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Alternative Pathways of Zeaxanthin Biosynthesis in a *Flavobacterium* Species

EXPERIMENTS WITH NICOTINE AS INHIBITOR

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In *Flavobacterium* R1519, nicotine blocks zeaxanthin biosynthesis by specifically inhibiting the cyclization reaction. Lycopene (at high nicotine concentrations, e.g. 7.5mM) and rubixanthin (at low nicotine concentration, e.g. 1mM) replace zeaxanthin as the main carotenoid. On removal of the nicotine lycopene is converted into β-carotene under anaerobic conditions and into zeaxanthin in the presence of O_2. The conversion *in vivo* of β-carotene into zeaxanthin was also demonstrated. Cyclization (an anaerobic process) thus precedes hydroxylation (O_2-requiring) in the biosynthesis of zeaxanthin. The conversion *in vivo* of rubixanthin into β-cryptoxanthin and into zeaxanthin was demonstrated, thus indicating the operation of alternative pathways of zeaxanthin biosynthesis. Several alternative biosynthetic pathways are considered and the results are also discussed in terms of reaction sequences of carotenoid 'half-molecules'.

The biosynthesis of carotenoids (for a review, see Goodwin, 1971) is generally considered to involve the condensation of two molecules of the C_{20} precursor geranylgeranyl pyrophosphate to yield phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψψ-carotene, I), via the intermediate prephytoene pyrophosphate (≡prelycopersene pyrophosphate) (Altman *et al.*, 1972; Qureshi *et al.*, 1972, 1973; Barnes *et al.*, 1973). This is followed by sequential desaturation of phytoene to give successively phytofluene (7,8,11,12,7′,8′-hexahydro-ψψ-carotene, II), ζ-carotene (7,8,7′,8′-tetrahydro-ψψ-carotene, III), neurosporene (7,8-dihydro-ψψ-carotene, IV) and finally lycopene (ψψ-carotene, V), which is thought to be the precursor of the cyclic carotenes, e.g. β-carotene (ββ-carotene, IX) (Fig. 1). An alternative desaturation sequence, via the unsymmetrical isomer of ζ-carotene, 7,8,11,12-tetrahydro-ψψ-carotene (VI) has been indicated in several micro-organisms (Davies, 1970, 1973; Weeks, 1971).

Although the pathway of biosynthesis of the carotene hydrocarbons, as outlined in Fig. 1, is reasonably well established, very little information is available on the introduction of other structural modifications, e.g. the oxygen functions of xanthophylls. Claes (1959) showed that if a *Chlorella* mutant was cultured heterotrophically in the dark and then illuminated in the absence of O_2 carotene hydrocarbons accumulated; when the cultures were placed in the dark and allowed access to O_2, these carotenes were apparently replaced by xanthophylls. Largely as a result of this work it has generally been accepted that the introduction of hydroxyl groups occurs at a late stage in the biosynthetic pathway, i.e. lutein (βε-carotene-3,3′-diol) and zeaxanthin (ββ-carotene-3,3′-diol, X) are generally considered to be formed by hydroxylation of α-carotene (βε-carotene) and β-carotene respectively.

The availability of strains of a non-photosynthetic *Flavobacterium* which synthesize large amounts of zeaxanthin has now provided an opportunity to study xanthophyll biosynthesis in detail. The stereochemistry of the bacterial zeaxanthin (3*R*, 3′*R*) (J. C. B. McDermott, F. J. Leuenberger, G. Britton & T. W. Goodwin, unpublished work) is the same as in higher plants (Bartlett *et al.*, 1969), and no differences have been observed in the stereochemistry of zeaxanthin biosynthesis in the bacterial and plant systems (J. C. B. McDermott, F. J. Leuenberger, T. J. Walton, G. Britton & T. W. Goodwin, unpublished work). The *Flavobacterium* strain R1519, used in the present work has previously been used in some preliminary and stereochemical studies (McDermott *et al.*, 1973*a*,*b*). This organism normally produces only zeaxanthin, no other likely intermediates being detected. Under abnormal conditions, i.e. in the presence of Ca^{2+} ions, some β-carotene and β-cryptoxanthin (ββ-caroten-3-ol, XI) are produced (McDermott

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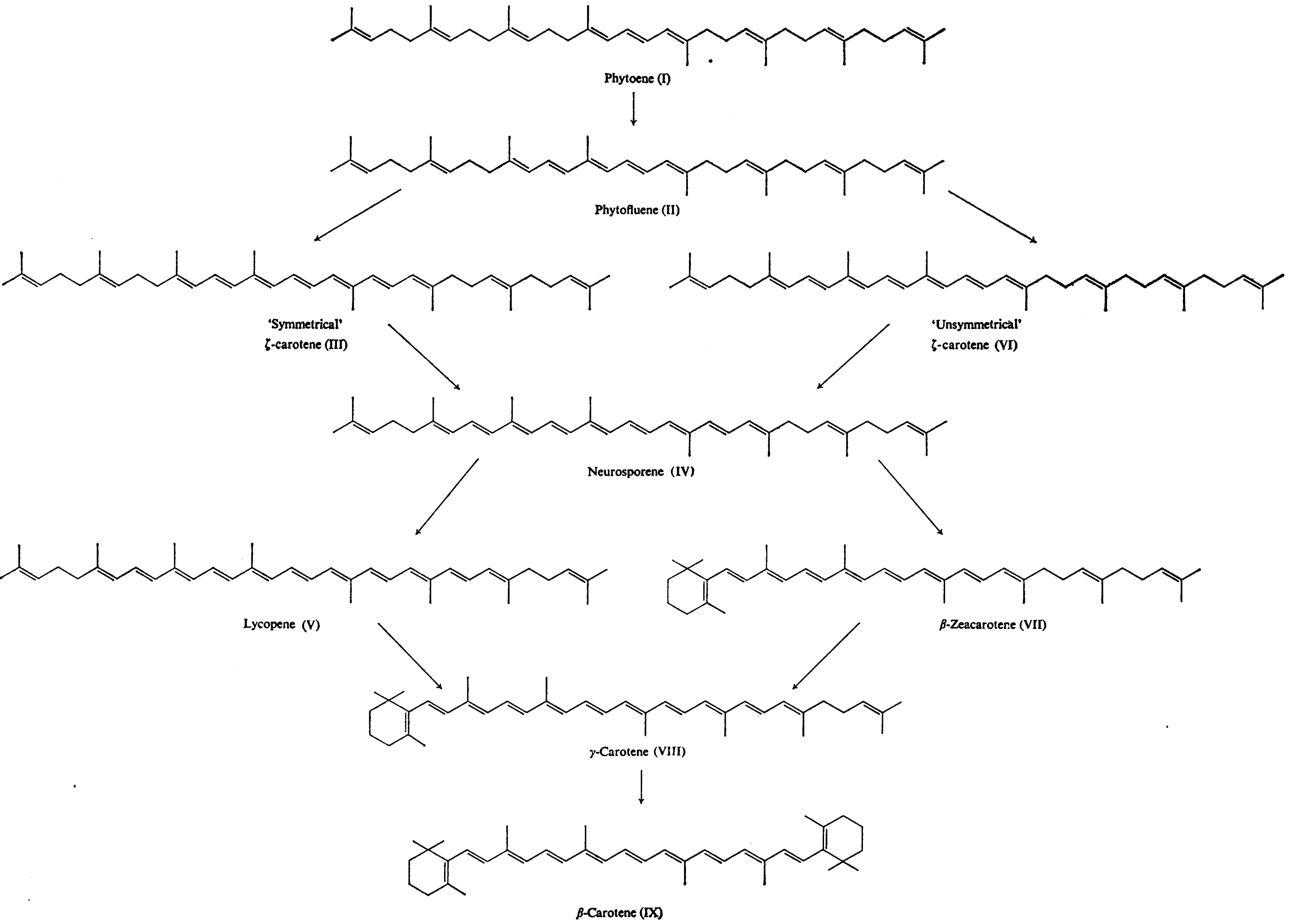

