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THE STEREOCHEMISTRY OF BIOSYNTHESIS OF DECAPRENOXANTHIN IN A CELL-FREE SYSTEM

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Key Word Index—*Flavobacterium dehydrogenans*; decaprenoxanthin; bacterial C_{50} carotenoid; biosynthesis; stereochemistry; cyclisation.

Abstract—Intact cells of *Flavobacterium dehydrogenans* grown on glucose or acetate did not incorporate mevalonic acid-[^{14}C]. After treatment with lysozyme the protoplasts were lysed by sonication in a dilute medium containing mevalonic acid-[^{14}C] and the cell-free system produced incorporated label into uncyclized C_{40}, monocyclic C_{45} and bicyclic C_{50} carotenoids of which decaprenoxanthin was the most abundant.

With mevalonate-[2-^{14}C,4*R*-4-$^{3}H_1$] the $^{14}C:^{3}H$ ratios of the carotenoids showed that the hydrogen atoms at C-2 and C-6 of the ring and that at C-3 of the 1-hydroxy, 2-methyl but-2-ene-4-yl residues of decaprenoxanthin were derived from the 4-*pro-R* hydrogen atom of mevalonic acid.

Mevalonate-[2-^{14}C,2*R*-2-$^{3}H_1$] and mevalonate-[2-^{14}C,2*S*-2-$^{3}H_1$] gave ratios which showed that the C-4 hydrogen atoms of decaprenoxanthin were derived from the 2-*pro-S* hydrogen atom of mevalonic acid.

INTRODUCTION

The stereochemistry of biosynthesis of decaprenoxanthin in a cell-free system

The stereochemistry of most of the biosynthetic reactions between mevalonic acid (MVA, 1) and the C_{40} carotenoids has been deduced from the retention or elimination of the appropriately labelled hydrogen atoms of the precursor [1, 2]. Recently Weeks and Liaaen-Jensen [3, 4] have described a new series of bacterial C_{50} carotenoids where a dimethylallyl residue appears to have been added to the C_{40} lycopene skeleton during each cyclization. Both ε and β rings occur in the C_{50} series and the absolute configurations at the C-2 positions, which carry the dimethylallyl groups, are identical in ε and β rings [5]. Furthermore, the absolute configuration of the group added to C-2 is the opposite to that at C-2 of the C_{40} β carotenoids where the *pro-R* hydrogen atom is added from the medium during cyclization [6]. In addition, the absolute configuration of the hydrogen atom at C-6 of the ε-ring of the C_{40} carotenoids is the opposite of that at C-6 of the ε-C_{50} compound. Thus it appears that the cyclases may operate with the mirror-image stereochemistry in the C_{40} and C_{50} carotenoids. It was of interest, therefore, to find whether or not the hydrogen atoms at C-2 and C-6 of the C_{50} carotenoid decaprenoxanthin (2) were derived from the 4-*pro-R* hydrogen atom of mevalonic acid [1] (as those of the C_{40} analogue are) and which of the hydrogen atoms on C-2 of mevalonic acid (1) was retained at C-4 of the carotenoid.

This information could help to discriminate between some of the possible cyclization mechanisms that have been proposed [2, 5, 7, 8]. Preliminary experiments showed that intact *Flavobacterium dehydrogenans* cells were incapable of taking up labelled mevalonic acid and incorporating it into carotenoids. It was necessary, therefore, to develop a cell-free system capable of this biosynthesis before the stereochemistry of the reactions could be investigated. We now report the preparation of such a system and some results obtained with it.

RESULTS AND DISCUSSION

(i) *Preparation of a cell-free system*

a. *Treatment of* F. dehydrogenans *cells. F. dehydrogenans* grows rapidly in an aerated glucose medium and produces a number of carotenoids when illuminated [3]. Growth on acetate is slower than on glucose (cell numbers double approximately every 14 hr on glucose, every 35 hr with acetate as carbon source). Neither cells growing logarithmically nor those in the stationary phase incorporated label into any carotenoid from added mevalonate (Table 1). Detergents, methanol, ethanol, digitonin and lysozyme were also used unsuccessfully.

