Phytochemistry, Vol. 22, No. 2, pp. 389–397, 1983.
Printed in Great Britain.

0031-9422/83/020389-09$03.00/0

THE RESISTANT POLYMER OF THE WALLS OF THE HYDROCARBON-RICH ALGA *BOTRYOCOCCUS BRAUNII**

C. BERKALOFF†, E. CASADEVALL, C. LARGEAU§, P. METZGER, S. PERACCA and J. VIRLET‡

Laboratoire de Chimie Biorganique et Organique Physique, E.R.A., C.N.R.S. 685, E.N.S.C.P., 11 rue Pierre et Marie Curie, 75231 Paris, Cedex 05, France; †Laboratoire de Botanique-Cytophysiologie Végétale, L.A., C.N.R.S. 311, E.N.S., 24 rue Lhomond, 75231 Paris, Cedex 05, France; ‡Département de Physicochimie, C. E. N., Saclay, B.P. 2, 91190-Gif sur Yvette, France

(*Revised received* 25 *June* 1982)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; hydrocarbon-rich alga; outer wall; electron microscopy; IR spectroscopy; ^{13}C NMR of solids; nature of the resistant polymer; comparison with sporopollenins.

Abstract—The outer walls of the green alga *Botryococcus braunii* (main sites of hydrocarbon production and accumulation) show a complex constitution. They comprise a biopolymer highly resistant to non-oxidative degradation. The resistant polymer accounts for *ca* 9% of the cell dry wt and appears, along with hydrocarbons, as one of the major constituents of the alga. In addition to chemical resistance, *B. braunii* polymer exhibits other properties: mode of deposition and fluorescence, often used to identify sporopollenins. (Class of wall components generally regarded as originating from polymerization of carotenoid derivatives.) Nevertheless further studies, using IR spectroscopy and high resolution ^{13}C NMR of solids, along with determination of elemental composition and unsaturation levels, indicate that the bulk of the resistant polymer from *B. braunii* outer walls does not derive from carotenoids; accordingly it cannot be considered, in this respect, as a sporopollenin. In fact the information obtained on the structure of this important constituent of the alga is consistent with its formation via oxidative polymerization of *B. braunii* dienic hydrocarbons.

INTRODUCTION

The green unicellular alga *Botryococcus braunii* is characterized by unusually high hydrocarbon levels (15–75% of dry wt) [1, 3]; although hydrocarbons are produced by a large range of photosynthetic organisms [4], they generally occur in minute amount (*ca* 0.1% in most algae) [4, 5]. In fact, *Dunaliella salina* is the only other hydrocarbon-rich alga known at the present time beside *B. braunii* [6]. On the other hand the latter, or at least a species which does not differ in any morphological feature from the existing *B. braunii*, was implicated in the formation of kerogens (insoluble organic matter from sedimentary rocks) showing a large oil potential [7–18].

Previous work [19] allowed us to show that *B. braunii* hydrocarbons accumulate in two distinct sites: cytoplasmic inclusions and cell outer walls. In addition to a polysaccharidic wall, *B. braunii* cells are surrounded by several outer walls where accumulation of most hydrocarbons (*ca* 95%) results in the formation of globules sometimes larger than the cells themselves. Moreover, it appears [20] that no hydrocarbon transfer takes place from the internal pool to the external one; accordingly the bulk of *B. braunii* hydrocarbons is not only stored but also produced within the outer walls. These walls exhibit a rather complex organization (Fig. 2.12 in ref. [19]) and comprise two distinct zones: the first one, Ta, in the apical part of the cells, is trilaminar and bears polysaccharidic fibrillar material. The second one, Tb, corresponds to the basal part of the cells; there hydrocarbons accumulate as globules and the trilaminar organization is no longer visible.

The important role that outer walls play in the high hydrocarbon production typical of *B. braunii* prompted us to study their constitution. In the present work we will examine chiefly the nature of the structural material building up the outer walls. This material which appears, along with hydrocarbons, as one of the major constituents of *B. braunii* is highly resistant to chemical treatments and shows some of the features typical of sporopollenins. (Sporopollenins would constitute a class of bio-polymers originating from oxidative polymerization of carotenoids and/or carotenoid esters [21–31]. Such polymers make up the outer wall, exine, of spores and pollens [32–36]; but they also occur in some algal [26, 37–43], bacterial [44] and fungal [26–28, 45, 46] walls.) However, further studies, using mainly IR and high resolution ^{13}C NMR of solids, indicate that the structure and origin of *B. braunii* outer wall resistant polymer differ from those generally associated with sporopollenins. On this basis the nature of the biosynthetic pathway yielding to this important constituent of the alga is discussed and, finally, the validity of the criteria often used to characterize sporopollenins is examined.

RESULTS AND DISCUSSION

Common features of B. braunii *outer walls and of sporopollenin-containing structures*

Resistance to chemical degradation. An unusually high resistance to non-oxidative treatments, and especially to

*Part 3 in the series "Hydrocarbon Formation in the Green Alga *Botryococcus braunii*". For part 2 see Largeau, C., Casadevall, E. and Berkaloff, C. (1980) *Phytochemistry* **19**, 1081.

§Author to whom correspondence should be addressed.

acetolysis, is one of the most conspicuous properties of sporopollenins [37, 47, 48]. In fact, as shown in a preliminary study [19], *B. braunii* outer walls are able to withstand acetolysis. However, it is documented [40] that some algal wall components can survive direct acetolysis while they are completely degraded by saponification (therefore, they do not satisfy the resistance conditions required to be classed as sporopollenins). Consequently, we have studied the effect, upon *B. braunii* outer walls, of the various chemical degradations usually carried out [32, 49] in order to identify and to isolate sporopollenins. (extraction with organic solvents, saponification, treatment by phosphoric acid). The purpose of this investigation was, first to ascertain whether a resistant polymer was actually present and, secondly, to gain information on the other outer wall constituents. To this end outer walls were removed from the cells [19] and, after each chemical treatment, examined using electron microscopy, elemental analysis and IR spectroscopy.

