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EFFECT OF VARIOUS INHIBITORS ON BIOSYNTHESIS OF NON-ISOPRENOID HYDROCARBONS IN *BOTRYOCOCCUS BRAUNII**

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Key Word Index—*Botryococcus braunii*; Chlorophyceae; biosynthesis; non-isoprenoid very long chain hydrocarbons; TCA; molecular oxygen; coupling with photosynthesis; elongation–decarboxylation mechanism.

Abstract—The green unicellular alga *Botryococcus braunii* (A race) produces unusually large amounts of very long chain, non-isoprenoid, unsaturated hydrocarbons. A previous study suggested that an elongation–decarboxylation mechanism was probably implicated in the biosynthesis of such products. The present results, about the influence of trichloroacetic acid on radioactivity incorporation from acetate and oleic acid, confirmed that an elongation–decarboxylation mechanism, related to that operating in some higher plants, is involved in the formation of *B. braunii* hydrocarbons. In this alga, TCA block(s) would occur in the early step(s) of the elongation process. It was also observed that the very long chain unsaturated fatty acids isolated from *B. braunii* are not intermediates in hydrocarbon biosynthesis. The bulk of this fraction originates from an independent pathway and several distinct elongation systems are probably implicated in the formation of *B. braunii* very long chain lipids. The considerable inhibition of the labelling of hydrocarbons, under partial anaerobic conditions allowing for photosynthesis and respiration, is also consistent with an elongation–decarboxylation mechanism. This important role of molecular oxygen may correspond, as in higher plants, to an oxidative activation step, taking place before the decarboxylation of the very long chain precursors of *B. braunii* hydrocarbons. It was also observed that the formation of these hydrocarbons is not closely related to photosynthesis. So, even after 1 day of dark culture, a substantial level of production is still maintained.

INTRODUCTION

Very long chain, non-isoprenoid, unsaturated hydrocarbons are produced, in large amounts, by the green unicellular alga *Botryococcus braunii*‡ [1–4]. Its total hydrocarbon fraction is dominated by unbranched dienes with odd carbon numbers from C_{25} to C_{31} [1, 2, 6–8] and by a C_{29} triene (Fig. 1) the structure of which was recently determined [9]. A previous study [10] showed that oleic acid is the direct precursor of the above dienes, hence the *cis* stereochemistry and the 9,10 location, relative to CH_3, of their internal double bond. In addition, it appeared that an elongation–decarboxylation mechanism is probably implicated in the biosynthesis of *B. braunii* dienic hydrocarbons.

While very long chain ($\geq C_{20}$) non-isoprenoid hydrocarbons are widely distributed in living organisms [11, 12], relatively few studies have been performed on their mechanism of formation and only in a limited number of species [13–17]. This is probably related to the very low hydrocarbon levels occurring in most organisms. However, these products were shown to derive from fatty acids and two distinct types of mechanism were observed in their biosynthesis:

—a head-to-head condensation mechanism in the bacterium *Sarcina lutea* [15, 18–23]

—an elongation–decarboxylation mechanism in various higher plants [14, 24–55] and in some insects [13, 56–60].

The general pattern of the latter mechanism involved in the formation of cuticular hydrocarbons is now well established. The direct precursor, generally a C_{16} or C_{18} fatty acid, is first elongated by successive addition of C_2 units, some of the resulting very long chain products are then decarboxylated and the corresponding hydrocarbons are finally released from the elongation–decarboxylation complex. However, some of

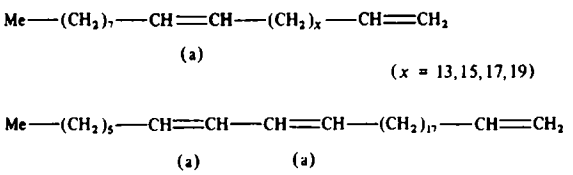

Figure 1. Structure of the major hydrocarbons of *B. braunii* A race (a: *cis* double bonds).

* Part 5 in the series "Hydrocarbon formation in the green alga *Botryococcus braunii*". For Part 4 see ref. [10].