Fig. 1. *Alternative pathways for formation of β-carotene in Flavobacterium*

For details see the text.

et al., 1973*b*). Another *Flavobacterium* strain R1560, also produces mainly zeaxanthin, but small amounts (approx. 5% of total carotenoid) of other carotenoids can be detected (Leuenberger *et al.*, 1973; F. J. Leuenberger, A. J. Schocher, G. Britton & T. W. Goodwin, unpublished work). These include β-carotene, β-zeacarotene (7′,8′-dihydro-βψ-carotene, VII), 3-hydroxy-β-zeacarotene (7′,8′-dihydro-βψ-caroten-3-ol, XIII) and β-cryptoxanthin, and phytoene, phytofluene, ζ-carotene, neurosporene and lycopene, showing the presence of a desaturation series similar to that found in higher plants and fungi (Fig. 1). The stereochemistry of hydrogen loss in the desaturations (McDermott *et al.*, 1973*a*) is the same as that found in plants (Williams *et al.*, 1967; J. R. Vose, G. Britton & T. W. Goodwin, unpublished work). The order of events (cyclization, hydroxylation) occurring after lycopene, however, remains in doubt, although the detection of β-carotene and β-cryptoxanthin may indicate the sequence lycopene → β-carotene → β-cryptoxanthin → zeaxanthin.

Recent work with *Mycobacterium* species (Howes & Batra, 1970; Batra *et al.*, 1973) has shown that nicotine inhibits the cyclization reaction of carotenoid biosynthesis, and the inhibitory effect appears to be easily reversible. In *Flavobacterium* any inhibition of cyclization by nicotine should result in accumulation of the pre-cyclization intermediate, thus indicating whether cyclization normally occurs before or after hydroxylation. After removal of the inhibitor, conversion *in vivo* of the accumulated pre-cyclization intermediate into the normal carotenoids could help to establish the pathway of zeaxanthin biosynthesis.

Preliminary work (McDermott *et al.*, 1973*b*) has shown that nicotine does inhibit the formation of zeaxanthin in *Flavobacterium* R1519, and the results of detailed studies of the effects of nicotine on zeaxanthin biosynthesis in this organism are now reported.

An outline of early stages of this work has been included in a previous publication (McDermott *et al.*, 1973*c*).

Materials and Methods

Flavobacterium strain R1519 (≡0147) was a kind gift from F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. Culture conditions for *Flavobacterium* and the methods used for extraction, purification, identification, quantitative assay and radioassay of the carotenoids have been described (McDermott *et al.*, 1973*b*).

Removal of nicotine and resuspension and reincubation of Flavobacterium cells

Flavobacterium R1519 was grown in a medium containing the required concentration of nicotine and harvested by centrifugation, washed twice with salts solution (4%, w/v, NaCl–0.5% $MgSO_4,7H_2O$), buffered salt solution (as above, buffered to pH 7.8 with 0.1 M-potassium phosphate), or culture medium and resuspended in the same solution. The pooled cell suspension was divided equally into the required number of portions, which were then extracted or incubated as described for each experiment in Tables 1–7.

In the series of experiments involving radioactive isotopic labelling, cultures were grown in medium containing [2-^{14}C]mevalonate, and nicotine. In the early experiments of this series (Table 4) the cells were washed with medium to remove nicotine and [2-^{14}C]mevalonate, and were then resuspended and reincubated in medium. In the later experiments (Tables 5 and 7) the cells were washed free from mevalonate with medium containing nicotine, reincubated for 5 h in medium containing nicotine to permit any intermediates to undergo conversion into lycopene and then washed with medium to remove nicotine, and reincubated as described in the Tables.

Resuspended cells were incubated aerobically in baffled conical flasks with shaking. In anaerobic reincubations, cell suspensions were maintained under N_2 in sealed vials. In each case, after the incubation was complete, the carotenoids were extracted, purified and assayed (McDermott *et al.*, 1973*b*).

Attempts to demonstrate the formation in vivo of γ-carotene from lycopene

(i) Cultures of *Flavobacterium* R1519 (10×50 ml) grown for 24 h at 20°C in medium containing nicotine (7.5 mM) were harvested by centrifugation and resuspended in 120 ml of medium containing nicotine (7.5 mM). The suspension was divided into eight 15 ml portions. The individual portions were washed twice with and resuspended in medium containing the required concentration of nicotine (0.1–7.5 mM) and then reincubated for 27 h at 20°C under N_2. The cells were harvested by centrifugation and the pigments extracted. The extracts were each chromatographed on thin layers of silica gel G, with 5% (v/v) benzene in light petroleum (b.p. 40–60°C) as developing solvent. β-Carotene (R_F 0.6) and lycopene (R_F 0.5) were recovered from these chromatograms, and were further purified and assayed spectrophotometrically (Table 8, Expt. A). No γ-carotene (R_F of marker 0.55) was detected in any of the extracts.

