a liquid scintillation spectrometer, whole cells being digested in 2 N NaOH before counting in NE 260 scintillant.

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