The combination of lysozyme treatment, osmotic shock on mixing the protoplast suspension with three times its volume of a solution of potential cofactors and substrates, followed by sonication, gave a cell-free system that incorporated labelled MVA into carotenoids (Table 1) so that the retention or loss of ^{3}H from variously tritiated mevalonates could be compared. It would appear, therefore, that it is necessary to remove the cell walls and to lyse the cell membranes as they constitute a formidable barrier to the penetration of mevalonic acid into the cells of *F. dehydrogenans*.

b. *Isolation of labelled carotenoids and tests of radiochemical purity.* Alumina column chromatography of ether extracts of ^{14}C carotenoids from such cell-free systems gave separate carotenoid fractions, samples of which were chromatographed on silica-gel thin layer plates and autoradiogrammed. The intensity of exposure caused by ^{14}C exactly coincided with the intensity of the yellow colour of the carotenoid zones, which suggested

Table 1. Incorporation of 3*R*-mevalonate-[2-^{14}C] into decaprenoxanthin by *F. dehydrogenans*

Treatment	^{14}C in decaprenoxanthin, dpm
Intact cells in nutrient broth	2
Intact cells in acetate medium	0
Intact cells in methanol (2%)	0
Intact cells in ethanol (2%)	1
Intact cells in Triton X-100 (0.5%) and sucrose (M)	2
Intact cells in digitonin	3
Protoplasts (after lysozyme treatment) in digitonin (4%)	11
Protoplasts (after lysozyme treatment lysed by osmotic shock and sonication)	70361

Cultures of *F. dehydrogenans* (100 ml) were incubated with MVA-[2-^{14}C] (1.1×10^7 dpm, 17.5 μCi/μmol) while subjected to a variety of treatments designed to assist incorporation of the precursor into carotenoids. Cells were grown for 48 hr at 30° with continual aeration and illumination.

that radioactive impurities, even at this stage of purification, were negligible or absent. The zones eluted from the silica-gel were subjected to HPLC and the main yellow fractions appeared as isolated peaks which crystallized on drying and gave visible spectra in hexane identical with published values [3] for the various C_{45} and C_{50} carotenoids.

A further test of radiochemical purity was carried out with decaprenoxanthin (**2**), the most abundant carotenoid in *F. dehydrogenans*, and its monohydroxy analogue. It was shown that all the radioactivity co-chromatographed with the respective mono- and diacetylated derivates after acetylation in pyridine–acetic anhydride; no other labelled compounds were detected. Thus the radioactivity measured was attributed entirely to the carotenoids.

As reported previously [9], insufficient aeration caused the accumulation of a range of uncyclized C_{40} carotenoids.

(ii) *Incorporation of mevalonate-*[2-^{14}C,4*R*-4-$^3H_1$] *into decaprenoxanthin*

a. *Retention of* 4-pro-R *hydrogen at* C-2 *and* C-6. The cell-free system was used to synthesize decaprenoxanthin (**2**) and its precursors from a sample of mevalonate-[2-^{14}C,4*R*-4-$^3H_1$] so that the retention or elimination of these hydrogen atoms to give what could become the C-2 and C-6 hydrogen atoms of **2**, could be followed during the cyclization reaction. The results in Table 2 show that the ^{14}C:^{3}H ratios of phytoene (**3**) 8:7.25 (Experiment 1) and lycopene (**4**) 8:7.54 (Experiment 2) were closely similar to that of the mevalonic acid (normalized to 1:1) from which they were derived. The slight decrease in the proportion of tritium could have been caused by the presence of an impurity in the mevalonate-[^{3}H] but it was too small to account for the change in ratio that would have occurred if a tritium atom were removed from any position of an uncyclized precursor molecule (8:6 rather than 8:8). The maintenance of the 1:1 ratio in phytoene (**3**) and lycopene (**4**) is as expected from the mechanism of biosynthesis of these compounds in higher plants [7]. The ratios of ^{14}C:^{3}H in the uncyclized precursors were used as a basis of comparison for the cyclized C_{45} and C_{50} compounds.

