Phytochemistry, 1980, Vol. 19, pp. 1081–1085. Pergamon Press Ltd. Printed in England.

THE BIOSYNTHESIS OF LONG-CHAIN HYDROCARBONS IN THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

C. LARGEAU*, E. CASADEVALL* and C. BERKALOFF†

*Laboratoire de Chimie Bioorganique et Organique Physique, E.R.A. CNRS 685, E.N.S.C. 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05; †Laboratoire de Botanique–Cytophysiologie Végétale, L.A. CNRS 311, E.N.S. 24 rue Lhomond, 75231 Paris Cedex 05, France

(*Received* 8 *June* 1979)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; algae; sites of hydrocarbon biosynthesis; hydrocarbon catabolism.

Abstract—The green colonial alga *Botryococcus braunii* has unusually high levels of hydrocarbons. Two distinct sites of hydrocarbon accumulation are present in the species: an internal pool present in cytoplasmic inclusions and an external pool in the trilaminar outer walls and associated globules. It is generally assumed that the hydrocarbons are produced within the cells and then excreted into the external pool to maintain the intracellular content at a normal value. Various feeding experiments showed, however, that the radioactivity of the external pool is much higher than the internal one. On the other hand, there was no decrease in the labelling of internal hydrocarbons in chase experiments. Therefore, an excretory process apparently does not take place in *B. braunii*. The outer wall, therefore, is the main site of hydrocarbon accumulation and also the place where the bulk of *B. braunii* hydrocarbons are produced. The outer wall also is involved in the matrix of colony formation and the above findings account for the sharp decrease of hydrocarbon production which is associated with the loss of colonial habit. The cultures were also shown to be unable, under usual growth conditions, to catabolize their own hydrocarbons. Such a feature, along with the extracellular location of the main site of production, may account for the abnormally high content of hydrocarbons typical of *B. braunii*.

INTRODUCTION

The green colonial alga *Botryococcus braunii* has hydrocarbon levels (*ca* 15% of dry wt) [1–3] conspicuously higher than those commonly observed in other unicellular algae (10–2000 ppm) [4, 5]. Moreover, the involvement of this species in the formation of various hydrocarbon-rich sediments is now well established [6–15]. It is generally assumed that hydrocarbons in *B. braunii* are synthesized within the cells and stored as cytoplasmic inclusions before being excreted into the outer wall [10, 15–17]. Earlier results showed that these compounds actually accumulate in both internal inclusions and in the outer walls [18] which contain 95% of the total. The presence of the same hydrocarbons in these two sites of accumulation is consistent with the occurrence of some active excretory process: the main role of the process would be to keep the hydrocarbon content within the cells to a normal value (0.75% of dry wt) in spite of the very high level in whole colonies [3, 18]. Nevertheless, some findings cast doubt on the involvement of excretion in *B. braunii*. For example, the relative abundance of the major hydrocarbons; the internal pool showing a higher percentage of shorter chain derivatives [18] indicating that, if there is a relation between the two pools, longer hydrocarbons must be excreted preferentially to the outer wall. Furthermore, electron microscopy does not reveal any special excretory structures [18, 19].

The above considerations prompted us to investigate the occurrence of hydrocarbon excretion in *B. braunii*. To this end, the relationship between the internal and external pools was examined by means of various radioactive feeding experiments. The localization of the sites of hydrocarbon biosynthesis was also examined; and the ability of *B. braunii* to catabolize its own hydrocarbons tested.

RESULTS AND DISCUSSION

Cultures of the alga, after incubation with a labelled precursor, were extracted selectively as previously described [18], to separate the external and internal hydrocarbon pools. Hydrocarbons were fractionated after addition of cold carriers, to obtain the unbranched long-chain dienic derivatives ($2\Delta C_{25}$, $2\Delta C_{27}$, $2\Delta C_{29}$, $2\Delta C_{31}$) which account for 80% of the external pool and 91% of the internal one in *B. braunii* [3, 18, 20–22]. The relative radioactivity (α) of the external and internal dienic long-chain hydrocarbons was determined and the variations of α with respect to various feeding conditions were examined to give information about possible transfers between the pools.

