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BIOSYNTHESIS OF THE RESISTANT POLYMER IN THE ALGA *BOTRYOCOCCUS BRAUNII*. STUDIES ON THE POSSIBLE DIRECT PRECURSORS

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ABSTRACT.—The outer walls of the green alga *Botryococcus braunii* contain alkadienic (*A* race) or terpenoid (*B* race) hydrocarbons and resistant biopolymers PRB-*A* and PRB-*B*, respectively, which structures are quite similar. In order to determine the origin of these biopolymers, various feeding experiments and analysis of outer wall lipids, especially very long-chain fatty acids (VLCFA) and hydrocarbons, were carried out. All these observations suggest the formation of PRB-*A* and PRB-*B* from the same precursor (oleic acid) and the same biosynthetic pathway: elongation of oleic acid into even VLCFA derivatives and polymerization of these unsaturated acids.

The green unicellular alga *Botryococcus braunii* Kützing (Chlorophyceae) is characterized by unusually high hydrocarbon levels (1, 2). The existence of two races of *B. braunii*, showing very close morphologies but producing different types of hydrocarbons, was recently demonstrated (3). One synthesizes linear, odd, unbranched alkadienes from C_{25} to C_{31} and C_{29}, C_{31} trienes (*A* race), while the other produces highly branched and unsaturated polyisoprenic hydrocarbons termed botryococcenes (4-7) (*B* race). These hydrocarbons are mainly located in the outer walls of the two races (8, 9). The outer walls also contain a biopolymer insoluble in organic solvents and highly resistant to nonoxidative chemical degradations: PRB-*A* and PRB-*B*, respectively, for the two races.[1]

These biopolymers have similar structures, as shown by ir, high resolution ^{13}C nmr of solids, and analysis of pyrolysis products (10, 11). Thus, only minor differences in the distribution of some series are noticed when pyrolyzates are compared. PRB-*A* and PRB-*B* are essentially built up from long (at least up to C_{31}), unbranched, hydrocarbon chains linked by ether bridges. These resistant biopolymers are important constituents of *B. braunii* (generally ca. 10% of the total biomass) (11). Moreover, they account for the geochemical importance of this species.

In fact, fossil *Botryococcus* make up most of the insoluble organic matter of some sedimentary rocks, termed torbanites, exhibiting a high oil potential (12).

This fossil organic matter was recently shown to originate chiefly from selective preservation of the resistant biopolymers of outer walls (13). Indeed, PRB-*A* and *B* on one hand, and immature torbanites on the other hand, showed similar structures, determined by the above mentioned techniques. Moreover, no significant variations in the chemical structure were detected for an immature torbanite submitted to the chemical treatments required for isolating PRB. Accordingly, it can be assumed that PRB is also unaffected by such treatments, and that isolated PRB is identical to the natural resistant biopolymer.

The dienic hydrocarbons produced by *A* race derive from oleic acid via an elongation-decarboxylation mechanism (14, 15): transformation of oleic acid into C_{28}, C_{30}, and C_{32} very long-chain fatty acid (VLCFA)[2] derivatives and subsequent decarboxyla-

[1]These biopolymers are isolated from the whole cells after extraction by organic solvents of all the lipidic material and successive treatments of the residue first with methanolic KOH, then with concentrated H_3PO_4 at 55°.

[2]VLCFA: fatty acids with a number of carbons ≥ 20.

tion yielding C_{27}, C_{29}, and C_{31} alkadienes. The above observations added to the structure of PRB-*A* and to the incorporation of (10-^{14}C) oleic acid in this biopolymer (11), suggested that PRB-*A* may result from polymerization of hydrocarbons or VLCFA derivatives.

In the present work, the pathways leading to PRB-*A* and PRB-*B*, and the nature of their possible precursors were examined.

RESULTS AND DISCUSSION

No structural relationship between the botryococcenes (highly branched hydrocarbons) and the resistant biopolymer of *B* race occurs, since the hydrocarbon chains building up the latter are characterized by a very low branching level. Indeed, feeding of the alga with [2-^{14}C]mevalonate, a presumed botryococcene precursor (16), does not result in significant labeling of PRB (Table 1).

