BIOSYNTHESIS OF CAROTENOIDS IN *RHODOMICROBIUM VANNIELII*

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1. Introduction

Rhodomicrobium vannielii is an atypical photosynthetic bacterium belonging to the family Rhodospirillaceae. In addition to distinct morphological features and nutritional requirements [1], its photosynthetic pigments are located in a peripheral lamellar system which differs from that of most other photosynthetic bacteria and resembles the organization of blue–green algae [2]. Furthermore, *Rhodomicrobium vannielii* synthesizes carotenoids that are typical of both anaerobic photosynthetic bacteria (Rhodospirillaceae) and aerobic photosynthetic organisms (e.g., Cyanophyceae). As in a large number of species of the family Rhodospirillaceae, acyclic carotenoids of the spirilloxanthin series are synthesized. However, unlike most photosynthetic bacteria and like aerobic photosynthetic organisms, *Rhodomicrobium* synthesizes small amounts of the aliphatic cyclic carotenoid, $\beta\beta$-carotene (β-carotene).

Rhodomicrobium is assigned to the family Rhodospirillaceae, although it has an unusual carotenoid composition*. Cyclic carotenoids are characteristically produced by the Chlorobiaceae (green and brown sulfur bacteria). The green members synthesize monocyclic aryl carotenoids such as ϕ,ψ-carotene (chlorobactene) while the brown representatives synthesize bicyclic aryl carotenoids (e.g., ϕ,ϕ-carotene (isorenieratene)).

The studies in this report were undertaken to ascertain whether cyclization of carotenoids to produce β-carotene occurs by the same biosynthetic pathway as in algae and higher plants or whether an alternate pathway is operating anaerobically. Therefore, this investigation concentrated on the hydrocarbons 7,8 dehydro-ψ,ψ-carotene (neurosporene), ψ,ψ,-carotene (lycopene), and β,ψ-carotene (γ-carotene) which have been shown to be intermediates in β-carotene production in algae, fungi and higher plants.

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* Some strains of *Rhodopseudomonas acidophila*, also in the Rhodospirillaceae, synthesize small amounts of β-carotene

2. Methods and materials

2.1. *Culture*

Rhodomicrobium vannielii was obtained from American Type Culture collection (ATCC #17100).

2.2. *Medium and culture conditions*

Sterilized medium was prepared in glass stoppered bottles as in [3]. In order to minimize the contribution of existing carotenoids in the organism, six 250 ml culture flasks were inoculated initially with 70 ml of a previously grown culture. Each flask contained a different nicotine concentration, mM: 0; 0.05; 0.1; 0.5; 1; 5. After 6 days, 175 ml of each of these cultures in turn was used as the inoculum for 1 liter cultures containing the same nicotine concentrations as above. The 6 cultures were grown anaerobically (e.g., culture flasks were filled to the top with medium) in the light (20 X 15 W tungsten bulbs at a distance of 100 cm) for 8–9 days at 25°C.

2.3. *Harvesting of the cells*

The medium was adjusted to pH 9 by the addition of 5 M NaOH and the cells were then flocculated by

the addition of a sufficient amount of 50% $CaCl_2$. The flocculated cells were recovered by filtration.

2.4. *Pigment extraction, saponification and chromatographic separation*

Cells were extracted and saponified simultaneously overnight with 5% KOH (w/v) in methanol. Succeeding extractions with acetone and transfer to petroleum ether (b.p. 30–60°C) were as in [4]. The saponified extract was chromatographed on a column of neutral alumina (Woelm, deactivated to Brockmann Grade III) and eluted as in [5]. The 4 fractions obtained after column chromatography were all rechromatographed on magnesium oxide: kieselguhr G (1:1 w/w) thin layer plates (0.5 mm thick). Fraction 1 from the column was developed in acetone : benzene : petroleum ether (b.p. 30–60°C) (1:1:8, v/v/v) while fractions 2, 3 and 4 were developed in acetone : benzene : petroleum ether (b.p. 30–60°C) (2:2:1, v/v/v).

