Phytochemistry, 1980, Vol. 19, pp. 1043–1051. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0601-1043 $02.00/0

SITES OF ACCUMULATION AND COMPOSITION OF HYDROCARBONS IN *BOTRYOCOCCUS BRAUNII*

C. LARGEAU*, E. CASADEVALL*, C. BERKALOFF† and P. DHAMELINCOURT‡

* Laboratoire de Chimie Bioorganique et Organique Physique, E.R.A. CNRS 685, E.N.S.C.P., 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France; † Laboratoire de Botanique-Cytophysiologie Végétale, L.A. CNRS 311, E.N.S., 24 rue Lhomond, 75231 Paris Cedex 05, France; ‡ Laboratoire de Spectrochimie Infrarouge et Raman, L.P. CNRS 2631, U.S.T. Lille, 59650 Villeneuve-D Ascq, France

(*Revised received* 8 *October* 1979)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; green algae; *in vivo* Raman spectrometry; electron microscopy; hydrocarbon composition and localization.

Abstract—Raman spectrometry and electron microscopy show that, in the hydrocarbon-rich alga *Botryococcus braunii*, hydrocarbons accumulate in two distinct sites; internally in cytoplasmic inclusions and externally in successive outer walls and derived globules. No other classes of lipid are present in noticeable amounts in the cytoplasmic inclusions and in the external globules. The same hydrocarbons are observed in the internal and external pools but with different relative abundances, the shorter hydrocarbons being more abundant in the internal pool. The bulk of *B. braunii* hydrocarbons (*ca* 95%) is located in the external pool. Such an extracellular location allows this species to exhibit both an unusually high hydrocarbon content (15% of dry wt) and a normal level (0.75%) within the cells. The hydrocarbon pattern and location of *B. braunii* were compared with that of other organisms; a close relation appears between higher plant epidermal cells and this green alga. The trilaminar outer walls of *B. braunii*, at whose contact external hydrocarbon globules accumulate, contain a sporopollenin-like compound.

INTRODUCTION

The green-colonial alga *Botryococcus braunii* has been widely studied because of its unusual features and also due to its involvement in the formation of hydrocarbon-rich sediments. It is now well documented that *B. braunii*, or at least a species which does not differ in any morphological characteristic from the existing alga, was implicated in the production of a wide range of such sediments [1–12]. Thus carboniferous deposits (boghead coals), various paleozoic oil-bearing rocks, as well as some tertiary sediments are assumed to derive their hydrocarbon content from this alga. Moreover the first steps of the formation of boghead coals can be observed, nowadays, in nature; so important blooms of *B. braunii* can give rise to a rubbery material, called Coorongite, which is considered as the 'peat stage' of boghead coals.

The morphology and the metabolism of *B. braunii* also show unusual characteristics. The cells are embedded in a thick envelope, termed the cup by Blackburn [13], which is impregnated with oil and exudes, when compressed, large drops of this material [14]. Refractive droplets are also observed in the cytoplasm [13–15]; Blackburn's findings suggest that some of them also contain an oily material. The same author has shown that, after cell division, the daughter cells secrete new cups which remain surrounded by the cup of the mother cell. Therefore, the rubbery matrix of the colony appears to be composed of the successive cups saturated with oil.

At the time the present work was in progress, Schnepf and Koch [16] published an ultrastructural study of *B. braunii*. The authors confirmed the existence of some droplets with an oily appearance in the cytoplasm of this species and showed that the wall of the alga consists (as in other green algae [17–19]) of an internal fibrillar layer, probably polysaccharidic, and an external thin trilaminar layer. In close contact with this external layer, and perhaps within it, numerous oil drops of varying sizes are also visible. According to Schnepf [16], the oily matrix which embeds the algal colonies would be formed by the external walls of the successive generations, partly fused one to another.

The oily material of the cups was first regarded as fatty acids [13]. However, recent reports [20, 21] indicate that hydrocarbons are their major constituents. It was also shown that *B. braunii* can occur in nature in two distinct physiological states [22] where hydrocarbons account respectively for 17% (green active cells) and 76% (brown resting stage) of the dry wt. The structure of the corresponding hydrocarbons was also determined [21–26], and the two stages shown to contain hydrocarbons of different structure and metabolic origin. However, as far as we are aware, the resting stage has not yet been obtained in laboratory cultures; in fact, old cultures generally accumulate carotenoids and turn red [15], similar to

the resting stage, but they still exhibit about the same hydrocarbon pattern as the green cells [22]. In the present work we are concerned only with green active cultures of *B. braunii*.

It is generally assumed that *B. braunii* hydrocarbons are produced within the cells, stored in the oily cytoplasmic globules and then excreted in the cups [6, 12–14]. However, no direct evidence about the presence of hydrocarbons in internal inclusions has been obtained up to now. Moreover, no information is available on the occurrence of excretory processes in *B. braunii*, and no trace of excretion was detected on microscope examination [16].

In view of the above considerations, we were prompted to examine, as part of a general study of hydrocarbon production in *B. braunii*, the following points. The occurrence of an internal site of hydrocarbon accumulation (cytoplasmic globules) in addition to the external one (cups) and the possible presence of other lipids in these structures, the relative abundance of these two hydrocarbon pools, if present, and their respective constituents and the relationship between the internal and external pools, in order to test the occurrence of some excretory process and the site(s) of hydrocarbon biosynthesis.