The first cyclized product isolated was a monocyclic C_{45} compound, identified as the major component of the P420 complex [3] from its chromatographic behaviour

Table 2. Incorporation of mevalonate-[2-^{14}C,4*R*-4-$^3H_1$] into decaprenoxanthin and related carotenoids

Sample	^{14}C (dpm)	^{3}H (dpm)	^{14}C:^{3}H	Observed ^{14}C:^{3}H normalized to MVA	Observed ^{14}C:^{3}H normalized to phytoene	Theoretical ^{14}C:^{3}H if 4-*pro-R* of MVA were retained at all positions	Theoretical ^{14}C:^{3}H if 4-*pro-R* of MVA were removed from one position during cyclization
Experiment 1							
MVA	110553	369596	1:3.34	1:1	1:1.10	—	—
Phytoene	302.1	916.0	1:3.03	8:7.25	(8.8)	8:8	8:8
Monocyclic C_{45} Carotenoids	682.2	2124	1:3.11	9:8.38	9:9.24	9:9	9:8
Decaprenoxanthin	521.8	1678	1:3.21	10:9.61	10:10.59	10:10	10:8
Experiment 2							
MVA	120612	61349	1:0.51	1:1	1:1.06	—	
Lycopene	5961	2882	1:0.48	8:7.54	(8:8)	8:8	8:8
Deshydroxy decaprenoxanthin	3412	6761	1:0.50	10:9.80	10:10.41	10:10	10:8
Decaprenoxanthin	37801	75063	1:0.50	10:9.80	10:10.41	10:10	10:8

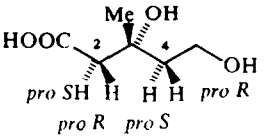
The optimal conditions described in Table 1 were used. MVA-[2-^{14}C] (17.5 μCi/μmol) and MVA-[4*R*-4-$^3H_1$] (60 μCi/μmol) were mixed and any lactone was hydrolysed. The cell free preparation (400 ml) was divided between 4 flasks and incubated at 30° under incandescent illumination for 48 hr. For Expt 1 24.8 μCi of mevalonate-[^{14}C] and 45.5 μCi of mevalonate-[^{3}H] were added. For Expt 2 9.9 μCi of mevalonate-[^{14}C] and 14.2 μCi of mevalonate-[^{3}H] were used. The 'P420 complex' carotenoids [3] are monocyclic C_{45} compounds with varying degrees of desaturation. The ^{14}C:^{3}H ratios show a slight excess of ^{3}H but demonstrate that no ^{3}H was lost from either C-2 or C-6 of decaprenoxanthin or from the isoprene unit added. The slight difference in ratio between the MVA and phytoene is attributed to a labelled impurity in the MVA.

MS and visible spectra. The ^{14}C:^{3}H ratio of 9:9.24 (i.e. 1:1.03 based on a normalized ratio of 8:8 in phytoene) showed that no tritium could have been lost either from the uncyclized precursor or from the isoprene group added.