TABLE 1. Feeding Experiment with Sodium DL [2-^{14}C]Mevalonate *B* Race Cultures[a]

Feeding time	PRB-*B*		Botryococcenes	
	Radioactivity dpm × 10^{-3}	Labeling[b]	Radioactivity dpm × 10^{-3}	Labeling[b]
1 day	1.1	0.010	16.4	0.149
6 days	1.8	0.016	39.4	0.358
Control, 6 days[c]	0.9	0.008	1.1	0.01

[a]Non axenic La Manzo strain.
[b]As percentage of the radioactivity fed, calculated only from the (3*R*) enantiomer.
[c]Control: algae killed by heat treatment (100°, 10 min) before incubation.

In fact, the closely related structures of PRB-*A* and PRB-*B* suggest that the same precursors and the same type of biosynthetic pathway may be implicated in their formation.

RELATIONSHIP BETWEEN PRB-*B* AND OLEIC ACID.—In order to test the supposed similarity of PRB-*A* and *B* biosynthesis, *B* race cultures were fed with [10-^{14}C]oleic acid. It appears (Table 2) that labeling is observed in PRB-*B* and that it increases with incubation time and largely exceeds the radioactivity of the control. A biosynthetic transformation of oleic acid into PRB-*B* occurs, therefore, during the culture.[3]

A smaller, but still significant incorporation also takes place in botryococcenes. In view of the terpenoid nature of these hydrocarbons, the latter labeling necessarily results from degradation, by *B. braunii*, of oleic acid into [2-^{14}C]acetate and subsequent incorporation into botryococcenes. Consequently, some labeled C_2 units should be also incorporated into PRB-*B*. In order to determine if oleic acid is actually a direct precursor of PRB-*B*, i.e., if it can be incorporated without prior degradation, it was, therefore, essential to assess the magnitude of oleic acid breakdown, and the resulting contribution of labeled acetate in PRB-*B* radioactivity. In fact, the following observations suggest a low contribution: While botryococcenes are largely predominant relative to

[3]After a 1-day incubation, the percentage of labeling in PRB is 0.75. The alga is then in the exponential growth phase, physiological state with intense metabolic activity, and specially hydrocarbon formation. At day 6, the alga is in a linear growth phase and most of the metabolic pathways including botryococcenes formation (16) slow down. In contrast, PRB biosynthesis remains important, since the % of labelling in PRB sharply increases up to 6.8 at this stage.

TABLE 2. First Feeding Experiment with [10-^{14}C]Oleic Acid in *B* Race Cultures[a] (incorporation in PRB-*B*)

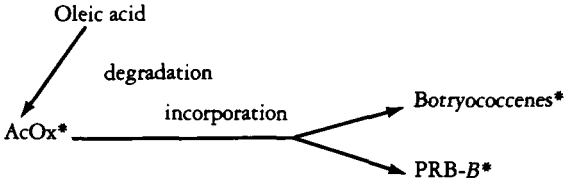
Feeding time	PRB-*B*		Botryococcenes	
	Radioactivity dpm × 10^{-3}	Labeling[b]	Radioactivity dpm × 10^{-3}	Labeling[b]
1 day	91.4	0.75	8.7	0.071
6 days	834	6.81	78.9	0.64
Control, 6 days[c]	13.9	0.11	4.4	0.036

[a]La Manzo axenic strain, culture conditions I.
[b]As percentages of the radioactivity fed.
[c]See note c Table 1.

resistant biopolymer (ca. × 5 in weight), the labeling observed in the former (exclusively derived from C_2 units) is markedly weaker (1:10).

When [U-^{14}C]acetate is fed to *B. braunii* cultures for 1 day (16), the incorporation in botryococcenes is considerably higher (ca. 12%) than the one reported here (Table 2).