2.5. *Identification of carotenoids*

Pigment bands were scraped from the plates, eluted with methanol, filtered, evaporated and redissolved in an appropriate organic solvent for measurement by absorption spectrophotometry: petroleum ether (b.p. 30–60°C) – β-carotene, γ-carotene, neurosporene, lycopene, 1-methoxy-3,4-didehydro-1,2-dihydro-ψ,ψ-carotene (anhydrorhodovibrin), 1′-methoxy-3′,4′-didehydro-1,2,1′,2′-tetrahydro-ψ,ψ-caroten-1-ol (rhodovibrin); benzene – 1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene (spirilloxanthin); acetone – 1,2-dihydro-ψ,ψ-caroten-1-ol (rhodopin). Individual carotenoids were then quantitated using $E^{1\%}_{1cm}$ values from [6,7]. The identity of the carotenoids was confirmed by their mass spectra: β-carotene – MS-M+ at *m/e* 536 (12%, $C_{40}H_{56}$), fragment ions at *m/e* 467, 444; γ-carotene – MS-M+ at *m/e* 536 (8%, $C_{40}H_{56}$), fragment ions at *m/e* 467, 443, 403; neurosporene – MS-M+ at *m/e* 538 (8%, $C_{40}H_{58}$), fragment ions at 487, 467, 443, 403, 69; lycopene – MS-M+ at *m/e* 536 (11%, $C_{40}H_{56}$), fragment ions at *m/e* 467, 444, 430, 378, 69; spirilloxanthin – MS-M+ at *m/e* 596 (3%, $C_{42}H_{60}O_2$), fragment ions at *m/e* 504, 490, 438,

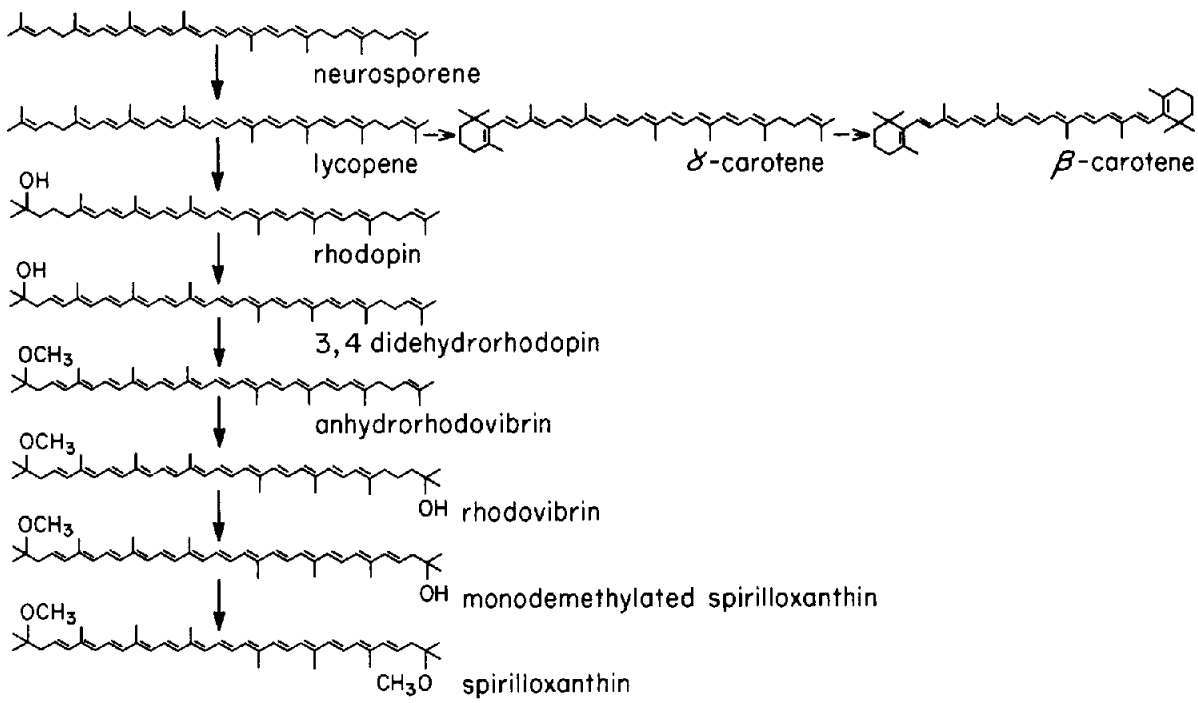

Fig.1. Pathway for spirilloxanthin biosynthesis in *Rsp. rubrum*. Solid arrows indicate biosynthetic pathway to spirilloxanthin. Dashed arrows indicate presumptive biosynthetic pathway to β-carotene in *Rm. vannielii*.

564, 523, 458, 417, 398, 384, 73; rhodopin – MS-M+ at *m/e* 554 (13%, $C_{40}H_{58}O$), fragment ions at *m/e* 485, 462, 448, 467, 536.