Similarly, the bicyclic decaprenoxanthin (10:10.59, Experiment 1; 10:10.41, Experiment 2) and its monohydroxy analogue (10:10.41) exhibited normalized ratios close to 10:10 showing no 4-*pro-R* tritium was lost during cyclization. If tritium had been lost from any position, the ratio would have been 10:8 or less. This was not observed so, during each cyclization, an isoprene group, containing one ^{14}C and one ^{3}H, was added and the hydrogen atoms from the 4-*pro-R* position of mevalonic acid (**1**) were retained at C-2 and C-6 of decaprenoxanthin (**2**). Retention of these two 4-*pro-R* hydrogen atoms of MVA in the C_{50} carotenoids also occurred during the formation of the ε ring of the C_{40} carotenoids.

b. *The occurrence of the* 4-pro-R *hydrogen of MVA in the side chain.* The addition of ^{14}C and ^{3}H in a ratio of 1:1 during each cyclization reaction showed that each isoprene residue added also carried a tritium derived from the 4-*pro-R* position of MVA. Retention of this hydrogen atom occurs when the 3-double bond of isopentenyl pyrophosphate (IPP) (**5**) is isomerized to the 2-position of dimethylallyl-pyrophosphate (DMAPP) (**6**) with the C-4 methyl group (i.e. that derived from C-2 of MVA) *trans*-(*E*) to C-1 of the DMAPP residue. When the isomerization occurs to leave this methyl group *cis* to C-1 (*Z*) then the hydrogen atom derived from the 4-*pro-R* position of MVA is lost to the medium [10].

The methyl group *trans*-(*E*) to C-1 of the DMAPP residue carries a hydroxyl group in decaprenoxanthin (**2**) and the origin of this *E* methyl group as C-2 of mevalonate is suggested by the retention of the 4-*pro-R* hydrogen of mevalonate (**1**) in the side chain.



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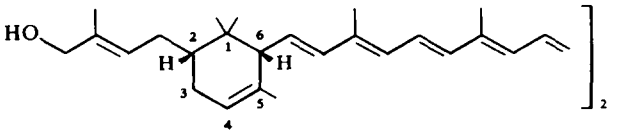

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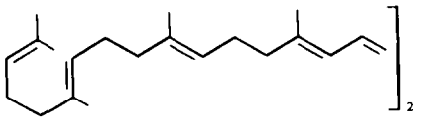

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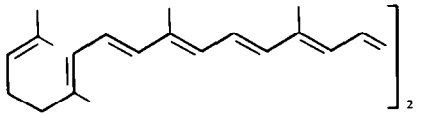

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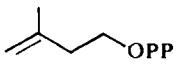

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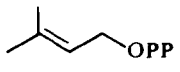

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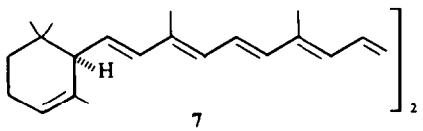

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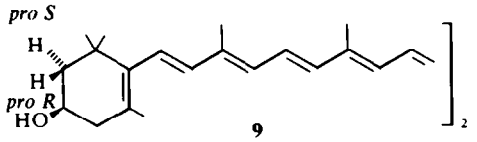

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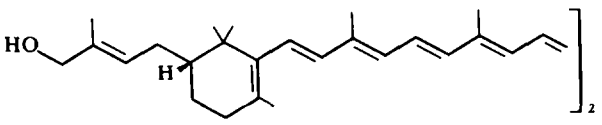

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c. *Correction of* ^{3}H *counts by spiking with standard* ^{14}C *and* ^{3}H *toluene.* The cause of the slight increase in the proportion of tritium from phytoene (3), through P420, to decaprenoxanthin (2), is unclear. The samples of ^{14}C and ^{3}H mevalonolactones were mixed and held in N KOH for several hours before feeding so differential hydrolysis cannot be involved, isotope effects would tend to decrease the ratio and counting errors and phosphorescence were discounted by counting each batch of samples on several occasions (no progressive changes in cpm were observed) and each sample was adjusted for quenching by a spike of a ^{3}H toluene standard followed by a ^{14}C toluene standard. Furthermore, the more highly radioactive solutions were counted at a range of dilutions to overcome any non-linearity of response. In addition, a further small correction to the ratios was carried out to adjust, in part, for the differential 'crossover' of ^{14}C counts into the ^{3}H channel with differing amounts of quenching.

