Effect of Inhibitors on Zeaxanthin Synthesis in a *Flavobacterium*

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SUMMARY

Several substances have been tested for inhibitory effects on carotenoid biosynthesis in *Flavobacterium* R1519, an organism which normally produces zeaxanthin (β,β -carotene-3,3'-diol) as essentially its only carotenoid. Diphenylamine, several mixed-function oxidase inhibitors (SKF 525A, SKF 3301A, SKF 7997A₃, metopirone base, diphenylpropylacetic acid, piperonyl butoxide), several alkaloids (atropine, brucine, hyoscyamine, pilocarpine, quinidine, quinine, scopolamine, strychnine), some substituted pyridines (pyridine, 3-picoline, 3-ethylpyridine, 5-ethyl-2-methylpyridine) and morpholine showed no specific inhibitory effects. In cultures grown in the presence of CaCl₂, β -carotene (10 %) and β -cryptoxanthin (10 %) partly replaced zeaxanthin. Nicotine specifically inhibited zeaxanthin synthesis. Lycopene (in 5 mM-nicotine) and rubixanthin (in 1 mM-nicotine) accumulated as the new main pigments; [2-¹⁴C]mevalonic acid was efficiently incorporated into both. Nicotine thus inhibited the cyclization reaction in carotenoid biosynthesis in *Flavobacterium* R1519 as in other organisms; in zeaxanthin biosynthesis cyclization appeared to precede hydroxylation. Similar inhibitory effects were produced by (+) and (–) nicotine and by *nor*-nicotine, but not by the closely related anabasine, myosmine, β -nicotyrine and *nor*-nicotyrine.

INTRODUCTION

The biosynthesis of carotenoid pigments in plants and micro-organisms has been extensively studied, and is the subject of several recent reviews (Porter & Anderson, 1967; Britton, 1971; Goodwin, 1971). The main outline of the pathway leading to the carotene hydrocarbons appears to be reasonably well established and, although the biosynthesis of the oxygenated carotenoids (xanthophylls) has not been studied in detail, it is generally assumed that these are formed from the carotenes at a late stage in the biosynthetic sequence. The availability of a *Flavobacterium* strain (R1519) which produces as virtually the only carotenoid large amounts of zeaxanthin ((3*R*,3'*R*)- β,β -carotene-3,3'-diol) (I) has provided the opportunity to study xanthophyll biosynthesis in detail. Thus it has been shown (J. C. B. McDermott, F. J. Leuenberger, G. Britton & T. W. Goodwin, unpublished results) that the stereochemistry of zeaxanthin biosynthesis in *Flavobacterium* R1519 is the same as in higher plant systems studied (Walton, Britton & Goodwin, 1969). However, under normal conditions, zeaxanthin constitutes at least 99 % of the total carotenoid in *Flavobacterium* R1519, so that it is not possible to identify any possible intermediates in zeaxanthin biosynthesis, or to try to establish precursor-product relationships between different carotenoids.

In order to overcome this difficulty, we have examined the effect on zeaxanthin production in *Flavobacterium* R1519 of several compounds considered as possible inhibitors of carotenoid synthesis, including a number of compounds known to inhibit the production of the

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normal carotenoids by other organisms. In particular, conditions were sought under which the formation of zeaxanthin was specifically inhibited, and other carotenoids, possibly intermediates in zeaxanthin biosynthesis, accumulated.

METHODS

Strains. *Flavobacterium* strains R1519 (= 0147), R1534, R1552 and R1560 were kind gifts from F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland.

Compounds tested as inhibitors of carotenoid production. Diphenylamine, nicotine hydrogen (+) tartrate, morpholine and 3-picoline were purchased from B.D.H. Ltd, Poole, Dorset, 5-ethyl-2-methylpyridine was obtained from Eastman Organic Chemicals, Rochester, New York, U.S.A., piperonyl butoxide and 3-ethylpyridine from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire and β -nicotyrine (Fluka) from R. Emmanuel Ltd, Wembley, Middlesex.

Samples of atropine sulphate, brucine sulphate, hyoscyamine sulphate, pilocarpine hydrochloride, quinidine sulphate, quinine sulphate, scopolamine hydrobromide and strychnine sulphate were obtained by our colleague Dr A. Ben-Aziz from Plantex Ltd, Nathanya, Israel, through the courtesy of Dr Pfeffer.

