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MECHANISM OF NON-ISOPRENOID HYDROCARBON BIOSYNTHESIS IN *BOTRYOCOCCUS BRAUNII**

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Key Word Index—*Botryococcus braunii*; Chlorophyceae; biosynthesis; hydrocarbon-rich alga; non-isoprenoid very long chain hydrocarbons; hydrocarbon direct precursor.

Abstract—The green unicellular alga *Botryococcus braunii* shows unusually high concentrations of non-isoprenoid very long chain hydrocarbons. The structure of such hydrocarbons, the relative efficiency of various long chain fatty acids as precursors, the relationship between fatty acid and hydrocarbon concentrations (over the different physiological stages of the alga achieved during batch cultures) and the preferential localization of fatty acids lead to the conclusion that all the major non-isoprenoid hydrocarbons of *B. braunii* derive from the same direct precursor, oleic acid. Feeding experiments, using doubly labelled oleic acid, show that the whole carbon chain of the latter is incorporated into final hydrocarbons; accordingly such compounds do not originate from a head-to-head condensation mechanism with oleic acid acting as donor. Various features (regarding chiefly the systematic occurrence of a terminal double bond in *B. braunii* hydrocarbon, their close specific activities after feeding and the large inhibition in their production achieved using dithioerythritol) show that the biosynthesis of *B. braunii* hydrocarbons probably takes place via an elongation-decarboxylation mechanism related to that operating in some higher plants.

INTRODUCTION

Non-isoprenoid hydrocarbons are widely distributed in living organisms [1, 2]. These compounds are assumed to be derived from fatty acids, yet relatively few studies have been carried out on their formation and they concern only a limited number of species [3–7]. One of the reasons for this situation is probably to be found in the very low amounts of such hydrocarbons occurring in most organisms. Indeed, just one major exception seems to be known at the present time: the green unicellular alga *Botryococcus braunii*. The latter species exhibits, in nature as well as in laboratory cultures, unusually high concentrations of non-isoprenoid hydrocarbons ranging from 15% to 40% of dry wt [8–11]‡ (instead of *ca* 0.1% in most algae [12, 13]). The major hydrocarbons of *B. braunii* show an odd carbon number and the general formula CH_3–$(CH_2)_7$–CH=CH–$(CH_2)_x$–CH=CH_2 (x = 13, 15, 17 and 19) [8, 9, 14–16]; a trienic C_{29} derivative is also generally detected. Taken together these compounds account for more than 95% of the total hydrocarbon fraction of the alga grown in laboratory cultures under various conditions [9, 10]. *B. braunii* appears, accordingly, as a suitable species to investigate the biosynthesis of non-isoprenoid hydrocarbons.

While relatively few studies on hydrocarbon§ biosynthesis have been performed, as mentioned above, two distinct mechanisms starting from fatty acids have been shown to occur: (a) the elongation-decarboxylation mechanism, observed in various higher plants [3, 21–52] but also in some insects [3–5] (hydrocarbons produced by the epidermal layer of cells are sometimes abundant in the surface waxes which build up the cuticle of higher plants and also in the cuticular lipids of insects) [1, 2]. In this mechanism the direct precursor, generally a C_{16} or C_{18} fatty acid derivative, is first elongated by successive addition of C_2 units derived from malonyl CoA, until the appropriate chain lengths are obtained. The very long chain derivatives produced are then decarboxylated to yield the corresponding hydrocarbons which are finally released from the elongation-decarboxylation complex [3]¶. The general pattern of the above mechanism is now well established. Thus, the direct conversion into hydrocarbons of exogenous very long chain fatty acids has been observed from various plant tissues and derived cell free

*Part 4 in the series "Hydrocarbon Formation in the Green Alga *Botryococcus braunii*". For part 3 see Berkaloff, C., Casadevall, E., Largeau, C., Metzger, P., Peracca, S. and Virlet, J. (1983) *Phytochemistry* **22**, 389.

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‡Still higher amounts of hydrocarbons (up to 75% dry wt) were reported in some *B. braunii* samples from nature; but, in this case the hydrocarbon fraction consists of highly unsaturated and branched derivatives, termed botryococcenes, which are probably of terpenic origin [8, 17–19]. Such hydrocarbons would be typical of the resting state, while the non-isoprenoid ones would be typical of active cells [8]. However, as far as we are aware, no substantial botryococcene formation was so far achieved from laboratory cultures [11, 20] and we will be concerned, in this paper, only with the non-isoprenoid hydrocarbons of the alga.

§Unless otherwise stated the term hydrocarbon will be now restricted, in this paper, to non-isoprenoid very long chain ($\geqslant C_{20}$) compounds.

¶The hydrocarbons thus formed are often further converted, in part, into secondary alcohols and/or ketones [22, 24, 26, 27, 33, 34, 47].

preparations [31, 35, 41, 48] and also from insects [53]. Nevertheless the biosynthetic pathway leading to hydrocarbons is not entirely elucidated and, especially, the nature of the intermediate(s) implicated in the decarboxylation process is not yet known [3–5]*. (b) The head-to-head condensation mechanism, first proposed in pioneering studies [54, 55], has been shown to occur, in a modified form, in the bacterium *Sarcina lutea* [56–61]. This mechanism involves the head-to-head condensation of two dissimilar fatty acid derivatives. One of the above derivatives (donor) is specifically decarboxylated following the condensation step, while the total carbon chain of the other (acceptor) is incorporated into the final hydrocarbons.

Various results suggest that, in the case of *S. lutea*, the acceptor moiety would be a vinylic ether. As a result a monoenic hydrocarbon would be obtained, after decarboxylation of the condensation product. Such a condensation is therefore entirely different from the one involved in the formation of corynomycolic acid by *Corynebacterium diphtericae* [62]. (In the latter two fatty acyl derivatives are implicated and, following condensation, a β-ketoacid is produced.) Almost all our present knowledge regarding the head-to-head condensation mechanism is restricted to the case of *S. lutea*. Moreover, the nature of the condensing moieties and of the primary condensation product are yet to be elucidated in this bacterium [3–5].

Studies regarding the biosynthesis of algal hydrocarbons are very limited. Furthermore they were chiefly related to blue–green species and mostly directed towards the study of the monomethyl alkanes which are typical of some of these algae [6, 63–65]. In *Nostoc muscorum* the formation of long chain hydrocarbons (n-C_{15} and n-C_{17}) is generally assumed to originate from direct decarboxylation of the corresponding acids [6]. However no information is available on the intermediate(s) involved in such a process and no definite evidences about its occurrence were provided [3–5].