Since hydrocarbons and PRB are non-metabolizable constituents, the specific activities of these end-products can be directly compared. If the radioactivity in PRB-*B* exclusively resulted (as in botryococcenes) from degradation C_2 units, close specific activities should be observed in these two compounds:



In fact, very important differences are noted after 6 days, between PRB-*B* (sp. act. ca. 10^5 dpm/mg) and botryococcenes (sp. act. ca. 2×10^3 dpm/mg), suggesting a major contribution of undegraded oleic acid in PRB-*B* labeling.

While some effects due to compartimentalization cannot be ruled out, all the following observations are consistent with a low contribution of degradatively derived acetate in PRB-*B* labeling. Accordingly, these results lead to the conclusion that oleic acid could be a direct precursor of PRB-*B*.

The nature of the intermediates implicated in the biosynthetic pathway between oleic acid and PRB-*B* was, therefore, examined. Taking into account the possible precursors of PRB-*A* and the similar structures of PRB-*A* and PRB-*B*, unbranched very long-chain hydrocarbons or fatty acid derivatives may operate as intermediates in this pathway.

POSSIBLE VERY LONG CHAIN PRECURSORS OF PRB-*B*.—No very long-chain unbranched hydrocarbons, even in trace amount, were detected in *B* race; indeed the total hydrocarbon fraction exclusively consists of botryococcenes.

On the contrary, unbranched VLCFA were detected in the two races, where they account for ca. 10% of total fatty acids. In fact, the same acids occur in *A* and *B* races,[4] and they show, moreover, a fairly similar distribution with oleic acid, the predominant

[4]However, the level of total fatty acids relative to biomass is higher in *A* (14%) than in *B* (2.5%), at the end of the culture.

acid (Table 3). In addition, the relative abundance of oleic acid tends to increase in *A* and *B* races when the cultures aged.

The above observations suggest that VLCFA derivatives may be implicated as intermediates in the transformation of oleic acid into PRB-*B* by the alga. Such intermediates should: (a) have a double bond with both the same stereochemistry and the same

TABLE 3. Analysis of Fatty Acids from the Outer Walls in Strains Belonging to the *A* and *B* Races (%)

Cultures	Fatty acids							
	C 30:1	C 30:2	C 28:1	C 28:2	C 26:0	C 26:1	C 24:0	C 24:1
A 1[a]	0.7	2.6	3.2	1.7	0.1	0.7	0.2	0.7
A 17[a]	0.6	1.4	2.0	0.8	0.1	0.5	0.2	0.4
M 1[b]	0.2	0.4	7.5	0.7	0.1	0.6	0.1	0.5
M' 21[c]	0.8	0.6	6.8	1.5	0.1	0.7	0.2	0.7
	C 22:0	C 22:1	C 20:1	C 18:0	C 18:1[d]	C 16:0	C 16:1[d]	
A 1[a]	1.6	0.9	1.6	1.5	78.8	5.2	0.5	
A 17[a]	0.5	0.8	1.5	0.9	85.6	4.2	0.4	
M 1[b]	0.2	0.3	0.6	2.7	59.7	25.4	1.0	
M 21[c,e]	0.4	0.4	2.7	0.8	71.3	11.9	1.2	

[a]Axenic Austin strain (*A* race) harvested 1 day (A1) and 17 days (A17), after inoculation.
[b]Axenic La Manzo strain (*B* race) harvested 1 day after inoculation (M1).
[c]Non axenic Paquemar strain (*B* race) harvested 21 days after inoculation (M 21).
[d]C 16:1 palmitoleic acid, C 18:1 oleic acid.
[e]+ C 32:2, trace.

location relative to the methyl end as the unsaturation of oleic acid (*cis*-$\Delta^{9,10}$), and (b) be labeled following feeding with radioactive oleic acid. Regarding the first feature, comparison with reference compounds [synthesized by a Kolbe reaction (15)] showed that the C 30:1, C 28:1, and C 26:1 VLCFA extracted from the two races actually possess a *cis*-$\Delta^{9,10}$ unsaturation.