Samples of *nor*-nicotine, β -nicotyrine, *nor*-nicotyrine, myosmine and anabasine picrates were kindly provided by Dr T. A. Scott, Department of Biochemistry, University of Leeds. (+) Nicotine di (-) tartrate was the kind gift of Dr R. B. Barlow, Department of Pharmacology, University of Edinburgh.

Samples of mixed-function oxidase inhibitors SKF525A, SKF3301A, SKF7997A₈, metopirone base and diphenylpropylacetic acid were the kind gifts of Dr P. D. G. Dean of this department.

Authentic carotenoids. β -Carotene, lycopene and zeaxanthin were kindly provided by F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland. β -Cryptoxanthin and rubixanthin respectively were obtained from the fruits of *Cyphomandra betaceae* and rose hips obtained from the University of Liverpool Botanic Gardens, Ness, through the courtesy of the Director, Mr J. K. Hulme. Both compounds were purified by standard techniques (Britton & Goodwin, 1971).

Culture conditions. *Flavobacterium* strains were grown in liquid culture on a medium containing (g/l): glucose, 5g; Bacto-yeast extract (Difco), 10g; Bacto tryptone, 10g; NaCl, 30g; MgSO₄.7H₂O, 5g; the final pH being adjusted to 7.2 by addition of a small amount of NaOH before autoclaving at 10 lb/in² for 10 min. Cultures were maintained on slopes of the above medium supplemented with 3 % Bacto-agar.

The bacteria were normally grown in liquid medium (50 ml) in 250 ml conical flasks modified by four indentations acting as baffles to increase aeration of the cultures. Cultures were inoculated either from slopes or with 1 ml of a 24 h liquid culture, and were shaken (160 rev./min) in an orbital incubator (Gallenkamp) illuminated with fluorescent light (about 200 ft-candles), usually for 40 to 44 h. Strain R1519 was grown at 19 to 21 °C, the other strains at 26 °C.

Addition of inhibitors. Appropriate amounts of CaCl₂.6H₂O were included in the liquid medium before autoclaving.

Solid alkaloid salts and mixed-function oxidase inhibitors were dissolved in sterile water containing sufficient dimethyl sulphoxide to give a final concentration of 0.4 % dimethyl sulphoxide in the cultures.

Diphenylamine (DPA) was dissolved in ethanol at concentrations such that 1 ml of ethanolic solution added to a 50 ml culture resulted in the required final DPA concentration.

Nicotine analogues supplied as salts were converted to the free bases before use.

All liquid additives and solutions were introduced into cultures by sterile pipette at the time of inoculation.

The final overall concentrations of inhibitors in cultures were as shown in Table 1.

Incubation with [2-¹⁴C]mevalonic acid. [2-¹⁴C]Mevalonic acid lactone (1 μ Ci/50 ml culture) (Radiochemical Centre, Amersham, Buckinghamshire) was converted into the potassium salt by the standard method (Britton & Goodwin, 1971). Sterile aqueous solutions of potassium mevalonate were added to *Flavobacterium* cultures by sterile pipette at the time of inoculation.

Extraction and purification of carotenoids. *Flavobacterium* organisms were harvested by centrifugation at 10000g for 10 min. Carotenoids were extracted from them with cold acetone, or in the case of nicotine-inhibited organisms containing lycopene, warm methanol followed by acetone. No mechanical disruption of the organisms was necessary. Debris was dried at 110 °C and its dry wt determined. The lipid extracts were transferred to diethyl ether–light petroleum (b.p. 40 to 60 °C) (1:1), washed with water, evaporated and chromatographed in light petroleum on a column of neutral alumina (Woelm) deactivated to Brockmann grade III by the addition of 6 % (w/w) water in the normal way (Britton & Goodwin, 1971). Three fractions (I–III) were usually collected, eluted respectively with 2 % (v/v) diethyl ether in light petroleum, 50 % diethyl ether in light petroleum and 2 % ethanol in diethyl ether. Fraction I contained carotenoid hydrocarbons, and was chromatographed on thin layers of silica gel G (Merck) with 0.5 % diethyl ether in light petroleum as developing solvent. Any β -carotene (R_F 0.6) or lycopene (R_F 0.5) present were eluted with diethyl ether and rechromatographed on thin layers of magnesium oxide–Kieselguhr G (1:1, w/w), β -carotene in 5 % acetone in light petroleum, and lycopene in acetone–benzene–light petroleum (2:2:1). The β -carotene chromatograms were examined under u.v. light for the presence of phytoene and phytofluene. In quantitative work, β -carotene and lycopene were estimated at this stage. For mass spectrometric and n.m.r. identification, and in radioisotope experiments, these compounds were given further purification by t.l.c. on silica gel G with 5 % benzene in light petroleum as developing solvent.