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‡The occurrence of two races of *B. braunii*, with a similar morphology but sharply differing in the nature of their hydrocarbons, was recently demonstrated [5]. Here we will be concerned with the race (A) exclusively producing non-isoprenoid hydrocarbons. Accordingly, the term hydrocarbon will be now restricted, in this paper, to very long chain non-isoprenoid products.

the main steps of the pathway, especially the decarboxylation and the nature of the activation implicated in CO_2 elimination, are still not elucidated [13, 14].

The major aim of the present work was, firstly, to test the involvement of an elongation–decarboxylation mechanism in the biosynthesis of *B. braunii* hydrocarbons and, secondly, to determine the influence of some culture parameters on this pathway. To this end we examined the effects of increasing concentrations of trichloroacetic acid (TCA) on hydrocarbon and very long chain fatty acid formation, the influence of partial anaerobic conditions on the hydrocarbons and the relationship between photosynthesis and hydrocarbon production.

RESULTS AND DISCUSSION

Influence of TCA on the formation of B. braunii *hydrocarbons and very long chain fatty acids*

The influence of TCA has been previously examined [26, 35, 51, 54, 55], in the cuticular waxes of some higher plants, *Brassica oleraceae*, *Pisum sativum*, *Vicia faba* and *Hordeum vulgare*, where hydrocarbon biosynthesis occurs via an elongation–decarboxylation mechanism. The relationships between TCA and the effects of some mutations were also studied in *B. oleraceae* [61]. In this species, TCA concentrations ranging from 0.6 to 20 mM affected several classes of cuticular lipids, including hydrocarbons, as revealed by large decreases in the labelling of the latter from radioactive acetate or from C_{10}–C_{18} fatty acids. It was concluded that TCA inhibits the elongation process leading to hydrocarbons, while the decarboxylation step is not affected. Furthermore, TCA does not necessarily provide a single elongation block and several steps are sometimes affected; hence, hydrocarbon inhibition may increase with chain length. On the contrary, in the tested higher plants, TCA does not affect elongation in the *de novo* synthesis of palmitic acid, the latter occurring in chloroplasts whose membranes are impermeable to TCA.

The formation of very long chain fatty acids (VLCFA; $\geqslant C_{20}$) is also inhibited by TCA in some higher plant cuticular waxes [26, 55, 61]. However, it was noted in *B. oleraceae* that the TCA concentrations required to inhibit the formation of these acids are considerably higher than those for hydrocarbon formation. Inhibition by TCA was also shown, in *B. oleraceae* and *H. vulgare*, to increase with VLCFA chain length.

The above observations on TCA influence confirmed the involvement of an elongation–decarboxylation mechanism in the formation of the cuticular hydrocarbons of some higher plants. They also demonstrated the lack of a precursor–product relationship between total VLCFA and hydrocarbons*. The former, while not true intermediates in hydrocarbon biosynthesis, may however be released by the elongation–decarboxylation complex. VLCFA could also originate from an entirely independent elongation system, either biochemically distinct or with a different location in the cells. In fact, the occurrence of several elongation systems, implicated in very long chain product formation, was noted in *H. vulgare* [54, 55].

In order to obtain significant information, the level of TCA added in *B. braunii* cultures should be high enough to reveal a possible inhibition but low enough to avoid a detrimental effect on the primary metabolism of the alga. We therefore examined, at first, the effect of increasing TCA concentrations on the O_2 consumption (dark respiration) and the O_2 production under saturating light (net photosynthesis) of *B. braunii* (Table 1).

With a short exposure time, photosynthesis and respiration are stimulated by TCA up to *ca* 1 mM. However, within that concentration range, photosynthesis tends to increase with increasing TCA while the reverse is noted for respiration. After a 1 day exposure *B. braunii* respiration is affected, even with the lowest concentration tested; this inhibition increases regularly with TCA concentration. On the contrary, photosynthesis remains nearly unaffected up to 0.2 mM of TCA, but it undergoes, thereafter, a very sharp decrease. Taking into account the above observations we used in subsequent experiments three TCA concentrations (0.04, 0.4 and 0.8 mM).