(iii) *Experiments with* 2R- *and* 2S-*tritiated mevalonic acid*

a. *Action of IPP/DMAPP isomerase.* If the same reactions that have been studied in the biosynthesis of other terpenoids [10] apply to the biosynthesis of decaprenoxanthin (2) in *F. dehydrogenans*, then the ^{14}C:^{3}H ratios which result from the incorporation of the 3*R*-mevalolate-[2-^{14}C,2*R*-2-^{3}H$_1$] and 3*R*-mevalonate-2-^{14}C,2*S*-2-^{3}H$_1$] into phytoene (3), lycopene (4) and decaprenoxanthin (2) can be used to deduce some features of the biosynthetic mechanism.

Any cycling of DMAP (6) back to IPP (5) will have the effect of randomizing the tritium, from what had been stereospecifically C-2 tritiated MVA, between the two positions in IPP, and removing up to one third of it. Isotope effects strongly favour the retention of tritium in this reaction [11] so although quite a significant proportion may be racemized a reltively small proportion only of the tritium may be lost.

The effect of racemization by the isomerase is to lower the proportion of tritium that is retained at any position during subsequent reactions and to raise the proportion of tritium retained by the molecule from positions occupied by tritium that should have been removed. The proportion of tritium derived from the 2*R* and 2*S* tritiated MVA samples, and retained in lycopene is slightly less than expected but the almost unitary losses of tritium during desaturation and cyclization to decaprenoxanthin show that in this cell-free system the activity of the IPP/DMAPP isomerase was slight in comparison with the condensing enzyme (Table 3).

The ^{14}C:^{3}H ratios of phytoene and lycopene synthesized by *F. dehydrogenans* in these experiments (Tables 2 and 3) from [2-^{14}C]- and [2-*R*-2-^{3}H$_1$[, [2*S*-2-^{3}H$_1$]- and [4*R*-4-^{3}H$_1$]-mevalonate are the same as those obtained with higher plants. Consequently, the reactions of terpenoid biosynthesis, at least those involving C-2 and C-4, up to and including the desaturation to lycopene, must have proceeded with the same stereochemistry as in higher plants.

b. *Cyclization.* During the first cyclization reaction to form a C_{45} carotenoid, one hydrogen atom is lost from C-4 (derived from C-2 of MVA), while the ratios should change predictably with the addition of one ^{14}C and one ^{3}H, with both the 2*R*- and the 2*S*-tritiated samples, and with the fusion of one isoprene unit to the carbon skeleton. Formation of decaprenoxanthin (2) occurs with the addition of one more ^{14}C and one more ^{3}H, again in both the 2*R*- and the 2*S*-tritiated samples, as the second

Table 3. Incorporation of mevalonate-[2-^{14}C, 2*R*-2-^{3}H$_1$] and mevalonate-[2-^{14}C, 2*S*-2-^{3}H$_1$] into decaprenoxanthin and related carotenoids

Sample	^{14}C (dpm)	^{3}H (dpm)	^{14}C:^{3}H	Observed ^{14}C:^{3}H normalized to MVA	Observed ^{14}C:^{3}H normalized to lycopene	Theoretical ^{14}C:^{3}H if 2-*pro-S* of MVA were retained at C-4	Theoretical ^{14}C:^{3}H if 2-*pro-R* of MVA were retained at C-4
(a)*							
MVA-[2*R*-2-^{3}H$_1$	22 248	67 588	1:3.04	(1:1)		1:1	1:1
Lycopene	102 114	257 862	1:2.53	8:6.64	(8:8)	8:8	8:8
Monocyclic C_{45} Carotenoids	7755	17 405	1:2.24	9:6.638	9:7.98	9:7.67 (min) 9:8.0 (max)	9:8.67 (min) 9:9.0 (max)
Decaprenoxanthin	11 566	23 322	1:2.02	10:6.65	10:8.00	10:7.33 (min) 10:8.0 (max)	10:9.33 (min) 10:10.0 (max)
(b)†							
MVA-[2*S*-2-^{3}H$_1$	375 726	2 317 584	1:6.17	1:1)		1:1	1:1
Lycopene	9903	44 668	1:4.51	8:5.84	(8:4)	8:4	8:4
Deshydroxy decaprenoxanthin	16 272	86 161	1:5.30	10:8.50	10:5.88	10:5.67 (min) 10:6.0 (max)	10:3.67 (min) 10:4.0 (max)
Decaprenoxanthin	15 335	82 624	1:5.39	10:8.70	10:5.98	10:5.33 (min) 10:6.0 (max)	10:3.33 (min) 10:4.0 (max)