Few studies have been reported on hydrocarbon biosynthesis in plants but in every case fatty acids in the range C_{14}–C_{18} were shown to be implicated in the formation of long-chain hydrocarbons [23]. Here, preliminary feeding experiments showed that palmitic

Table 1. Variations in the labelling of hydrocarbons in *Botryococcus braunii* in relation to the duration of feeding*

	Duration of feeding (labelling in dpm × 10^{-6})							
	1.5 hr	3.5 hr	7 hr	1 day	2 days	5 days	12 days	35 days
Total lipids	370	267	212	199	154	125	109	97
External dienic hydrocarbons	6.8	5.8	7.5	8.9	14.4	20.4	22	24.4
Internal dienic hydrocarbons	0.21	0.21	0.38	0.42	0.75	1.07	1.53	2.9
Ratio: external/internal (α)	32	28	20	21	19	19	14	8
Contribution (%) of hydrocarbons to total lipid labelling	1.9	2.2	3.7	4.6	9.8	17.2	21.6	28.1

*Each culture was incubated with 1 mCi of palmitic acid-[9,10-^{3}H]. Control experiments indicate that no noticeable amount of radioactivity was incorporated in *B. braunii* hydrocarbons when the cultures were heated at 100° for 10 min immediately after inoculation into labelled medium. On the other hand, successive PLC with various solvent systems did not result in significant variations of the sp. act. of external and internal hydrocarbon fractions obtained on normal feeding, and replication of the experiments shows that the scattering of α values is fairly low (*ca* 10%).

acid-[9,10-^{3}H] was rapidly incorporated into *B. braunii* hydrocarbons and this was used in all experiments.

Effect of the duration of feeding on hydrocarbon labelling

The results on feeding experiments of increasing length are shown in Table 1. It can be seen that the radioactivity of the external pool is much higher than that of the internal one in every case, especially during short periods of feeding.

The activity of both pools rises with time, the increase being more pronounced for internal hydrocarbons.

If excretion was actually occurring, one would expect that the bulk of hydrocarbon activity, in the case of short feeding, should be located within the internal pool ($\alpha < 1$). Thereafter there should be a progressive migration towards the external pool giving an increase in α. The results obtained are completely contrary to this but they do not enable us to exclude entirely the occurrence of some hydrocarbon excretion in *B. braunii*. Indeed, the above findings are compatible with two possible situations: (a) lack of transfer between the internal and the external pools of hydrocarbons: and (b) the occurrence of a rapid excretory process, so that the label is partitioned between the two sites of accumulation as soon as it is incorporated into hydrocarbons: and thus $\alpha > 1$ could be observed even after short times of feeding.

However, as stressed previously [18], the abundance of hydrocarbon-bearing inclusions is not uniform within the cells. A few cells contain many of these structures while others have none. This casts doubt on the occurrence of a fast excretory process and some pulse-chase feedings were carried out to test the above assumptions.

Variations in α associated with pulse–chase experiments

After feeding *B. braunii* colonies with tritiated palmitic acid, they were resuspended in a fresh medium containing unlabelled palmitic acid and growth allowed to continue. The values of α from such cultures were then compared. If fast hydrocarbon excretion were taking place, a sharp decrease of activity should be observed within the internal pool and hence a considerable increase of α. It is found, in fact (Table 2), that variations in α after transfer are low. Moreover, radioactivity in the hydrocarbon pools still

Table 2. Variations of α during pulse–chase experiments*

	Controls†				Cultures transferred to an unlabelled medium			
Culture conditions (days labelled: unlabelled)	Contribution of hydrocarbons to total lipid radioactivity (%)	Labelling of external dienic hydrocarbons	Labelling of internal dienic hydrocarbons	α	Contribution of hydrocarbons to total lipid radioactivity (%)	Labelling of external hydrocarbons	Labelling of internal hydrocarbons	α
Culture I (1:1)	4.1	4.05	0.19	21.3	11.9	4.85	0.24	20.2
Culture II (5:5)	16.6	9.75	0.53	18.4	20.1	10.5	0.61	17.2
Culture III (35:12)	27.3	12.85	1.5	8.6	30.3	12.7	1.55	8.2

* Label is given in dpm × 10^{-6}. All figures are averages from two experiments.

† The culture was analysed just before transfer into a cold medium.

increases during the 'chase' (period), particularly in the internal pool and a small but significant decrease of α is thus actually observed.

The results of these experiments are consistent with the lack of long-chain hydrocarbon migration from the internal pool to the external one and in agreement with previous observations (alga morphology, hydrocarbon distribution within the two pools) which suggested that no excretion of these compounds occurs in *B. braunii* [18, 19]. The outer wall is therefore not only the main site of hydrocarbon accumulation, but also, probably, the place where the bulk of the *B. braunii* hydrocarbons are produced.