The influence of the above TCA concentrations on hydrocarbon formation was determined using [2-^{14}C]acetate and [10-^{14}C]oleic acid. The latter allowed the determination of the influence of TCA on the production of *B. braunii* dienic hydrocarbons from their direct precursor. The results obtained from acetate feeding should reflect, in addition, a possible effect on oleic acid formation. It is noted (Table 2), firstly, that hydrocarbon labelling is affected whatever the precursor and, secondly, that inhibition substantially increases with TCA concentration. For a given concentration, a more pronounced influence is observed for [2-^{14}C]acetate. However, this difference between acetate and oleic acid tends to disappear at high TCA concentrations. It therefore appears that TCA affects both the transformation of oleic acid into hydrocarbons and the formation of this direct precursor (the influence of TCA on oleic acid is confirmed, see

Table 1. Influence of TCA on *B. braunii* dark respiration (DR) and net photosynthesis (NP) (% relative to control cultures without TCA added in the medium)

	Culture duration (hr)	TCA concentration (mM) 0.02	0.1	0.2	1	2
DR	1		125	108	100	92
	24	86	77	77	54	*
NP	1		114	143	157	64
	24	100	103	91	†	*

*A total loss of viability was observed in these cultures.
†O_2 absorption (photosynthesis weaker than respiration).

*This lack of relationship was also supported, in *B. oleraceae*, by the time course of radioactivity incorporation in VLCFA and hydrocarbons, and by the chemical form of VLCFA [26]. In the case of *Allium porrum* it was assumed, chiefly from time course experiments, that VLCFA actually operate as intermediates in hydrocarbon biosynthesis [43]. In addition it was demonstrated that VLCFA are mainly produced in the endoplasmic reticulum and hydrocarbons in the plasmalemma [47]. On these grounds the occurrence of two distinct systems (one for elongation and one for decarboxylation) was postulated in the formation of *A. porrum* hydrocarbons. However, the results of the above time course experiments may be also accounted for by the wax movements which were shown to occur between cuticle and epidermal cells [44].

Table 2. Influence of TCA on the labelling of *B. braunii* hydrocarbons*

		TCA concentration (mM)			
		Control	0.04	0.4	0.8
[2-^{14}C]Acetate	L†	9.5	8.1	6.4	6
	I‡		14.7	32.6	36.8
[10-^{14}C]Oleic acid	L†	7.4	7.1	6.1	4.8
	I‡		4.1	17.6	35.1

*Each culture was fed with 10 μCi of [2-^{14}C]acetate or 5 μCi of [10-^{14}C]oleic acid; the radioactivity incorporated in total hydrocarbons was determined after 1 day of incubation; average values from two experiments.

†Label incorporated into total hydrocarbons (as % of fed radioactivity).

‡Inhibition (%) calculated with respect to the corresponding control without TCA. Accordingly, the relative labelling efficiency observed for a given TCA concentration, from acetate and oleic acid, is independent of the difference in the absorption rate of the two exogeneous precursors.

below, by the examination of fatty acid labelling). However, the latter inhibition is relatively more important at low TCA concentrations while the former predominates at high TCA concentrations.

We also examined the influence of TCA on the radioactivity distribution in *B. braunii* major hydrocarbons (Table 3). Taking into account the fairly low accuracy in the measurement of the area of radioactivity peaks from GC-RC traces, it appears that distribution of label is not significantly changed with TCA, even at high concentration. GC-RC also allowed the measurement of the specific radioactivity of each compound (dpm/mg). This value depends on the amounts of cold hydrocarbon pre-existing in the algae when incubated. However, it was previously observed [4] that the relative abundance of *B. braunii* major hydrocarbons does not show important variations during batch cultures. Accordingly, significant specific radioactivities can be calculated here and it appears, for a given culture, that all the main hydrocarbons of the alga exhibit similar specific radioactivities. Addition of TCA to the culture results in large inhibition of hydrocarbon formation from exogeneous precursors; but label distribution and specific radioactivities show that *B. braunii* hydrocarbons are similarly affected whatever their chain length and structure.

