Short Communications

Carotenoid Biosynthesis in a *Flavobacterium* sp.: Stereochemistry of Hydrogen Elimination in the Desaturation of Phytoene to Lycopene, Rubixanthin and Zeaxanthin

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[2-¹⁴C,(2*R*)-2-³H₁]- and [2-¹⁴C,(2*S*)-2-³H₁]-Mevalonates were rapidly incorporated into phytoene, lycopene, rubixanthin and zeaxanthin in a *Flavobacterium* system obtained by disruption of the bacterial cells by shaking with glass beads. Four hydrogen atoms arising from the 2-*pro-S*-hydrogen atoms of mevalonate were lost in the desaturation of phytoene to lycopene, rubixanthin and zeaxanthin. The desaturation of phytoene involves *trans*-elimination of hydrogen in the introduction of the double bonds at C-7, C-11, C-7' and C-11'.

Since the preparation by Cornforth and Popják and their collaborators (Cornforth *et al.*, 1966*a,b*; Donninger & Popják, 1966) of stereospecifically tritiated species of mevalonic acid, the stereochemistry of steps in the biosynthesis of many terpenoid compounds, especially of several reactions in carotenoid biosynthesis, has been established (Britton, 1971; Goodwin, 1971). Thus the loss of all tritium from [2-¹⁴C,(4*S*)-4-³H₁]mevalonate and the retention of all tritium from [2-¹⁴C,(4*R*)-4-³H₁]mevalonate in the formation of phytoene and lycopene showed that the carotenoids were biosynthesized by the all-*trans*-isoprenoid pathway, via geranylgeranyl pyrophosphate (Goodwin & Williams, 1966; Williams *et al.*, 1967*a*). Use of [2-¹⁴C,(5*R*)-5-³H₁]mevalonate and [2-¹⁴C,5-³H₂]mevalonate has enabled the stereochemistry of hydrogen loss in the formation of phytoene (7,8,11,12,7',8',11',12'-octahydro- ψ -*h*-carotene) from geranylgeranyl pyrophosphate to be defined (Williams *et al.*, 1967*b*; Buggy *et al.*, 1969), and has shown that introduction of the hydroxyl groups at C-3 and C-3' in zeaxanthin (β -carotene-3,3'-diol) occurs by direct replacement of hydrogen by hydroxyl (Walton *et al.*, 1969; J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work).

One outstanding problem in carotenoid biosynthesis is the stereochemistry of hydrogen loss in the introduction of double bonds at C-7, C-11, C-7' and C-11' in the series of desaturation reactions in the conversion of phytoene into the normal unsaturated carotenoids. The earlier work with [2-¹⁴C,(5*R*)-5-³H₁]- and [2-¹⁴C,5-³H₂]mevalonates established that in the desaturation reactions the hydrogen atoms lost from C-7, C-11, C-7' and C-11' of phytoene were those that arose from the 5-*pro-R*-hydrogen atom of meva-

lonate, the 5-*pro-S*-hydrogen atom being retained at each stage (Williams *et al.*, 1967*b*; J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work). Early attempts to establish the stereochemistry of hydrogen loss from C-8, C-12, C-8' and C-12' in the introduction of these double bonds, however, met with considerable difficulty, because of loss of the stereospecificity of labelling, presumably (Goad, 1970) through the isopentenyl pyrophosphate-dimethylallyl pyrophosphate equilibration during the comparatively long (48 h) incubation with the tomato systems used (R. J. H. Williams, G. Britton & T. W. Goodwin, unpublished work).

The development of a crude preparation obtained by disruption of a *Flavobacterium*, strain R-1519, capable of very rapid and efficient incorporation of mevalonate into the main carotenoid, zeaxanthin, has provided an opportunity to investigate the stereochemistry of desaturation in short incubations (90 min), during which time little or no randomization of label should occur.