In the present report we will discuss and compare the results obtained by electron microscopy, *in vivo* Raman spectroscopy, selective extraction and analysis of hydrocarbons.

RESULTS AND DISCUSSION

Ultrastructure

The strain of *B. braunii* examined exhibits '*in vivo*' characteristics more closely related to Chodat's description [14] than that of Blackburn [13]. The matrix of the colonies is not so compact as in Blackburn's strain. The cells are attached to each other by a refringent material which sometimes stretches itself between two or three distinct clumps of cell (Fig. 1d). Associated with almost every colony are one or more refringent droplets frequently as large (or larger) than the cell (Fig. 1). The cell walls themselves also bear refringent thickenings. The refringent material is stained by lipid reagents, Sudan red or OsO_4.

In ultrathin sections, the *B. braunii* strain used appears similar to the Göttingen strain described by Schnepf [16]. The cell wall (Figs. 4 and 5) possesses an internal fibrillar layer and an external trilaminar sheath (TLS), as observed in other green algae [17–19]. Selective staining with the Thiéry reaction [27] allowed us to confirm the polysaccharidic nature of the fibrillar layer (Fig. 9). The refringent lipid material observed '*in vivo*' appears in ultrathin sections uniformly stained and generally homogeneous. (Fig. 2). It forms numerous appressed droplets in the lumen of the TLS or in close contact with it (Figs. 4 and 7). It also forms several thimble-shaped layers which surround each cell and adhere to those of neighbouring cells, forming the matrix of the colony (Figs. 2 and 3). Each of these layers appears in ultrathin section after double fixation with glutaraldehyde and OsO_4 as a string of droplets of various size, connected by thin strands of an apparently homogeneous material (Fig. 2). Treating the samples with ruthenium red *en bloc* immediately after OsO_4 fixation showed that these thin strands have the same trilaminar structure as the TLS of the cell (Figs. 6–8). This finding, at first sight, seems to confirm the statement of Schnepf [16] that these surrounding layers are the successive TLS of each cell generation. However, it should be noted that each cell is usually surrounded by 3 (Fig. 4) or more layers (up to 6), and thimble-shaped layers surrounding more than two cells are only rarely observed. Thus, we think that each cell can secrete several successive TLS. This hypothesis is further supported by the existence of an analogous stacking of trilaminar layers at the surface of some isolated liverwort spores or pollen grains [28].

In the central part of the colony no positive Thiéry staining is observed between the successive TLS, which suggests a rapid disorganization and dissolution of the successive polysaccharidic layers. The assembly of the TLS probably follows the process described in other algae by Atkinson [17]. We also observed in many cells the presence of fragments of membranes appressed under the internal polysaccharidic wall (Fig. 3). Analogous membranes appear numerous in some cells in vacuole-like structures (Figs. 3 and 5). The relations between these membranes and their role in wall formation remains to be ascertained.

As previously observed [16], that part of the TLS situated in front of the apical part of the cell does not contain oil droplets. Instead, its external surface bears irregular fibrillar structures orientated roughly perpendicular to the surface. Our observations show that these structures are more conspicuous in the samples treated *en bloc* by ruthenium red (Figs. 3, 5–8). This fibrillar material distends the outermost trilaminar layers, which are frequently disrupted at the apical pole of the cell (Fig. 5). Some fragments of these external TLS are frequently seen in the sections, still carrying on one side fibrillar material which therefore appears to be attached to the TLS (Fig. 8). With ruthenium red treated samples, one can see that this more or less disorganized fibrillar material forms an almost continuous area around the colony (Fig. 3). This envelope is barely visible in samples not treated by ruthenium red (Fig. 2). The staining of the fibrillar material after ruthenium red treatment is in accordance with its acid polysaccharide nature.

In addition to the external lipids, some oil droplets are observed in the cells. According to our observations, their presence seems to be related to a special physiological state of cellular metabolism, since they are abundant in some cells but absent from others (Fig. 2).

In vivo *Raman microprobe studies*

The oily cytoplasmic droplets show the same staining reactions as those of the lipid material (Fig. 2) saturating the outer wall and giving rise to extracellular globules. This observation is consistent with the assumption that hydrocarbons accumulate not only in the TLS but also in the cells. However, as already emphasized, no direct evidence concerning the occurrence of intracellular hydrocarbon accumulation in *B. braunii* has been obtained up to now. In order to gain further information about hydrocarbon distribution the colonies were examined using a Raman microprobe. This recent technique [29] provides the opportunity to obtain the Raman spectrum of a limited volume