(ii) In a similar experiment, *Flavobacterium* R1519 was grown in medium containing nicotine (7.5 mM) and [2-^{14}C]mevalonate (1 μCi/50 ml of culture). The cells were harvested, washed and reincubated anaerobically as above and the pigments extracted. Carrier γ-carotene (200 μg) was added to each extract, and the γ-carotene was isolated and purified in each case by successive t.l.c. on silica gel G with 5% (v/v) benzene in light petroleum (b.p. 40–60°C), MgO–Kieselguhr G (1:1, w/v) with 20% (v/v) acetone in light

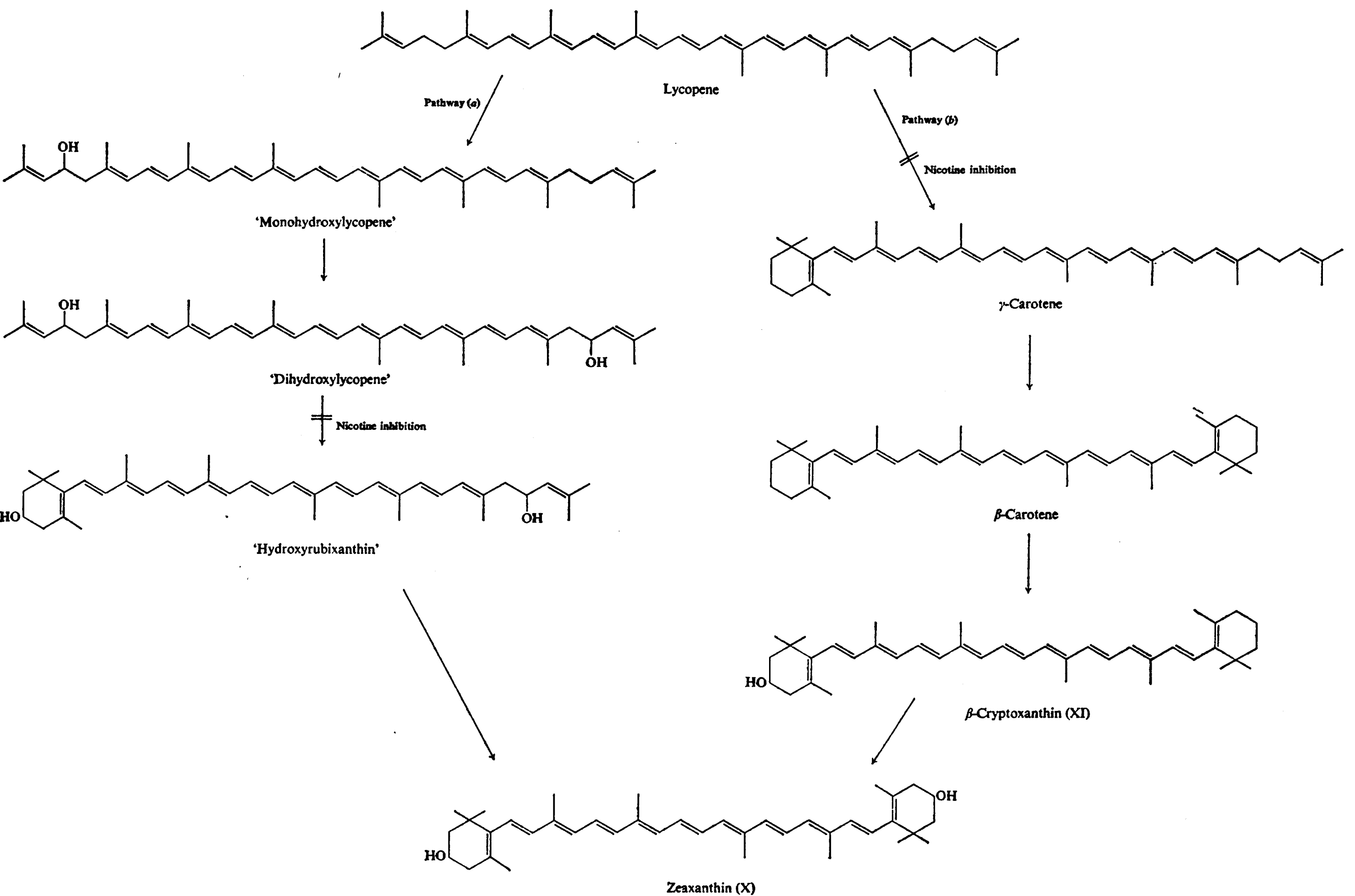

Fig. 2. *Expected effects of the cyclization inhibitor nicotine on zeaxanthin biosynthesis*

Pathway (*a*) if hydroxylation precedes cyclization; pathway (*b*) if cyclization precedes hydroxylation.

Table 1. *Carotenoid changes in nicotine-grown Flavobacterium R1519 after removal of the nicotine by washing*

Expt. (A), *Flavobacterium* R1519 (3×50ml) was grown aerobically for 40h in medium containing nicotine (7.5mM). The nicotine was removed by washing with salts solution (4%, w/v, NaCl–0.5% $MgSO_4,7H_2O$) and the cells were resuspended in this solution (45ml). Three portions (15ml) of the cell suspension were treated as below. Expt. (B), *Flavobacterium* R1519 (4×50ml) was grown for 41h in medium containing nicotine (7.5mM), and the cells were harvested, washed and resuspended under the same conditions as in Expt. (A). In all Tables, percentage values refer to the percentage of total carotenoid.

	Carotenoid composition					
	Lycopene		β-Carotene		Zeaxanthin	
Treatment	(μg)	(%)	(μg)	(%)	(μg)	(%)
Expt. (A)						
(i) Control cells (not reincubated)	90	87	0	0	2	2
(ii) Reincubation aerobic (40h)	5.5	15	7.1	19	15.0	41
(iii) Reincubation anaerobic (40h)	0	0	114	87	2.3	2
Expt. (B)						
(i) Control cells (not reincubated)	278	62	40	9	3	1
(ii) Reincubation anaerobic (22h)	81	23	214	61	17	5
(iii) Reincubation anaerobic (22h), then aerobic (8h)	140	21	364	55	58	9
(iv) Reincubation aerobic (8h)	157	28	251	45	63	11

petroleum, and silica gel G with 0.5% (v/v) diethyl ether in light petroleum. The γ-carotene samples were assayed for radioactivity (McDermott *et al.*, 1973*a*) but no radioactivity was detected in any sample.