The influence of TCA on fatty acids was also considered. The incorporation of [2-^{14}C]acetate in this fraction is not inhibited in the range of concentrations tested. On the contrary a fairly regular rise in the labelling of total fatty acids is noticed with increasing concentrations of TCA (Table 4). GC and GC/MS showed that the same acids occur in the control and in the cultures with TCA. Radioactivity location was determined from GC-RC

Table 3. Influence of TCA on label distribution in *B. braunii* hydrocarbons (A: feeding with [2-^{14}C]acetate; O: feeding with [10-^{14}C]oleic acid)*

		Radioactivity distribution (%)†				
		C_{25}	C_{27}	C_{29}	C_{29T}	C_{31}
	Control	2.4	10.5	37.4	28.3	21.4
A	0.04‡	1.9	10.9	37.1	30.2	19.9
	0.4‡	2.1	10	38.4	27.8	21.6
	0.8‡	2.7	11.2	38.2	25.8	22.1
	Control	3	12.7	38.1	25.3	20.9
O	0.04‡	2.7	10.7	40.9	23.7	22
	0.4‡	2	12.5	40	25	20.5
	0.8‡	3.4	12.5	40.1	24.3	19.7

*See Table 2, note *, for feeding conditions. Average values from five measurements.

†C_{29T}:C_{29} trienic hydrocarbon (Fig. 1); general formula of the C_{25}–C_{31} dienic hydrocarbons see also Fig. 1. Taken together these hydrocarbons account for *ca* 95% of the mass and radioactivity peaks of the fraction.

‡TCA concentration (mM).

Table 4. Influence of TCA on the radioactivity of *B. braunii* fatty acids after feeding with [2-^{14}C]acetate*

	Total label†	Radioactivity distribution (%)‡									
		C_{16}	$C_{18:1}$	$C_{20:1}$	$C_{22:1}$	$C_{24:1}$	$C_{26:1}$	$C_{28:2}$	$C_{28:1}$	$C_{30:2}$	$C_{30:1}$
Control	23	5	76.4	1.6	0.6	0.8	1.2	2.4	7.1	3.7	1.1
0.04§	27	6.2	71.4	1.8	1.1	1.5	2	3.2	8.2	3	1.5
0.4§	28	8.1	66	4.1	0.6	0.8	1.9	3.2	9.2	3.7	2.3
0.8§	34	8.6	68.2	2.5	1	1.1	2.3	3.5	7.2	3.8	1.8

*See Table 2, note *.

†Label incorporated in total fatty acids (as % of fed radioactivity).

‡The radioactivity distribution reported here is calculated on the basis of the identified acids.

§TCA concentration (mM).

traces. (No radioactivity peaks were clearly associated with the mass peaks of the minor C_{18}–C_{26}, saturated even fatty acids. On the contrary, several small radioactivity peaks were not definitely identified; some were shown to correspond to degradation products of oleic acid generated during the isolation procedure of the total fatty acid fraction). The incorporated label is mainly located in even carbon numbered monounsaturated acids, including very long chain products up to C_{30}; a major part of the radioactivity is always located in oleic acid. However, TCA tends to decrease the relative labelling of oleic acid while a reverse change is noticed with palmitic acid. The radioactivity of the latter increases both in absolute and in relative value (as % of total fatty acid labelling). It therefore appears that no inhibition of the *de novo* synthesis of palmitic acid takes place, in *B. braunii*, with the amounts of TCA tested.

The radioactivity of total VLCFA increases regularly, in absolute value, with TCA concentration (from *ca* 5 $\times 10^5$ dpm in the control to *ca* 7.6×10^5 dpm in the culture with 0.8 mM of TCA). However, this stimulation of VLCFA labelling is similar to that achieved for total fatty acids and the former account, both in the control and in the cultures with TCA, for *ca* 15% of the radioactivity of the fatty acid fraction. Regarding radioactivity distribution in VLCFA, no pronounced variations are observed in the presence of TCA and the labelling of the C_{28} monoenic acid is always predominant. Moreover, for a given culture, the identified fatty acids exhibit similar mass and radioactivity distribution on GC-RC and thus fairly close specific radioactivities. These observations are consistent with the lack of a relationship between TCA and VLCFA labelling and chain length.