Extraction of cell outer walls with organic solvents results in the complete elimination of hydrocarbon globules. However, the basal zone of the wall Tb remains distinct from the apical zone Ta. The latter does not undergo any structural variations, but elimination of polysaccharidic fibrils; it appears trilaminar with two electron-opaque layers (55 Å thick) enclosing an electron-transparent centre (50 Å thick). In sharp contrast zone Tb, initially carrying hydrocarbon globules, is less regular and substantially thicker (up to 1000 Å); moreover, it does not show clearly a trilaminar organization. The appearance of the two zones remains unchanged following the subsequent treatments. Therefore, if one excludes hydrocarbon globules and polysaccharidic fibrils, the outer walls retain not only their shape but also their original size and organization after chemical degradation.

After a drastic fragmentation, the outer walls were also subjected to the above treatments; their resistance, as well as the final ultrastructure of zones Ta and Tb, are identical with that of intact walls. We examined, similarly, the behaviour of whole cells: following phosphoric acid treatment, the cell content and the internal polysaccharidic wall are entirely removed; as a result, the residue thus obtained is indistinguishable from the one originating from treatment of intact isolated outer walls.

B. braunii outer walls are able to survive the successive chemical degradations generally used for sporopollenin isolation. But the latter are not the only class of plant biopolymers exhibiting such a resistance. Lignins can also withstand the above treatments; however, most lignins are soluble in dioxane while sporopollenins are insoluble [50]. In fact, the resistant polymer from *B. braunii* outer wall is actually insoluble in this solvent; moreover, it does not show any staining reaction typical of lignins (see Experimental).

It is well documented that sporopollenins are altered by strongly oxidizing agents [49, 51–53]. This affords a simple way to distinguish sporopollenins from silica which is an important constituent of some algal walls; moreover, in these walls, silica is sometimes associated with a sporopollenin-type resistant polymer [37, 54]. However, the resistant polymer from *B. braunii*, which is completely degraded following chromic acid action, does not contain any silica.

In summary, if one considers the effect of all the above chemical agents, it appears that *B. braunii* resistant polymer shows the same properties as the wall constituents classed as sporopollenins. In addition to hydrocarbons and resistant polymer, *B. braunii* outer walls comprise numerous compounds which are gradually removed following chemical treatment. The resulting variations in IR spectra and elemental composition (Table 1) provide some information on the most abundant of these compounds.

Saponification causes a sharp decrease in nitrogen level,

Table 1. Elemental composition (%) of classical sporopollenins and of *B. braunii* (*B.b.*) outer walls at various degradation stages

Sample	Origin	Chemical treatments	C	H	O	N	P	Ash	Formula†
A	*B.b.* outer wall	1	61.1	10.2	15	3.85	2.66	7	—
B	*B.b.* outer wall	1+2	61.3	8.35	19	0.92	1.85	8	—
C	*B.b.* outer wall	1+2+3	71.35	10.3	8.8	0.52	0.38	8	$C_{90}H_{156}O_8$*, ‡
D	*B.b.* outer wall	1+2+3+4	69.9	10.25	11.5	0.41	0.23	7	—
E	*B.b.* outer wall	1+2+3+5	71	10.35	8.9	0.51	0.43	8	—
F	*B.b.* outer wall	1+2+3+6	71.1	10.15	9	0.45	0.32	8	—
G	Synthetic sporopollenin	—	66.1	7.2	27	—	—	—	$C_{90}H_{110}O_{28}$ §
H	Synthetic sporopollenin	1+2+3	65.6	7	27.6	—	—	—	—
I	*L. clavatum* spore	1+2+3	62.9	8	26.3	0.12	0.05	3	$C_{90}H_{138}O_{28}$‖, ¶

1, Extraction by organic solvents; 2, saponification; 3, treatment by H_3PO_4; 4, acetolysis; 5, sodium dodecyl sulphate treatment; 6, phenol treatment.

*The resistant polymer of *B. braunii* outer walls is isolated at this stage.

†In order to facilitate comparison with previous results, the formulae are arbitrarily based on a 90 carbon unit [33] and the low levels of N and P are not taken into account.

‡Average values from *B. braunii* samples of various origin: whole cells, intact isolated outer walls, outer wall fragments (low scattering between the samples).

§The elemental composition of synthetic sporopollenin obtained from β-carotene may vary in relation to reaction conditions [27]. Values previously reported: $C_{90}H_{130}O_{27}$–$C_{90}H_{132}O_{32}$ [23, 25–27].

‖Formulae previously reported: $C_{90}H_{144}O_{27}$ [21, 23] and $C_{90}H_{143}O_{25}$ [30, 31].

¶Sporopollenins are isolated at this stage.

along with important changes in the IR spectrum, originating mainly from hydrolysis and elimination of outer wall proteins. A large decrease in the broad band at 3500–3000 cm^{-1} (N–H stretching) and also in the absorption centered at 1650 cm^{-1} (amide I band) occurs and peaks at 1540 cm^{-1} (amide II band) and 1290 cm^{-1} (amide III band) disappear completely.

Treatment with phosphoric acid, which takes place after saponification, results chiefly in a large decrease in phosphorus content, paralleled, in the IR spectrum, by a nearly complete disappearance of the broad band at 1200–950 cm^{-1} (free and bonded P=O stretching, P–O–C stretching).