Attempts to demonstrate the formation in vivo of rubixanthin from lycopene

In an experiment similar to Expt. (i) above, cultures of *Flavobacterium* R1519 were grown in medium containing nicotine (7.5mM), washed as above, and portions of the cells were resuspended and incubated in medium containing various concentrations of nicotine (0.1–7.5mM) under aerobic conditions (15ml of cell suspension in a 50ml baffled conical flask) for 27.5h at 20°C. The pigments were extracted and lycopene, rubixanthin and zeaxanthin were purified and assayed quantitatively. (Table 8, Expt. B).

Results

Flavobacterium R1519 grown in the presence of nicotine (7.5mM) contained as its main pigment lycopene, identified by a comparison of its electronic, n.m.r. and mass spectra, and chromatographic properties with those of authentic lycopene. At lower nicotine concentrations (approx. 1mM) the main pigment was similarly identified as rubixanthin (βψ-caroten-3-ol, XII). A graph showing the variation of carotenoid composition with nicotine concentration has been published (McDermott *et al.*, 1973*c*). No hydroxylycopenes were detected at any nicotine concentration. Nicotine, which inhibits the cyclization reaction in carotenoid biosynthesis in a *Mycobacterium* species (Howes & Batra, 1970; Batra *et al.*, 1973), had the same effect in *Flavobacterium* R1519. In both organisms lycopene accumulated as the apparent pre-cyclization precursor. Since nicotine did not inhibit carotenoid hydroxylation in *Flavobacterium* R1519 (see Table 5, Expt. B and the Discussion section), the accumulation of lycopene rather than hydroxylycopene showed that hydroxylation did not normally precede cyclization (Fig. 2).

Table 2. *Variation with time of the carotenoid composition of nicotine-grown Flavobacterium R1519 after removal of the nicotine by washing*

Flavobacterium R1519 (4×50ml) was grown and treated as in Table 1. Four portions (50ml) of cell suspension were incubated anaerobically for different times.

	Carotenoid composition					
Incubation time	Lycopene		β-Carotene		Zeaxanthin	
(h)	(μg)	(%)	(μg)	(%)	(μg)	(%)
0	205	82	0	0	4	2
3.5	62	37	80	48	9	6
20	30	15	136	69	8	4
44	25	14	130	71	5	3

Experiments with high nicotine concentrations

To confirm the biosynthetic sequence of lycopene being converted into β-carotene then into zeaxanthin, experiments were devised to try and demonstrate this conversion *in vivo*. In the first experiments, cultures of *Flavobacterium* R1519, grown in the presence of

Table 3. *Carotenoid changes in nicotine-grown Flavobacterium R1519 resuspended in buffered salts solution after removal of the nicotine by washing*

Flavobacterium R1519 (4×50ml) was grown aerobically for 40h in medium containing nicotine (7.5mM). The nicotine was removed by washing (twice) with salts solution (4% NaCl–0.5% $MgSO_4$,$7H_2O$) buffered to pH7.8 with 0.1M-potassium phosphate. The cells were resuspended in this solution (60ml) and four portions (15ml) of the suspension were treated as follows.

	Carotenoid composition					
	Lycopene		β-Carotene		Zeaxanthin	
Treatment	(μg)	(%)	(μg)	(%)	(μg)	(%)
(i) Control cells (not reincubated)	170	93	0	0	2	1
(ii) Reincubation anaerobic (24h)	45	32	72	52	7	5
(iii) Reincubation aerobic (24h)	22	13	39	24	82	50
(iv) Reincubation anaerobic (24h) then aerobic (24h)	40	29	64	48	17	12

Table 4. *Carotenoid changes in nicotine-grown Flavobacterium R1519 resuspended in buffered culture medium after removal of the nicotine by washing*

Flavobacterium R1519 (15×50ml) was grown aerobically for 42h in medium containing nicotine (7.5mM) and [2-^{14}C] mevalonic acid (as potassium salt, 15μCi). The nicotine and mevalonate were removed by washing (twice) with culture medium (buffered to pH7.8 with 0.1M-potassium phosphate). The cells were resuspended in this buffered medium (60ml) and four portions (15ml) of the suspension were treated as described below.

	Carotenoid composition								
	Lycopene			β-Carotene			Zeaxanthin		
Treatment	(μg)	(%)	(^{14}C radio-activity; d.p.m.)	(μg)	(%)	(^{14}C radio-activity; d.p.m.)	(μg)	(%)	(^{14}C radio-activity; d.p.m.)
(i) Control cells (no reincubation)	309	81	13570	0	0	0	3	1	110
(ii) Reincubation aerobic (24h) with nicotine (7.5mM)	383	69	25420	0	0	0	30	5	1890
(iii) Reincubation aerobic (24h) without nicotine	36	8	1170	18	4	1460	336	79	12750
(iv) Reincubation anaerobic (24h) without nicotine	32	9	3170	222	63	17770	7	2	410

nicotine (7.5mM), were harvested and the cells washed free from nicotine and resuspended and incubated in salts solution (see the Materials and Methods section) with no carbon source present, to minimize the possibility of carotenoid synthesis *de novo*. In each case (Table 1) formation of β-carotene at the expense of the accumulated lycopene occurred under anaerobic conditions, and some production of zeaxanthin was observed in aerobic incubations. The admission of air into anaerobic cell resuspensions that had been allowed to produce β-carotene from accumulated lycopene did not result in any appreciable formation of zeaxanthin. Anaerobic formation of β-carotene was essentially complete after 20h (Table 2). These results were confirmed by similar experiments in which nicotine-grown cells were washed and resuspended in salts solution buffered with 0.1M-potassium phosphate to pH7.8, the normal pH value of 40h *Flavobacterium* cultures (Table 3).

In these experiments, after the prolonged reincubation under non-physiological conditions (lack of carbon source), the total carotenoid content was often greatly diminished, especially in aerobic reincubations. In later experiments therefore the cells were washed and resuspended in full growth medium, and radioactive isotopic labelling with [2-^{14}C]mevalonate was used to distinguish between conversions *in vivo* and synthesis *de novo*.

The first of these modified experiments (Table 4) showed an increase in the amount and radioactivity of zeaxanthin (aerobic reincubation) and β-carotene (anaerobic reincubation) at the expense of the accumulated lycopene, but the incorporation of similar amounts of radioactivity into lycopene during

Table 5. *Changes in carotenoid composition and radioactivity in nicotine-grown Flavobacterium R1519 after removal of the nicotine by washing*

Flavobacterium R1519 (16×50ml) was grown aerobically for 24h in medium containing nicotine (7.5mM) and potassium [2-^{14}C]mevalonate (16μCi). The mevalonate was removed by washing twice with culture medium containing nicotine (7.5mM) and the cells were resuspended in this nicotine-containing medium and incubated for 5h (Expt. A) and 16h (Expt. B). The cells were then harvested, washed (twice) with medium to remove the nicotine and resuspended in culture medium. Portions (15ml) of the cell suspension were treated as below.