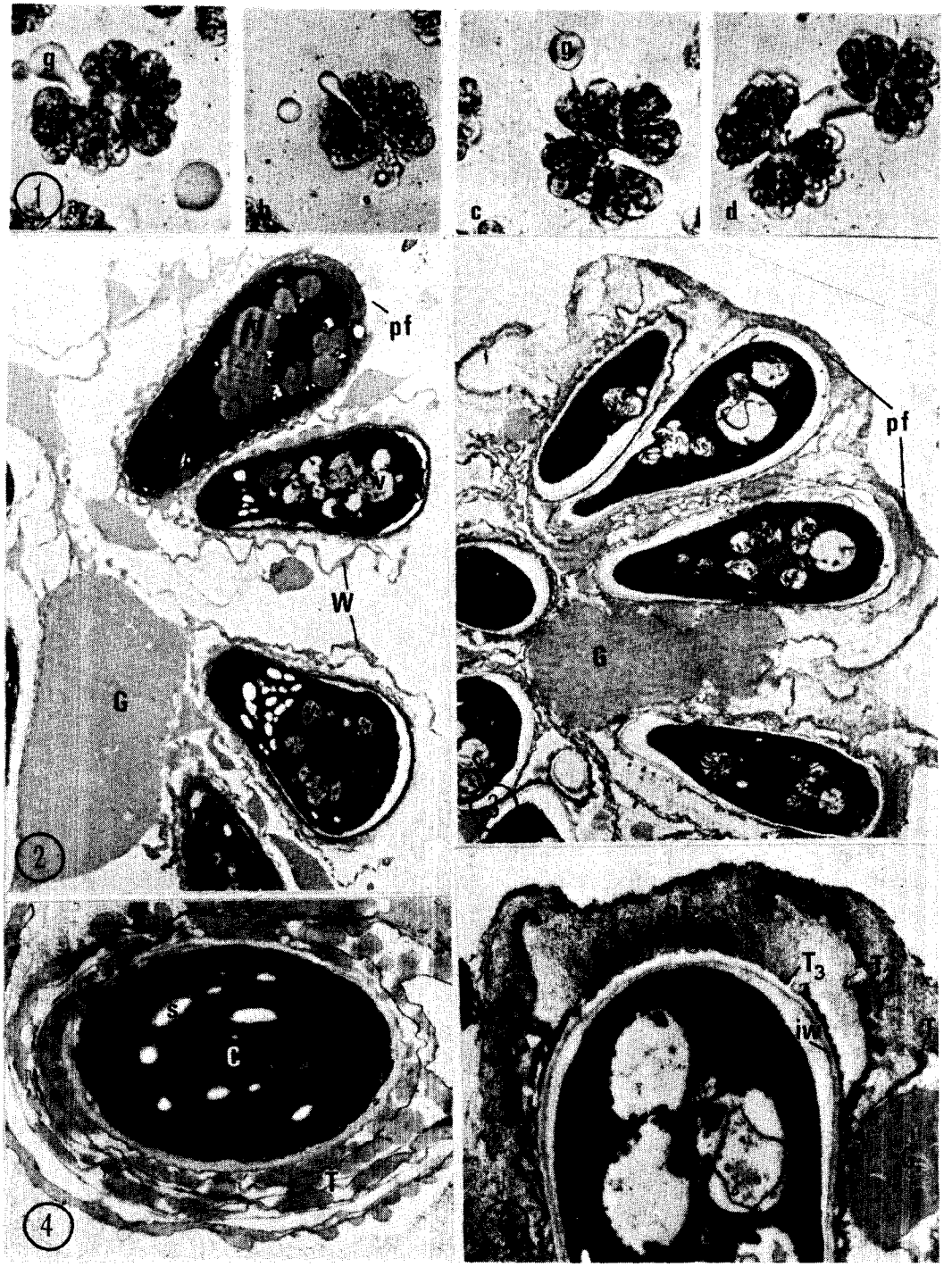
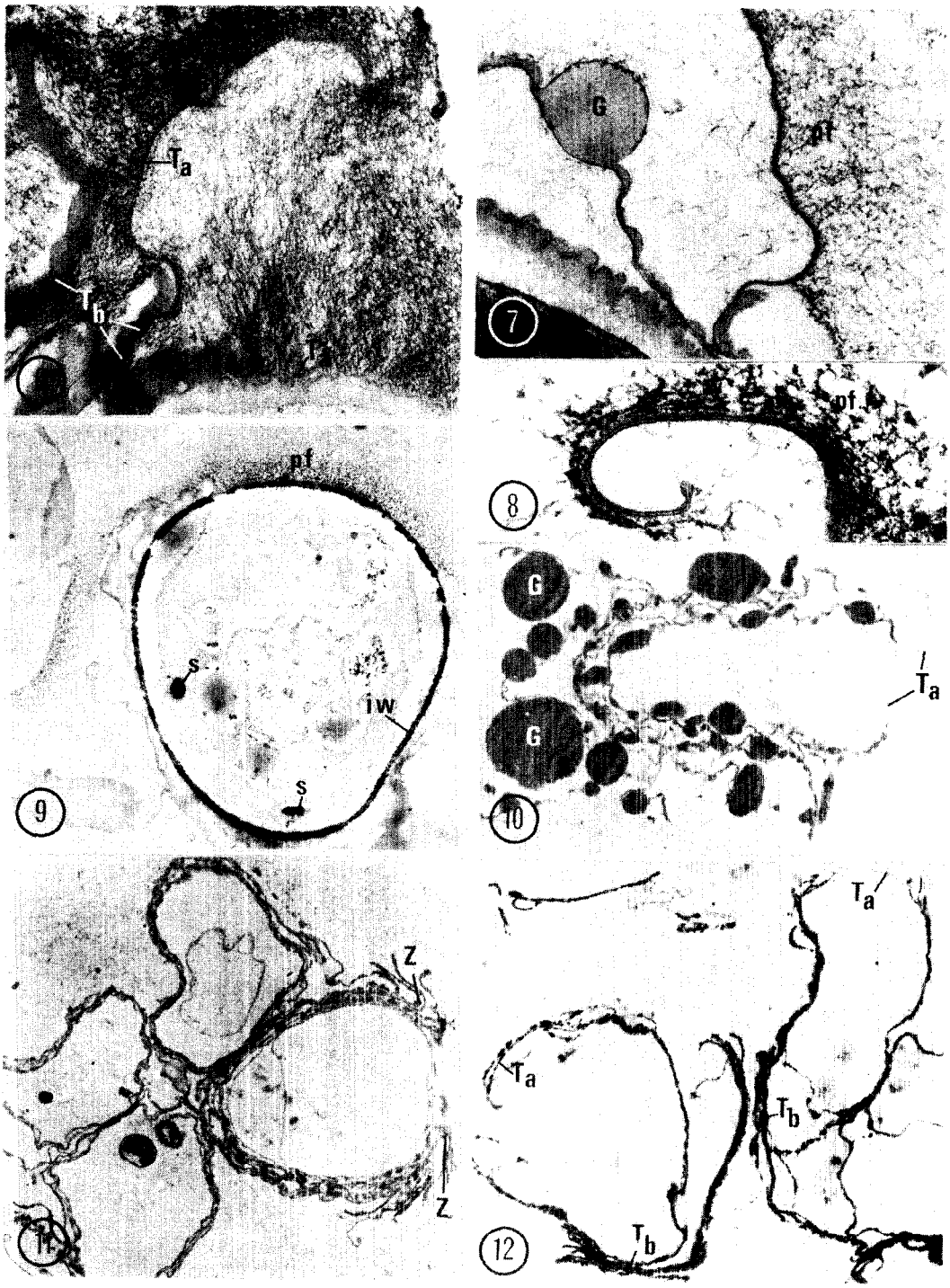