In the present work, we examined the nature of the fatty acid(s) operating as direct precursor(s),† and also the type of mechanism implicated in the biosynthesis of the major hydrocarbons of *B. braunii*.

RESULTS AND DISCUSSION

Nature of the direct precursor

Due to their non-isoprenoid structure *B. braunii* hydrocarbons should derive from fatty acids. Preliminary experiments allowed us to test the above assumption [66]. Indeed, when *B. braunii* cultures are fed with [9,10-^{3}H]palmitic acid a part of the label is recovered in the hydrocarbons. However, such a result alone does not show that palmitic acid is actually the direct precursor of *B. braunii* hydrocarbons. The acid fed could be incorporated into the latter through elongation and/or desaturation, or through degradation into shorter derivatives. Accordingly a comparative study was carried out in order to determine the relative efficiency of hydrocarbon labelling after parallel feedings with various fatty acids.

In most cases where an elongation-decarboxylation pathway has been shown to occur in higher plants, palmitic or stearic acid derivatives are involved as the direct substrates of the elongation complex [22, 23, 25, 26, 29, 30, 40, 41, 44, 51]. Regarding head-to-head condensations it seems that palmitic acid derivatives are implicated, as direct precursors, both in corynomycolic acid production by *Corynebacterium diphtericae* [62] and in monobranched alkane formation by *Sarcina lutea* [60, 61]. Accordingly the comparative study, on fatty acid efficiency as precursors of *B. braunii* hydrocarbons, was at first restricted to C_{16} and C_{18} acids. The ^{14}C label of these acids was located at such a position (terminal methyl or middle of the carbon chain) that it would be retained, whatever the mechanism operating during conversion into hydrocarbons. On the other hand, it appears that catabolic breakdown of the acids fed was small and that the degradation products of these acids do not play a significant role in hydrocarbon labelling. Therefore the amounts of radioactivity incorporated into hydrocarbons actually reflect the relative efficiency of the considered acids. It appears (Table 1) that the typical behaviour previously reported in the case of feedings using only palmitic acid [66] (labelling into the two hydrocarbon pools of the alga, but radioactivity levels conspicuously higher in the external pool) is again observed. The efficiency of incorporation into external hydrocarbons is increased, first as a result of chain lengthening (× 2 from palmitic to stearic acid) and, secondly, owing to chain desaturation (× 4.7 from stearic to oleic acid). This latter change gives way also to a substantial radioactivity increase into internal hydrocarbons.

With regard to the higher efficiency, as hydrocarbon precursor, of stearic acid compared with palmitic acid, it was also previously observed in several higher plants [22, 23, 45]. Moreover the reported increases in labelling are close to those achieved here‡. Nevertheless, concerning the relative efficiency of stearic and oleic acid, entirely different behaviours are obtained in *B. braunii* and in higher plants. With the latter, oleic acid, although it is actually taken up by the cells, appears as a very poor precursor (hydrocarbon labelling decreasing by a factor of *ca* 100 with respect to stearic acid) [22]. In sharp contrast, oleic acid is a better precursor in the case of *B. braunii*. However, it is well documented that the epidermis of the higher plants considered above produces mainly alkanes (starting from palmitic or stearic acid as direct precursor) while *B. braunii* hydrocarbons are unsaturated. Furthermore, if one considers the structure of the major hydrocarbon of the alga it is to be noticed that they all exhibit a 9,10 double bond and previous IR examinations suggested that this bond has a *cis* configuration [16]. NMR spectroscopy allowed us to ascertain the above assumption: a coupling constant ^3J H_9–H_{10} of 10 Hz is observed on ^{1}H NMR and allylic carbons 8 and 11 show a ^{13}C chemical shift of δ27.22; such figures are typical of a *cis*

*However some results obtained, using a cell free preparation from *Pisum sativum* epidermis, suggest that α-hydroxyl very long chain fatty acids could be involved as intermediates in the decarboxylation [35].

†The direct precursor is either the fatty acid which serves as direct substrate for the elongation system (elongation-decarboxylation mechanism) or one of the two condensing acids (head-to-head condensation mechanism).

‡It is known that fatty acid uptake decreases as their chain length increases [22]. Such a feature could lead to an underestimation of the efficiency of stearic relative to palmitic acid. Consequently the differences in efficiency of these acids could be still higher than the one derived from feeding.

Table 1. Relative efficiency of label incorporation into *B. braunii* hydrocarbons from various fatty acids

	Radioactivity (dpm × 10^{-3})†		Labelling of total hydrocarbons (as % of the radioactivity fed)§
	External hydrocarbons	Internal hydrocarbons	
[16-^{14}C] Palmitic acid*	11.4‡	1.57‡	0.06‡
[18-^{14}C] Stearic acid*	22.6	1.60	0.11
[10-^{14}C] Oleic acid*	107.1	3.86	0.5

*Incubation for 5 days with 10 μCi of ^{14}C acid.

†Hydrocarbons obtained after purification using column chromatography. When these samples are further fractionated (prep. TLC on $AgNO_3$-impregnated silica gel) *ca* 95% of the radioactivity is retained in the unsaturated hydrocarbons typical of the alga. Analysis by radio GC showed that the label is located in major hydrocarbons which exhibit close specific activities.

‡The incorporation of palmitic acid into hydrocarbons obtained here is substantially lower, whatever the pool, than the one previously reported [66]. Such a variation would arise chiefly from differences in the physiological state of cultures used as inocula in feeding experiments.

§The acids fed show similar specific activities (see Experimental). On the other hand, hydrocarbon specific activity (dpm/mg) increases in the following way: 1230 from palmitic acid, 2410 from stearic acid and 11450 from oleic acid (hydrocarbon concentrations were determined by GC with *n*-triacontane as int. reference).

double bond. Consequently it appears that *B. braunii* hydrocarbons possess an unsaturation exhibiting both the same localization and the same stereochemistry as that of the double bond of oleic acid*. These features, added to the results on labeling efficiency, suggest that oleic acid acts probably as the direct precursor of *B. braunii* major hydrocarbons. Under such conditions palmitic acid and stearic acid incorporation into hydrocarbons would require, at first, their conversion, via elongation and/or desaturation, into oleic acid (various algal species are known to perform direct desaturation of usual fatty acids [71–74]). This conversion would account, as a result of dilution into endogenous pools, for a lower efficiency of palmitic and stearic acids and, especially, of the former.