	Carotenoid composition								
	Lycopene			β-Carotene			Zeaxanthin		
Treatment	(μg)	(%)	(^{14}C radioactivity; d.p.m.)	(μg)	(%)	(^{14}C radioactivity; d.p.m.)	(μg)	(%)	(^{14}C radioactivity; d.p.m.)
Expt. (A)									
(i) Control cells (not reincubated)	682	77	28650	0	0	0	40	4	360
(ii) Reincubation aerobic with nicotine (7.5mM)	1038	71	28800	0	0	0	56	4	330
(iii) Reincubation, limited aeration, without nicotine	102	10	2430	403	40	17220	348	34	8700
Expt. (B)									
(i) Control cells (not reincubated)	824	92	64990	0	0	0	25	2	1150
(ii) Reincubation 24h anaerobic without nicotine	621	52	37980	421	35	19970	43	4	2160
(iii) Reincubation 24h, anaerobic without nicotine, then 24h aerobic without nicotine	195	20	8320	36	4	2080	675	68	50630
(iv) Reincubation 24h anaerobic without nicotine, then 24h aerobic with nicotine (7.5mM)	746	54	53502	4	0	620	518	37	24130

further incubation in the presence of nicotine showed that these increases could be accounted for by synthesis *de novo* from radioactive biosynthetic intermediates formed before the [2-^{14}C]mevalonate was removed. In later experiments this effect was overcome by washing the cells free from [2-^{14}C]mevalonate and reincubating with nicotine for a further 5–16h so that the labelled intermediates were incorporated into the accumulated lycopene. Continued reincubation in the presence of nicotine then resulted in no further increase in the radioactivity found in lycopene (Table 5, Expt. A). In cells reincubated with limited aeration and with no nicotine present the amount of lycopene fell to a low value, and substantial amounts of β-carotene and zeaxanthin were formed; the radioactivity previously associated with the accumulated lycopene was now found in the β-carotene and zeaxanthin. This experiment clearly demonstrated the conversion *in vivo* of the accumulated lycopene into β-carotene and zeaxanthin.

The work was then extended in an attempt to show the stepwise conversion *in vivo* of lycopene into β-carotene and then into zeaxanthin. Cultures were grown as above, with nicotine and [2-^{14}C]mevalonate and, after removal of the mevalonate, were incubated for a further 16h in the presence of nicotine. At this stage [treatment (i), Table 5, Expt. B], lycopene constituted over 90% of the total pigment and contained over 90% of the total carotenoid radioactivity. The nicotine was then washed out and the cells were reincubated anaerobically with no nicotine present whereupon β-carotene (with 35% of total carotenoid and 30% of total carotenoid radioactivity) was formed and the amount of radioactivity in lycopene fell by approx. 30–40%. Exposure of one portion of these cells to air, in the absence of nicotine, resulted in a large increase (to approx. 70% of total carotenoid) in the amount of zeaxanthin present and zeaxanthin also became the most radioactive carotenoid. The amounts and radioactivities of lycopene and β-carotene fell to very low values, suggesting that the zeaxanthin had been formed from the lycopene and β-carotene. The second portion of the cells in which β-carotene had been allowed to accumulate was incubated in the presence of air and nicotine (7.5mM). The amount and radioactivity of β-carotene decreased to a negligible value, and a concomitant increase was observed in the amount and radioactivity of zeaxanthin. The relative amount and radioactivity of lycopene remained essentially unchanged.