When the cultures are fed with oleic acid, most of the radioactivity of the total fatty acid fraction corresponds to unmetabolized oleic acid (including the molecules adsorbed on the surface of the cells). In this case, the labelling of total fatty acids, and the derived figures, are not significant. Accordingly we exclusively considered the relative radioactivity of the major VLCFA (Table 5). As observed above, from acetate, the radioactivity is only located in even carbon numbered unsaturated VLCFA and the labelling of the C_{28} monoene is predominant (the very low radioactivity associated with the C_{22} and C_{24} monoenic VLCFA did not allow measurement of their relative labelling; the measurement of radioactivity in the $C_{20:1}$ VLCFA is made difficult by the presence of degradation products from oleic acid). Here also the total radioactivity of VLCFA tends to increase with TCA concentration; no clear influence of TCA on label distribution was noted and, for a given culture, the different VLCFA show fairly close specific activities.

Several common features appear when the present observations are compared with previous reports on higher plant cuticular hydrocarbons and fatty acids. Fairly low TCA levels substantially decrease hydrocarbon production from the direct precursor. In sharp contrast, the *de novo* synthesis of palmitic acid and the formation of VLCFA are not inhibited by these TCA concentrations*. Nevertheless, differences are noticed regarding TCA effect on the direct precursor. In higher plants, like *B. oleraceae*, the synthesis of this precursor (palmitic acid) is not lowered while, in *B. braunii*, TCA inhibits both the transformation into hydrocarbon of the direct precursor (oleic acid) and its production from acetate. However, the relative importance of the latter factor is strongly reduced when TCA concentration increases. It was previously noted, in higher plants, that the location of TCA elongation blocks vary according to species. Thus, several late elongation steps are affected in *V. faba* and, accordingly, inhibition increases with hydrocarbon chain length [51]. In contrast in *B. oleraceae*, some TCA inhibition takes place in the first step of the elongation [26], or in the C_{28}–C_{30} step [61]. In the case of *B. braunii*, within the range of concentrations which can be used without detrimental effects on primary metabolism, all the major hydrocarbons are similarly affected as shown by label distribution and specific radioactivities. TCA block(s) would therefore occur, in this alga, in the early step(s) of the elongation process.

Taken together, the above observations are consistent with the occurrence of an elongation–decarboxylation mechanism in the biosynthesis of *B. braunii* hydrocarbons. Also it appears that the unsaturated VLCFA isolated from the alga, and unaffected by TCA, are not true intermediates in hydrocarbon formation. This was confirmed by examination of fatty acids in different races of *B. braunii*. Similar structures and distributions of VLCFA were thus obtained from strains producing completely different types of hydrocarbons. The elongation–decarboxylation complex implicated in the transformation of oleic acid into hydrocarbons could release, as in insects and higher plants, low amounts of VLCFA [26, 56]. However, such products are, at most, minor constituents of the total VLCFA fraction in *B. braunii*. The bulk of this fraction originates from an independent elongation pathway. This pathway is less sensitive to TCA, as was also observed in *B. oleraceae* [26], than the elongation–decarboxylation system leading to hydrocarbons. As previously noted with *H. vulgare* epicuticular waxes, several distinct systems are therefore probably involved in the formation of very long chain lipids in *B. braunii*.

Table 5. Influence of TCA on radioactivity distribution in *B. braunii* major long chain fatty acids after feeding with [10-^{14}C]oleic acid*

	Radioactivity distribution (%) in VLCFA				
	$C_{26:1}$	$C_{28:1}$	$C_{28:2}$	$C_{30.1}$	$C_{30:2}$
Control	15.9	43.5	14.5	8.4	17.7
0.04†	15.9	51.2	9.2	11	12.7
0.4†	21.2	40	13.6	11.1	14.1
0.8†	17	34.9	19.2	12.9	15.9

*See Table 2, note *.
†TCA concentration (mM).