In order to test the supposed role of oleic acid as direct precursor, we then examined if a precursor–product relationship was actually occurring between this acid and the hydrocarbons of the alga. During a previous study [11], we investigated the effect of the physiological state of *B. braunii* on hydrocarbon production. To this end batch cultures were carried out and their growth curves were obtained. We observed first (Fig. 1) an exponential growth phase A followed, due to the progressive decrease in nutrient levels, by a linear phase B, a deceleration phase C and, finally, a stationary phase D where growth stops. During these closed cultures all the intermediate physiological states, ranging from active growth to resting state, are successively obtained. The examination of

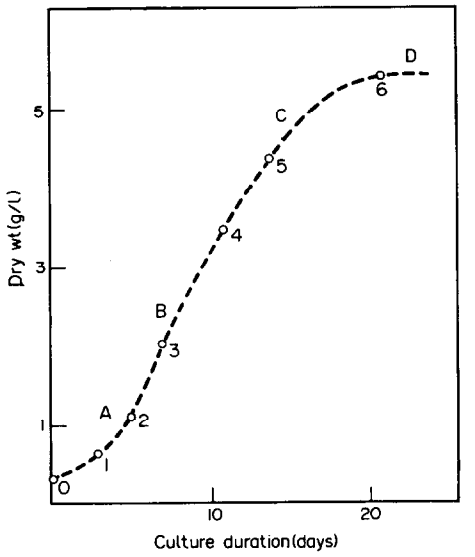

Fig. 1. Growth curve of *B. braunii* in batch cultures. Nature of the various phases and location of samples 0–6.

samples 0–6 (Fig. 1) indicates that, in this case, a constant relation is not held, along the whole growth curve, between the production of total biomass and hydrocarbon production. Indeed, the latter is particularly efficient in the exponential and early linear stages (hydrocarbon production is then higher than the one of the other constituents of the biomass) and a reverse situation takes place in the following stages. The same samples as those used for studying hydrocarbons were analysed, in the present work, so as to examine their fatty acids. It is noted (Table 2) that oleic acid is present within all the samples.

*Such relationships regarding hydrocarbon and fatty acid unsaturations have been previously observed with some other organisms [25, 67–70] in the case of polyunsaturated derivatives. This observation led Knights *et al.* [16] to assume, from the location of the double bond and from its supposed stereochemistry based on IR spectrum, that oleic acid could play some role in *B. braunii* hydrocarbon formation.

Table 2. Variations in amounts of oleic acid in relation to physiological state during batch cultures of *B. braunii*

Sample	Duration of cultures (days)	Total biomass (g/l)	Total fatty acid content†	Oleic acid*		
				Total amount†	As % of total biomass	As % of total fatty acids
0‡		0.27	15	trace	<0.05	<1
1§	3	0.58	18	"	"	"
2‖	5	1.13	28.5	"	"	"
3¶	7	2.17	34.5	1.1	0.05	3.2
4**	11	3.04	134	5.4	0.18	4
5††	14	4.49	140	6.2	0.14	4.4
6‡‡	21	5.45	146	39.5	0.72	27.1

*Oleic acid was identified, like other fatty acids, using GC and GC/MS of Me esters.
†mg/l of culture.
‡Inoculum (cells in exponential stage).
§Middle of the exponential phase.
‖Transition from exponential to linear phase.
¶Middle of the linear phase.
**Late linear phase.
††Early deceleration phase.
‡‡Transition from deceleration to stationary phase.

However, its concentration changes markedly in relation to the physiological state of the alga. Thus, during the exponential and early linear phases oleic acid concentrations are at very low values. Afterwards they increase, at first fairly slowly and, finally, very sharply during the late deceleration phase. Furthermore, such increases are observed not only if one considers the total amount of oleic acid, but also its relative percentage with respect to total biomass or to other *B. braunii* fatty acids. Along the late linear and the deceleration phases oleic acid accumulation is therefore higher than those of the other algal constituents including other fatty acids. As a result oleic acid becomes, finally, the major fatty acid of the alga. Regarding the other major acids we observe, in all the samples, the usual *n*-saturated derivatives (C_{12}, C_{14}, C_{16} and C_{18}) [75]. Some unsaturated and saturated very long chain fatty acids were also detected; they exhibit even carbon number ranging from C_{20} to C_{30}.

If we compare, now, the variation in hydrocarbon and oleic acid levels, it is found that oleic acid content remains very low as long as hydrocarbon production is high (exponential and early linear phases) but when hydrocarbon production sharply decreases (deceleration stage) oleic acid concentration rises considerably. These opposite changes show clearly that a relationship of the precursor–product type occurs, along the various physiological states achieved in a batch culture, between *B. braunii* hydrocarbons and oleic acid*.

The occurrence of the above relation in several growth experiments is consistent with the involvement of oleic acid, as direct precursor, in the biosynthesis of *B. braunii* hydrocarbons. However such a relation may be fortuitous and this assumption was further tested by determining the location of oleic acid in the alga cells. A previous work [9, 66] allowed us to show that most (*ca* 95 %) of *B. braunii* hydrocarbons are produced and stored within the trilaminar outer walls surrounding the cells. These external hydrocarbons are synthesized from substances, intracellular in origin, excreted towards the outer walls. However, the nature of the excreted intermediate is not known. As shown below, it seems likely that an elongation-decarboxylation mechanism occurs in *B. braunii* hydrocarbon biosynthesis. In such a mechanism the fatty acid acting as direct precursor serves as substrate for an elongation-decarboxylation complex which releases final hydrocarbons [3–5]. Under these conditions the substance supplied to the outer walls by the cells could be only the direct precursor itself or a shorter intermediate. As shown above, during the deceleration phase of batch cultures, hydrocarbon production stops earlier than the one of oleic acid. If the latter is actually the direct precursor of *B. braunii* hydrocarbons, its accumulation during the deceleration phase should originate from the lack of hydrocarbon production. Consequently the oleic acid so accumulated should be located in outer walls. In fact it is possible to discriminate, owing to selective extractions using solvents of increasing polarity, the intracellular oleic acid from the outer wall-located one. Samples of *B. braunii* corresponding to the late deceleration stage were thus examined. Selective extraction indicates that 93 % of the oleic acid they have accumulated is located in the outer walls.