Fraction II, which contained monohydroxycarotenoids, was chromatographed on silica gel G with 50 % diethyl ether in light petroleum as developing solvent, and any coloured band present was eluted with diethyl ether and rechromatographed on MgO–Kieselguhr G with 40 % acetone in light petroleum to separate β -cryptoxanthin (R_F 0.8) and rubixanthin (R_F 0.4). These were assayed quantitatively at this stage and, when necessary, further purified by t.l.c. on silica gel G with 40 % diethyl ether in light petroleum as solvent, for mass spectrometry or radioassay.

Fraction III (dihydroxycarotenoids) was chromatographed on thin layers of silica gel G with diethyl ether as developing solvent, and the zeaxanthin band obtained was rechromatographed on MgO–Kieselguhr G (1:1) in 30 % acetone in light petroleum, for quantitative assay. For mass spectrometry and radioisotope experiments the zeaxanthin was further purified by t.l.c. on silica gel G with 8 % (v/v) methanol in benzene or 25 % (v/v) ethyl acetate in carbon tetrachloride as developing solvents.

Acetylation. Rubixanthin and zeaxanthin samples were acetylated by the acetic anhydride–pyridine method (Britton & Goodwin, 1971), and the acetates were purified by t.l.c. first on silica gel G in 20 % diethyl ether in light petroleum, and then on MgO–Kieselguhr G (1:1) with 25 % acetone in light petroleum (rubixanthin acetate) or 20 % acetone in light petroleum (zeaxanthin diacetate).

Determination of spectra. Electronic absorption spectra were determined, in light petroleum

(hydrocarbons) or ethanol (xanthophylls), with a Unicam SP 800 recording spectrophotometer. Carotenoids were estimated quantitatively from the spectra by the method of Davies (1965), using the $E_{1\text{ cm}}^{1\%}$ values given by Davies.

Mass spectra were determined by Mrs A. M. Ball in an A.E.I. MS12 instrument, at 70 eV, 200 to 220 °C.

Electron microscopy. Electron microscopic examination of *Flavobacterium* R1519 cells was kindly undertaken by Mrs J. Wood through the courtesy of Dr C. Green of this department.

Radioassay. Purified samples were dissolved in 5 ml toluene, bleached by u.v. irradiation and transferred to clean vials, to which 5 ml of liquid scintillator solution (10 g 2,5-diphenyloxazole and 0.6 g 1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene/l of toluene) were added. The samples were assayed for radioactivity in a Beckman LS 200 liquid scintillation counter.

RESULTS AND DISCUSSION

Several substances have been shown to inhibit production of the normal carotenoids in one or more species of bacteria and fungi. The effects of some of these substances on growth, and carotenoid biosynthesis in *Flavobacterium* R1519, an organism which normally produces zeaxanthin as virtually its only carotenoid, have been studied (Table 1).

The inhibitor best known and most widely used in carotenoid studies is diphenylamine (DPA). DPA has been shown to inhibit production of the normal carotenoids, and to cause an accumulation of the probable biosynthetic intermediate, phytoene (7,8,11,12,7',8',11',12'-octahydro- ψ,ψ -carotene) (IV) in many organisms, e.g. *Neurospora crassa* (Turian, 1957), *Phycomyces blakesleeanus* (Goodwin, Jamikorn & Willmer, 1953), *Blakeslea trispora* (Thomas & Goodwin, 1967), *Verticillium albo-atrum* (Valadon & Mummery, 1966), *Rhodospirillum rubrum* (Goodwin & Osman, 1954), *Mycobacterium phlei* (Goodwin, 1953; Rilling, 1965), *Flavobacterium dehydrogenans* (Weeks, 1971). Moreover, in some cases, removal of the DPA by washing allows synthesis of the normal carotenoids to proceed, often at the expense of the accumulated phytoene (Goodwin & Osman, 1954; Valadon & Mummery, 1966; Davies, Villoutreix, Williams & Goodwin, 1963). From studies of the kinetics of synthesis of the normal carotenoids in cultures of *Rhodospirillum rubrum* from which the DPA had been removed by washing, Liaaen-Jensen, Cohen-Bazire, Nakayama & Stanier (1958) were able to postulate schemes for the biosynthesis of spirilloxanthin, the normal major carotenoid of *R. rubrum*.