Variations in the IR spectrum and in elemental composition, which occur during the isolation of the resistant polymer, reveal that *B. braunii* outer walls contain substantial amounts of proteins and phosphorus derivatives*. Such a complex constitution fits well with the occurrence of an active biosynthetic activity, yielding external hydrocarbons [20], within the outer walls.

At the end of successive chemical degradations, similar IR spectra (Fig. 1C) and elemental compositions are obtained from residual outer walls whatever the starting material (whole *B. braunii* cells, intact isolated outer walls or outer walls fragments). Most of the initial nitrogen and phosphorus are removed at this stage; however, small amounts are still present in the final residue. Acetolysis gives way to a further decrease in nitrogen and phosphorus levels (Table 1) but, even after prolonged treatments, such elements do not entirely disappear. Similarly spore and pollen exines retain a low level of nitrogen [56] and phosphorus [57] after chemical degradation.

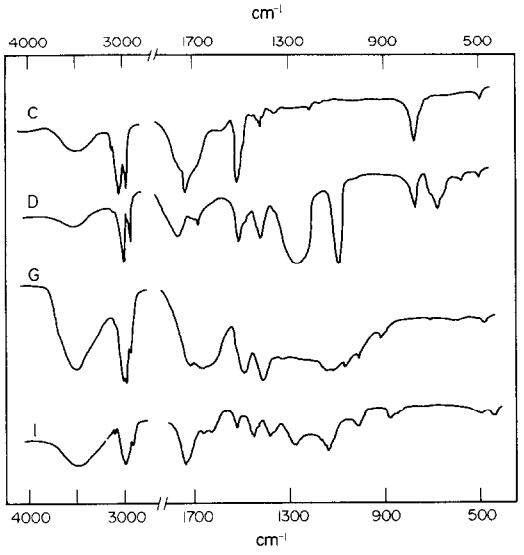

Fig. 1. IR spectra of *B. braunii* resistant polymer and of classical sporopollenins. C, D, G and I as in Table 1 (C, resistant polymer; D, acetolysed resistant polymer; G, synthetic sporopollenin; I, *L. clavatum* sporopollenin.

*In spite of the technique used for *B. braunii* outer wall isolation, a small part of these proteins and phosphorus compounds may derive from the few intact cells and accompanying bacteria possibly present in the outer walls fraction. Large protein levels have been previously observed within the walls of various green algae [55]; however, in these cases, the whole cell wall (comprising the polysaccharidic wall and perhaps, in some species, an external wall) was analysed.

Outer wall organization. Deposition of the resistant polymer. The systematic occurrence of low nitrogen and phosphorus levels in sporopollenins would directly result from the process of deposition characterizing such polymers. Sporopollenins are supposed to be deposited extracellularly on specific surfaces promoting polymerization [35, 58, 59]. In spore exines [60] and also in the inner part of pollen exines, [61–63] sporopollenin would polymerize on lamellae of unit membrane dimension which could be composed of lipoproteins, lipopolysaccharides, mucopolysaccharides or lipids in bilayer configuration [64–69]. Gradual sporopollenin accumulation protects lamella constituents from external chemical agents. Such a deposition accounts for the presence of residual nitrogen and phosphorus derivatives within sporopollenins, in spite of chemical treatments which should degrade any other wall component.

Sporopollenin deposition on the lamellae gives rise to a trilaminar organization and spore exines consist of stacking of such lamellae. However, as sporopollenin accumulation progresses, this organization becomes obscured and can finally disappear, in mature exines, leading then to an apparently homogeneous material [60, 66, 70]. It seems that deposition of substances classed as sporopollenins takes place in a similar manner, within the outer walls of various algae [37, 39–41, 71–73], to yield trilaminar structures. However, algal walls are characterized, in most cases, by the occurrence of only one outer trilaminar sheath (TLS) instead of the stacking typical of exines. It seems that just one clear exception is known at the present time: the subaerial green alga *Phycopeltis epiphyton* [42] which shows a definite resistant multilayered outer wall. Such a wall may also occur in the isolated phycobionts *Trebouxia* [43].

If we consider now the case of *B. braunii* outer walls, the following features are noted. The apical part Ta shows a trilaminar organization identical to the one of algal walls containing a sporopollenin-like constituent. The dimensions of the three layers making up zone Ta are also similar to those observed in various algal TLS [37, 39, 72, 73]. As stressed above, the part of the outer wall carrying initially hydrocarbons (zone Tb) is considerably thicker and does not show a clear internal organization. Accordingly this latter zone could originate from a large accumulation of resistant polymer resulting in the disappearance of the initial trilaminar structure.

The organization of *B. braunii* outer walls is, therefore, related to those observed in algae which are supposed to contain sporopollenin. However, *B. braunii* is characterized by the occurrence of several outer walls surrounding each cell. (Such a feature has been seldom observed up to now and always with algae living in special conditions where the outer walls may provide protection against desiccation [42, 43].)

In unicellular algae it seems that a sporopollenin-like material occurs only when a trilaminar outer wall is present [37]. However, the occurrence of a TLS does not necessarily imply the presence of a resistant polymer. Thus, a parallel examination of the blue-green alga *Gloeocapsa alpicola* allowed us to show that the trilaminar outer walls of this species are completely disorganized following treatment by sodium dodecyl sulphate (SDS) or

phenol (substances able to extract [74, 75] lipopolysaccharides and lipoproteins). In the case of a TLS, made up of a sporopollenin-like material sandwiching an initial lamella, the components of the latter should be protected from external reagents, and treatments by SDS or phenol should not affect the walls. Such behaviour is actually observed from *B. braunii* where the action of SDS or phenol (taking place after phosphoric acid treatment, i.e. after isolation of the resistant polymer) does not result in any variation in outer wall organization, composition (Table 1) and IR spectra. In the case of *G. alpicola*, the lack of resistant polymer suggested by wall disorganization using SDS or phenol was confirmed with the usual chemical agents. Thus, following phosphoric acid treatment, neither organized outer walls nor amorphous resistant polymer are obtained from *G. alpicola* walls.