Sites of hydrocarbon formation

The existence of a single site of hydrocarbon production in *B. braunii* located within the cells has been generally assumed along with the occurrence of ready excretion of the products [10, 15–17]. However, according to our results, it seems more likely that external long-chain hydrocarbons originate from a biosynthetic pathway, the terminal steps of which, at least, occur in the outer wall.

Such an extracellular location appears at first sight surprising, particularly in view of the unusual abundance of the external pool. But, as emphasized in the previous part of this work [18], numerous common features related to hydrocarbon accumulation are observed between colonies of *B. braunii* and the epidermis of higher plants. It is now well documented [23–25] that cutin (a polymer of hydroxy-fatty acids which is found in higher plant cuticles) is finally assembled and some steps of the formation of monomers take place outside the cells. On the other hand, cuticular wax constituents, and particularly long-chain hydrocarbons, are known to be synthesized within the epidermis and not in the underlying tissues [26, 27]. From these data it is often assumed that waxes are produced within epidermal cells and then excreted to the cuticle [28, 29] although very little experimental evidence exists to support such an assumption. Cassagne and Lessire have clearly shown [30] that some exchange of waxy derivatives takes place between epidermal cells and the cuticle, and their results suggest the occurrence of hydrocarbon excretion in epidermal cells of *Allium porrum* [31]. However, their results do not exclude the possibility that part of the biosynthesis of cuticular alkanes may take place, as in the case of *B. braunii*, outside the epidermal cells.

The formation of the bulk of *B. braunii* hydrocarbons in the outer wall also affords an explanation of the following feature: loss of the colonial habit in the alga is always accompanied by a sharp decrease in the production of hydrocarbons. For example, the physiological state originating from a culture of the brown resting stage is characterized by large green single cells showing very low hydrocarbon levels [3]. On the other hand, a treatment carried out in order to obtain an axenic strain resulted in single cell cultures which did not synthesize long-chain hydrocarbons.Two suggestions were put forward [32] to explain this behaviour: (a) contaminating bacteria might be implicated, at least in part, in the formation of long-chain hydrocarbons; or (b) the extracellular part of the algae matrix could synthesize hydrocarbons. The latter assumption is consistent with evidence presented here. Morever, as we have shown previously [18], the outer walls of the cells play a prominent role in the formation of the matrix of the colonies. The disappearance or atrophy of such a structure should therefore result in the loss of colonial behaviour, and could also account for the parallel decrease in hydrocarbon level.

Catabolism of the hydrocarbons in B. braunii

It is now well established that a wide range of micro-organisms can use exogeneous hydrocarbons as the sole carbon source [33, 34]. But, in fact, very few species are able to degrade their own long-chain hydrocarbons in order to use them as an energy reserve [35]. Similarly, it is generally assumed that the hydrocarbon constituents of higher plant waxes are end products of metabolism [36–38]; however, they might be occasionally subjected to a low turnover and in *A. porrum* some cuticular alkanes undergo reabsorption and further metabolism within epidermal cells [31]. According to Belcher [39] the slow growth observed in *B. braunii* may be due, in part, to its inability to

Table 3. Effect upon radioactivity of hydrocarbons of successive transfers of the alga to labelled media

	Transfer*			
	Culture I	Culture II	Culture III	Control
	Labelling dpm $\times 10^{-6}$†			
External pool	6.65	12.2	18.1	7.05
Internal pool	0.22	0.42	0.59	0.26
Ratio (α)	30	29	30.7	27

*Culture I was analysed after incubation for 1.5 hr with 1 mCi of palmitic acid-[9, 10-^{3}H]. Culture II was transferred to a fresh medium containing 1 mCi of tritiated palmitic acid and incubated for a further 1.5 hr. Culture III was transferred to a fresh medium with 1 mCi of tritiated palmitic acid and analysed after a further 1.5 hr of incubation. The control was incubated without transfer for 4.5 hr with 1 mCi of tritiated palmitic acid.

metabolize further the abundant lipid material produced by the cells.

The present results indicate that *B. braunii*, under normal culture conditions, cannot catabolize its own long-chain hydrocarbons. No decrease of hydrocarbon radioactivity was observed after transfer into a cold medium for a prolonged period (Table 2) and in fact the activity of both pools increased slowly while the contribution of hydrocarbons to the radioactivity of the total lipid fraction also increased.