DPA thus acts by inhibiting the desaturation reaction of carotenoid biosynthesis (Turian, 1957; Rilling, 1965), and, because of this effect, has proved to be very useful in studies of carotenoid biosynthesis.

However, at no DPA concentration was any specific inhibition of zeaxanthin synthesis observed, or any production of phytoene detected, in *Flavobacterium* R1519 (Table 1); DPA is thus not a suitable inhibitor for use in studies of zeaxanthin biosynthesis in *Flavobacterium* R1519.

One outstanding problem in zeaxanthin biosynthesis is whether hydroxylation occurs at an early or a late stage. A substance which specifically inhibited the hydroxylation reaction would therefore be very useful in causing the accumulation of the prehydroxylation intermediate. Stereochemical studies (J. C. B. McDermott, F. J. Leuenberger, G. Britton and T. W. Goodwin, unpublished results) indicated that the hydroxylation probably involves a mixed-function oxidase type of enzyme, thus suggesting the use of a mixed-function oxidase inhibitor. Hammond & White (1970) have reported that several such inhibitors

Table 1. Summary of the effects of various substances on growth and carotenoid synthesis in *Flavobacterium* R1519

Substance	Concentration	Inhibition of growth	Carotenoids present
Diphenylamine	1×10^{-3} M, 1×10^{-4} M 1×10^{-5} M, 1×10^{-6} M 1×10^{-7} M,	Complete Partial None	None Zeaxanthin (100 %) Zeaxanthin (100 %)
SKF 525A	0.01 %, 0.005 %	Complete	None
SKF 3301A	0.01 %, 0.005 %		
Metopirone base	0.01 %, 0.005 %		
Diphenylpropylacetic acid	0.01 %, 0.005 %		
Piperonyl butoxide	0.04 %, 0.01 %		
SKF 7997-A ₃	0.01 %, 0.005 %	Slight	Zeaxanthin (100 %)
CaCl ₂ ·6H ₂ O	2.0 % 1.0 %		
(–)Nicotine	0.5 %, 0.1 %	None	Very little, mainly zeaxanthin
	5.0 mM	Very slight	Zeaxanthin (80 %), β -carotene (10 %), β -cryptoxanthin (10 %)
	3.75 mM	None	Zeaxanthin (100 %)
	1.0 mM	None	Lycopene (95 %), traces zeaxanthin, rubixanthin
(+)Nicotine			Lycopene (54 %), rubixanthin (42 %), zeaxanthin (trace)
			Rubixanthin (60 %), zeaxanthin (35 %), lycopene (trace)
	5.0 mM	Very slight	Lycopene (80 %), rubixanthin (5 %), zeaxanthin (10 %)
Nicotyrine	2.5 mM	Partial	Zeaxanthin (100 %)
Nor-nicotyrine	0.65 mM	Partial	Zeaxanthin (100 %)
Nor-nicotine	3.75 mM	Slight	Rubixanthin (84 %), zeaxanthin (12 %)
Myosmine	2.5 mM	Partial	Zeaxanthin (100 %)
Anabasine	5.0 mM	Partial	Zeaxanthin (100 %)
Quinine sulphate	5.0 mM	Complete	None
Quinidine sulphate			
Brucine sulphate			
Atropine sulphate	5.0 mM	Partial	Zeaxanthin (100 %)
Hyoscyamine sulphate			
Pilocarpine hydrochloride			
Scopolamine hydrobromide			
Strychnine sulphate	5.0 mM	Slight	Zeaxanthin (100 %)
3-Picoline			
Morpholine			
Pyridine			
3-Ethylpyridine	5.0 mM	Complete	None
5-Ethyl-3-methylpyridine			

prevented the formation of the normal polar carotenoids in *Staphylococcus aureus*, and led instead to the accumulation of carotene hydrocarbons.

The effect of some of these compounds (SKF 525A, SKF 3301A, metopirone base, diphenylpropylacetic acid, piperonyl butoxide and SKF 7997-A₃) on zeaxanthin production in *Flavobacterium* R1519 was therefore studied (Table 1), but in no case was it possible to demonstrate specific inhibition of zeaxanthin synthesis and accumulation of any other carotenoid in its place. It thus appears that mixed-function oxidase inhibitors are not of use as inhibitors of zeaxanthin biosynthesis in cultures of *Flavobacterium* R1519.

Earlier work during studies of the stereochemistry of zeaxanthin biosynthesis in *Flavobacterium* R1519 had established that the inclusion of small amounts of Ca²⁺ in the culture

medium resulted in a slight decrease in zeaxanthin production and allowed the detection of small amounts of β -carotene (β, β -carotene) (II) and β -cryptoxanthin (β, β -caroten-3-ol) (III). The effect of Ca^{2+} at different concentrations and under different incubation conditions was therefore studied.