*A weak stimulation of palmitic acid and VLCFA labelling was even noted with *B. braunii*. Inhibition of VLCFA formation was previously observed in some higher plants but the corresponding TCA concentrations were considerably higher than those required for entirely inhibiting hydrocarbon formation. Here no significant observations can be carried out on *B. braunii* VLCFA, using such high TCA levels, since the primary metabolism of the alga is then strongly affected.

Influence of molecular oxygen on the formation of B. braunii *hydrocarbons*

The influence of partial anaerobic conditions, allowing for both photosynthesis and respiration, was previously examined in *B. oleraceae* [28]. Low oxygen levels of *ca* 3% were thus shown to inhibit very strongly the production of cuticular hydrocarbons while fatty acid elongation did not seem to be affected; in fact, a high O_2 concentration (20%) was required to achieve a normal hydrocarbon production. From these observations it was assumed that an oxidation step of the very long chain precursors may precede their decarboxylation into hydrocarbons.

Continuously illuminated *B. braunii* cultures were carried out under a (99:1) N_2–CO_2 flow. Bubbling rate, CO_2 level and light flux density were identical to those in normally aerated control cultures. Photosynthesis and respiration then occurred and the algae were submitted to partial anaerobic conditions. The resulting effects on hydrocarbon production were determined with [10-^{14}C]oleic acid (Table 6). Oxygen was eliminated from the cultures prior to radioactive precursor addition, by vigorous bubbling for 2 hr with the gas mixture. A very large inhibition in hydrocarbon labelling was observed both from short and prolonged feedings. (Due to this inhibition accurate GC-RC analyses could not be carried out. The total hydrocarbon fractions obtained from CC were therefore further purified by TLC on $AgNO_3$–silica gel, so as to remove possible labelled contaminants from *B. braunii* hydrocarbons. Radioactivity incorporation was measured on these purified products, which always accounted for *ca* 90% of the radioactivity of the total hydrocarbon fraction.)

The high sensitivity of *B. braunii* and *B. oleraceae* hydrocarbons to partial anaerobic conditions confirms the involvement in the former of an elongation–decarboxylation mechanism. Molecular oxygen seems to play a specific and important role in this type of biosynthetic pathway and the occurrence of a high concentration of oxygen in the cell environment appears to be essential. This may reflect the involvement of molecular oxygen in the activation step taking place before decarboxylation of the very long chain precursors of non-isoprenoid hydrocarbons [28, 38, 62].

Relationship of hydrocarbon production to photosynthesis in B. braunii

It was previously shown that the formation of cuticular hydrocarbons, via an elongation–decarboxylation mechanism, is not related to photosynthesis in *B. oleraceae*, *N. tabacum* and *A. porrum* [26, 28, 39, 40, 53]. Thus, during short feeding experiments of a few hr, the labelling of these hydrocarbons is not substantially affected in the dark*. However, some variations were sometimes noticed in the relative radioactivity of the different classes of cuticular lipids [63, 64]. On the contrary a close relationship was observed between photosynthetic reactions, like photophosphorylation and photosynthetic production of NADPH and the *de novo* synthesis of fatty acids.

Table 6. Effect of partial anaerobic conditions on *B. braunii* hydrocarbons*

	C(1)	A(1)	C(2)	A(2)
Hydrocarbon labelling†	1.46	0.11	2.38	0.22
Inhibition‡		92.5		90.8

*C: control cultures normally aerated with (99:1) air–CO_2; A: cultures under partial anaerobic conditions. 5 μCi of [10-^{14}C]oleic acid were added in each culture and the incubation was carried out for 3 hr (1) or for one day (2).

†Radioactivity of *B. braunii* purified hydrocarbons, as % of fed radioactivity.

‡as % with respect to the normally aerated control.