A similar situation was observed during prolonged feeding experiments (Table 1), the radioactivity of the hydrocarbons as a part of the total lipid fraction rose steadily with time and reached *ca* 30% after 35 days. On the other hand, the activity associated with the two hydrocarbon pools is relatively high as early as 1.5 hr of incubation. This suggests that a rapid incorporation of the bulk of the 'available' labelled acid occurs easily. This point was confirmed by feeding experiments involving successive transfers after short incubation into a fresh medium containing labelled palmitic acid (Table 3). Each transfer shows a nearly constant increase in the radioactivity of the hydrocarbons. In sharp contrast, the control culture grown without transfer exhibits a much more slower increase in radioactivity. Such behaviour results, very likely, from rapid incorporation and partitioning of the bulk of the 'available' acid between the various constituents of the cells; afterwards the activity of the hydrocarbon pools shows only a slow increase originating mainly from trapping of labelled catabolic products.

In conclusion, no excretory process involving hydrocarbon transfer from the internal pool to the external one is observed in *B. braunii*. The alga is not only able to store [18] but also to synthesize the bulk of its hydrocarbons in the outer wall. Such a feature, added to the absence of hydrocarbon catabolism accounts for the unusually high content of hydrocarbons typical of that species.

EXPERIMENTAL

The strain of *Botryococcus braunii* used throughout this work was obtained from the Cambridge Culture Collection (No. LB 807/1 DROOP, 1950, Maddingley Bricks Pits, U.K.) and grown in a modified CHU 13 medium [18]: *ca* 1 mCi of palmitic acid-[9, 10-^{3}H], 10–30 mCi/mM, was added to 70 ml of fresh medium in each culture flask. The bulk of the acid was dispersed by repeated sonication, and the remainder dissolved in 0.1 ml pentane before addition to the culture medium. However, the results (Tables 1 and 3) suggest that only a part of the acid (dissolved or sufficiently dispersed) is readily metabolized by the cells. This behaviour may be due to the particular structure [16, 18] of the colonies (the cell are embedded in outer walls and mucilage) which hinders the assimilation of external nutrients [39]. After autoclaving, 10 ml of a dense culture of *B. braunii*, entering the exponential phase, were inoculated in each flask and the growth carried out under the conditions previously described [18]. The cultures were stopped after various times of feeding and the cells (harvested under sterile conditions and washed with H_2O so as to remove any tritiated acid which may be adsorbed on the colonies) were then subjected to different treatments: immediately analysed (time-course expts); resuspended in fresh cold medium containing palmitic acid (200 mg/l) where growth was continued (pulse–chase expts).

The colonies, harvested after the various feedings, were dried under vaccum and subjected to successive extractions with solvents of increasing polarity in order to separate the external (hexane extract) and the internal ($CHCl_3$–MeOH extract) hydrocarbons [18]. After addition of cold carriers, the crude hydrocarbon fractions thus obtained were further purified (CC on neutral Al_2O_3) and fractionated (PLC, $AgNO_3$–Si gel) as already described [18], to obtain the series of long-chain dienic derivatives predominant from the external and internal hydrocarbon pools. The radioactivity of the external and of the internal dienic hydrocarbons was then determined by liquid scintillation counting, using 0.4% butyl-PBD in toluene. Control expts showed that no significant variations in the sp. act. of the external, or internal, dienic long-chain hydrocarbons were observed after successive PLC ($AgNO_3$–Si gel, 1:9, 0.5 mm thick, detection by spraying rhodamine-6G in Me_2CO and examining under UV light) with different solvent systems: hexane–Et_2O (9:1), hexane–C_6H_6 (4:1), $CHCl_3$–EtOH (49:1), $CHCl_3$.

Acknowledgements—This work was supported by the C.N.R.S. as a part of the 'Programme Interdisciplinaire de Recherche et de Développement de l'Energie Solaire'.