At low Ca^{2+} concentrations (below 0.5 %, w/v, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$) no effect on growth or carotenogenesis was observed; 2 % $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ markedly inhibited growth and carotenogenesis. At 1 % $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, some change in carotenoid composition occurred, especially if the bacteria were cultured in normal conical flasks, rather than 'baffled' flasks, in order to diminish the aeration. Under these conditions, β -carotene and β -cryptoxanthin each constituted about 10 % of the total carotenoid content, but growth was somewhat lessened.

The accumulation of β -carotene and β -cryptoxanthin suggests that these compounds may be intermediates in zeaxanthin biosynthesis, though they could be side products which appear only under abnormal conditions. However, the inhibition of zeaxanthin biosynthesis was not sufficient to be of use in further series of experiments.

Recently two compounds have been shown, in several biological systems, to inhibit production of the normal carotenoids and cause an accumulation of lycopene (ψ, ψ -carotene) (V). In several higher plants, especially citrus species, and fungi (*Phycomyces blakesleeanus*, *Blakeslea trispora*), 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA) caused the accumulation of lycopene in place of the normal carotenoid complement (Coggins, Henning & Yokoyama, 1970; Yokoyama, Coggins & Henning, 1971; Hsu, Yokoyama & Coggins, 1972; Yokoyama, De Benedict, Coggins & Henning, 1972). A similar effect was observed in cultures of *Mycobacterium marinum* grown in the presence of nicotine (Howes & Batra,

1970); the normal main pigment, β -carotene, was replaced by lycopene. CPTA and nicotine are therefore thought to inhibit the cyclization reaction in carotenoid biosynthesis, and the accumulation of lycopene rather than neurosporene has been taken to indicate that lycopene is the substrate of this reaction. If cyclization is inhibited by nicotine or CPTA in *Flavobacterium* 0147, then production of zeaxanthin should be prevented, and in its place the precyclization intermediate would be expected to accumulate. Identification of the intermediate as a hydroxy compound or a hydrocarbon should then indicate whether hydroxylation occurs before or after cyclization.

Nicotine was therefore tested for any inhibitory effect on zeaxanthin formation in *Flavobacterium* 0147, initially at the concentration (5 mM) used by Howes & Batra (1970). It was immediately apparent that a change in pigmentation had occurred, since the cultures grown in the presence of nicotine were dull reddish-pink in contrast to the yellow of normal cultures. This was confirmed by chromatographic examination of the pigment extracts of the two cultures. Whereas the normal organisms contained zeaxanthin as essentially the only pigment, only traces of zeaxanthin were detected in nicotine-grown organisms. In these the main pigment was a hydrocarbon, which was identified as lycopene by comparison of its chromatographic properties, electronic absorption spectrum and mass spectrum with those of authentic lycopene. No other carotenoids were detected in more than trace amounts; in particular, no compound was detected with the chromatographic properties expected for the mono- or dihydroxylycopene which might have been expected if hydroxylation normally occurred before cyclization. These findings thus suggest that cyclization precedes hydroxylation in the biosynthetic pathway.

Because the inhibition of zeaxanthin synthesis was virtually complete at 5 mM-nicotine the effect of a lower concentration (1 mM) was examined. At this nicotine concentration, zeaxanthin synthesis was again largely inhibited, but only a very small amount of lycopene was present. The major carotenoid which replaced zeaxanthin had the chromatographic properties of a monohydroxycarotenoid, and was identified as rubixanthin (β, ψ -caroten-3-ol) (VI) by comparison of its chromatographic properties, electronic absorption spectrum and mass spectrum with those of rubixanthin isolated from rose hips, and by similar comparison of its acetate with rubixanthin acetate. This could suggest that rubixanthin is an intermediate in zeaxanthin biosynthesis, but probably indicates that, under the abnormal conditions of nicotine culture, inhibition of cyclization and hydroxylation at one end of the carotenoid molecule is essentially complete before appreciable inhibition of cyclization occurs at the other end of the molecule.

The effects of 7.5 mM-nicotine on growth and on the time course of pigment production (Fig. 1, 2) were that the total organism dry wt was always slightly lower, but the total pigment produced was higher, in the nicotine-grown cultures than in normal cultures. The total carotenoid concentration ($\mu\text{g/g}$ dry wt) was usually more than 30 % greater in nicotine-grown than in normal cultures. Such a stimulatory effect of nicotine on total carotenoid production has also been observed in cultures of several photosynthetic bacteria (A. Ben-Aziz, R. K. Singh, G. Britton & T. W. Goodwin, unpublished results).