From the above results regarding the structure of the hydrocarbons, the higher efficiency of oleic acid relative to palmitic and stearic acids in hydrocarbon labelling, the relationship of the precursor–product type observed from batch culture between hydrocarbon and oleic acid concentration and the preferential accumulation of this acid in

*The variation, against batch culture duration, in 22:6 fatty acid [76] and 21:6 hydrocarbon concentrations [69] suggest that a similar relationship takes place in the diatom *Skeletonema costatum*.

outer walls when hydrocarbon production ends one can conclude that oleic acid is the direct precursor implicated in the biosynthesis of the major hydrocarbons of *B. braunii*. Such a feature is also consistent with the following observations. Oleic acid is generally assumed to be produced in chloroplasts [77] and then exported towards other cell compartments [78, 79]; *B. braunii* hydrocarbons accumulate exclusively in the basal part of the outer walls [9], i.e. in the portion of the wall laying in a close proximity to the chloroplast. Oleic acid would play a prominent role in the regulation of various lipid biosynthesis. Thus the switching mechanism from the ACP track to the CoA track, which would be required for further metabolism, including elongation to very long chain fatty acid derivatives, is supposed to operate chiefly via oleic acid [80, 81]. Accordingly the involvement of oleic acid, as direct precursor of *B. braunii* major hydrocarbons, fits well with the large amount of hydrocarbons typical of this species.

Mechanism of biosynthesis

Feeding experiments with doubly labelled fatty acid (^3H in the alkyl chain and ^{14}C at the carboxyl atom) determine if the conversion is associated with a decarboxylation process or if the intact carbon chain of the acid is incorporated into hydrocarbons. When *B. braunii* cultures are fed with [9,10-^3H, 1-^{14}C]oleic acid, two situations can be encountered. With an elongation-decarboxylation mechanism (Fig. 2a), or a head-to-head condensation where oleic acid operates as acceptor (Fig. 2b), the $^3H:^{14}C$ ratio should not exhibit any variations when the starting acid is converted into hydrocarbons. With a head-to-head condensation, where oleic acid acts as donor and is decarboxylated (Fig. 2c), the $^3H:^{14}C$ ratio in hydrocarbons should increase significantly relative to oleic acid.

However, none of the above situations was observed and a systematic decrease in the $^3H:^{14}C$ ratio occurred between the acid fed and the hydrocarbons produced by the alga. (The results of an experiment typical of these series of feedings are reported in Table 3.) Therefore such experiments, alone, do not provide any information on the fate of the fatty acid carbon chain during hydrocarbon biosynthesis.

If we consider, now, the previously reported doubly labeling experiments related to hydrocarbon biosynthesis, it appears that the results obtained fit generally well with the mechanism which is known to operate [47]. Nevertheless, some cases exhibit an unexpected decrease in the $^3H:^{14}C$ ratio [26, 45]. Such a decrease is particularly pronounced in *P. fuliginosa* hydrocarbons [53] (it is similar to the variations observed here). The above feedings were chiefly carried out using [1-^{14}C, U-^3H] or [1-^{14}C, 2-^3H] acids and it was generally assumed that the observed decrease in $^3H:^{14}C$ ratios, between the starting fatty acid and final hydrocarbons, would be mainly due to degradation of the former into acetate units. The acetate units so formed lost most of their tritium [45, 47]; accordingly, their subsequent reincorporation into hydrocarbons would cause the $^3H:^{14}C$ ratio to decrease.

Following the first series of incubation with [9,10-^3H, 1-^{14}C]oleic acid, further experiments were carried out using doubly labeled [9,10-^3H, 10-^{14}C] acid. In the latter case ^{14}C is located at such a position that it would be retained, whatever the type of mechanism, during direct conversion of oleic acid into hydrocarbons. As a result the comparison of the two series of feeding should allow, firstly the establishment of the origin of the decrease in $^3H:^{14}C$ ratios and, secondly, to derive the corrective factor which is necessary to determine the fate of the carbon chain of oleic acid during its conversion into hydrocarbons.

It appears that the efficiency of ^{14}C incorporation into external hydrocarbons is not significantly affected by the location (at C-1 or C-10) of the label. Such a lack of dependence is observed, not only if one takes into account the total labelling of hydrocarbons (Table 4), but also their specific activities (Table 5) which are the only objective measure of the efficiency of incorporation [40]. (Specific activities of *B. braunii* major external hydrocarbons exhibit low and randomly directed changes with ^{14}C location in the starting oleic acid.)* Such results indicate that degradation products from the acid fed do not play a significant role in the labelling of *B. braunii* hydrocarbons. Indeed, if a part of the label originated from acetate units produced by exogenous oleic acid degradation, the [1-^{14}C]acid should be a better hydrocarbon precursor, unless fast and complete degradation of oleic acid into acetate units occurred [58]. However, in this latter case, the tritium lost during doubly labelling experiments should be considerable [45, 47] and the decrease in $^3H:^{14}C$ ratio a lot higher than those observed here. We can therefore assume that the label from oleic acid is not incorporated into hydrocarbons via C_2 units (such a feature is consistent with previous findings according to which acetate originating from degradation may be segregated from fatty acid-synthesizing sites [82, 83] and could not be implicated in hydrocarbon biosynthesis) [84]. On the other hand, if some long chain fatty acids derived from the partial degradation of oleic acid were operating in this biosynthesis, then the [10-^{14}C] derivative should exhibit higher efficiency as a precursor [58]. Accordingly the results observed here show that no substantial amount of the oleic acid fed to *B. braunii* is at first partly degraded into shorter molecules and, subsequently, incorporated into hydrocarbons. In addition, radio GC of the total fatty acids of the alga, following feeding with [10-^{14}C] acid, show only the presence of low levels of radioactivity in acids shorter than C_{18} ($< 10\%$ of the label occurring in total fatty acids). This radioactivity is chiefly located in C_{16} (7%), C_{14} (2%) and C_{12} ($< 1\%$) acids. One observes also the lack of label in C_{17} acids; such a feature indicates that α-oxidation processes, which are sometimes detected during fatty acid feeding [22], are not operating in *B. braunii*. These oxidations of the acid fed could give way to the production of even carbon number hydrocarbons (derived from the odd fatty acids produced by α-oxidation) which are sometimes observed in addition to the usual odd hydrocarbons [35, 42, 43, 85]. The lack of α-oxidation is ascertained, in *B. braunii*, by the inability of radio GC to show any labelled even carbon number hydrocarbon. Only the odd hydrocarbons typical of the alga are labelled after all the above feedings. Taken together our results indicate that the incorporation of radioactivity from oleic acid into the

*The mass and radioactivity levels of internal hydrocarbons are too low to enable an accurate determination of their total labeling and specific activities. However it appears (Table 4) that, in this case also, the ^{14}C radioactivity of the hydrocarbon fraction does not depend largely on label location in the acid fed.