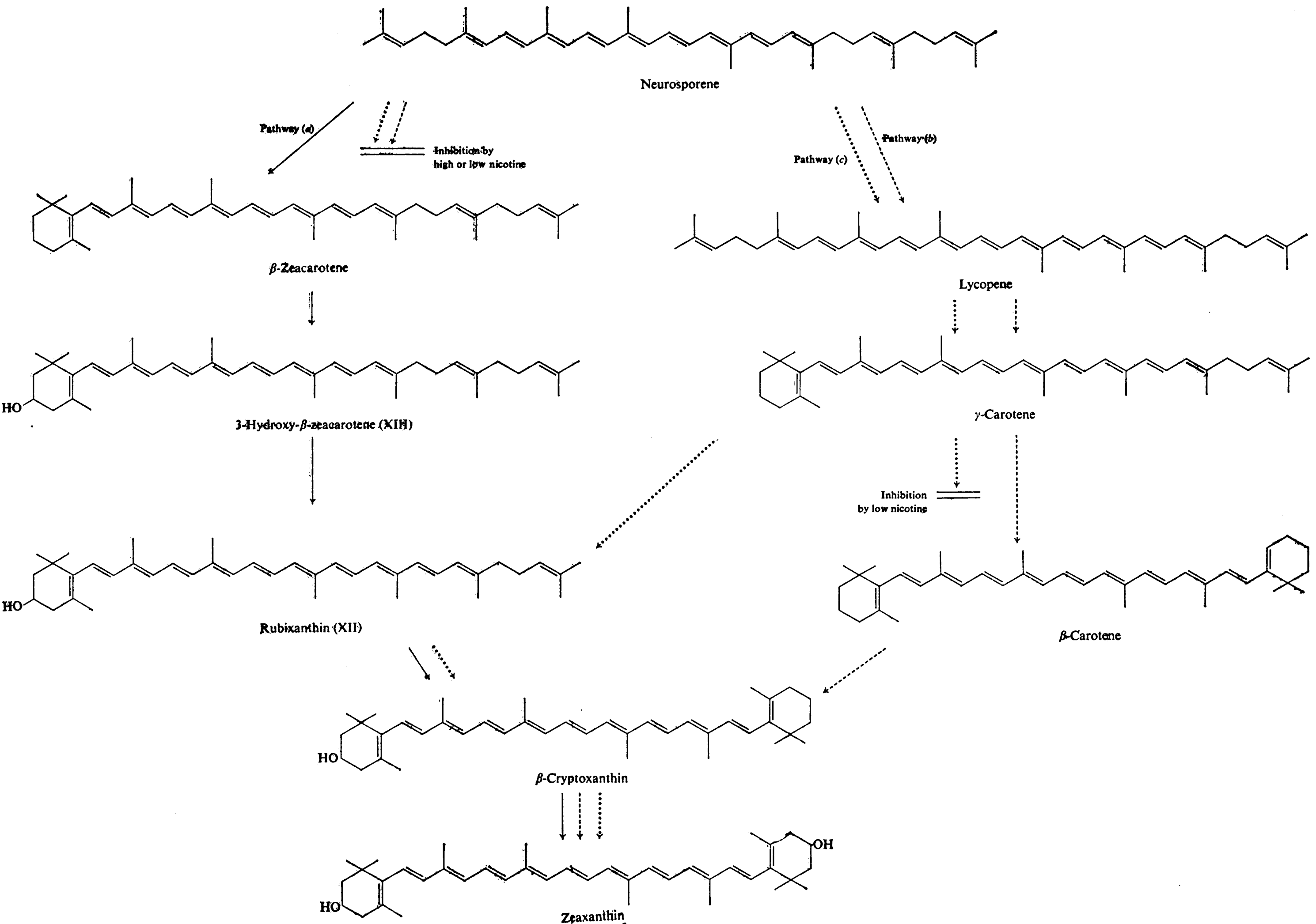

Fig. 3. *Alternative pathways of zeaxanthin biosynthesis from neurosporene in Flavobacterium*

Pathway (*a*) via β-zeacarotene if no inhibitor is present; pathway (*b*) diversion to lycopene in the presence of nicotine, and the conversion of lycopene into zeaxanthin via β-carotene on removal of the inhibitor; pathway (*c*) via lycopene and rubixanthin as demonstrated by experiments with low nicotine concentrations.

Table 6. *Carotenoid changes in nicotine-grown* (1 mM) *Flavobacterium R*1519 *resuspended in buffered salts solution after removal of the nicotine by washing*

Experimental details are as outlined in Table 3, except that the organism was originally cultured in medium containing 1 mM-nicotine.

	Carotenoid composition					
	Rubixanthin		β-Cryptoxanthin		Zeaxanthin	
Treatment	(μg)	(%)	(μg)	(%)	(μg)	(%)
Expt. (A)						
(i) Control cells (not reincubated)	103	61	1	1	65	38
(ii) Reincubation aerobic (48h)	72	39	1	1	110	60
Expt. (B)						
(i) Control cells (not reincubated)	317	83	0	0	64	17
(ii) Reincubation anaerobic (24h)	172	65	62	23	31	12

Table 7. *Carotenoid changes in nicotine-grown* (1 mM) *Flavobacterium R*1519 *resuspended in culture medium after removal of the nicotine by washing*

Experimental details are as outlined in Table 5, Expt. (A), except that the organism was originally cultured in medium containing 1 mM-nicotine.

	Carotenoid composition								
	Rubixanthin			β-Cryptoxanthin			Zeaxanthin		
Treatment	(μg)	(%)	(^{14}C radio-activity; d.p.m.)	(μg)	(%)	(^{14}C radio-activity; d.p.m.)	(μg)	(%)	(^{14}C radio-activity; d.p.m.)
(i) Control cells (not reincubated)	348	41	27690	12	1	450	482	57	23170
(ii) Reincubation aerobic (44h) with nicotine (1 mM)	350	26	25930	3	0	120	988	73	32780
(iii) Reincubation aerobic (44h)	194	19	1410	3	0	150	814	81	44700
(iv) Reincubation anaerobic (44h)	279	30	19630	67	7	3590	565	60	26000

This experiment clearly establishes that zeaxanthin was formed at the expense of the accumulated β-carotene, since formation of zeaxanthin from lycopene, and any carotenoid synthesis *de novo* beyond the lycopene stage, were prevented by the presence of nicotine. Under the conditions used therefore the pathway lycopene→β-carotene→zeaxanthin was operating. This experiment also demonstrated that the hydroxylation reaction was not inhibited by nicotine, since the formation of zeaxanthin from β-carotene was able to proceed in the presence of the inhibitor.

Experiments with low nicotine concentrations

The identification of rubixanthin as the main carotenoid present in cultures of *Flavobacterium* R1519 grown in the presence of lower concentrations of nicotine (1 mM) suggested the possibility of an alternative pathway of zeaxanthin biosynthesis via rubixanthin (Fig. 3, pathway *c*). A series of experiments was therefore performed in which *Flavobacterium* R1519 grown in the presence of 1 mM-nicotine was reincubated under various conditions after removal of the inhibitor. The results of these experiments are shown in Tables 6 and 7.

In the first experiments, the cells were washed free from nicotine, resuspended and reincubated in salts solution buffered to pH 7.8 with 0.1 M-potassium phosphate. Aerobic reincubation (Table 6, Expt. A) resulted in a considerable decrease in the rubixanthin content, with a similar increase in the zeaxanthin content. An anaerobic reincubation (Table 6, Expt. B) showed a substantial production of β-cryptoxanthin, undetected in the control cells, at the expense of the accumulated rubixanthin. In the confirmatory experiment (Table 7) in which radioactively labelled rubixanthin was accumulated by growth of the bacteria in the presence of nicotine (1 mM) and [2-^{14}C]mevalonate, aerobic reincubation resulted in a considerable increase in the amount and radioactivity of zeaxanthin, and a concomitant decrease in the amount of rubixanthin and the radioactivity associated with it.

Some increase in the amount and radioactivity of β-cryptoxanthin at the expense of rubixanthin was observed in the anaerobic reincubation. These experiments clearly demonstrated the conversion of accumulated rubixanthin into β-cryptoxanthin under anaerobic conditions, and into zeaxanthin in the presence of O_2, and indicated the operation of an alternative pathway of zeaxanthin biosynthesis.

It was thought that the rubixanthin that accumulated in the presence of low concentrations of nicotine would be formed from lycopene via γ-carotene (βψ-carotene, VIII), so attempts were made to demonstrate these conversions. Nicotine at high concentrations inhibited cyclization in both halves of the carotenoid molecule, resulting in the accumulation of lycopene, whereas the main effect of lower nicotine concentrations was to inhibit cyclization of one-half of the molecule, resulting in the accumulation of rubixanthin. When *Flavobacterium* cultures were grown with 7.5mM-nicotine to cause an accumulation of lycopene, and then washed and reincubated aerobically with low concentrations of nicotine, no significant increase in the rubixanthin content was observed at any nicotine concentration used, although some zeaxanthin was formed at the expense of the lycopene (Table 8, Expt. B). The small fluctuation in the amount (always low) of rubixanthin could easily be accounted for by synthesis *de novo* occurring during the reincubations. Similarly anaerobic reincubation in a range of low nicotine concentrations resulted in limited formation of β-carotene at the expense of some of the accumulated lycopene (Table 8, Expt. A). No γ-carotene was detected at any of the nicotine concentrations used. When the accumulated lycopene was labelled by growth of the cultures in the presence of [2-^{14}C]mevalonate and 7.5mM-nicotine, and the washed cells were reincubated anaerobically in low concentrations of nicotine, no radioactivity was recovered in γ-carotene isolated after the addition of unlabelled carrier material. Thus it was not possible to demonstrate the conversion *in vivo* of lycopene into γ-carotene or rubixanthin under these conditions.