3. Results

As stated previously, *Rhodomicrobium vannielii* synthesizes both acyclic and aliphatic cyclic carotenoids. The pigment complex of *Rhodomicrobium* was proposed to be typical of several photosynthetic bacteria [7] and the biosynthetic pathway leading to spirilloxanthin suggested to be that of the normal spirilloxanthin series present in *Rhodospirillum rubrum* [8] (fig.1). Lycopene, accumulated in the presence of nicotine, can be converted into rhodopin, the next intermediate of the acyclic pathway leading to spirilloxanthin [9].

No data have been presented concerning the biosynthesis of β-carotene. Since nicotine has been shown to inhibit cyclization and hydroxylation of lycopene to β-carotene and rhodopin, respectively, in *Rhodomicrobium* [9,10], varying nicotine concentrations were used to study the effect on β-carotene production and accumulation of intermediates. Table 1 contains those carotenoids which have been shown to be intermediates in the production of β-carotene in aerobic organisms. When the percentage of these individual hydrocarbons (fig.2) is plotted against nicotine con-

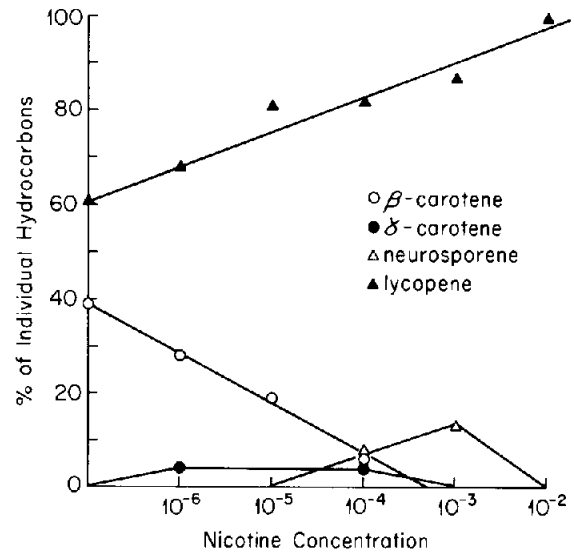

Fig.2. Effect of nicotine on β-carotene biosynthesis.

Table 1
Effect of nicotine on β-carotene biosynthesis

Nicotine conc. (M)		0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Dry wt (g) of culture		3.87	5.13	5.58	4.99	4.70	4.03
Carotenoid content (μg/g dry wt, % total carotenoid, %hydrocarbons)							
β-Carotene	(μg/gm d.w.)	23	70	23	16	–	–
	(% total)	8	4	3	2	–	–
	(% HCs)	39	28	19	6	–	–
γ-Carotene	(μg)	–	10		10		–
	(% total)	–	1		1	–	–
	(% HCs)		4		4	–	–
Neurosporene	(μg)	–	–	–	18	30	–
	(% total)	–	–	–	2	3	–
	(% HCs)	–	–	–	8	13	–
Lycopene	(μg)	36	170	99	198	332	55
	(% total)	13	10	13	23	30	46
	(% HCs)	61	68	81	82	87	100
Xanthophylls	(μg)	225	335	618	621	728	65
	(% total)	79	85	84	72	67	54

Table 2
Effect of nicotine on spirilloxanthin biosynthesis

Nicotine conc. (M)		0	5×10^{-5}	10^{-4}	5×10^{-4}	10^{-3}
Dry wt. (g)		0.661	0.726	1.005	0.878	0.223
Carotenoid content (μg/g dry wt, % total carotenoid)						
Lycopene	(μg)	165	160	602	395	197
	(%)	10	13	24	31	33
Rhodopin	(μg)	1083	773	1532	741	368
	(%)	67	64	60	57	55
Anhydrorhodovibrin	(μg)	33	22	18	27	9
	(%)	2	2	1	2	1
Rhodovibrin	(μg)	38	39	77	49	–
	(%)	2	3	3	4	–
Spirilloxanthin	(μg)	107	112	222	42	–
	(%)	7	9	9	3	–
Other	(μg)	197	101	124	36	76
	(%)	12	8	5	3	12

centration, it can be seen that the decrease in the amount of β-carotene is mirrored by the increase in lycopene. γ-Carotene and neurosporene also increase at intermediate nicotine concentrations. As expected, xanthophyll production decreases (table 1).

A second experiment was conducted specifically to monitor accumulation of intermediates in spirilloxanthin biosynthesis. It is possible that monohydroxy carotenoids such as rhodopin may serve as alternate substrates for cyclization, a concept proposed [11]. As can be seen from the values in table 2, plotted in fig.3, the decrease in rhodopin closely reflects the increase in lycopene. Spirilloxanthin biosynthesis is inhibited by nicotine concentrations greater than 5×10^{-4} M and rhodopin biosynthesis by still higher nicotine concentrations. No intermediates (e.g., rhodopin) which could serve as alternative substrates for cyclization were seen to accumulate.