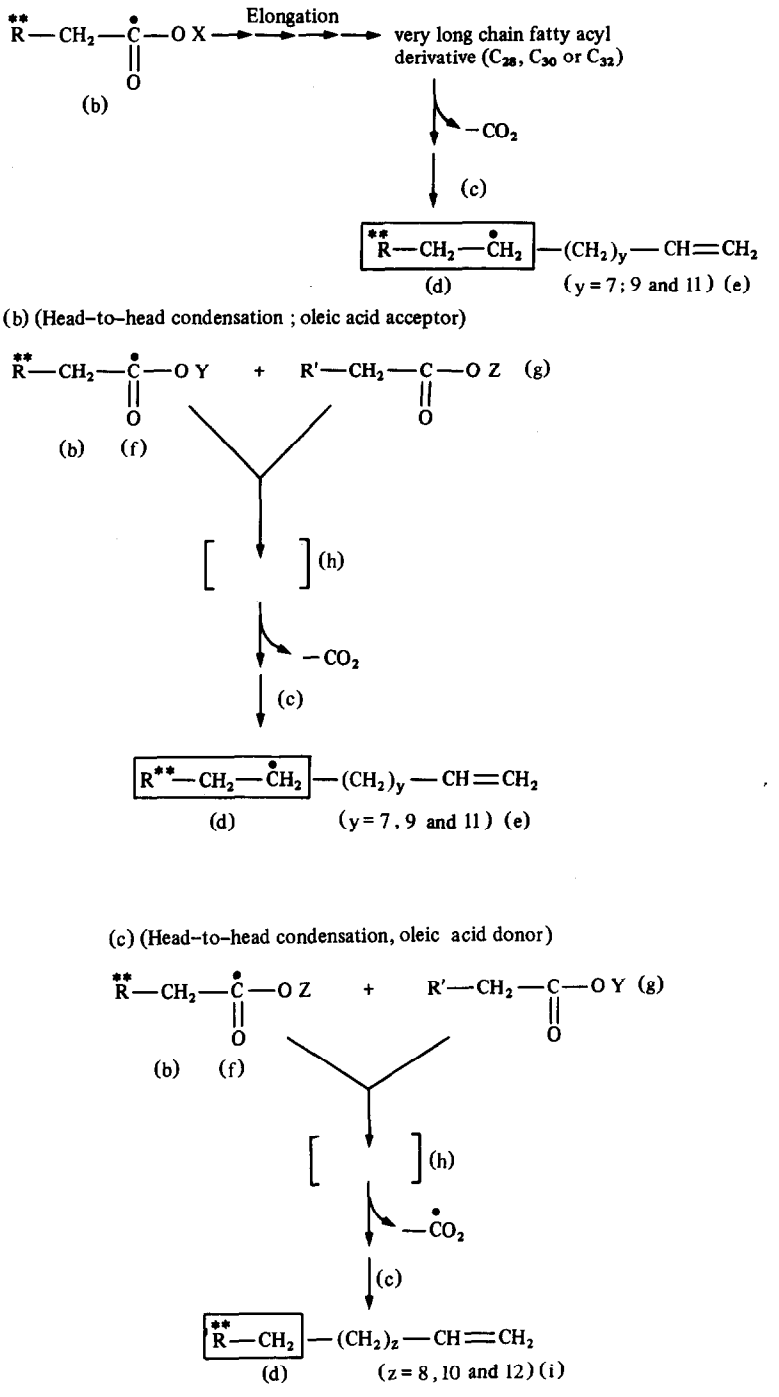

Fig. 2. Possible fate of [1-^{14}C] and [9,10-^{3}H] from oleic acid in relation to the mechanism involved in hydrocarbon biosynthesis (a). (a) * = tritium, ● = ^{14}C. (b) Oleic acid derivatives; $\overset{**}{R} \Rightarrow CH_3-(CH_2)_7-\overset{^3H}{\underset{|}{C}}=\overset{^3H}{\underset{|}{C}}-(CH_2)_6$. (c) Some intermediate steps occur, sometimes, between the decarboxylated product and the final hydrocarbons. (d) Part of the carbon chain originating from oleic acid. (e) y = 7, 9 and 11 (respectively in the major C_{27}, C_{29} and C_{31} dienic hydrocarbons of *B. braunii*). (f) Oleic acid would not necessarily react as fatty acyl derivative; it could undergo, at first, some transformations (like the acceptor palmitic acid in *S. lutea*) [5]. However, such transformations would not alter the ^{3}H : ^{14}C ratio of the carbon chain and they would not interfere with possible variations related to the mechanism. (g) C_{10}, C_{12} or C_{14} derivatives operating as donor (b) or acceptor (c). (h) primary product from condensation. (i) z = 8, 10 and 12 (respectively in the major C_{27}, C_{29} and C_{31} dienic hydrocarbons of the alga).

Table 3. Conversion of doubly labeled [9, 10-^{3}H, 1-^{14}C] oleic acid into *B. braunii* hydrocarbons*

	Radioactivity (dpm × 10^{-3})†		
	^{3}H	^{14}C	^{3}H:^{14}C ratio
External hydrocarbons‡	10^4	1.5×10^3	6.7
Internal hydrocarbons‡	206.5	28.3	7.3

*Incubation for 5 days with [9, 10-^{3}H, 1-^{14}C] oleic acid (50 μCi of ^{14}C and 0.58 mCi of ^{3}H; ^{3}H:^{14}C ratio = 11.45 in the acid).

†Radioactivity in the total hydrocarbon fraction obtained after purification using column chromatography. A further separation (prep. TLC on $AgNO_3$ impregnated silica gel), which affords the unsaturated hydrocarbons typical of the alga, does not show significant variations in radioactivity levels or in ^{3}H:^{14}C ratios. Radio GC demonstrated that nearly all the label of the total hydrocarbon fractions is located in the characteristic hydrocarbons of the alga.

‡Radio GC of the above fractions does not show any mass or radioactivity peak in the range C_{15}–C_{17} (small amounts of a ΔC_{17} alkene were observed in *B. braunii* [14, 15, 90], but such a compound is not always detected) [8, 9]. Similar results are obtained when palmitic acid is fed. It appears therefore that *B. braunii*, unlike various blue–green algae [63, 65], is not able to decarboxylate exogenous long chain C_{16} and C_{18} fatty acids at least under our culture conditions.

*^{3}H was always located on CH_2 groups in previous studies [26, 45, 47, 53] concerned with hydrocarbon formation from doubly labelled fatty acids.

†Further control experiments showed that a tritium loss, amounting to a value very close to the above corrective factor, was occurring, during sterilisation by autoclaving of the culture medium, in the case of [9,10-^{3}H]oleic acid (see Experimental).

‡Such a feature was already suggested from the similar level of hydrocarbon labelling achieved after feeding with 1-^{14}C and 10-^{14}C oleic acids (Table 4).

hydrocarbons of the alga is not obscured by side degradation reactions affecting the carbon chain of the exogenous acid with further rebuilding into hydrocarbons of the shorter derivatives thus formed.