Resistant polymer level in B. braunii. As stated above the appearance of zone Tb suggests that a large deposition of resistant polymer takes place in *B. braunii* outer walls. Various quantitative studies allowed us to ascertain the above assumption. Thus, it appears that the resistant polymer accounts for *ca* 10% of the whole dry wt in the case of *B. braunii* cultures from various origin.

A large range of sporopollenin levels is observed [25, 36, 49] in pollen (from 1.8 to 32%) and also in fungal spores [27, 28, 46]. Regarding algae, it seems that only one quantitative study has been carried out up to now with *Chlorella fusca*; this species shows a low level (0.6%) of sporopollenin-like material [37]. In sharp contrast, *B. braunii* appears not only as a hydrocarbon-rich species, but shows also a high level of resistant polymer. This result is consistent with the appearance of zone Tb and also with the ability of each *B. braunii* cell to become surrounded by several outer walls. In fact, if one takes into account the presence of ash in the residue following phosphoric acid treatment (Table 1), it appears that the content of resistant polymer is *ca* 9.2%. Moreover, the walls still retain, at this stage, small amounts of nitrogen and phosphorus derivatives which are protected from degradation by the sandwiching polymer layers. The overestimate due to the presence of such residual compounds cannot be accurately assessed; nevertheless we know that it is low. Therefore, it is possible to conclude that the resistant polymer is one of the major constituents of *B. braunii*. Such a feature fits well with previous observations [76], according to which a large part of *B. braunii* biomass is unaccounted for after cell analysis. In fact, the corresponding unidentified constituents should be ascribable mainly to the resistant polymer from the outer walls.

Fluorescence of B. braunii *outer walls*. Fluorescence under UV illumination is also an important feature which can be observed [42–44, 62, 77, 78] from sporopollenins alone (auto-fluorescence), or after treatment with a fluorescent dye, e.g. primuline (secondary fluorescence). The ability of the outer walls to fluoresce was tested under the above conditions. Following excitation by UV (300–400 nm) secondary fluorescence, as well as autofluorescence, was obtained at each stage of degradation from native to acetolysed *B. braunii* outer walls, in the range 450–600 nm.

Identification of the resistant polymer

The above results show that *B. braunii* outer wall polymer satisfies the criteria often used to identify sporopollenins [41, 44, 73] (i.e. resistance to chemical non-oxidative degradation, mode of deposition, UV fluorescence). Sporopollenins are generally supposed to originate from oxidative polymerization of carotenoids and/or carotenoids esters [21 31]; indeed, the occurrence of walls containing a sporopollenin-like polymer is correlated with the presence of carotenoid derivatives in various algal [37] and fungal [27, 28, 46] species. On the other hand it is well-known that *B. braunii* contains carotenoids and that, sometimes, large carotenoid accumulations occur in this alga [79]. All the above findings led us, therefore, to regard *B. braunii* resistant polymer as a sporopollenin. However, such an assumption appears incorrect if one considers the following points.

IR study. The spectrum of *B. braunii* resistant polymer (Fig. 1C) is similar to the spectra reported for various sporopollenins.* On the other hand previous papers [37, 56] stress that sporopollenins can exhibit rather important spectral changes between species. However, in spite of these general variations, the spectrum from *B. braunii* polymer shows some systematic differences with respect to classical sporopollenins. In order to specify such differences, a synthetic sporopollenin was prepared by oxidative polymerization of β-carotene [25 27] and a natural sporopollenin was isolated from *Lycopodium clavatum* spores (the latter has been thoroughly studied and most of the previous work leading to the theory of sporopollenin formation from carotenoids, was carried out using *L. clavatum* sporopollenin).

The use of IR spectroscopy to gain information on the structure of *B. braunii* resistant polymers, or more generally of sporopollenins, could lead to incorrect conclusions as a result of alterations originating from the various chemical treatments required to isolate such compounds [56]. In order to determine if these treatments alter the initial structure, synthetic sporopollenin was subjected to the whole range of chemical agents used to isolate natural sporopollenins and *B. braunii* resistant polymer (extraction, saponification, phosphoric acid). It appears that no variations in the IR spectrum, or in the elemental composition of synthetic sporopollenin (Table 1), takes place following these treatments. It seems likely, therefore, that the basic structure of sporopollenins is not altered during isolation. In the same way, the spectra of *B. braunii* resistant polymer (which exhibits, as shown below, a chemical resistance still even greater than classical sporopollenins) should also reflect the initial structure of the bio-polymer.

On the contrary, acetolysis of synthetic sporopollenin causes considerable changes in the IR spectra due to partial acetylation of hydroxyl groups (see Experimental). Similar variations are observed from *B. braunii* polymer (Fig. 1D) and from *L. clavatum* sporopollenin [37]; moreover, their magnitude varies largely in relation to experimental conditions. Accordingly spectra from acetolysed samples are not suitable to discuss the structure of sporopollenins and *B. braunii* resistant polymer.

If we compare now the IR spectrum of *B. braunii* polymer (Fig. 1C) with those of classical sporopollenins (synthetic from β-carotene and natural from *L. clavatum*) (Figs. 1G, 1I) the following features are noticed.

(1) *B. braunii* polymer contains fewer hydroxyl groups

*Only a limited number of the biopolymers classed as sporopollenins have been studied by IR spectroscopy [25, 28, 37, 42–44, 46, 49, 56].

as shown by: (a) the lack of noticeable absorption in the range 1100–1000 cm^{-1} (hydroxyl deformation and C–O stretching), while strong peaks appear in that zone with classical sporopollenins (Figs. 1G, 1I) [25, 56]; and (b) the decrease in the broad band at 3700–3100 cm^{-1} (polymeric hydroxyl stretching) with respect to absorption at 3000–2800 cm^{-1} (methyl and CH_2 stretching).