Fig. 1(a–d). Light micrographs of colonies of *B. braunii* observed *in vivo*. Note the refringent globules of hydrocarbons (g), still largely connected to the colonies in a, b; free or nearly free in c. In d, two clusters of cells are bound by a strand of refringent material. ×1080. Figs. 2–5. Electron micrographs. Fig. 2. A colony fixed by glutaraldehyde and OsO_4. The cells are surrounded by several outer wall layers (W), the more external of which are disrupted at their apical end, where the polysaccharidic fibrils (pf) are hardly visible. One of the cells is filled with internal hydrocarbon globules (g), the other contain different vacuole types (v). ×5400. Fig. 3. A colony fixed by glutaraldehyde and OsO_4–ruthenium red. The peripheral polysaccharidic substances (pf) are much more conspicuous than in Fig. 2, and appear as an almost continuous layer around the colony. After this treatment, the external globules (G) are somewhat distorted. ×5400. Fig. 4. Transverse section in the basal part of a cell. The cytoplasm is entirely occupied by the chloroplast (C). Note the successive hydrocarbon-bearing trilaminar sheaths (T) which surround the cell, (s) = starch. ×18 450. Fig. 5. Longitudinal section of the apical part of a cell. Three successive trilaminar sheaths are clearly visible; two of them (T_1, T_2) are disrupted in their apical part, the last one (T_3) is still directly appressed on the internal wall (iw). The ruthenium red stained material (pf) appears as fibrils orientated perpendicularly to the apical part of each successive TLS. ×14 400.



Figs. 6–12. Electron micrographs. Fig. 6. Detail of the transition zone between hydrocarbon-containing (Tb) and fibril-carrying (Ta) TLS. ×29 700. Figs. 7 and 8. Two details of the TLS. In Fig. 7, a part of a TLS is filled with hydrocarbon inclusions, the other is devoid of such inclusions and bears fibrils (pf) on one side. In Fig. 8, the extremity of a TLS is rolled up as frequently observed in the sections. ×41 400. Fig. 9. Thiéry reaction for polysaccharides. Note that only the internal wall (iw), the apical fibrils (pf) and chloroplastic starch (s) give a positive Ag grain contrast. ×10 800. Fig. 10. Sample of a crude 'outer wall' fraction. Numerous hydrocarbon droplets (G) fill the successive TLS, except in their apical part (Ta) where they appear very thin. ×5400. Fig. 11. 'Outer wall' fraction after extraction into acetone. Hydrocarbon droplets are removed from the trilaminar sheaths. Note the abrupt fold of the disrupted sheaths in the transition zone (Z). ×4500. Fig. 12. 'Outer wall' fraction after acetolysis. The general outline of the wall is conserved. The successive sheaths appear attached together in their basal part (Tb). The apical part (Ta) remains separate and appears thinner than the basal part. ×8100.

(*ca* 1 μm^3) directly on the living cell. The Raman spectra of various types of globules (free globules, globules bound to outer walls, globules connecting two colonies) were thus recorded and compared with those of the total hydrocarbon fraction of the alga (extracted and purified according to standard methods). The reference spectrum (Fig. 13) is consistent with the occurrence of long-chain unsaturated hydrocarbons, particularly the 2800–3000 cm^{-1} region and the 1650–1670 band (double bond stretching). The spectra of the extracellular globules (Fig. 13) appear identical to the reference spectrum, not only with respect to band shape and frequency, but also in terms of the relative intensities of the bands. The oily lipidic material arising from the outer wall, and building up the extracellular globules, is thus shown to consist nearly exclusively of hydrocarbons. The specific intensity of such characteristic hydrocarbon bands ($\nu_{C=C}$, ν_{C-H}) is low compared with that of the other classes of lipids (e.g. $\nu_{C=O}$, ν_{OH}). Hence it is possible to ensure that the latter compounds occur in negligible amount within the globules.