Materials and methods

[2-¹⁴C,(2*R*)-2-³H₁]Mevalonate was prepared by mixing [2-¹⁴C]mevalonate with [(2*R*,3*R*)-2-³H₁ + (2*S*,3*S*)-2-³H₁]mevalonate in the approximate ratio 1:6. [2-¹⁴C,(2*S*)-2-³H₁]Mevalonate was prepared by mixing [2-¹⁴C]mevalonate with [(2*S*,3*R*)-2-³H₁ + (2*R*,3*S*)-2-³H₁]mevalonate in the approximate ratio 1:6. The ¹⁴C- and ³H-labelled mevalonates (as benzene solutions of the lactone) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). The mevalonates were converted into the potassium salt before use (Britton & Goodwin, 1971).

Flavobacterium strain R-1519 was a gift from F. Hoffmann-La Roche and Co., Basle, Switzerland.

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Cultures (50ml) were grown in the liquid medium previously described (McDermott *et al.*, 1973a) in 250ml conical flasks modified by three or four indentations to act as baffles to increase aeration. After 24h growth in an orbital incubator at 160rev./min and 20°C, the cells were harvested, washed with 0.1M-Tris-HCl buffer, pH 6.7, and resuspended in this buffer. The cells from four culture flasks, resuspended in buffer (15ml), were transferred to a homogenizing flask (50ml) containing ATP (10mg), MgCl₂ (15μmol), glass beads (0.10–0.11mm diameter; 5g) and [2-¹⁴C,(2*R*)-2-³H₁]- or [2-¹⁴C,(2*S*)-2-³H₁]-mevalonate (1μCi of ¹⁴C, 6μCi of ³H; no carrier added). In a second experiment nicotine (2mm) was included in the reaction mixture. The flask was flushed with N₂, stoppered and shaken for 2min in a Braun MSK homogenizer at 4000rev./min, the temperature being maintained at 0–2°C by liquid-CO₂ cooling. The reaction mixture was incubated in an orbital incubator (160rev./min) at 20°C anaerobically for 30min, then aerobically for 60min. Carrier phytoene, and in the second experiment lycopene plus rubixanthin (approx. 500μg each), was added and the pigments were extracted and purified by column chromatography and t.l.c. as previously described (McDermott *et al.*, 1973a). A portion of the zeaxanthin was acetylated by the acetic anhydride-pyridine method and the acetate purified as previously described (McDermott *et al.*, 1973a).

Carotenoid samples were decolorized by u.v. light and assayed for ³H and ¹⁴C radioactivity by the standard method (Britton & Goodwin, 1971) in a Beckman LS200 liquid-scintillation counter.

Results and discussion

Disruption of the cells of *Flavobacterium* strain R-1519 with glass beads gave a crude system that was capable of incorporating mevalonate efficiently and rapidly into terpenoids, particularly zeaxanthin (up to 10% incorporation in 3h).

The *Flavobacterium* system was incubated with [2-¹⁴C,(2*R*)-2-³H₁]- and [2-¹⁴C,(2*S*)-2-³H₁]-mevalonates, and the two carotenoids present, phytoene and zeaxanthin, were purified and assayed, together with zeaxanthin diacetate, for ³H and ¹⁴C radioactivity. The results (Table 1a) show that essentially all the tritium from [2-¹⁴C,(2*R*)-2-³H₁]-mevalonate was retained in zeaxanthin, whereas approximately half of the tritium from [2-¹⁴C,(2*S*)-2-³H₁]-mevalonate had been lost. The ¹⁴C/³H atomic ratio of approximately 8:4 for zeaxanthin, compared with 8:8 for phytoene, showed that four labelled hydrogen atoms from [2-¹⁴C,(2*S*)-2-³H₁]-mevalonate had been lost in the formation of zeaxanthin, i.e. introduction of the double bonds at C-7, C-11, C-7' and C-11' involves loss of hydrogen atoms arising from the 2-*pro-S*-hydrogen atom of mevalonate from each of

Table 1. Incorporation of [2-¹⁴C,(2*R*)-2-³H₁]-mevalonate and [2-¹⁴C,(2*S*)-2-³H₁]-mevalonate into carotenoids by a *Flavobacterium* preparation

