Biosynthesis of Spheroidene and Hydroxyspheroidene in *Rhodopseudomonas* Species: Experiments with Nicotine as Inhibitor

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Neurosporene replaces spheroidene and hydroxyspheroidene as the main carotenoid in *Rhodopseudomonas spheroides* and *Rhodopseudomonas gelatinosa* grown in the presence of nicotine. On removal of the nicotine, spheroidene and hydroxyspheroidene are formed at the expense of the accumulated neurosporene. This shows that nicotine inhibits introduction of the C-1 tertiary hydroxyl groups, and supports the postulated pathway neurosporene → spheroidene → hydroxyspheroidene.

Photosynthetic bacteria, especially members of the Athiorhodaceae, are characterized by the presence of acyclic carotenoids, usually containing tertiary hydroxyl and methoxyl groups at C-1 and C-1', and often with double bonds at C-3,4. In particular *Rhodopseudomonas spheroides* and *Rhodopseudomonas gelatinosa* accumulate as their main carotenoids spheroidene [1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- $\psi\psi$ -carotene (IV)] and hydroxyspheroidene [1'-methoxy-3',4'-didehydro-1,2,7,8,1',2'-hexahydro- $\psi\psi$ -caroten-1-ol (V)] respectively when grown under anaerobic conditions (Van Niel, 1947; Goodwin *et al.*, 1955; Eimhjellen & Liaaen-Jensen, 1964). Kinetic studies have indicated the biosynthetic pathway for spheroidene and hydroxyspheroidene from neurosporene outlined in Scheme 1 (Liaaen-Jensen *et al.*, 1961; Liaaen-Jensen, 1963; Eimhjellen & Liaaen-Jensen, 1964), although the occurrence of other compounds, especially 11',12'-dihydro-spheroidene (1-methoxy-3,4-didehydro-1,2,7',8',11',12'-hexahydro- $\psi\psi$ -carotene), has led to the suggestion that alternative pathways may operate in photosynthetic bacteria (Davies, 1969).

The introduction of the hydroxyl group at C-1 is considered to be a hydration reaction (Liaaen-Jensen, 1963; Britton, 1971; Goodwin, 1971), which may involve protonation at C-2 as the first stage, analogous to the first stage of the cyclization reaction in the biosynthesis of cyclic carotenoids. The successful use of nicotine as an inhibitor of the cyclization reaction in *Mycobacterium* species (Howes & Batra, 1970) and in a *Flavobacterium* (J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work) suggested an investigation of nicotine as a possible inhibitor of the hydration reaction of carotenoid biosynthesis in photosynthetic bacteria. In particular the effects of nicotine on spheroidene and hydroxyspheroidene

synthesis in *Rps. spheroides* and *Rps. gelatinosa* have been studied.

Materials and methods

Rps. spheroides N.C.I.B. 8253 and *Rps. gelatinosa* N.C.I.B. 8290, obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K., were cultured on a malate–glutamate medium (Lascelles, 1956). Cultures (1 litre) were normally grown anaerobically in light at 28°C, and the appropriate amounts of nicotine were added, aseptically, at the time of inoculation. After 6 days of growth, cells were harvested by centrifugation, and carotenoids were extracted and purified by standard methods (Britton & Goodwin, 1971). Pigments were assayed quantitatively by the spectrophotometric method (Davies, 1965). $E_{1\text{cm}}^{1\%}$ values used were those quoted by Davies (1965), i.e. neurosporene 2990, spheroidene 2630 and hydroxyspheroidene 2543.

In further series of experiments cells were grown for 6 days in the presence of nicotine (7.5 mM for *Rps. spheroides*, 4.0 mM for *Rps. gelatinosa*), harvested, washed twice with fresh medium to remove the nicotine and finally divided into three portions. One portion was analysed (control) and the other two portions were reincubated in fresh medium, one with nicotine (7.5 mM for *Rps. spheroides*, 4.0 mM for *Rps. gelatinosa*), the other without nicotine. After reincubation for 24 h anaerobically in the light at 28°C the cells were harvested and analysed as before.

Neurosporene, spheroidene and hydroxyspheroidene from the two species were characterized by their chromatographic properties and by their electronic absorption and mass spectra.