Hydrocarbons produced in feeding experiments with [9,10-^{3}H, 10-^{14}C]oleic acid show a large decrease in ^{3}H:^{14}C ratio. Yet, as already mentioned, ^{14}C is here located at such a position that it cannot be lost, during the *direct* incorporation of the acid. On the other hand we have shown above that degradation products from the oleic acid fed do not play a significant role into *B. braunii* hydrocarbon labelling. Consequently the large decrease observed in ^{3}H:^{14}C ratios must be due chiefly to tritium losses which are not directly related to the biosynthesis pathway leading from oleic acid to hydrocarbons. Such a loss could result, in the present case, from the location of tritium at positions 9 and 10 in the acid fed. These tritium atoms, due to their vinylic nature, could undergo rather easily some exchange reactions*. Anyway, the decrease in ^{3}H:^{14}C ratio observed here from [9,10-^{3}H, 10-^{14}C] acid enabled us to assess a corrective factor. This term reflects first that ^{3}H:^{14}C ratios undergo, under our feeding conditions, a systematic decrease (by a factor of *ca* 1.8) between the oleic acid used and the final hydrocarbons and, secondly, that such a decrease is entirely independent of the steps involved in the biosynthesis of *B. braunii* hydrocarbons†.

Possible variations in ^{3}H: ^{14}C ratios, directly originating from oleic acid conversion into hydrocarbons, would be obscured by the large tritium loss discussed above. But, when the corrective factor is applied to the results obtained from feeding with [9,10-^{3}H, 1-^{14}C]oleic acid, it appears that the ^{3}H:^{14}C ratio shows just relatively low variations when the acid is converted into hydrocarbons (Table 6). The experimental values are slightly lower than the corrected ones (e.g. 3.22 instead of 3.64 in experiment Ia). However, such a variation is a lot smaller and contrary to that expected if oleic acid was incorporated via a decarboxylation step. Thus feeding with doubly labelled oleic acids show that the carbon chain of this compound is incorporated intact into hydrocarbons‡. Accordingly the occurrence, in *B. braunii*, of a head-to-head condensation like in Fig. 2c, where oleic acid acts as donor, can be ruled out.

Table 4. Feeding experiments with doubly labelled [9, 10-^{3}H, 1-^{14}C] and [9, 10-^{3}H, 10-^{14}C] oleic acid*

			External hydrocarbon labelling (dpm × 10^{-3})			Internal hydrocarbon labelling (dpm × 10^{-3})		
		R_0†	^{3}H	^{14}C	R_1†	^{3}H	^{14}C	R_2†
[9, 10-^{3}H, 1-^{14}C]	Ia	6.55	333.3	103.5	3.22	20.2	6.1	3.31
Oleic acid	IIb	6.22	334.7	116	2.89	19.45	5.15	3.78
[9, 10-^{3}H, 10-^{14}C]	Ic	5.25	322	97.9	3.29	23	7	3.28
Oleic acid	IId	2.99	181.2	111.4	1.63	6.67	5.4	1.24

*In each experiment 10 μCi of ^{14}C are fed for 5 days with, respectively, 65.5 μCi of ^{3}H (Ia), 62.2 μCi of ^{3}H (IIb), 52.5 μCi of ^{3}H (Ic) and 29.9 μCi of ^{3}H (IId). Previous feeding experiments showed that incorporation levels can change significantly as a result of variations in the physiological state of the inocula (a similar behaviour was also observed [31, 33] from higher plants). Accordingly the results reported here concern parallel cultures starting from the same inoculum for Ia, Ic and IIb, IId.

†R_0, R_1 and R_2 correspond, respectively, to ^{3}H:^{14}C ratios in the starting oleic acid, in external hydrocarbons and in internal ones.

Table 5. Effect of ^{14}C location in the oleic acid fed on the specific activity of external hydrocarbons

	Specific activity of the major hydrocarbons*				
Oleic acid	$2\Delta C_{25}$	$2\Delta C_{27}$	$2\Delta C_{29}$	$3\Delta C_{29}$	$2\Delta C_{31}$
[1-^{14}C]	1.25	1.56	1.48	1.56	2.21
[10-^{14}C]	1.54	1.27	1.82	1.79	2.43

*Specific activities based on arbitrary units (see Experimental); mean values from two feedings. Precision ±10%

Table 6. Variations in $^{3}H:^{14}C$ ratio during conversion of [9, 10-^{3}H, 1-^{14}C] oleic acid into *B. braunii* hydrocarbons

		$^{3}H:^{14}C$ ratio in hydrocarbons	
Feedings*	$^{3}H:^{14}C$ ratio in oleic acid†	External	Internal
I_a	3.64	3.22	3.31
II_b	3.45	2.89	3.78

*See Table 4 for feeding conditions.

†Corrected figures taking into account the mean tritium loss independent of hydrocarbon biosynthesis (*ca* 1.8) observed, from [9, 10-^{3}H] oleic acid, following autoclaving.

An elongation-decarboxylation (Fig. 2a) and a head-to-head condensation where oleic acid operates as acceptor (Fig. 2b) are both consistent with the above results. However, some features suggest that the former is probably occurring in *B. braunii*. Thus it is noticed that all the major hydrocarbon of the alga show a terminal double bond the position of which, relative to the second 9,10 unsaturation [16], indicates that it is located at the opposite end of the carbon chain originating from oleic acid. Therefore if mechanism 2b was occurring one should assume either the terminal double bond is introduced into a preformed monoenic hydrocarbon (however, no traces of very long chain monoenic hydrocarbons, then supposed to be the primary product of hydrocarbon biosynthesis, are observed in *B. braunii*) [8, 9, 14, 15] or that the double bond is already present in C_{10}, C_{12} and C_{14} fatty acids operating as donors in the formation of the major C_{27}, C_{29} and C_{31} hydrocarbons; no monoenic acids of this type were detected from *B. braunii**.

On the contrary, the systematic presence of the terminal double bond seems to fit well with the involvement of an elongation-decarboxylation mechanism 2a. Such an unsaturation would be then located at the end of the chain where decarboxylation takes place. As decarboxylations show a very high energy of activation it is likely that they require the presence of some activating group in order to make easier CO_2 elimination†. With regard to the elongation-decarboxylation mechanism, the occurrence of an activation via oxidation could be consistent with previous observations related to the inhibition, under anaerobic conditions, of *B. oleraceae* hydrocarbon production [21, 25]. Moreover, the implication of an α-hydroxy C_{32} fatty acid, as intermediate in biosynthesis of *n*-C_{31} alkane, was postulated [35] in the case of *P. sativum*. From the above findings one can therefore assume that the systematic presence of a terminal double bond in *B. braunii* major hydrocarbons would originate from the activation taking place prior to decarboxylation (either directly in the case of an activation via a β–γ double bond, or indirectly through elimination of an other activating group like OH). Under such conditions the double bond location indicates that decarboxylation takes place at the end of the molecule opposite to the carbon chain derived from oleic acid. In fact only an elongation-decarboxylation mechanism is compatible with such a situation‡.