	(i) With [2- ¹⁴ C,(2 <i>R</i>)-2- ³ H ₁]-mevalonate				(ii) With [2- ¹⁴ C,(2 <i>S</i>)-2- ³ H ₁]-mevalonate			
	¹⁴ C (d.p.m.)	³ H (d.p.m.)	Radioactivity ratio (³ H d.p.m./ ¹⁴ C d.p.m.)	Normalized atomic ratio ¹⁴ C: ³ H	¹⁴ C (d.p.m.)	³ H (d.p.m.)	Radioactivity ratio (³ H d.p.m./ ¹⁴ C d.p.m.)	Normalized atomic ratio ¹⁴ C: ³ H
(a) Incubation at 20°C for 90min								
[2- ¹⁴ C,(2 <i>R</i>)-2- ³ H ₁]-mevalonate	19068	121774	6.39	—	—	—	—	—
[2- ¹⁴ C,(2 <i>S</i>)-2- ³ H ₁]-mevalonate	—	—	—	—	6326	39774	6.29	—
Phytoene	8360	51958	6.20	8:8.00	1888	11622	6.16	8:8.00
Zeaxanthin	1663	9672	5.82	8:7.51	1911	6462	3.38	8:4.29
Zeaxanthin diacetate	1112	6400	5.75	8:7.42	1720	5921	3.44	8:4.37
(b) Incubation at 20°C for 90min with nicotine (2mm)								
[2- ¹⁴ C,(2 <i>R</i>)-2- ³ H ₁]-mevalonate	7580	43806	5.78	—	—	—	—	—
[2- ¹⁴ C,(2 <i>S</i>)-2- ³ H ₁]-mevalonate	—	—	—	—	6528	41234	6.32	—
Phytoene	502	2864	5.74	8:8.00	1878	11622	6.19	8:8.00
Lycopene	1978	10920	5.52	8:7.70	1760	6412	3.64	8:4.70
Rubixanthin	607	3270	5.39	8:7.51	225	815	3.62	8:4.68
Zeaxanthin	1469	7703	5.24	8:7.30	700	2565	3.66	8:4.73

C-8, C-12, C-8' and C-12'. In the short incubation time used (90 min) very little randomization of tritium labelling from C-2 of mevalonate seemed to have occurred.

A second experiment in which the *Flavobacterium* system was incubated with [2-¹⁴C,(2*R*)-2-³H₁]- and [2-¹⁴C,(2*S*)-2-³H₁]-mevalonates in the presence of nicotine (2 mM) allowed the investigation to be extended to other carotenoids. Nicotine at the concentration used (2 mM) caused a partial inhibition of zeaxanthin biosynthesis, and lycopene (*ψψ*-carotene) and rubixanthin (*ββ*-caroten-3-ol) accumulated (McDermott *et al.*, 1973*b*). Therefore in this second incubation the ¹⁴C/³H atomic ratios for lycopene and rubixanthin were obtained, in addition to those of phytoene and zeaxanthin. The results (Table 1*b*) show that the 2-*pro-S*-hydrogen atom from mevalonate was that lost in each desaturation reaction in the biosynthesis of lycopene, rubixanthin and zeaxanthin.

It has previously been shown that the hydrogen atom lost from C-7, C-11, C-7' and C-11' in the introduction of the double bonds is in each case that which originates from the 5-*pro-R*-hydrogen atom of mevalonate (Williams *et al.*, 1967*b*; J. C. B. McDermott, G. Britton & T. W. Goodwin, unpublished work). The present results therefore establish the stereochemistry of these elimination reactions, and show that an overall *trans* loss of hydrogen atoms occurs in the formation of each double bond introduced in going from phytoene to the unsaturated carotenoids lycopene, rubixanthin and zeaxanthin. The stereochemistry is the same as that recently established for the introduction of the Δ⁴-bond (equivalent to the Δ⁷-bond in carotenoids) in abscisic acid (Milborrow, 1972).

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