Throughout this work it was found that the extraction of lycopene from nicotine-inhibited cells was much more difficult than the extraction of zeaxanthin from normal cells, or of zeaxanthin, rubixanthin and β -cryptoxanthin from nicotine-inhibited cells. The lycopene thus appeared to be much more firmly bound within the organisms. No apparent differences were revealed, however, by electron-microscopic examination of normal and 5 mM-nicotine-inhibited organisms, not even in the walls and membranes, where carotenoids of non-photosynthetic bacteria are generally believed to be located (Sistrom, 1959; Salton &

Fig. 1. Comparison of variation of lipid-free organism dry wt with growth time for normal and nicotine-grown (7.5 mM) cultures of *Flavobacterium* R1519.

Fig. 2. Comparison of variation of total carotenoid with growth time for normal and nicotine-grown (7.5 mM) cultures of *Flavobacterium* R1519.

Ehtisham-Ud-Din, 1965; Stoeckenius & Rowen, 1967; Work & Griffiths, 1968; Steensland & Larsen, 1969; Eberhardt, 1971).

The inhibition of zeaxanthin production was observed only with *Flavobacterium* R1519, a low temperature (20 to 22 °C) bacterium derived from a Norwegian parent, and not with several other zeaxanthin-producing *Flavobacterium* strains (R1534, R1552, R1560) derived from a higher temperature (26 to 28 °C) African parent.

Table 2. Incorporation of [2-¹⁴C]mevalonic acid into carotenoids in normal and nicotine-inhibited cultures of *Flavobacterium* R1519

Cultures (50 ml) were grown for 40 h with [2-¹⁴C]potassium mevalonate (1 μ Ci), and nicotine as indicated.

Nicotine added	Lycopene		Rubixanthin		Zeaxanthin	
	μ g	d.p.m.	μ g	d.p.m.	μ g	d.p.m.
None	—	—	—	—	372	40 240
1.0 mM	—	—	174	13 840	241	11 584
7.5 mM	309	1 3570	70	3 790	3	110

Flavobacterium R1519 efficiently incorporated [2-¹⁴C]mevalonate into zeaxanthin in normal cultures and into lycopene and rubixanthin in nicotine-inhibited cultures. (Table 2).

Several alkaloids, substituted pyridines and compounds structurally similar to nicotine were examined for inhibitory effects on zeaxanthin synthesis, to determine whether the inhibition of cyclization was specific to nicotine or other related compounds acted similarly (Table 1).

No specific inhibitory effect on zeaxanthin biosynthesis was observed for any of the alkaloids or substituted pyridines tested.

Several compounds with structures very similar to nicotine (VII) were then tested. Most of these did not affect carotenoid biosynthesis, zeaxanthin remaining virtually the only pigment in cultures grown in the presence of anabasine (VIII), myosmine (IX), nicotyrine (X) and *nor*-nicotyrine (XI). In cultures grown in the presence of 3.75 mM-*nor*-nicotine (XII) however, rubixanthin (84 %) replaced zeaxanthin as the main carotenoid. *Nor*-nicotine was thus also an inhibitor of carotenoid cyclization, although at this concentration, the degree

of inhibition was less than with nicotine. Concentrations of any of these compounds above 2 to 3 mM seriously inhibited bacterial growth.

The work on the inhibition by nicotine had all been with the naturally occurring (–) enantiomer; it was found that zeaxanthin biosynthesis was inhibited to approximately the same extent by the unnatural (+) isomer.

Thus the inhibition of carotenoid cyclization by nicotine had considerable specificity. Although both optical isomers of nicotine were inhibitory, absence of the *N*-methyl group (as in *nor*-nicotine) reduced the inhibition, and the minor modifications in anabasine, myosmine, nicotyrine and *nor*-nicotyrine resulted in a complete loss of inhibition. Unfortunately, only small amounts of these nicotine-like compounds were available, allowing in most cases only a single small-scale experiment, so that this potentially interesting aspect of nicotine inhibition could not be studied further.

The nicotine inhibition in *Flavobacterium* R1519 of the cyclization reaction of carotenoid biosynthesis, especially in combination with isotopic labelling, promises to be very useful for elucidating details of the pathways of zeaxanthin biosynthesis in *Flavobacterium* R1519.

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