Table 8. *Carotenoid changes in nicotine-grown* (7.5mM) *Flavobacterium R*1519 *resuspended in lower nicotine concentrations*

Expt. (A), anaerobic reincubation. *Flavobacterium* R1519 was grown aerobically for 24h at 20°C in medium containing nicotine (7.5mM). The cells were harvested and washed, and portions were resuspended in medium (15ml) containing various nicotine concentrations and reincubated anaerobically (under N_2) for 27h at 20°C. Expt. (B), aerobic reincubation. Experimental details were as in Expt. (A), except that cell suspensions were reincubated for 27.5h aerobically.

Nicotine concentration in reincubation (mM)	Carotenoid composition			
	Lycopene		β-Carotene	
	(μg)	(%)	(μg)	(%)
Expt. (A)				
0	172	60	115	40
0.1	159	77	46	22
0.4	206	89	26	11
0.7	255	95	15	5
1.0	186	97	6	3
1.5	290	99	2	1
2.0	249	100	1	0
7.5	259	100	0	0

	Carotenoid composition					
	Lycopene		Rubixanthin		Zeaxanthin	
	(μg)	(%)	(μg)	(%)	(μg)	(%)
Expt. (B)						
0	394	62	34	5	204	32
0.1	558	78	47	6	106	15
0.4	557	82	42	6	77	11
0.7	661	87	40	5	54	7
1.0	842	92	42	5	35	4
1.5	835	92	45	5	29	3
2.0	823	93	40	5	24	3
3.0	841	94	33	4	18	2
5.0	800	96	20	3	13	2
7.5	899	95	32	3	13	1

Discussion

These studies have demonstrated that in *Flavobacterium* R1519 as in the *Mycobacterium* system used by Batra and his co-workers (Howes & Batra, 1970; Batra *et al.*, 1973) the cyclization reaction of carotenoid biosynthesis was inhibited by nicotine, but they have also shown that the introduction of hydroxyl groups at C-3 and C-3′ in the biosynthesis of zeaxanthin was not inhibited. The fact that the compound that accumulated in the presence of nicotine was the hydrocarbon lycopene rather than a hydroxylated acyclic carotenoid clearly demonstrated that in xanthophyll biosynthesis the hydroxyl groups were not introduced until after cyclization had taken place.

In *Flavobacterium* R1519, the inhibitory effect of nicotine was readily removed by washing, whereupon synthesis of cyclic carotenoids was able to proceed at the expense of the carotenoids that accumulated during nicotine inhibition. This fact was used to demonstrate experimentally the formation of zeaxanthin from lycopene via β-carotene, and from rubixanthin, thus indicating that two alternative pathways were possible (Fig. 2, pathway *b*, and Fig. 3, pathway *c*). The obvious interpretation of these results is that lycopene is the immediate precursor of the cyclic carotenoids. [For details of the arguments that have been used to support lycopene or neurosporene as the immediate precursor of cyclic carotenoids, see Goodwin (1971).] On removal of the inhibitor, cyclization of lycopene occurred to yield, under anaerobic conditions, β-carotene; in the presence of O_2, this was hydroxylated to give zeaxanthin (Fig. 2, pathway *b*). The accumulation of rubixanthin at low nicotine concentrations apparently indicated that inhibition of cyclization of one half of the acyclic precursor was largely complete before any appreciable inhibition of the second cyclization occurred.

Further consideration of the experimental facts, however, leads to other interpretations, in which cyclization is considered normally to occur at the neurosporene level. Davies (1973) stated that in a cell-free *Phycomyces blakesleeanus* system β-carotene could be formed either from neurosporene via β-zeacarotene or from lycopene via γ-carotene with comparable efficiency. A scheme (Fig. 3, pathway *a*) may be proposed in which neurosporene normally cyclizes to give β-zeacarotene; this product is hydroxylated (to give 3-hydroxy-β-zeacarotene) and desaturated to yield rubixanthin, which then undergoes cyclization and hydroxylation to give β-cryptoxanthin and zeaxanthin. Neurosporene and all the postulated intermediates in this scheme have been isolated from a related high-zeaxanthin-producing *Flavobacterium* strain, R1560 (Leuenberger *et al.*, 1973; F. J. Leuenberger, A. J. Schocher, G. Britton & T. W. Goodwin, unpublished work). At high nicotine concentration, cyclization of neurosporene to β-zeacarotene would be inhibited, but desaturation of the 7′,8′ bond should not be affected. The pathway would then be diverted and lycopene would accumulate rather than neurosporene. The high nicotine concentration would prevent any further metabolism of the lycopene. On removal of the inhibitor cyclization of the lycopene to β-carotene and the subsequent hydroxylation to give zeaxanthin could proceed (Fig. 3, pathway *b*).

Lower nicotine concentrations would be sufficient to inhibit cyclization of neurosporene, which would then be desaturated as before to lycopene. The nicotine concentration, however, may be too low to inhibit cyclization of the newly formed 'lycopene level' end group, which could be cyclized and hydroxylated to give rubixanthin (Fig. 3, pathway *c*). Removal of the inhibitor would then allow cyclization and hydroxylation to occur, resulting in the formation of zeaxanthin.

The present results can be used as a basis for the proposal of several alternative pathways of zeaxanthin biosynthesis. However, a more satisfactory interpretation of this situation can perhaps be obtained by considering that the whole of the biosynthesis of zeaxanthin, from neurosporene or an earlier intermediate, takes place on an enzyme complex with two sites, each capable of desaturating, cyclizing and hydroxylating a suitable substrate (Fig. 4). Each site would deal with one-half of the carotenoid molecule so that sequences of events occurring at each site must be considered rather than biosynthetic pathways. Under normal conditions desaturation, cyclization and hydroxylation occur in rapid sequence at both sites so that zeaxanthin is produced with virtually no intermediates being detected. Under abnormal conditions, e.g. nicotine inhibition or lack of O_2, some reactions are unable to proceed so that the normal sequence of reactions is altered and abnormal products are found. The earlier interpretations of the present work may then be modified as follows.