The feeding with [10-^{14}C]oleic acid was carried out under conditions II (Table 4). When the radioactivity of fatty acids was examined, it appeared that VLCFA showed a substantial incorporation, but some labeling in botryococcenes revealed, as above, some degradation of oleic acid. Nevertheless, no radioactivity was detected in palmitic acid. This is consistent with the absence of incorporation of degradatively derived acetate, via de novo synthesis, into palmitic acid. Moreover, the high specific activities of the VLCFA (ca. 50 times that of botryococcenes for monounsaturated VLCFA) (Table

TABLE 4. Second Feeding Experiment with [10-^{14}C]Oleic Acid in *B* Race Cultures.[a] (incorporation in VLCFA)

Feeding time	Σ VLCFA		PRB-*B*	
	Radioactivity dpm × 10^{-3}	Labeling[b]	Radioactivity dpm × 10^{-3}	Labeling[b]
1 day	366.3	1.0	132.1	0.36
Control, 1 day[c]	0	0	14.7	0.04

[a]La Manzo axenic strain, culture conditions II.
[b]As percentage of the radioactivity fed.
[c]See note c Table 1.

TABLE 5. Second Feeding Experiment with [10-^{14}C]Oleic Acid in *B* Race Cultures: Specific Activities of VLCFA and Terpenoid Hydrocarbons (radio-gc)[a]

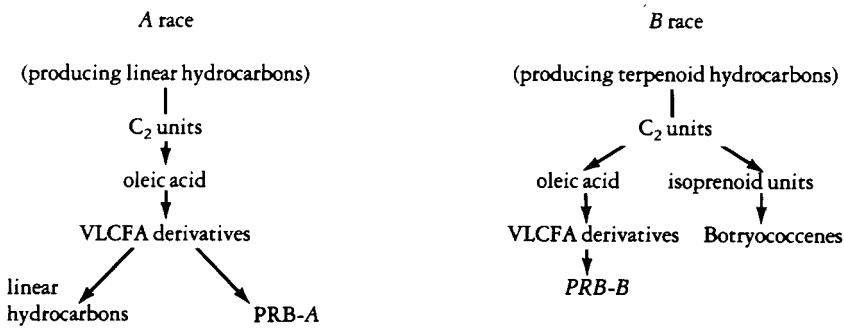
Botryococcenes	VLCFA			
	C 28:2	C 28:1	C 26:1	C 24:1
0.009	0.66	0.44	0.48	0.40

[a]Arbitrary units: sp. act. = area of the radio peak/area of the mass peak. The sensitivity of mass and radioactivity measurements were always kept at a constant level.

5) seemed to indicate that most of their labeling was incorporated by direct conversion of oleic acid.

Taken together, the above results regarding very long-chain fatty acid structure and labeling suggest that VLCFA with a *cis*-$\Delta^{9,10}$ double bond relative to the terminal methyl may be implicated in the biosynthesis of PRB-*B*.

CONCLUSION.—The resistant biopolymer occurring in *B. braunii* (*B* race) probably derives from oleic acid. The intermediates involved in the transformation of this acid into PRB-*B* are apparently linear VLCFA derivatives. The structural similarities of PRB-*A* and PRB-*B* (polymer backbone based on long methylenic chains up to 31 carbons) added to the probable involvement of a common precursor (oleic acid) and to the presence of the same VLCFA in the two races, suggest that PRB-*A* and PRB-*B* are biosynthesized via the same pathway, for which the following steps are proposed:



Accordingly, polymerization of very long-chain fatty acid derivatives probably accounts for the formation of the resistant polymers associated with the typical structure (occurrence of outer walls) and the geochemical importance of *B. braunii*.

EXPERIMENTAL

STRAINS.—The axenic strain of *A* race was provided by the Austin culture collection (807/1). Laboratory cultures of *B* race were obtained from samples collected in the West Indies (wild strains isolated in the laboratory, not yet deposited). When the first experiment (feeding with MVA) was carried out, these cultures were unialgal, but not axenic. Afterwards, one strain of *B* race (La Manzo) was rendered axenic by serial dilutions and used in subsequent feeding experiments.