The same procedures as those described under Table 2 were used except MVA-[2*R*-2-^{3}H$_1$] or MVA-[2*S*-2-^{3}H$_1$] (both 60 μCi/μmol) were mixed with the MVA-[2-^{14}C]. The theoretical ^{14}C:^{3}H ratios were based on the assumption that 1/3 of the ^{3}H was lost from what had been C-2 of MVA when the methyl group of the side chain was hydroxylated (min). Operation of an isotope effect would tend to raise the observed value to a maximum figure where no tritium was lost (max).

*The incubation was performed with 24.8 μCi of mevalonate-[^{14}C] and 45.5 μCi of mevalonate-[^{3}H].

†The incubation was performed with 19.8 μCi of mevalonate-[^{14}C] and 98 μCi of mevalonate-[^{3}H].

isoprene unit is added. The loss of a second hydrogen atom from C-4 of the new ring will also occur. Thus it should be possible to find whether the *pro-R* or the *pro-S* hydrogen is lost during the formation of the 4-α double bond by comparing the ^{14}C:^{3}H ratios before and after each cyclization. The data in Table 3 show that in **2** the 2-*pro-S* tritium of MVA was retained and the 2-*pro-R* tritium was lost during each cyclization. This result is the opposite of the tentative result cited by Goodwin [2] for the analogous position in β,ε-carotene.

DISCUSSION

We have established that the C-2 and C-6 hydrogen atoms of decaprenoxanthin (**2**) are derived from the 4-*pro-R* hydrogen atoms of mevalonate (**1**) although the one at C-6, at least, has the opposite absolute configuration [5] to that at C-6 in β,ε-carotene (**7**). The isoprene residue at C-2 of decaprenoxanthin (**2**) is *trans* to the C-6 hydrogen atom and has the same absolute configuration as the isoprene group at C-2 of the β rings of the C_{50} carotenoid (**8**) produced by *Corynebacterium poinsettiae* [13]. It would appear, therefore, that the stereochemistry at C-2 is the same for both β and ε cyclizations in the C_{50} carotenoids. The isoprene groups added to the *Corynebacterium* carotenoid, during cyclization, have the opposite stereochemistry at C-2 compared with the *pro-R* hydrogen atoms which are added during the β cyclization of the precursor of zeaxanthin (**9**) [6].

If the analogous ε-cyclization mechanism in the C_{40} series occurs with the same stereochemistry as that of the C_{40}-β series, then the hydrogen atom at C-2 (2-*pro-R*) would be *trans* to the C-6 hydrogen atom in the C_{40}-ε series also.

The hydrogen atom at C-4 of decaprenoxanthin (**2**) is derived from the 2-*pro-S* hydrogen atom of mevalonate (**1**) and while the result for ε-carotene has not been determined unambiguously, nevertheless, Goodwin [2], reporting the tentative results of Vose, Britton and Goodwin, suggested that the C-*pro-R* is retained at C-4 in the ε-ring of β,ε-carotene.

In the retention of hydrogen atoms from the 4-*pro-R* position of MVA at C-2 and C-6, the cyclization mechanisms of the C_{40} and C_{50} carotenoids are alike. However, in the absolute configuration at C-6 in the two ε series, in the absolute configuration of the group added at C-2 in the two β series, and probably in the hydrogen eliminated from C-4 in the two ε series, the stereochemistry of cyclization in the bacterial C_{50} carotenoid series is the mirror image of that in the C_{40} carotenoid series.