Changes in the hydrocarbon fraction of several organisms were reported when cultures aged [50, 56, 64, 85]. In sharp contrast the nature and the relative abundance of *B. braunii* hydrocarbons are not affected by the large variations in physiological state encountered during batch cultures [11]. This feature must reflect a tight relationship between biosynthesis of the various hydrocarbons in *B. braunii*; it is in agreement with the occurrence of an elongation-decarboxylation mechanism (where a multienzymatic system performs all the reactions from the direct precursor and releases final hydrocarbons [3–5]).

The major external hydrocarbons of the alga exhibit rather close specific activities (Table 5). Due to the involvement of the complex, all the hydrocarbons originating from the same direct precursor, through an elongation-decarboxylation mechanism, should exhibit close specific activities whatever their chain length. This behaviour is actually observed in different higher plants where such a mechanism occurs [32, 48, 51]§.

Taken together the above features suggested that an elongation-decarboxylation mechanism operates in *B. braunii*. In order to test this assumption the influence of dithioerythritol on the biosynthesis of *B. braunii* hydrocarbons was investigated. It is well documented that some thiols inhibit hydrocarbon formation in various higher plants where an elongation-decarboxylation has been shown to occur. These thiols block specifically the decarboxylation of the very long chain fatty acyl derivatives [32, 40, 48, 51, 52]. Such an action has been observed from dithioerythritol (DTE) and dithiothreitol (DTT) which show similar effects¶. Previous studies revealed that the

*Moreover no C_{10}, C_{12} and C_{14} acids, with a substituent capable of yielding via elimination a terminal double bond, were observed.

†Thus, if one considers the head-to-head condensation occurring in *S. lutea* [3–5] it appears that decarboxylation takes place from a fatty acid derivative activated by a β–γ double bond.

‡The presence of 1-alkenes was previously observed in various higher plants and algae [12, 15, 21, 86]. However no study concerned with hydrocarbon biosynthesis was carried out on such species. Therefore it is not known if there is some relation, in these cases, between the terminal double bond and the involvement of an elongation-decarboxylation process.

§In the case of a head-to-head condensation, one free intermediate, at least, would exist between each primary condensation product and the corresponding hydrocarbons [3–5]. As a result the specific activities of the hydrocarbons derived from the same precursor can vary with chain length (label dilution in endogenous pools of various size corresponding to the intermediates involved).

¶Some *B. oleraceae* and *P. sativum* mutants exhibit evidence for genetic blocks in hydrocarbon biosynthesis close to the chemical blocks achieved using DTE or DTT [87–89].

extent of hydrocarbon inhibition depends strongly on DTE concentration. Moreover, low thiols levels could actually give way to a significant increase in hydrocarbon production [32, 51]. Therefore it was essential to use DTE concentrations high enough to reveal the occurrence of a possible inhibition, but which do not reach the toxicity level where the general metabolism of the alga is affected. In order to assess the suitable concentration we examined, first, the effect of increasing DTE levels on *B. braunii* primary metabolism. To this end we measured on one hand the algal oxygen consumption in the absence of light (dark respiration) and its oxygen production in the light (net photosynthesis). The results (Table 7) show with low DTE concentrations a significant stimulation of net photosynthesis while dark respiration remains nearly constant, with intermediate DTE concentration, a net photosynthesis close to the one of the control and a dark respiration noticeably lower, with high DTE concentrations there was a considerable decrease in net photosynthesis, the latter disappearing at 2×10^{-2} M/l. Finally, as DTE concentrations increase further, O_2 absorption is observed (photosynthesis lower than respiration, the cells are then exhausting rapidly storage compounds). With respect to dark respiration, one observes a dramatic increase in the range of high DTE concentrations.

It appears that the primary metabolism of *B. braunii* is not largely affected in culture where the DTE concentration does not exceed 4×10^{-3} M/l. This latter concentration was therefore used in the following feedings. During these experiments the algae are first subjected to a 20 hr pretreatment, in the culture medium containing DTE, prior to addition of labelled oleic acid in this medium. Hydrocarbon radioactivity is determined, after one day of incubation, and compared to that in control cultures. It appears (Table 8) that the presence of DTE results in an extensive inhibition (> 95%) of external hydrocarbon labelling. This inhibition level is similar to those previously reported in *P. sativum* [32], *A. porrum* [40] and *H. vulgare* [51, 52] cuticular hydrocarbons. The actual inhibition is probably still higher than the experimental values reported here. Since parallel *B. braunii* cultures, incubated after a glutaraldehyde pretreatment, provide an external hydrocarbon fraction weakly labelled (*ca* 20000 dpm after feeding with 50 μCi of [9,10-^{3}H]oleic acid) although glutaraldehyde should completely block the metabolism of the alga as a result of protein denaturation. Similarly, control feedings carried out on boiled tissues from different higher plants, lead usually to low residual labelling [31, 39, 41] of hydrocarbons. Therefore, it is likely that DTE inhibition of hydrocarbon formation is nearly complete in the case of *B. braunii* as in the various higher plants previously examined. Regarding the latter species it is well documented that hydrocarbon biosynthesis occurs via an elongation-decarboxylation pathway and that DTE specifically inhibits mechanisms of this type.

All the above results support therefore the occurrence of an elongation-decarboxylation mechanism in *B. braunii*. Such a conclusion is also consistent with previous observations [9] about the presence of similar hydrocarbon chain length distributions in *B. braunii* and in various higher plants. Nevertheless *B. braunii* differs from

Table 7. Effect of DTE concentration on the primary metabolism of *B. braunii*

	DTE concentration in culture media (M/l)					
	10^{-4}	10^{-3}	2×10^{-3}	4×10^{-3}	2×10^{-2}	10^{-1}
Dark respiration*	100	85	50	50	100	650
Net photosynthesis*	140	120	100	80	0	<0

*As % of the control culture without DTE added. Measurement carried out after 20 hr of culture in the DTE-containing medium.

