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OLEIC ACID IN THE BIOSYNTHESIS OF THE RESISTANT BIOPOLYMERS OF *BOTRYOCOCCUS BRAUNII*

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Key Word Index—*Botryococcus braunii*, *A* and *B* races; Chlorophyceae; biosynthesis; pyrolysis; label location; resistant biopolymers; polymeric network formation; oleic acid; very long chain fatty acid derivatives.

Abstract—The incorporation of radioactivity into the resistant biopolymers of the *A* and *B* races of *Botryococcus braunii* (PRB *A* and PRB *B*) was examined after feeding with oleic acid. The location of the label in the biopolymers was determined by pyrolysis and confirmed that PRB *A* and PRB *B* are biosynthesized via similar pathways. However, a large part of the exogeneous oleic acid is diverted towards the formation of non-isoprenoid hydrocarbons in the *A* race; due to this competition the incorporation yield, very high in PRB *B*, is much lower in PRB *A*. It also appears that two pools of oleic acid are implicated in PRB *A* and PRB *B* formation. Oleic acid plays a major role in the biosynthesis of such resistant materials, especially as precursor of the long hydrocarbon chains building up the polymeric network. Very long chain fatty acid derivatives, originating from oleic acid elongation, are probably involved as intermediates in the formation of these chains.

INTRODUCTION

The green microalga *Botryococcus braunii* is characterized by a very large content of hydrocarbons [1–6] whose structures vary according to race [7]. Thus, the *A* race produces non-isoprenoid dienic and trienic hydrocarbons, odd numbered from C_{25} to C_{31}, whilst the *B* race yields C_{30} to C_{37} highly unsaturated isoprenoid hydrocarbons, termed botryococcenes. The bulk of the hydrocarbons is biosynthesized and stored in the outer walls of the two races [8–10]. These outer walls also contain a biopolymer insoluble in organic solvents and unusually resistant to non oxidative treatments, termed PRB *A* and PRB *B* in the *A* and *B* races, respectively (*) and accounting for *ca* 10% of the total biomass [11, 12]. In sharp contrast to hydrocarbons, the resistant biopolymers of the two races exhibit closely related structures [11–14]. Long hydrocarbon chains (up to C_{31}), saturated and unbranched, probably linked by ether bridges, build up the polymeric network. A relatively small number of hydroxy and ester groups also occur in PRB *A* and PRB *B*. These groups are efficiently protected, from chemical degradations, by the three dimensional network of hydrocarbon chains. Consequently the esters are not affected by the drastic saponification and acidic treatments required for isolating PRB; they are only cleaved on pyrolysis thereby releasing free fatty acids. PRB also play a major role in *B. braunii* fossilization and account for the high oil potential of the derived organic matter [13, 14].

The occurrence of resistant materials based on polymethylenic chains may be quite widespread in green microalgae. This type of constituent was also recently observed in the cuticle of higher plants [15] and in the wall of lichen phycobionts [16]. However, it seems that the mechanism of biosynthesis of this new group of biopolymers was only examined in the case of *B. braunii*. Previous studies indicated that oleic acid is a precursor of PRB *A* and PRB *B* [13,17]. However, taking into account the structural features of PRB, the labelling observed in the two biopolymers, following feeding with oleic acid, could reflect either a simple incorporation into the protected esters (analyses of the fatty acids released on PRB *A* and PRB *B* pyrolysis show that oleate and higher homologues afford a large contribution in these esters), or a more important role of oleic acid via the formation of the hydrocarbon chains of the polymeric network. In fact, comparative analysis of the saponifiable lipids in the two races [17] suggested that C_{24} to C_{30} very long chain fatty acids derivatives, originating from the elongation of oleic acid, occurring in the two races, may be implicated as intermediates in PRB biosynthesis. (If these derivatives are to be involved in the formation of the saturated chains of the network, then the double bond inherited from oleic acid should disappear. This may result either from a simple reduction or, more likely, from the introduction of the oxygenated functions implicated in the cross linking of the chains.)