The size of the intracellular oily droplets is just sufficient to allow direct *in vivo* examination and their spectra (Fig. 13) show some background due to the fluorescence from the chloroplasts. Nevertheless, it is clearly seen that these spectra are also identical with the reference one.

The above findings thus provide conclusive evidence on the occurrence of two distinct sites where hydrocarbons accumulate in *B. braunii*. Moreover, no noticeable amounts of other classes of lipids are detected along with hydrocarbons, which thus appear to possess specific sites of accumulation in green active cells. (Previous observations on old carotenoid-rich cultures suggested that the carotenoids accumulate in the same sites than hydrocarbons [14, 15]. But, in the present study, only green cells entering the stationary phase, and without noticeable accumulation of carotenoids, were examined.)

Separation and analysis of internal and external hydrocarbon pools

The spectroscopic study does not provide precise information about the nature of the hydrocarbons accumulating in the internal (cytoplasmic inclusions) and external (outer walls) sites. Likewise the quantitative distribution of the total hydrocarbon fraction between these locations was not determined. A mechanical treatment of the colonies, which does not result in

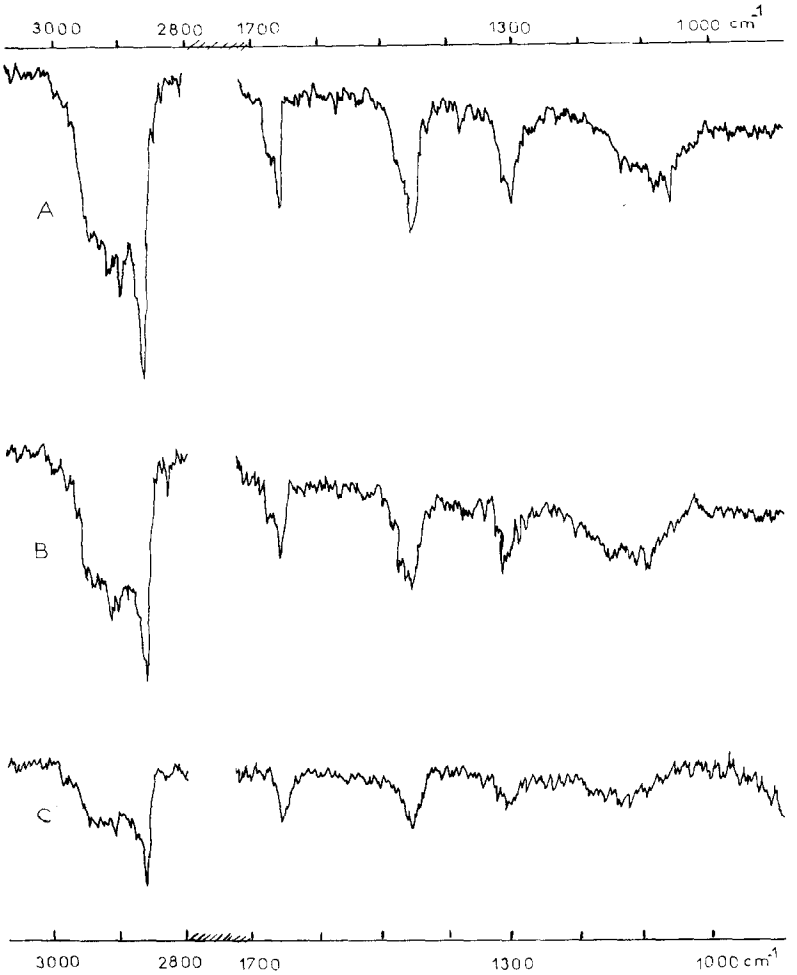


Fig. 13. Spectroscopic study of the sites of hydrocarbon accumulation using a Raman microprobe. (A) Reference spectrum (total hydrocarbon fraction extracted from the culture). (B) *In vivo* spectrum of an external globule. (C) *In vivo* spectrum of a cytoplasmic inclusion.

any cell disruption allowed us to separate, after centrifugation, the following fractions: the supernatant ('outer walls' fraction), which comprises exclusively (Fig. 10) globules together with hydrocarbon-saturated outer wall fragments, and appears as a white cloudy liquid and the pellet ('cells' fraction) which consists of whole living cells still enclosed in their wall and of very few globules.

These fractions were subjected to successive extractions with solvents of increasing polarity. Such a treatment causes any class of lipids to be fractionated according to the following parameters: the localization within the cell (selective permeability of membranes towards various solvents), and the strength of the interactions with other cell constituents [30–34]. In the case of non-polar lipids such interactions are weak. Accordingly, it can be assumed that location is the main parameter which controls the efficiency of hydrocarbon extraction with different solvents and an appropriate extraction scheme was designed (hexane; Me_2CO; $CHCl_3$–MeOH; H_2O–MeOH–KOH) for *B. braunii*.