(2) *B. braunii* polymer contains fewer methyl groups; the lower methyl–CH_2 ratio is due to the shape of the absorption curve at 3000–2800 cm^{-1}. The latter consists of four distinct bands and there is a partial overlapping between the peaks associated with symmetric and asymmetric C–H stretching of CH_2 (respectively, 2926 and 2853 cm^{-1}) and of methyl groups (respectively, 2962 and 2872 cm^{-1}). The low level of methyl groups compared to CH_2 in *B. braunii* resistant polymer is thus reflected by: (a) the low width at half height of the peak centered at 2930 cm^{-1} (45 cm^{-1} in *B. braunii* polymer against 60 cm^{-1} in the case of synthetic sporopollenin); (b) the lack of a shoulder at 2960 cm^{-1} (present in synthetic and *L. clavatum* sporopollenins); and (c) the increase of the trough separating the two peaks between 2930 and 2855 cm^{-1}. The relative magnitude of the band at 1455 cm^{-1} (which results both from methyl and CH_2 asymmetric bending) and at 1375 cm^{-1} (chiefly methyl symmetric bending) supports the lower methyl level. Thus the intensity ratio 1455 : 1375 is, respectively, 1.25 and 1.8 for synthetic and *L. clavatum* sporopollenins while it amounts up to 4.20 in the case of *B. braunii* polymer.

(3) *B. braunii* polymer shows a weak absorption at 1160 cm^{-1} which is probably due to the presence of ether bridges. (If one considers the structure generally assumed for sporopollenins [21–31], such an absorption should be observed in them, but it is often obscured by broad bands due to hydroxyl groups.) The occurrence of ether groups in *B. braunii* polymer, as well as in synthetic and *L. clavatum* sporopollenins, is also supported by the presence of a band at 450 cm^{-1} (C–O–C bending) (Fig. 1).

(4) *B. braunii* polymer is characterized by a high intensity absorption at 720 cm^{-1}. Such a band has never been reported in previous papers related to classical sporopollenins and it is actually absent (Figs. 1G, 1I) in spectra from synthetic and *L. clavatum* sporopollenins. This peak may originate from di-substituted *cis* double bonds (C–H out of plane deformation) or from a long methylenic chain $(CH_2)_n$ with $n \geqslant 4$ (skeletal vibrations). The occurrence of a peak at 3010 cm^{-1} (vinylic C–H stretching) indicates that *B. braunii* polymer comprises some unsaturations. On the other hand the following points are observed: (a) the presence of a band at 1310 cm^{-1} (*cis* double bond, C–H in plane deformation); and (b) lack of absorptions at 970–960 cm^{-1} (*trans* double bond, C–H out of plane deformation) and at 840–790 cm^{-1} (trisubstituted double bond, C–H out of plane deformation).

Taken together the above features show that some of the double bonds of the polymer possess a *cis* stereochemistry; the absorption at 720 cm^{-1}, therefore, originates, at least partly, from such unsaturations.

Sporopollenins should not present either *cis* double bonds nor long methylenic chains in so far as they are derived from carotenoids: on the one hand the basic skeletal structure of such polymers is built from isopentyl units and on the other carotenoid unsaturations comprise *trans* conjugated, tri- or tetrasubstituted double bonds. The lack of absorption at 720 cm^{-1} was, therefore, expected from synthetic and natural sporopollenins.

The IR study suggests, therefore, that important structural differences exist between the classical sporopollenins examined here and the resistant polymer of *B. braunii* outer walls. The occurrence, in the latter, of a peak at 720 cm^{-1} and the lack of absorptions ascribable to *trans* or trisubstituted double bonds, added to the low level of hydroxyl and methyl groups, are particularly important since they suggest that *B. braunii* polymer is not actually derived from carotenoids.

Elemental composition. As a result of the limited ways so far available for sporopollenin study, the determination of their elemental composition was largely used. Biopolymers regarded as sporopollenins show, in fact, a considerable scattering in composition [25–27, 56] from $C_{90}H_{158}O_{44}$ (*Pinus silvestris* pollen exine) to $C_{90}H_{115}O_{10}$ (*Aspergillus niger* fungal walls). Moreover, large differences are observed between closely related samples; thus the sporopollenin of *Pinus radiata* pollen exine ($C_{90}H_{148}O_{25}$) differs markedly from those of *P. silvestris* [23]. Some authors assumed that such variations would reflect chiefly different levels of hydroxyl and ether groups [25, 26, 32]. On the other hand a high ash content in the polymer could cause the oxygen content to be overestimated [28].

Regarding *B. braunii* resistant polymer, it appears (Table 1) that the elemental composition is nearly independent of the origin of the sample. The average figure thus observed ($C_{90}H_{156}O_8$) indicates that *B. braunii* polymer is characterized both by a high hydrogen level (similar to the maximum value reported in compounds classed as sporopollenins), and by an oxygen level markedly lower than values reported for biopolymers considered as sporopollenins. The comparison of *B. braunii* polymer composition with those of classical sporopollenins, like synthetic and *L. clavatum* polymers (Table 1), gives further support to the assumption that the basic structure of the former is different from that of sporopollenins.

Level of unsaturation. Sporopollenins, in view of their supposed formation from carotenoids, could retain a high unsaturation level. Such a feature is actually observed in the case of *L. clavatum* polymer which reacts easily with bromine to afford, via addition on double bonds, a bromosporopollenin containing up to 50% by weight of bromine [80].