Carotenoids other than those specifically listed in table 2 included hydrocarbons (β-carotene, γ-carotene and neurosporene) and unidentified xanthophylls. The hydrocarbon content was considered to be insignificant; while the majority of unidentified carotenoids appeared to be oxygenated (e.g., hydroxylated and/or methoxylated) derivatives of lycopene, as determined on the basis of their absorption spectra.

4. Discussion

In *Rhodomicrobium vannielii* the anaerobic production of small amounts of cyclic β-carotene as an end product rather than as an intermediate (e.g., the

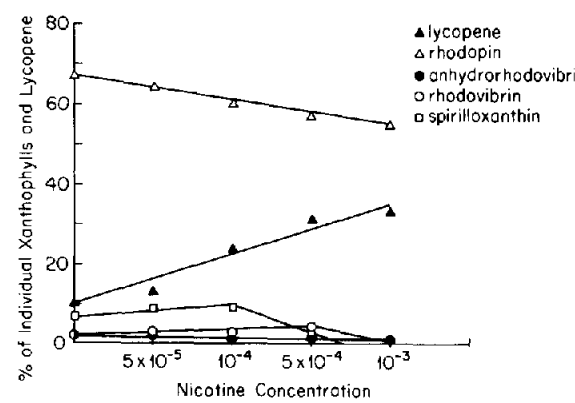

Fig.3. Effect of nicotine on spirilloxanthin biosynthesis.

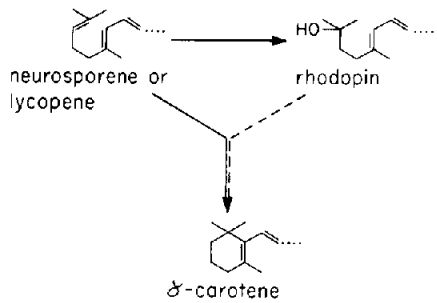

Fig.4. Possible pathways for cyclization of carotenoids. Solid arrow indicates pathway for aerobic organisms. Dashed arrow indicates a possible alternate pathway in anaerobic organisms.

presumptive biosynthesis of isorenieratene in the brown sulfur bacteria) makes its carotenoid composition unusual. This characteristic along with certain morphological features suggests that *Rhodomicrobium* may be an evolutionary intermediate between photosynthetic bacteria and blue–green algae.

Two different possible pathways for cyclization of carotenoids have been proposed [11] (fig.4). In aerobic organisms the hydrocarbons lycopene, and in some cases neurosporene, are the immediate precursors of cyclization. In anaerobic bacteria it is possible therefore that cyclization might also proceed through an hydroxylated intermediate (e.g., rhodopin) with subsequent dehydration to yield a cyclic product.

This investigation supports the hypothesis that cyclization in *Rhodomicrobium vannielii* occurs by the same pathway as aerobic organisms. γ-Carotene is detectable at low nicotine concentrations whereas lycopene accumulates at higher nicotine concentrations. These results are also consistent with the postulated role of nicotine as an inhibitor of cyclization.

The mechanism by which nicotine inhibits carotenoid biosynthesis is unknown. However, as the nicotine concentration is increased there is a differential inhibition first of γ-carotene, then of γ-carotene and lycopene, either binding to or reacting with the respective catalytic sites for cyclization. At low nicotine concentrations the cyclase responsible for the production of bicyclic β-carotene is blocked; γ-carotene is then detected. At higher nicotine concentrations, the cyclase responsible for the production of monocyclic γ-carotene also is inhibited; lycopene accumulation results. At still higher nicotine concentrations neurosporene was seen to accumulate. However, it is believed that lycopene rather than neurosporene is the substrate for cyclization. The increase in neurosporene may result from lycopene accumulation rather than nicotine inhibition. Finally, nicotine concentrations above 10^{-2} M interfered with the growth of the organism.

Although there is no evidence that rhodopin is the substrate for cyclization, it is difficult to entirely rule out this possibility, since rhodopin accounts for such a high percentage of total carotenoids (67% in the control) and since β-carotene represents such a small percentage of total carotenoids (8% in the control). These data support the hypothesis that the pathway for β-carotene biosynthesis in *Rhodomicrobium vannielii* takes place in a manner similar to that of many aerobic organisms:

Lycopene → γ-carotene → β-carotene.

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