Table 8. Effect of DTE on the incorporation of oleic acid into *B. braunii* external hydrocarbons

	Control culture*	Culture with DTE†	Inhibition (%)
Amount of [10-^{14}C] oleic acid fed (dpm $\times 10^{-6}$)	92.3	110.8	—
Labelling of external hydrocarbons‡ (dpm $\times 10^{-3}$)	649	12.2	98
Amount of [9, 10-^{3}H] oleic acid fed (dpm $\times 10^{-6}$)	100	100	
Labelling of external hydrocarbons‡ (dpm $\times 10^{-3}$)	443.6	14.9	96.6

*Identical to the corresponding inhibited cultures (same inocula, culture conditions and feeding duration with oleic acid) but without DTE added.

†Pretreatment for 20 hr in a culture medium containing 4×10^{-3} M/l of DTE; afterwards radioactive oleic acid is added and the cells incubated for 24 hr in the same medium.

‡Variations in labelling efficiency observed between the two controls results, first from the tritium loss discussed above and, secondly, from the use of different inocula in the two series of culture.

higher plants if one considers the specificity of the elongation-decarboxylation system. Thus, in *B. oleracea*, this system shows a high specificity for *n*-saturated long chain fatty acids and excludes oleic acid [22, 46]. In sharp contrast, oleic acid is involved as starter in the biosynthesis pathway leading to the large hydrocarbon concentrations typical of *B. braunii*.

EXPERIMENTAL

The *B. braunii* strain used throughout this work was obtained from the Cambridge Culture Collection (N°LB807/1 DROOP, 1950, Maddingley Bricks Pits, U.K.).

Culture and study of hydrocarbons. Extraction of int and ext pools, purification using CC and prep. TLC on $AgNO_3$–silica gel, analysis on GC and GC/MS were carried out as previously described [9].

Analysis of total fatty acids. In order to ensure that very long chain fatty acids, if present, would be actually recovered in the total lipids, the dried algal biomass was subjected to the following extractions for 4 hr under N_2. Boiling $CHCl_3$–MeOH (2:1), boiling $CHCl_3$–MeOH–conc HCl (128:64:1) and boiling toluene. The corresponding extracts were combined after neutralization. The total lipid fraction thus obtained was saponified following usual methods and the fatty acids recovered using successive continuous extraction for 24 hr with hexane and Et_2O. The nature and the relative abundance of fatty acids were determined using GC (Apiezon L 10%) and GC/MS (SE 52 capillary column) after conversion into their Me esters using MeOH–BF_3 complex under N_2.

Feeding expts. The algae were fed with [16-^{14}C]palmitic acid (40–50 mCi/mM), [18-^{14}C]stearic acid (35–45 mCi/mM) [9,10-^{3}H]oleic acid (40–50 Ci/mM), [1-^{14}C]oleic acid (45–55 mCi/mM) and [10-^{14}C]oleic acid (45–55 mCi/mM). The labelled acids were dissolved in a mixture of Et_2O (5 ml) and Tween 20 (0.05 ml), Et_2O was then eliminated under N_2 and 3 ml of H_2O finally added. The mixture was then sonicated (× 4) so as to obtain a stable emulsion. The latter was injected into the fresh culture medium before autoclaving. Figures which have to be compared were always obtained from parallel cultures starting from the same inoculum in order to avoid variations originating from differences in the physiological state of inoculated cells. Moreover these parallel cultures were always inoculated with close concns in algal biomass. Hydrocarbons and total fatty acids were extracted and purified as above and their radioactivity determined by liquid scintillation counting using 0.4% butyl-PBD in toluene.

Radio GC of purified hydrocarbons and total fatty acid fraction was carried out using an Apiezon L 10% column; the sensitivity of radioactivity and mass measurements were always kept at a constant level. The sp. act. of *B. braunii* major hydrocarbon reported, from radio GC, are based on arbitrary units (sp. act. = area of the radio peak/area of the mass peak).

NMR of hydrocarbons. High resolution ^{13}C NMR spectra were obtained at 25.17 MHz using standard pulse methods, Fourier transform technique and D_2O as ext. reference. The spectra were obtained using a 20 μsec pulse width, a 0.45 pulse delay and a 0.666 sec acquisition time. The samples were dissolved in $CDCl_3$ and TMS used as ref. Continuous wave ^{1}H NMR spectra were also obtained from the above solns.

Localization of oleic acid was determined using selective extraction. The lipids of the outer walls were first recovered by successive extraction (1 hr and 3 hr) with a solvent of low polarity (stirring of dried algal biomass into hexane at room temp); afterwards the internal lipids were extracted by a $CHCl_3$–MeOH–conc HCl mixture (128:64:1) (stirring at room temp for 24 hr). The fatty acids of these two fractions were analysed and the oleic acid concentrations determined as above.

Tritium losses. Following the occurrence of some unexpected decreases in ^{3}H:^{14}C ratios, between the oleic acid used for feeding and the hydrocarbons produced by *B. braunii*, various controls were carried out. They show that (i) no traces of labelled impurities were present in the oleic acids fed; thus no variations occur in their radioactivity after purification on TLC (silica gel; hexane–Et_2O–HOAc, 80:20:1); (ii) a large tritium loss takes place during sterilization of [9,10-^{3}H]oleic acid (the decomposition of tritiated compounds is well documented and it seems that several factors can control the magnitude of this complex process, however it appears that high temps and high sp. act. favour such a degradation [91]. Known amounts of tritiated oleic acid were therefore autoclaved, in culture medium, under the conditions used for the preparation of feeding expt; afterwards the acid was extracted by Et_2O and purified, as above, using TLC. The radioactivity of the oleic acid thus recovered shows that *ca* 50% of the initial tritium was lost during sterilisation).

DTE effect on primary metabolism of B. braunii. The inoculum was divided into equal samples which were inoculated and grown for 20 hr under usual conditions but in culture media containing, in addition to mineral nutrients, increasing DTE concns. O_2 consumption of algal cells was then determined in the absence of light (dark respiration); afterwards their O_2 production was measured in the light (net photosynthesis) under such conditions that neither light intensity nor CO_2 supply are limiting [92]. All the above O_2 determinations were carried out using a Clark polarographic electrode and a thermostated measuring cell.

Influence of DTE on efficiency of oleic acid conversion into hydrocarbons. The feedings and the determination of hydrocarbon radioactivity were performed as usual. The inocula were divided into three equal portions. Part 1 was, at first, grown for 20 hr in a culture medium containing 4×10^{-3} M/l of DTE. Thereafter ^{3}H or ^{14}C oleic acid was added and the growth was carried on for 24 hr with the labelled precursor. Part 2 was used as control, grown and fed, under the same conditions as above, but without DTE added. Part 3 was inoculated into a culture medium containing 2% of glutaraldehyde; after 20 hr the labelled oleic acid was added and the feeding performed as above.

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