CULTURE CONDITIONS.—The algae were grown on a chemically defined medium (17) at 25° with continuous illumination (light flux density 235 $\mu E\ m^{-2}s^{-1}$ under air-lift conditions as previously described (18) (air 1% CO_2, aeration rate 20 liters/h per liter of culture). Dense cultures in the exponential growth phase were used as inocula.

FEEDING EXPERIMENTS.—Sodium DL [2-^{14}C]mevalonate (MVA) (39.5 mCi/mmol) (C.E.A.) and [10-^{14}C]oleic acid (45-55 mCi/mmol) (C.E.A.) were used in these experiments. Oleic acid was dispersed, after addition of 10 mg of Tween 20, in 3 ml fractions of growth medium by repeated sonication and then

combined with the remaining fresh medium. The same amounts of radioactivity and of algal biomass were added to the culture flasks and to the controls.

Feeding with MVA was carried out in 5 cm i.d. cylindrical tubes containing about 500 ml of culture (10 μCi of tracer per tube, initial algal concentration ca. 0.1 g/liter). Two different sets of conditions were used in feeding with oleic acid.

Conditions I: 3 cm i.d. cylindrical tubes containing 100 ml of culture; 5.5 μCi of acid per tube; algal intitial concentration 0.55 g/liter.

Conditions II: 5 cm i.d. cylindrical tubes containing 320 ml of culture, 16.5 μCi of tracer per tube; initial biomass 1.16 g/liter.

ISOLATION OF PRB.—This was carried out as previously described (10, 11). In all these cultures the resistant biopolymer accounts for about 4% of the dry biomass.

ANALYSIS OF OUTER WALL LIPIDS.—Outer wall lipids were extracted twice with hexane (17) (stirring at room temperature for 2 h) from algae dried under vacuum.

They were fractionated on a silica gel column into a hydrocarbon fraction (eluted with hexane) and a polar fraction (eluted with $CHCl_3$-MeOH, 1:1). The total hydrocarbon fraction of *B* race (Paquemar strain) was examined by gc (SE 52 capillary column) for the possible presence of very long-chain unbranched hydrocarbons. Botryococcenes were purified on tlc (10% $AgNO_3$ silica gel; hexane-Et_2O, 90:10). The total fatty acids were recovered after saponification with 0.9 N KOH (boiling for 4 h under N_2 in MeOH-toluene-H_2O, 20:2:1). Continuous extraction with toluene (1 day) was performed to ensure that even the VLCFA of the sample were actually recovered. The total fatty acid fraction was finally esterified (ca. 1% acetyl chloride in boiling anhydrous MeOH, 20 min, under N_2). The methyl esters were purified by tlc (silica gel, hexane-Et_2O-HOAc, 90:10:1) and analyzed by gc (SE 52 capillary column) and gc/ms.

RADIOACTIVITY INCORPORATION.—Liquid scintillation was used for counting PRB-*B*, botryococcenes, and total fatty acids, using 0.4% butyl PBD in toluene. Analysis by radio-gc (Panax-radiogas detector system) was also carried out with SE 30 (hydrocarbons) and CS5 (fatty acid methyl esters) columns. The incorporation into VLCFA (Table 5) was calculated from the integration of radioactivity peaks and from the radioactivity of the purified total fatty acid methyl ester fraction. Radio-gc of this fraction showed a negligible labeling for C 30:1 and C 30:2 acids. In addition, due to the weak values of the radioactivities of C 22:1 and C 20:1 acids, accurate measurements were made difficult by tailing of the very large peak corresponding to unmetabolized oleic acid. Thus, only C 28:2, C 28:1, C 26:1, and C 24:1 acids allowed for significant radioactivity measurement and for determination of specific activities (Table 5).

Due to its insolubility in organic solvents, PRB cannot be examined by radio-gc.

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