The hydrocarbons of the 'outer wall' fraction are completely recovered after a short extraction in hexane. The hydrocarbon content of subsequent extracts, obtained with more polar solvents, is lower than the minimal amount detectable using GLC. The very ready extraction of the hydrocarbons from the 'outer wall' fraction would result from their location outside the plasmalemna, and reveals also the absence of noticeable physicochemical interactions with the other constituents of the TLS. However, analysis of the culture medium separated before mechanical treatment, affords a small amount of hydrocarbons (corresponding to free globules, and readily extracted by hexane) which account for only 3.2% of the hydrocarbons recovered from the 'outer wall' fraction. This result shows that the external hydrocarbons are normally associated with the colonies; this is consistent with the location of most of the hydrocarbon droplets within the lumen of the TLS or in close connection with it, as suggested by ultrastructural observations (Fig. 7). It is also to be noted that the ready extraction of *B. braunii* external hydrocarbons may be compared to the similar behaviour of higher plant cuticular waxes [34, 35]. Hydrocarbon extraction from the 'outer wall' fraction results in the almost complete elimination of the globules and of the oily thickenings (Fig. 11), and principally the trilaminar framework of the outer walls remains.

From the 'cell' fraction a significant amount of hydrocarbons is rapidly extracted by hexane. It is thought to originate from the globules, and from the outer walls, still present in the fraction. This assumption is confirmed by chemical analysis (exactly the same qualitative and quantitative hydrocarbon distribution to that in the 'outer wall' hexane extract being observed). But, in marked contrast with the 'outer wall' fraction the $CHCl_3$–MeOH extract, in this case, contains hydrocarbons. These hydrocarbons should correspond, therefore, to those occurring in the cytoplasmic oily droplets. It cannot be ruled out that a small part of the $CHCl_3$–MeOH extract can originate from hydrocarbons bound to membranes; however, such compounds were always shown to be present in very small amounts [31, 34] and their contribution should be negligible. We observed also that *B. braunii* cells, unlike some microorganisms [37], do not provide any additional hydrocarbons on alkaline treatment. Similarly [46] no increase of neutral lipid yield was obtained when *B. braunii* was extracted with an acidic solvent.

The selective extraction allowed us to confirm the occurrence of two distinct accumulation pools and to separate the external hydrocarbons from the internal ones. Analysis of the two hydrocarbon mixtures thus obtained (Table 1) showed that the major hydrocarbons observed in the external and internal site of accumulation *are the same*. They comprise, as shown using PLC on $AgNO_3$–Si gel and GC–MS, a series of unbranched diene derivatives of odd carbon number, ranging from C_{25} to C_{31} and a C_{29} triene. However, the relative abundance of these long-chain hydrocarbons varies in relation to their localization and, in the internal pool, shorter hydrocarbons are more abundant than in the external pool. (Similarly the yeast *Candida utilis* shows different hydrocarbon fractions

Table 1. Analysis of the two hydrocarbon pools occurring in *B. braunii*

	Relative abundance (%) of the pools(*)	Hydrocarbon content of each pool (as % of culture dry wt) (*)	Relative abundance * and nature of the main hydrocarbons present in each pool (†)				
			$2\Delta C_{25}$	$2\Delta C_{27}$	$2\Delta C_{29}$	$2\Delta C_{31}$	$3\Delta C_{29}$
External pool (‡)	3	0.5	trace	14	45	21	20
External pool (§)	92	13.8	trace	14	45	21	20
Internal pool (‖)	5	0.75	3	33	38	17	9

* Mean figures from 3 experiments.

† Only the main hydrocarbons are listed here, taken together they account for at least 95% of each hydrocarbon fraction. They exhibit MS spectra very closely related to those already reported for the hydrocarbons of green active *B. braunii* [22]. According to previous reports [25], $2\Delta C_{27}$ = heptacosa-1,18-diene, $2\Delta C_{29}$ = nonacosa-1,20 diene, $2\Delta C_{31}$ = hentriaconta-1,22-diene.

‡ Hydrocarbons of the free globules present in the culture medium (separated before mechanical treatment and extracted by hexane).

§ Hydrocarbons saturating the outer walls and giving rise to the associated globules (hexane extracts).

‖ Hydrocarbons of the cytoplasmic inclusions ($CHCl_3$–MeOH extract).

in the cell wall, the plasmalemna and the cell content [36]. On the contrary two distinct hydrocarbon pools, with identical composition, are observed [37] in the bacterium *Sarcina lutea*.) The quantitative differences between the external and internal pools were not reflected in the corresponding Raman spectra which are essentially the same. This can be attributed to the close relation existing, in terms of band shape and frequency, between the spectra of all the major hydrocarbons of *B. braunii*; such spectra should mainly exhibit differences with regard to relative band intensities. However, the changes in quantitative composition observed between the internal and external pool do not lead to a great variation of the average chain length and of the average degree of unsaturation which are respectively, 29.14 and 2.2 for external hydrocarbons, 28.56 and 2.09 for internal ones. Therefore it is not surprising that very closely related Raman spectra were observed for the two sites of hydrocarbon accumulation.