EXPERIMENTAL

Growth of cells. A culture of *Flavobacterium dehydrogenans* was obtained from the American Type culture collection (ATCC 13930). It was maintained on nutrient agar slopes containing peptone (Oxoid) (5 g/l.), yeast extract (Difco) (3 g/l.), K_2HPO_4 (1.5 g/l.), KH_2PO_4 (1.5 g/l.) and agar (15 g/l.) pH 7.2. Two media were used for the growth of 5 and 10 l. cultures of *F. dehydrogenans:* nutrient broth (as above without agar), in which populations reached stationary phase within 24 hr at 30°, and with OAc as sole C source. NaOAc (6.8 g/l.) NH_4Cl (2.0 g/l.) $MgSO_4$ (2.0 g/l.) K_2HPO_4 (3.0 g/l.) KH_2PO_4 (3.0 g/l.) and B-complex vitamins (Sigma) thiamine HCl (100 μg/l.) biotin (0.5 μg/l.) riboflavin (100 μg/l.) folic acid (510.0 μg/l.) pyridoxine (200 μg/l.) and *p*-amino benzoate (5 μg/l.). Cultures took 4–5 days to reach stationary phase. Carotenoid production followed growth, when cultures were illustrated and aerated. Media were sterilized by autoclaving before vitamins were added.

Extraction of carotenoids. Cultures at stationary phase ($OD_{700\,nm} > 1.8$) were centrifuged at 0° for 30 min at 8500 *g*. The cell pellet was then resuspended in MeOH (100 ml/l. of culture medium) and extracted with continual stirring for 30 min at 55° with antioxidant 2,6-di-*t*-butyl-4-methyl-phenol (500 mg/l.). These and all subsequent manipulations were carried out in dull light. The methanolic cell suspension was then mixed with an equal vol. of Et_2O and sat aq. NaCl was added to give phase separation. The yellow etheral layer was collected and evapd at 40°. A second Et_2O extraction removed any yellow pigment remaining.

Isolation of carotenoids. The residue, redissolved in Et_2O (5 ml), was applied to an activity III Al_2O_3 column (260 × 20 mm) from which the carotenoids were eluted with EtOAc–hexane–MeOH (50:190:10). 8 × 15 ml fractions were collected, dried under N_2 and stored at 0°. Fractions 4–8 contained decaprenoxanthin and were combined, redissolved in redistilled *iso*-PrOH (3 ml), and chromatographed in a Waters HPLC μporosil analytical column. The carotenoids (25 μl injections) were separated with *iso*-PrOH hexane (1·97: 4 ml/min: 2000 psi) and absorption was monitored at 440 nm. All solvents were re-distilled, degassed and filtered before use.

The carotenoids were identified by their absorption spectra [3] in hexane. In addition, decaprenoxanthin was identified by chemical ionisation MS ($M + 1/e = 705$), NMR, and by co-chromatography with authentic material.