The purpose of the present work was to determine the role of oleic acid in the biosynthesis of PRB *A* and PRB *B* and to examine the mechanism of formation of their hydrocarbon chains. To this end the precise location of

*The successive basic and acid drastic chemical treatments used for isolating PRB [11] entirely degrade all the other known biopolymers except sporopollenins. The latter group builds up the outer wall, exine, of spores and pollens and originates from oxidative polymerization of carotenoids and/or carotenoid esters. However PRB exhibit, when compared to sporopollenins, a markedly different chemical structure and biosynthetic origin [11].

the label incorporated into PRB, following feeding of the two races with oleic acid, was determined via analysis of pyrolysis products.

RESULTS AND DISCUSSION

Incorporation of oleic acid into PRB A *and PRB* B

Two parallel cultures of *A* and *B* races were fed, for six days, with 5 μCi of [10-^{14}C] oleic acid. Low amounts of incorporation and sp. act. were noted for PRB *A* while PRB *B* was strongly labelled (Table 1). Such a large difference cannot be accounted for by variations in the rate of production of PRB; the final amount of resistant biopolymer produced was similar in the two sets of experiments. So it appears that the contribution of exogeneous oleic acid is sharply lowered in PRB *A* formation when compared to PRB *B*. Oleic acid is a precursor of the non-isoprenoid hydrocarbons produced in large amount by the *A* race [18]. Furthermore, the inhibiting effect of dithioerythritol (DTE) on oleic acid incorporation in both PRB *A* and hydrocarbons suggested that their biosynthetic pathways comprise several common steps [13]. On the other hand, no biosynthetic relationship occurs in the *B* race, between PRB *B* and botryococcenes [17]. Accordingly, the markedly lower labelling of PRB *A* probably reflects a large diversion of the exogeneous oleic acid towards hydrocarbon production in the *A* race.

*The thermal cracking of these chains occurs via a radical mechanism involving radical rearrangements and fragmentations. Homologous series of hydrocarbons, with a maximum carbon number equal to the length of the polymeric chains, are therefore obtained. From saturated chains, these series correspond to alkanes (radical stabilization via H• addition) and to monoenes, especially 1-alkenes (stabilization via H• elimination).

†Under the present pyrolysis conditions, designed for reducing secondary reactions, the fatty acids are not decarboxylated and further transformed into hydrocarbons.

Pyrolysis of labelled PRB A *and PRB* B

Due to the very high resistance of PRB, selective chemical degradations could not be used for precisely locating the radioactivity incorporated from oleic acid. However, previous studies on PRB structure indicated this can be achieved by 400° pyrolysis under an helium flow. Such a thermal treatment affords large amounts of a complex mixture of medium volatility products which can be fractionated for identification. PRB pyrolysates so obtained are dominated by hydrocarbons and also contain ketones and highly polar constituents including fatty acids. The origin of these different groups was previously established [12, 14] on the basis of spectroscopic examinations of pyrolysis residues by FT IR and solid state ^{13}C NMR, the distribution of the main homologous series and the general mechanisms of pyrolyses established for synthetic polymers. Accordingly it was shown that hydrocarbons originate from the methylenic chains of the polymeric network * and fatty acids from the cleavage of the protected esters.† Consequently, the distribution of radioactivity in the different fractions of the 400° pyrolysates should clarify the role of oleic acid in the biosynthesis of PRB *A* and PRB *B*.

In order to eliminate adsorbed compounds, PRB were first heated at 300° for 20 min. The resulting weight loss is low (*ca* 10%) and the trapped products, as well as the low volatility compounds recovered by extraction of the heated samples, show a low radioactivity (Table 2). Accordingly, the bulk of the label incorporated in PRB does not correspond to the adsorption of oleic acid derivatives but to the involvement of this acid in the formation of the biopolymers.

Pyrolysis of PRB at 400° results in a very important weight loss (75–85% relative to the unheated material) and most of the radioactivity is then released. Very low amounts of label are retained in the extractable compounds and in the insoluble residue (Table 2), while a very large amounts of radioactivity is recovered in the trapped, medium volatility, products. Highly volatile products, especially hydrocarbons, are also generated on PRB pyrolysis and can be trapped with liquid nitrogen.