The bulk of the hydrocarbons occurring in *B. braunii* (*ca* 95%) is located in the external pool, i.e. in the outer walls and associated globules. Owing to this extracellular location the hydrocarbon level within the cells is low (0.75% relative to culture dry wt). Such a level is comparable to those generally reported for green unicellular algae [38]. The localization in the outer wall of the bulk of their hydrocarbons allow *B. braunii* cultures to exhibit both an unusually high hydrocarbon content (15% of the dry wt) and a normal level inside the cells. However, the abundance of the cytoplasmic inclusions changes substantially from one cell to another. The value quoted for hydrocarbon concentration in the internal pool is therefore a mean value relating to the whole culture, and some cells will exhibit higher internal hydrocarbon concentrations. The results reported here on the composition of the external pool are close to those previously reported [22–24] after total extraction of *B. braunii* (and which do not discriminate between different hydrocarbon localizations); this point is consistent with the prominent contribution of the derivatives located in the external pool to the total hydrocarbon fraction of the alga.

Comparison with other organisms

Microscopic algae generally show a hydrocarbon pattern with odd/even predominance and most of them have C_{15} or C_{17} *n*-alkanes as major hydrocarbons. A bimodal distribution, with chain-length maxima at C_{15}–C_{17} and C_{27}–C_{29}, is also often observed [38]. Therefore *B. braunii* does not exhibit a completely unusual pattern and it is mainly its very high hydrocarbon content which distinguishes this alga from other species. Regarding hydrocarbon localization in microscopic algae, it does not seem that any studies have been reported up to now. The occurrence of lipids was shown in the TLS of some green algae but a complete analysis of this fraction was not carried out [17].

Hydrocarbon composition and location in yeast are fairly well documented but these organisms do not exhibit any common feature with *B. braunii*. Their hydrocarbons are mainly located in the plasmalemna [36], no dominance of odd chains is evident and hydrocarbon concentrations are always very low [38].

The ready extraction of *B. braunii* external hydrocarbons is comparable to that observed with higher plant epicuticular waxes. This is mainly due to the similar location of such waxes (outside epidermal cells) and of *B. braunii* external hydrocarbons. In higher plant epidermis, the bulk of the hydrocarbons is present in the cuticle [39, 40]; similarly 95% of *B. braunii* hydrocarbons accumulate in the outer wall. Odd, unbranched hydrocarbons with chain length ranging from C_{25} to C_{33} are widespread in higher plants [41] where they can account for up to 90% of cuticular waxes [42]; moreover C_{29} and C_{31} compounds are commonly the major constituents of cuticular hydrocarbons. Thereby *B. braunii* exhibits a hydrocarbon pattern very closely related to that observed in plant cuticles.

The TLS forming the outer wall of *B. braunii* closely resembles that observed [17–19] in some strains of other green algae (*Chlorella fusca, Scenedesmus quadricauda* and *Prototheca moriformis*), although in such algae no hydrocarbons saturating the TLS are reported. The presence of a sporopollenin-like material in the TLS of the latter species was shown by Atkinson *et al.* [17]. Sporopollenin, the constituent of the coating of pollens and spores, is characterized by an extreme resistance to chemical degradation [43]; in contrast to other components of cell walls it can withstand prolonged acetolysis [44]. In order to test the occurrence of sporopollenin in *B. braunii* the 'outer wall' fraction was acetolysed after hydrocarbon extraction. The TLS of *B. braunii* is thus shown to contain an acetolysis-resistant material (Fig. 12); moreover IR examination of the residue affords a spectrum similar to those previously reported for sporopollenins of various origins [17]. On account of some analogies between cutins and sporopollenins [45], the outer wall of *B. braunii*, with its TLS containing sporopollenin-like material and its accumulation of wax-related hydrocarbons, exhibits interesting relationships with the cuticle of the epidermal cells of vascular plants.

EXPERIMENTAL

The *B. braunii* strain used throughout this work was obtained from the Cambridge Culture Collection (No. LB 807/1 DROOP, 1950, Maddingley Bricks Pits, U.K.).

Culture conditions. The alga was grown in a modified CHU 13 medium [22]; 4-fold strength, chelated with citric acid and including trace elements. The concns were as follows (g/l.): KNO_3 (0.2), K_2HPO_4 (0.04), $MgSO_4 \cdot 7\ H_2O$ (0.1), $CaCl_2 \cdot 6\ H_2O$ (0.08), Fe citrate (0.01), citric acid (0.1); micro elements: B, Mn (both at 0.5 ppm), Zn (0.05 ppm), Cu, Co, Mo (0.02 ppm). The pH was adjusted to 7.5 with KOH before autoclaving. Growth was carried out at 20° and 2000 1*x* (12 hr illumination per day). All cultures were routinely harvested, by centrifugation, when they entered the stationary phase. The cultures were unshaken and non-aerated.

Electron microscopy. Algae were collected by centrifugation and fixed for 2–15 hr in 1% glutaraldehyde buffered at pH 7.4 with 0.1 M cacodylate buffer, washed in the same buffer and post-fixed for 1–5 hr in 1% OsO_4 in cacodylate or veronal–acetate buffer (0.1 M). In some cases, 0.15% of ruthenium red was added to the OsO_4 fixative. The samples were embedded in agar, dehydrated in EtOH, transferred to propylene oxide and embedded in Araldite. Sections were

double stained with uranyl acetate and Pb citrate and observed on an EMU 300 Philips electron microscope. Specific staining of polysaccharides was performed according to the technique of ref. [27]: sections were treated for 20 min with 1% periodic acid, washed with dist H_2O, floated for 72 hr on 1% thiosemicarbazide in 1% aq. HOAc, washed with 10% aq. HOAc and with dist H_2O, and then stained with 1% aq. Ag-proteinate for 30 min.