The radioactivity incorporated into *B. braunii* hydrocarbons from [10-^{14}C]oleic acid was determined, for cultures carried out in the dark and for normally illuminated cultures, after various times of incubation (Table 7). On the shortest incubation time hydrocarbon labelling is not significantly affected in the dark. However, some inhibition is observed after 3 hr and it becomes important for 1 day cultures. This latter inhibition of hydrocarbon formation probably reflects a general decrease in the metabolic activity of *B. braunii*, caused by the interruption of photosynthetic reactions and the resulting progressive depletion of storage products, since the alga is grown in a medium containing no organic carbon source. The possible influence of dark on radioactivity distribution was also considered for 1-day-old cultures (Table 8). No significant variations in distribution of label

Table 7. Influence of dark on *B. braunii* hydrocarbon production*

		Duration of incubation 1	2	3
I	†	0.4	1.7	13.2
D	†	0.4	1.2	4.8
	‡	0	29.4	63.6

*I: illuminated cultures; D: dark cultures. 5 μCi of [10-^{14}C]oleic acid were incorporated in each culture and incubated for 30 min (1), 3 hr (2) or 1 day (3).

†Radioactivity incorporated (as % of fed label) in hydrocarbons.

‡Inhibition (%) with respect to normally illuminated cultures.

Table 8. Influence of dark on radioactivity distribution in *B. braunii* major hydrocarbons*

	Label distribution (%) C_{25}	C_{27}	C_{29}	C_{29T}	C_{31}
Control	1.6	10.6	30.6	32.2	25
Dark culture	2.3	10.4	30	32.9	24.3

*See Table 3, note †, for hydrocarbon formula.

*In sharp contrast the production of internal hydrocarbons in the parenchyma cells of *A. porrum* leaves is strongly and rapidly affected [39, 40]. The occurrence of two sites of hydrocarbon biosynthesis was thus demonstrated since cuticular hydrocarbons are produced in the epidermal layer of cells and are therefore not dependent on light.

were noted with respect to the control and, for a given culture, all the hydrocarbons showed fairly close specific radioactivities. Consequently, the biosynthesis of all the major hydrocarbons of *B. braunii* is similarly affected by prolonged dark culture. As previously observed in higher plants, the present results indicate that hydrocarbon formation is not related to photosynthesis in *B. braunii*. Even after 1 day of dark culture a substantial level of hydrocarbon production is still maintained in this alga.

Taken together the above observations confirm that an elongation–decarboxylation mechanism is implicated in the biosynthesis of *B. braunii* hydrocarbons. Furthermore, they provide some information on the conditions required for large scale cultures of *B. braunii*. Thus, an efficient aeration of such cultures appears essential to avoid the appearance of partial anaerobic conditions and the resulting considerable decrease in hydrocarbon production. On the contrary, fairly long dark periods, as under natural illumination, should not adversely affect this production.

EXPERIMENTAL

An axenic strain of *B. braunii*, obtained from the Austin Culture Collection (807/1 DROOP, 1950, Maddingley Bricks Pits, U.K.) was used throughout this work.

Preparation of inocula and batch cultures under air lift conditions were carried out as already described [4]. In each expt the inhibited cultures and the corresponding controls were initiated from the same inoculum to avoid variations due to differences in the physiological state of the inoculated cells. Inocula corresponding to cells in the early exponential stage were always used. Cultures with TCA were always grown for 5 hr, with the inhibitor, before addition of the labelled precursor in the medium.

Measurements of net photosynthesis (under light and CO_2 satn) and dark respiration using a Clark-type electrode; feeding with [2-^{14}C]acetate (48 mCi/mM) or [10-^{14}C]oleic acid (45–55 mCi/mM); extn of external hydrocarbons and purification on CC and prep. TLC on $AgNO_3$–silica gel; extn of fatty acids under conditions allowing for the recovery of very long chain products; identification of hydrocarbons and fatty acids as Me esters by GC and GC/MS; radioactivity determination by scintillation counting and GC–RC; were carried out as previously described [2, 10, 65].

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