Table 1. Incorporation of ^{14}C oleic acid into PRB *A* and PRB *B**

	A1	A2	B1	B2
Oleic acid fed (dpm × 10^{-6})	11.6	11.4	11.1	11.1
PRB radioactivity (dpm × 10^{-6})†	0.103	0.088	1.56	1.34
Incorporation yield (%)‡	0.89	0.77	14	12
PRB weight (mg)	19.4	19.9	17.8	18.1
Sp. act. (dpm/mg)	5300	4400	87600	74000

*The physiological state of the inoculum influences *B. braunii* cultures. Parallel experiments on each race (A1, A2 and B1, B2) were therefore carried out using the same inoculum, corresponding to cells in the early exponential stage.

†As previously demonstrated, the incorporation of oleic acid into PRB is a direct process and does not result from a prior degradation into C_2 units [17].

‡Radioactivity determinations were carried out from suspended PRB and so the counting yield is probably lowered relative to solutions. However, the resulting underestimation of incorporation yields should be similar for the different samples and the reported values reflect the relative labelling of PRB *A* and PRB *B*. Incorporation yields in control cultures, killed by 2% glutaraldehyde before incubation, were below 0.1%.

Table 2. Radioactivity distribution after pyrolysis of PRB *A* and PRB *B**

		Radioactivity (dpm × 10^{-3})		
		A†	B1	B2
300° Pyrolysis	Trapped‡	6.1	13.2	3.1
	Extracted§	9.9	54.7	45.2
400° Pyrolysis	Trapped‡	272	1690	1520
	Extracted§	0.7	16.7	17.4
	Residue‖	0.85	7.4	5

*Unlabelled PRB (*ca* 500 mg) was added before the pyrolysis of each sample, in order to increase the accuracy and the yield of recovery during the subsequent fractionation of the pyrolysates.

†Due to the low incorporation in PRB *A*, the resistant material obtained in expts A1 and A2 was combined before pyrolysis.

‡Medium volatility products trapped by bubbling the effluent in $CHCl_3$ at −5°. In 400° pyrolysis they account for 57 to 65% of the total weight loss.

§Low volatility products generated by cracking but not swept along by the helium flow; recovered by extraction of the heated samples with $CHCl_3$–MeOH (2:1).

‖Insoluble final residue.

Table 3. Radioactivity distribution in trapped products of 400° pyrolysis

	A		B1		B2	
	R*	S†	R*	S†	R*	S†
Hydrocarbons	210	1120	1510	8000	1360	7420
Ketones	32.3	720	70.3	1690	99	2830
Fatty acids	2.4	310	3.04	460	2.67	590
Other polar products	17	410	90.4	2830	90.4	2580

*Radioactivity in each fraction (dpm × 10^{-3}). *Ca* 98% of the labelling of the crude trapped pyrolysates was recovered after fractionation. Hydrocarbons, ketones, fatty acids and non acidic polar products account for *ca* 70, 15, 3 and 12%, respectively by weight of these pyrolysates.

†Sp. act. (dpm/mg).

Table 4. Radioactivity distribution in the different groups of hydrocarbons

	A		B1		B2	
Subfraction	R*	S†	R*	S†	R*	S†
1	33.3	774	212.1	7370	193.8	5750
2	27.2	880	265.6	7380	207.8	7450
3	45.2	1190	352	9070	367.6	8280

*Radioactivity (dpm × 10^{-3}); measurements were directly carried out on the TLC plates. Radioactivity was also determined in the areas of the plates located below the bands corresponding to subfractions 1, 2 and 3. GC of the products recovered in these areas indicated that they correspond mainly to tailing of the separated bands. Some radioactivity (*ca* 10% of total label) was also recovered in the area of the starting line and is probably due to oxidative degradation of the hydrocarbons.

†Sp. act. (dpm/mg).

However, in sharp contrast to the medium volatility constituents, such low M_r compounds are hardly related to the structure of the biopolymers and afford little information on the precise location of the label. Only the former constituents were therefore further considered for label distribution.