Chemical examination of the various fixation baths showed that they did not contain any significant amount of hydrocarbons; the algal hydrocarbons are thus retained during the successive treatments of fixation and dehydration.

Extraction and analysis of internal and external pools of hydrocarbons. Colonies were resuspended in H_2O and ground in a Thomas tissue homogenizer for 5 min at max speed. Microscopical examination showed that such treatment did not cause any disruption of the cells. Grinding resulted in the separation of some outer walls impregnated with hydrocarbons and the liberation of numerous free hydrocarbon globules. After centrifugation (500 g, 10 min) the globules and the freed outer walls were recovered in the supernatant which appeared as a white cloudy liquid. The grinding treatment was repeated $\times 7$ and the supernatants combined. The culture was thus separated into the 'outer wall' fraction and the 'cell' fraction. These two fractions were dried under vacuum and extracted by solvents of increasing polarity according to the following sequence: hexane 5 min; hexane 10 min; Me_2CO 10 min; $CHCl_3$–MeOH (1:1) 6 hr; KOH 10% in MeOH–H_2O (4:1) 2 hr. All extractions, except the last one, were carried out at room temp. with efficient stirring. The extracts were separated from the residue by centrifugation and the solvent removed under vacuum. During alkaline extraction the medium was refluxed for 2 hr; after cooling some H_2O was added, the MeOH removed and the resulting soln was then neutralized and extracted with Et_2O.

The dried extracts were fractionated on 30 g of neutral Al_2O_3 (grade II) prewashed with hexane. Elution with hexane (200 ml) afforded the hydrocarbons, if any, present in the extract. Hexane eluates were then analysed by GLC (10% Apiezon L and 10% SE 30, at 280° isothermal). Before analysis some *n*-tricosane was added as int. standard, in order to determine the amount of hydrocarbons in each extract. Only two extracts had a significant hydrocarbon content: the first hexane extracts of the 'cell' and 'outer wall' fractions (external hydrocarbons); and the $CHCl_3$–MeOH extract of the 'cell' fraction (internal hydrocarbons). The relative abundance of the major constituents of these distinct hydrocarbons pools were determined using GLC.

Both hydrocarbon mixtures were further examined by PLC on $AgNO_3$–Si gel, (1:9) (0.5 mm thick, *ca* 10 mg of sample applied to the layer, detection by spraying rhodamine 6G in Me_2CO and examining under UV light, solvent system hexane–Et_2O (9:1), products eluted with dry Et_2O from the adsorbent). Dienic hydrocarbons were thus shown to account for the bulk of internal and external hydrocarbons, but a spot ascribable to a trienic derivative was also observed. Examination by GC–MS (SE 30, 10%) was also carried out and the same major hydrocarbons (2Δ C_{25}, 2Δ C_{27}, 2Δ C_{29}, 2Δ C_{31} and 3Δ C_{29}), but with different relative abundance, were shown to be present in the two pools. The MS obtained were in agreement with previous findings [22–25] on the structure of the major hydrocarbons obtained after total extraction of *B. braunii* and they were very closely related to those previously reported for the main hydrocarbons of green active *B. braunii* [22].

Occurrence of sporopollenin-like material in outer walls. The 'outer wall' fraction, obtained as above, was extracted with various organic solvents: Me_2CO, MeOH, $CHCl_3$–MeOH (2:1); and then acetolysed, 10 min at 100° in conc H_2SO_4–Ac_2O (1:9), according to ref. [17]. Ultrastructural examination of the residue shows that *B. braunii* contains an acetolysis-resistant material. IR of the residue afforded spectra similar to those reported for samples of spropollenin from various origins: ν_{max}^{CsBr} cm^{-1}: 3480, 2920, 2850, 1670, 1475, 1200, 1040.

Raman microprobe. Spectra were obtained with a Jobin-Yvon Molecular Optic Laser Examiner. This microanalysis technique is based upon vibrational spectra of materials to furnish information on the identity of molecular constituents of microsamples and their distribution [29]. Photons generated by a laser are used to excite the sample causing Raman lines of the compounds to be emitted. Using these lines each compound can be identified or (and) located by obtaining a micrographic image which gives its distribution in the sample (Raman image). The basic configuration of the instrument results from the coupling of a light microscope to a spectrometer/spectrograph equipped with sensitive monochannel and multichannel optical detection systems. The laser beam can be focused into a microscopic spot (point illumination) to analyse a volume of sample down to 1 μm^3 or spread over a large area (global illumination) to obtain a Raman image. The analysis is performed directly inside culture media and does not require special preparation of samples.

Acknowledgements—This work was supported by the CNRS as a part of the Programme Interdisciplinaire de Recherche et de Développement de l'Energie Solaire. We thank Mr. B. Rousseau (electron microscopy), Mr. J. Barbillat and J. Laureyns (Raman spectrometry) for technical assistance. We are grateful to Prof. Giraud (E.N.S.) for reading the manuscript.

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