IR examination, following bromination of *L. clavatum* sporopollenin and *B. braunii* polymer, allowed us to show that the same major variations occur in these two products: (a) disappearance of the peak at 3010 cm^{-1} (vinylic C–H stretching); (b) changes in the shape of the broad band at 1800–1550 cm^{-1} attributed to the disappearance of the absorption at 1680–1600 cm^{-1} (C=C stretching); and (c) appearance of a weak peak at 550 cm^{-1} (C–Br stretching). It can thus be assumed that the double bonds were completely brominated in the two samples. Nevertheless the amount of bromine added is markedly lower for *B. braunii* polymer (17% by wt) than for *L. clavatum* sporopollenin (47%). Accordingly the former shows an unsaturation level considerably lower than that of classical sporopollenins.

One observes also that the absorption at 720 cm^{-1} is retained after bromination of *B. braunii* polymer; however, its intensity decreases compared to the band at 3000–2800 cm^{-1} (methyl and CH_2 groups). As stressed above, the peak at 720 cm^{-1} originates from *cis* double

bonds and also, possibly, in part from long methylenic chains. Bromination shows that *B. braunii* polymer contains both the above structures which contribute together to the absorption at 720 cm^{-1}.

The low level of unsaturation of *B. braunii* polymer is also consistent with its very high resistance to chemical degradation. It is well-known that sporopollenins can be completely degraded by strongly oxidizing agents [49, 51–53]; however resistance to oxidation varies largely in relation to the species examined [50]. Regarding *B. braunii* polymer, we know that it is degraded by chromic acid; however it can withstand several strong oxidizing agents, e.g. sodium hypochlorite and potassium permanganate. Numerous sporopollenins are also altered by ethanolamine [50, 52, 56, 66, 81] (a basic agent which can promote oxidative degradation [25]), but *B. braunii* polymer is able to survive prolonged treatment by such a reagent. This polymer appears, therefore, still more resistant to oxidative degradation than classical sporopollenins.

Sporopollenins are known to resist acetolysis; nevertheless some algal walls containing a sporopollenin-like polymer show, following acetolysis, a pronounced decrease in thickness leading to the disappearance of the trilaminar organization [37]. Concerning pollens, some acetolysed exines do not retain their initial structure and afford only an amorphous mass of sporopollenin [27, 36, 46, 49]. In sharp contrast, as stressed above, the organization of *B. braunii* outer walls remains unaltered, not only after isolation of the resistant polymer, but also after acetolysis. These features regarding chemical resistance are in agreement with the low unsaturation level of *B. braunii* polymer since double bonds are preferentially broken by oxidation or acetolysis.

High resolution ^{13}C *NMR of solids.* All the above findings regarding IR spectra, elemental composition and unsaturation levels, strongly suggest that *B. braunii* polymer does not derive from carotenoids and, consequently, that it does not fit in with the usual definition [21–31] of sporopollenins. High resolution ^{13}C NMR of solids allowed us to ascertain this assumption.

The precise structure of *B. braunii* polymer, as well as the one of sporopollenins, is difficult to determine as a result of the following features: (a) from such highly resistant compounds one cannot easily obtain partial degradation products which could provide precise information on the native polymer [26, 33, 49]; and (b) due to insolubility in organic solvents they cannot be examined using ^{1}H and ^{13}C NMR in solution. On the contrary high resolution ^{13}C NMR of solids, using new techniques [82–84] of cross-polarization and magic angle spinning at high speed, appears particularly suitable to study these substances.

The spectrum of *B. braunii* resistant polymer, obtained under such condition, affords the following information. (a) *B. braunii* polymer comprises mainly saturated carbons, without heterosubstituents, which give rise to the prominent broad band ranging from δ 40 to 20. This band shows a well-defined maximum at *ca* δ 29 ascribable to long methylenic chains; on the other hand the polymer contains few methyl group attached to aliphatic carbons (very weak band at δ 15. (b) One observes also the presence of two weak bands at δ 75–60 (corresponding to carbons bearing alcohol and ether functions) and at δ 130–110 (C=C bonds).

This preliminary study using ^{13}C NMR provides only semiquantitative results; however, when added to those from IR, bromination and elemental analysis, a fairly precise picture of *B. braunii* resistant polymer structure appears. This polymer is mainly built from long unbranched, or weakly branched* hydrocarbon chains containing some *cis* double bonds along with hydroxyl groups; the above chains are probably linked by ether bridges. Such a structure, in view of the *cis* stereochemistry of double bonds and of the low unsaturation and branching levels, cannot derive from carotenoids. Accordingly, the resistant polymer from *B. braunii* outer walls cannot be regarded, in this respect, as a sporopollenin.† In fact the above findings suggest that the bulk of *B. braunii* resistant polymer could derive from the hydrocarbons of the alga via oxidative polymerization. In our standard culture conditions, *B. braunii* major hydrocarbons are dienic derivatives [3, 19, 85–88] of general formula:

$$Me-(CH_2)_7-\underset{H}{C}=\underset{H}{C}-(CH_2)_x-CH=CH_2$$

(x = 13, 15, 17 and 19).

Consequently they possess both the long methylenic chain and the *cis* double bonds typical of the structure of the resistant polymer. Such hydrocarbons contain also the unsubstituted allylic positions which would be required for oxidative polymerization [25, 26]. All the above features point to the formation of *B. braunii* resistant polymer chiefly from hydrocarbons. Moreover, geochemical studies on fossil *Botryococcus* [Largeau, C. *et al.*, unpublished results] give further support to this assumption. On the other hand such an origin is also consistent with the high level of resistant polymer typical of *B. braunii* since the latter would derive directly from the abundant hydrocarbons synthesized by the alga.‡ Accordingly, the pathway leading to the bulk of *B. braunii* resistant polymer would be entirely distinct from the one

*According to the hypothetical structure of *L. clavatum* sporopollenin [30, 31], some methyl groups from the starting carotenoids would be transformed into CH_2OH during oxidative polymerization. Consequently a low methyl level does not show definitely that highly branched chains are not present. However, in the case of *B. braunii* polymer, the low content of hydroxyl groups enable us to exclude the possibility that numerous methyl groups are converted into CH_2OH. Thus, it is possible to correlate the methyl level with chain branching.