At each site on the enzyme complex, nicotine can inhibit the cyclization reaction but not desaturation or hydroxylation. The accumulation of lycopene in the presence of nicotine shows that hydroxylation is not possible before cyclization of the 'half-molecule' substrate has occurred. On removal of the inhibition cyclization of both half-molecules occurs, normally followed rapidly by hydroxylation, resulting in the formation of zeaxanthin; if no O_2 is present, the product is β-carotene. The substrate for the cyclizing enzyme site may thus be considered to be a carotenoid 'half-molecule' at the lycopene level of desaturation.

At lower nicotine concentrations, only one of the two cyclization sites is inhibited. Cyclization and hydroxylation can occur at the second site, resulting in the production of rubixanthin. On removal of the inhibitor cyclization and hydroxylation of the rubixanthin can take place and zeaxanthin is produced.

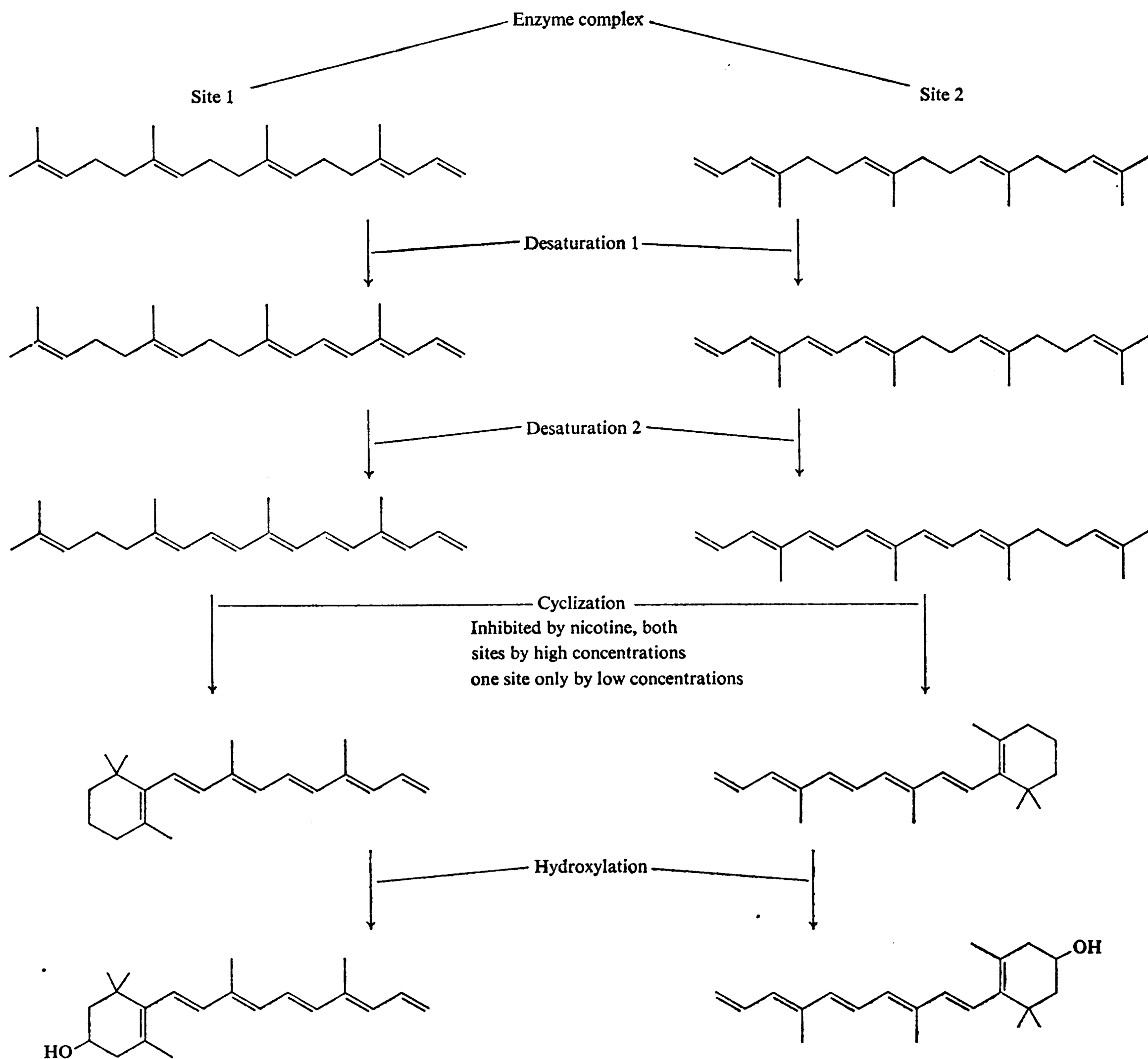

Fig. 4. *Sequence of reactions occurring in the carotenoid 'half-molecules' at the two sites of an enzyme complex involved in the biosynthesis of zeaxanthin*

If one half-molecule reached the required (lycopene) desaturation level before the second half-molecule (i.e. equivalent to the formation of neurosporene), then cyclization would yield β-zeacarotene, which could be hydroxylated and desaturated to rubixanthin before cyclization and hydroxylation of the second half-molecule to give zeaxanthin. Low concentrations of nicotine would be sufficient to block the cyclization of the first half-molecule by inhibiting the first cyclization site. Desaturation could, however, still occur in the second half-molecule which, if insufficient nicotine were available to inhibit the second cyclization, would then be cyclized and hydroxylated leading to the production of rubixanthin. Removal of the inhibitor would again allow zeaxanthin to be formed from rubixanthin.

The failure to demonstrate the conversion *in vivo* of lycopene into γ-carotene or rubixanthin is difficult to explain in terms of either pathways or enzyme-complex reaction sequences. It could be due to the fact that washing removes the nicotine from both sites on the enzyme complex, and never from only one site. Accumulated lycopene would therefore be converted into β-carotene or zeaxanthin and not into γ-carotene or rubixanthin.

Although it is clear from this work that in the

sequence of reactions that a carotenoid 'half-molecule' undergoes, desaturation precedes cyclization which in turn precedes hydroxylation, the question of the relative sequences of events occurring under normal conditions in the two halves of the carotenoid molecule remains unanswered and must await detailed enzyme studies.

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