REFERENCES

1. Maxwell, J. R., Douglas, A. G., Eglinton, G. and McCormick, A. (1968) *Phytochemistry* **7,** 2157.
2. Swain, F. M. and Gilby, J. M. (1964) *Pubbl. Stn. Zool. Napoli* **33,** 361.
3. Brown, A. C., Knights, B. A. and Conway, E. (1969) *Phytochemistry* **8,** 543.
4. Weete, J. D. (1976) *Chemistry and Biochemistry of Natural Waxes* (Kolattukudy, P. E., ed.) p. 350. Elsevier Amsterdam.
5. Stransky, K., Streibl, M. and Storm, F. (1968) *Collect. Czech. Chem. Commun.* **33,** 416.
6. Bertrand, C. and Renault, B. (1892) *Bull. Soc. Hist. Nat. Autun* **4,** 160.
7. Bertrand, C. and Renault, B. (1893) *Bull. Soc. Hist. Nat. Autun.* **6,** 321.
8. David, T. W. E. (1889) *Linn. Soc. N.S.W.* **6,** 483.
9. Zalessky, M. D. (1926) *Rev. Gen. Bot.* **38,** 31.
10. Temperley, B. N. (1936) *Trans. R. Soc. Edinburgh* **58,** 855.
11. Traverse, A. (1955) *Micropaleontology* **1,** 343.
12. Cane, R. F. (1969) *Geochim. Cosmochim. Acta* **33,** 257.
13. Douglas, A. G., Eglinton, G. and Maxwell, J. R. (1969) *Geochim. Cosmochim, Acta* **33,** 569.
14. Douglas, A. G., Eglinton, G. and Maxwell, J. R. (1969) *Geochim. Cosmochim. Acta* **33,** 579.
15. Cane, R. F. and Albion, P. R. (1973) *Geochim. Cosmochim. Acta* **37,** 1543.
16. Chodat, M. R. (1896) *J. Bot. Genève* **10,** 333.
17. Blackburn, K. B. (1936) *Trans. R. Soc. Edinburgh* **58,** 841.
18. Largeau, C., Casadevall, E., Berkaloff, C. and Dhamelincourt, P. (1980) *Phytochemistry* **19,** 1043.
19. Schnepf, E. and Koch, W. (1978) *Bot. Jahrb.* **99,** 370.
20. Gelpi, E., Oro, J., Schneider, H. J. and Bennett, E. O. (1968) *Science* **161,** 700.
21. Gelpi, E., Schneider, H., Mann, J. and Oro, J. (1970) *Phytochemistry* **9,** 603.

22. Knights, B. A., Brown, A. C., Conway, E. and Middleditch, B. S. (1970) *Phytochemistry* **9,** 1317.
23. Kolattukudy, P. E., Croteau, R. and Buckner, J. S. (1976) *in Chemistry and Biochemistry of Natural Waxes* (Kolattukudy, P. E., ed.) p. 290. Elsevier, Amsterdam.
24. Kolattukudy, P. E. and Walton, T. J. (1972) *Biochemistry* **11,** 1897.
25. Kolattukudy, P. E., Walton, T. J. and Kushwaha, R. P. S. (1973) *Biochemistry* **12,** 4488.
26. Kolattukudy, P. E. (1968) *Plant Physiol.* **43,** 375.
27. Cassagne, C. (1970) *C. R. Acad. Sci. Ser. D* **270,** 3055.
28. Herbin, G. A. and Robins, P. A. (1969) *Phytochemistry* **8,** 1985.
29. Weete, J. D., Leek, G. L., Peterson, C. M., Currie, H. E. and Branch, W. D. (1978) *Plant Physiol.* **62,** 675.
30. Cassagne, C. and Lessire, R. (1974) *Arch. Biochem. Biophys.* **165,** 274.
31. Cassagne, C. and Lessire, R. (1975) *C. R. Acad. Sci. Ser. D* **280,** 2537.
32. Murray, J. and Thomson, A. (1977) *Phytochemistry* **16,** 465.
33. Davis, J. B. (1967) in *Petroleum Microbiology.* Elsevier, Amsterdam.
34. Jurtshuk, P. and Cardini, G. E. (1971) *Crit. Rev. Microbiol.* **1,** 239.
35. Bird, C. W., Lynch, J. M. and Pirt, S. J. (1974) *Chem. Ind. (London)* **15,** 617.
36. Jordan, R. C. and Chibnall, A. C. (1933) *Ann. Botany (London)* **57,** 163.
37. Kolattukudy, P. E. (1970) *Annu. Rev. Plant Physiol.* **21,** 163.
38. Eglinton, G. and Hamilton, R. J. (1967) *Science* **156,** 1322.
39. Belcher, J. H. (1968) *Arch. Microbiol.* **61,** 335.