†However, we know that two distinct zones of *B. braunii* outer walls are in fact built up of resistant polymer: the apical zone Ta which exhibits a trilaminar structure and the basal zone Tb where such an organization is obscured. Electron microscopy shows that Tb is considerably thicker and that most of *B. braunii* resistant polymer is located in this latter zone. Consequently, the results discussed here reflect chiefly the nature of the polymer contained in Tb. Therefore, one cannot exclude the possibility that the resistant polymer making up the trilaminar structure, visible in Ta and obscured in Tb, may have a different origin, possibly related to that of sporopollenins. If actually occurring, such a difference would not appear due to the very low level of this constituent in the total resistant fraction.

‡It is noteworthy that outer wall hydrocarbons are rapidly extracted [19] during the first step of the process leading to the isolation of the resistant polymer. Accordingly the latter is actually a constituent of the living alga and cannot originate from physicochemical hydrocarbon polymerization taking place during the various chemical treatments.

assumed to be implicated in sporopollenin production [21–31]. The latter would start from carotenoid derivatives while the former requires, as precursors, non-terpenic hydrocarbons which themselves derive from long chain fatty acids [20].

Identification of sporopollenins

As shown above, *B. braunii* resistant material does not derive from carotenoids; therefore, it cannot be regarded, in this respect, as a sporopollenin. Nevertheless it exhibits the properties often used [41, 44, 73] to characterize this group of biopolymers (resistance to non-oxidative agents, deposition, UV fluorescence). Consequently, it appears that such features alone are insufficient to identify sporopollenins; they have necessarily to be complemented by elemental composition, IR spectroscopy, bromine addition and, especially, by ^{13}C NMR of solids so as to obtain information on the polymeric structure. Previous results regarding the whole range of biopolymers classed as sporopollenins (rather large scattering in elemental composition [25–27, 56], in chemical resistance [48, 50, 68], in staining [61, 81, 89] and in UV fluorescence [77, 78]) suggested that they do not actually constitute a homogeneous group of compounds originating from the same biosynthetic pathway (oxidative polymerization of carotenoid derivatives).* In fact the classification of all these biopolymers as sporopollenins reflected, chiefly, the inadequacy of the criteria generally used, which afforded no precise information on the structure.† Therefore, in addition to compounds actually corresponding to sporopollenins (e.g. the polymers from *L. clavatum*, *P. silvestris*, *Mucor mucedo* and *Lilium henryii* which were subjected to thorough studies, including labelling, from radioactive carotenoids [26–28] and/or careful analysis of oxidation products [22, 26, 27, 49]) the 'sporopollenin' group, as defined at the present time, should comprise numerous biopolymers of different origin, some of them being possibly related to *B. braunii* resistant material.

Conclusion

The outer walls of *B. braunii*, the main site of hydrocarbon accumulation and production, show a complex constitution. They contain a polymer highly resistant to non-oxidative agents. This resistance is typical of sporopollenins, a group of biopolymers generally regarded as originating from oxidative polymerization of carotenoid derivatives; *B. braunii* resistant polymer is also closely related to sporopollenins by its mode of deposition and its UV fluorescence.

Compounds classed as sporopollenins were previously reported in the outer walls of various algal species. However, *B. braunii* is characterized by its ability to produce several successive outer walls and, also, by a high level of resistant polymer (accounting for *ca* 9% of the culture dry wt); the latter appears, consequently, as one of the major constituents of the alga.

Further studies on the structure of *B. braunii* resistant polymer, using IR spectroscopy and high resolution ^{13}C NMR of solids along with determination of elemental composition and of unsaturation levels, show that this compound does not derive from carotenoids but, probably, from the hydrocarbons of the alga via oxidative polymerization. Consequently, *B. braunii* resistant polymer does not fit in with the usual definition of sporopollenins. Nevertheless, it satisfies criteria often used to identify this class of biopolymers. It appears that such criteria are not sufficient and it seems likely that the group of biopolymers classed at the present time as 'sporopollenins' comprises, in addition to products actually derived from carotenoids, various compounds of different origin, some of them possibly related to the resistant polymer observed in *B. braunii* outer walls.

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.). Algal cultures and electron microscopy examination were carried out as previously reported [19]. IR spectra were obtained from CsBr pellets.

Separation of B. braunii *outer walls.* Outer wall fraction with negligible levels of bacteria (the Cambridge strain is not bacteria-free) and of whole algal cells were obtained by successive centrifugation (500 *g*, 10 min) of large culture vols. Under such conditions outer walls and accompanying hydrocarbon globules are recovered in the upper part of the supernatant which appears as a white cloudy liquid.

Isolation of the resistant polymer. Extraction by organic solvents. Successive reflux for 3 hr with efficient stirring in Me_2CO, MeOH, $CHCl_3$–MeOH (2:1) and Et_2O. After each extraction the insoluble residue was recovered by centrifugation and the supernatant discarded. *Saponification.* Reflux for 6 hr in MeOH–H_2O (90:10) with 6% KOH. After cooling the residue was separated by centrifugation and extracted (reflux for 1 hr with stirring) in H_2O, EtOH, Me_2CO and Et_2O. *Treatment by* H_3PO_4. Temp. 55° for 13 days without stirring; at the end of the treatment a large excess of pentane was added and the residue separated by centrifugation, washed with H_2O until neutralization and finally extracted (reflux for 1 hr with stirring) successively with H_2O, MeOH, Me_2CO and Et_2O. The resistant polymer from *B. braunii* outer walls was obtained at this stage and its level determined. All the above treatments were also carried out starting from whole cells and also from outer wall fragments (obtained by passing, × 4, an outer wall fraction through a French pressure cell at 1430 atm).