Radioassay. The ^{14}C and ^{3}H in samples were measured simultaneously by liquid scintillation counting with the scintillant described previously [14]. The machine gave the following efficiencies ^{14}C (62.02%), ^{3}H (21.98%), ^{3}H in the ^{14}C channel (0.1), ^{14}C in the ^{3}H channel (12.8%). All experimental samples were counted to give a maximum error of 1% or less and each batch was counted successively at least 2× to confirm the absence of phosphorescence. Decolourization by UV in cold BuOH reduced the quenching of coloured compounds but did not eliminate it, so all samples were spiked first with standard toluene-[U-^{3}H] (10 μl, 124 μCi/mol) and subsequently with toluene-[U-^{14}C] (10 μl, 23.5 μCi/mol). Correction was then made for any quenching. However it was observed that a series of compounds expected to contain a constant proportion of ^{14}C to ^{3}H showed a slightly raised tritium content of up to 1.00:1.06 and the excess was *ca* proportional to the degree of quenching. A standard curve of β-carotene and toluene-[^{14}C] showed that the quenching of ^{14}C crossover into the ^{3}H channel was relatively greater in quenched samples and so the calibration curve was used to give a final, adjusted figure. In these, as in previous experiments with mevalonic acid prepared by the same synthetic route, the nominally 4*R*-tritiated material 3*R*-MVA-[4*R*-4-$^{3}H_1$] and was present as a racemic mixture with 3*S*-MVA-4*S*-4-$^{3}H_1$]. The MVA-[2-^{14}C] is racemic also and because 5-phospho mevalonic kinase accepts the 3-(*R*) enantiomer only, the molecules incorporated are the 3-(*R*)-[2-^{14}C] and 3-(*R*)-[4(*R*)-4-$^{3}H_1$] species. The analogous situation applies with the 2*R* and 2*S* samples. It is assumed that in *F. dehydrogenans* the same selectivity occurs as in all other organisms that have been investigated.

Preparation of a cell-free system. Harvested cells were resuspended in 0.05 M Tris-HCl buffer, pH 8.0, and washed 2×. The cell pellet was resuspended in 1M sucrose–0.02 M Tris HCl, pH 7.8 (25 ml/l. culture medium). Lysozyme (0.75 mg/ml sucrose resuspension soln) was added and the soln was stirred gently at 37° for 20 min. The protoplasts were collected by centrifugation at 30000 *g* for 40 min and resuspended in sucrose (0.25 M) 0.02 M Tris–HCl, pH 7.8 (75 ml/l. culture medium) containing $MnCl_2$ (3 mM), glutathione (5 mM), EDTA (0.06 mM), thiamine (0.2 μM), ATP (6 μM), NADH (1.0 μM), NADPH (1.0 μM), NADP (1.0 μM). This soln was stirred for 30 min at 0° and sonicated (75 watts) at 0° for 1 min.

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REFERENCES

1. Popják, G. and Cornforth, J. W. (1966) *Biochem. J.* **101**, 553.
2. Goodwin, T. W. (1971) *Biochem. J.* **123**, 293.
3. Weeks, O. B. and Garner, R. J. (1967) *Arch. Biochem. Biophys.* **121**, 35.
4. Liaaen-Jensen, S., Hertzberg, S., Weeks, O. B. and Schweiter, U. (1968) *Acta Chem. Scand.* **B22**, 1171.
5. Andrewes, A. G., Liaaen-Jensen, S. and Weeks, O. B. (1975) *Acta Chem. Scand.* **B29**, 884.
6. Britton, G., Lockley, W. J. S., Patel, N. J., Goodwin, T. W. and Englert, G. (1977) *Chem. Commun.*, 655.
7. Britton, G. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W., ed.) p. 255. Academic Press, London.
8. Bu'Lock, J. D., Austin, D. J., Snatzke, G., and Hruban, L. (1970) *Chem. Commun.*, 255.
9. Weeks, O. B. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry*. (Goodwin, T. W., ed.) p. 291. Academic Press, London.
10. Cornforth, J. W., Cornforth, R. H., Donninger, C. and Popják, G. (1965) *Proc. Roy. Soc. Ser. B.* **163**, 492.
11. Goad, L. J. and Goodwin, T. W. (1972) in *Progress in Phytochemistry* (Reinhold, L. and Liwchitz, Y., eds.) Vol. 3, p. 113. Interscience, London.
12. Milborrow, B. V. (1975) *Phytochemistry* **14**, 123.
13. Andrewes, L. J. and Borch, G. (1974) *Acta Chem. Scand.* **B28**, 737.
14. Milborrow, B. V. (1972) *Biochem. J.* **128**, 1135.