Results and discussion

The amounts of the three main carotenoids, neurosporene, spheroidene and hydroxyspheroidene,

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are given in Table 1, expressed as $\mu\text{g/g}$ dry wt. of cells and as a percentage of total carotenoid content. In *Rps. spheroides* nicotine inhibits synthesis of spheroidene and hydroxyspheroidene and causes an accumulation of neurosporene (I). The increase in neurosporene with increasing nicotine concentration parallels the decrease in spheroidene and hydroxyspheroidene. This suggests that nicotine inhibits the hydration reaction in spheroidene and hydroxyspheroidene biosynthesis, and indicates that neurosporene is the pre-hydration intermediate. This is

confirmed by the results with *Rps. gelatinosa*. In this case, however, at lower nicotine concentrations the spheroidene content increases as hydroxyspheroidene decreases, indicating that the second hydration, of spheroidene to hydroxyspheroidene, is largely inhibited before any substantial inhibition of the first hydration (of neurosporene) occurs. The results thus suggest that spheroidene is a precursor of hydroxyspheroidene.

The effect on carotenoid composition of removal of the inhibitor was then studied. With *Rps. spheroides*,

Scheme 1. Postulated pathway for biosynthesis of spheroidene and hydroxyspheroidene in *Rps. spheroides* and *Rps. gelatinosa*

Table 1. *Effect of nicotine on carotenoid composition in Rps. spheroides and Rps. gelatinosa*

	Neurosporene		Spheroidene		Hydroxyspheroidene	
	($\mu\text{g/g}$ dry wt.)	(%)	($\mu\text{g/g}$ dry wt.)	(%)	($\mu\text{g/g}$ dry wt.)	(%)
(1) <i>Rps. spheroides</i>						
(a) Cells grown for 6 days with or without nicotine						
No nicotine	39	0.4	9421	88	710	7
2.5mM-Nicotine	1686	22	5627	72	124	2
5.0mM-Nicotine	3504	49	3471	49	—	—
7.5mM-Nicotine	4633	75	1434	23	—	—
(b) Cells grown for 6 days with nicotine (7.5mM), then nicotine removed by washing						
Cells not reincubated	1487	53	1275	45	—	—
Cells reincubated for 24h with nicotine (7.5mM)	1617	49	1570	48	6	0.2
Cells reincubated for 24h without nicotine	468	17	2074	76	174	6
(2) <i>Rps. gelatinosa</i>						
(a) Cells grown for 6 days with or without nicotine						
No nicotine	1	0.1	115	9	1115	87
3.0mM-Nicotine	486	21	1135	49	374	16
4.0mM-Nicotine	710	31	1243	55	147	6
5.0mM-Nicotine	914	41	1147	51	72	3
(b) Cells grown for 6 days with nicotine (4.0mM), then nicotine removed by washing						
Cells not reincubated	504	27	858	46	320	17
Cells reincubated for 24h with nicotine (4.0mM)	460	28	730	45	212	13
Cells reincubated for 24h without nicotine	22	1	217	13	1153	72

cells grown in the presence of nicotine (7.5mM) accumulated neurosporene as their main carotenoid. After removal of the nicotine and reincubation, the neurosporene content had decreased considerably, and this decrease was accompanied by an increase in the amounts of spheroidene and hydroxyspheroidene. Also, with *Rps. gelatinosa*, neurosporene and spheroidene decreased, and the hydroxyspheroidene content rose almost to the value in cells grown under normal conditions (no nicotine).

This therefore suggests that, on removal of nicotine, the hydration reaction is able to proceed, and spheroidene and hydroxyspheroidene are produced at the expense of the accumulated neurosporene. Also, especially in *Rps. gelatinosa*, it is apparent that hydroxyspheroidene is produced at the expense of the accumulated spheroidene.

The postulated intermediates between neurosporene and spheroidene, i.e. chloroxanthin [1,2,7',8'-

tetrahydro- $\psi\psi$ -caroten-1-ol (II)] and the previously undetected demethylated spheroidene [3,4-didehydro-1,2,7',8'-tetrahydro- $\psi\psi$ -caroten-1-ol (III)], have been identified in cultures of both species (A. Ben-Aziz, H. C. Malhotra, R. K. Singh, G. Britton & T. W. Goodwin, unpublished work). It is also worthy of note that no 3,4-didehydroneurosporene was detected in nicotine-grown cultures of *Rps. spheroides* or *Rps. gelatinosa*. This suggests that introduction of the double bond at C-3,4 cannot occur until the C-1,2 bond is hydrated.

This work therefore provides direct support for the operation of the biosynthetic pathway from neurosporene to spheroidene and hydroxyspheroidene outlined in Scheme 1.

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