Label distribution in trapped medium volatility constituents of 400° pyrolysates

As stressed above, the complex mixtures trapped during 400° pyrolysis of PRB *A* and PRB *B* are composed of hydrocarbons (derived from the chains of the polymeric network), of ketones and of polar products (including fatty acids derived from the protected esters). It is noted (Table 3) that the label is chiefly located in the hydrocarbon fraction which also exhibits the highest sp. act. On the contrary the fatty acid fraction is characterized by a low amount of labelling and sp. act. This is not due to poor recovery during the extraction required for separating fatty acids from other polar constituents. In fact prolonged Soxhlet extractions with toluene afford only a low amount of additional fatty acids with a similarly weak sp. act. Thus, the fatty acid moiety of the protected esters of PRB *A* and PRB *B* originates from a pool where exogeneous oleic acid is not easily incorporated. In sharp contrast oleic acid is more efficiently incorporated (via a second pool, and after elongation and disappearance of the double bond) into the chains of the polymeric network of PRB *A* and PRB *B*, as shown by the preferential location of the label in the hydrocarbon fraction and by its higher sp. act.

The thermal cracking of C–C bonds in the network of PRB *A* and PRB *B* generates, as discussed above, radicals which undergo chain reactions. As a result a fairly complex mixture of saturated and monoenic hydrocarbons is obtained. It is dominated by C_{13} to C_{31} *n*-alkanes and *n*-1-alkenes but also comprises several homologous series of *trans* and *cis* olefins [12, 14]. Label distribution between these different groups was examined after fractionation using TLC on silica gel–$AgNO_3$. The three subfractions obtained correspond to *n*-alkanes (1), *trans*-alkenes (2) and *n*-1-alkenes and *cis*-alkenes (3). It is noted (Table 4) that the sp. act. of subfraction 3 tends to be higher. However, the label is quite homogeneously distributed between the different groups of hydrocarbons derived from the polymeric network. This confirms the involvement of oleic acid in the formation of the hydrocarbon chains building up this structure.

Taken together the relationships observed here between PRB *A* and PRB *B*, regarding the relative label distribution in the different constituents of the pyrolysates, support previous assumptions about the occurrence of similar pathways in the formation of these resistant biopolymers from oleic acid. However, in the *A* race, a large part of the exogeneous oleic acid is diverted to-

wards non-isoprenoid hydrocarbon formation. The total incorporation yield, very high in PRB *B* is, due to this competition, much lower in PRB *A*. It appears that two distinct pools of oleic acid are involved in PRB *A* and PRB *B* biosynthesis. One pool, implicated in the formation of the protected esters derived from oleic acid and from its higher homologues, is not easily accessible to exogeneous oleic acid. The second pool is involved in the formation of the long, saturated, hydrocarbon chains building up the polymeric network and most of the label incorporated into PRB is located in these chains. Accordingly oleic acid would be, first, elongated into monoenic very long chain fatty acid derivatives; afterwards the double bond would disappear due to the cross linking of the chains resulting in the formation of the polymeric network. Oleic acid, therefore, plays a major role in PRB *A* and PRB *B* biosynthesis. These observations are also consistent with the involvement of very long chain fatty acid derivatives, originating from oleic acid elongation, as intermediates in the formation of the long hydrocarbon chains which are the major structural feature of PRB *A* and PRB *B*.

EXPERIMENTAL

The axenic strain of the *A* race was obtained from the Austin Culture Collection (807/1). The *B* race strain was isolated [7] and made axenic [10] from a sample collected in Martinique, French West Indies (La Manzo strain). Preparation of inocula and batch culture under air lift conditions, feeding with [10-^{14}C] oleic acid (45–55 mCi/mM), isolation of PRB, pyrolysis, fractionation and identification of pyrolysate constituents, were all carried out as previously described [6, 13, 14, 18].

Radioactivity was determined by liquid scintillation counting using 0.4% butyl-PBD in toluene. Resistant biopolymers were finely dispersed and counting was carried out for 0.1 min, immediately after shaking, in order to limit settling. Radioactivity distribution on TLC was measured using a plate scanner.

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