Chemical tests on B. braunii *resistant polymer. Acetolysis.* According to Atkinson [37]; the duration of the treatment varied from 5 to 30 min. Acetolysis results in considerable changes in IR spectra which originate from partial acetylation of OH groups: decrease of the broad absorption at 3700–3100 cm^{-1} (polymeric OH stretching), appearance of peaks at 1750 cm^{-1} (C=O stretching), 1230 cm^{-1} (asymmetric stretching of C–O acetate), 1050 cm^{-1} (symmetric stretching of C–O acetate) and 620 cm^{-1} (O–$\overset{O}{\overset{\|}{C}}$ bending) and an increase of the band at 1375 cm^{-1} with respect to that at 1455 cm^{-1} reflecting a higher level of methyl groups relative to CH_2. The same IR variations are observed also on acetolysis of synthetic and *L. clavatum* sporopollenins.

Insolubility in dioxane. Verified by prolonged reflux (24 hr) in dioxane–1% 11 M HCl followed by several washings and centri-

*In fact spore exines themselves are built of two distinct materials. The first one, synthesized by the tapetum, could originate from carotenoids; while the second one, formed by grains when still in tetrad, derives from precursors which are unlikely to be carotenoids.

†Due to this lack of structural information numerous assumptions on sporopollenin origin have been proposed. [68].

fugation in MeOH. *Action of ethanolamine.* Reflux for 24 hr in pure ethanolamine with stirring followed by MeOH washing and centrifugation. *Action of SDS and phenol.* Reflux for 30 min in 4% SDS with stirring and washing with H_2O; afterwards the polymer was stirred in PhOH–H_2O (1:1) at 70° for 30 min and washed with Me_2CO–H_2O.

Oxidative degradation. The resistant polymer from *B. braunii* outer walls is entirely degraded by CrO_3 (2 g in 4 ml H_2O–HOAc, 50:50) after 7 days at room temp. However, this polymer can survive treatment by $KMnO_4$ (1.5 g in 30 ml H_2O, room temp. for 7 days) and by conc. ClO_3Na (1 hr at 100°).

Examination of Gloeocapsa alpicola. This blue-green alga (strain 1430/1 from the Cambridge Culture Collection) was grown according to ref. [90]. Treatments of the whole cells by SDS or PhOH, under the same conditions as above, give complete degradation of the outer trilaminar walls. In the same way, no resistant polymer was recovered after the successive treatments used in the isolation of *B. braunii* resistant polymer.

Phloroglucinol test for lignin. The samples were placed on a slide in a large drop of a satd aq. soln of phloroglucinol in 20% HCl. After a few min the lignin-containing reference appeared red-violet while samples from *B. braunii* did not stain.

UV fluorescence study. Samples were observed with a fluorescence microscope fitted with UG1 + BG 38 filters (UV excitation *ca* 365 nm). Different barrier filters were tested which, respectively, transmit radiations of wavelength higher than 410, 440, 470 and 530 nm. For study of secondary fluorescence, samples were first treated for 5 min in an aq. neutral soln of primuline (0.005%).

Sporopollenin of L. clavatum. Obtained from spores subjected to the successive chemical treatment used for the isolation of *B. braunii* resistant polymer.

Synthetic sporopollenin. 1 g β-carotene was dissolved in CH_2Cl_2 (500 ml) and 0.5 ml BF_3–Et_2O was added to the soln; O_2 was bubbled in the stirred soln for 8 days. Afterwards 0.5 ml BF_3–Et_2O was again added and the reaction continued for 3 days. Most of the solvent was then removed under vacuum; a large excess of Et_2O was added and the polymer was separated by centrifugation. The crude polymer was purified by successive extraction (reflux for 1 hr) in Me_2CO, $CHCl_3$–MeOH (1:1) and Et_2O.

Bromination of B. braunii *resistant polymer and of* L. clavatum *sporopollenin.* Bromine (2 g) was added to a suspension of the sample (*ca* 100 mg) in CCl_4 (8 ml) and the mixture kept at room temp. for 6 days. The reaction was carried out in the dark in order to minimize bromine introduction via substitution. (However, addition on double bonds does not necessarily account for all the introduced bromine and one cannot exclude that a small part of the latter originates from substitution.) A large excess of Et_2O was added to the reaction mixture and the polymer separated by centrifugation. Its dry wt was determined after successive washing (stirring at room temp.) with Et_2O, Me_2CO, H_2O, MeOH and pentane.

^{13}C NMR of solids. CP–MAS (cross-polarization and magic angle spinning) ^{13}C NMR was performed on a home-built spectrometer at a 12.07 MHz frequency. The H_1 field for ^1H was 7.5 G and that for ^{13}C was 30 G. Single contact cross-polarization pulse sequence was used. The spectrum resulted from data accumulated from 5600 scans; the cross-polarization time and the pulse repetition time being, respectively, 2 msec and 2 sec. The rotor was machined from Kel-F and the vol. of the sample cavity was nearly 0.7 cm^3; the rotation was *ca* 3 kHz. Chemical shifts are related to tetramethylsilane and were determined relative to external adamantane.

Acknowledgements—This work was supported by the CNRS as a part of the 'Programme Interdisciplinaire de Recherche et de Développement de l'Energie Solaire' and by Société National e Elf Aquitaine Production. We thank Mr. B. Rousseau (electron microscopy) and Mrs. Voyron (IR spectroscopy) for technical assistance.

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