Retention of the 4-*pro-R* Hydrogen Atom of Mevalonate at C-2,2' of Bacterioruberin in *Halobacterium halobium*

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Intact cells of *Halobacterium halobium* fed with (3*R*,4*R*)-[2-¹⁴C,4-³H₁]mevalonic acid were found to incorporate label into acyclic C₄₀ and C₅₀ carotenoids, of which bacterioruberin was the most abundant. The ¹⁴C/³H ratios of the isolated carotenoids demonstrated that the 4-*pro-R* hydrogen atom of mevalonic acid was retained at the C-2 and C-2' positions of bacterioruberin. Diphenylamine was found to inhibit the production of bacterioruberin.

The absolute configuration of the bacterial C₅₀ ϵ -carotenoid, decaprenoxanthin (Fig. 1, I), at the polyene chain to ϵ -ring junction (C-6-*S*) (Andrewes *et al.*, 1975) is the opposite to that at the equivalent position in the higher plant C₄₀ ϵ -carotene (C-6'-*R*) (Eugster *et al.*, 1969). Recent experiments, in which (3*R*,4*R*)-[2-¹⁴C,4-³H₁]mevalonic acid was incorporated into decaprenoxanthin (Fahey & Milborrow, 1978), showed that the 4-*pro-R* hydrogen atom of mevalonic acid was retained at C-6 of (I) as it is at C-6' of the C₄₀ β,ϵ -carotene (Goodwin & Williams, 1965; Williams *et al.*, 1967). Furthermore, both decaprenoxanthin and its C₅₀ β -analogue, C.p. 450, (Andrewes *et al.*, 1974), bear isoprene residues at C-2,2' that also appear to have been added with the opposite stereochemistry, at the equivalent position, of the hydrogen atom added during the

cyclization of the C₄₀ β -analogue (Britton *et al.*, 1977). Thus the C₄₀ and C₅₀ rings appear to have been formed by cyclases operating with opposite stereochemistry.

Bacterioruberin [2,2'-bis-(3-hydroxy-3-methylbutyl)-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- ψ,ψ -carotene-1,1'-diol] (Fig. 1, II), the principal carotenoid of *Halobacterium halobium* (Baxter, 1960), is an acyclic C₅₀ compound whose structure not only suggests that it is formed by the addition of an isoprene unit to C-2,2' but that it also has the same absolute configuration at these positions (J. E. Johansen & S. Liaaen-Jensen, quoted in Liaaen-Jensen, 1976; Johansen & Liaaen-Jensen, 1977) as those of the cyclic C₅₀ carotenoids.

It was of interest, therefore, to determine whether the 4-*pro-R* hydrogen atom of mevalonic

Fig. 1. Carotenoid structures

acid has been retained at C-2,2' during the isopentenylolation of the acyclic bacterioruberin as it is during the formation of the cyclic C₃₀ carotenoids.

Experimental

Materials

(3*RS*)-[2-¹⁴C]mevalonolactone (17.5 Ci/mol) and (3*R*,4*R*)-[4-³H₁]mevalonolactone plus (3*S*,4*S*)-[4-³H₁]mevalonolactone (250 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [U-³H]Toluene (124 μCi/mol) and [U-¹⁴C]toluene (23.5 μCi/mol) were obtained from Packard Instruments, Downers Grove, IL, U.S.A. All other reagents and solvents were the best available grade.

Culture conditions

A culture of *H. halobium* was obtained from the School of Microbiology, University of New South Wales, Sydney, Australia. It was maintained on agar slopes containing peptone (Oxoid) (10 g/l), sodium citrate (2 g/l), KCl (3 g/l), MgSO₄·7H₂O (20 g/l), NaCl (250 g/l) and agar (15 g/l), pH 7.2. The medium used was as described above without agar, and was sterilized by autoclaving for 10 min at 112°C and 60 kPa.

Bacteria were grown at 26°C in conical flasks shaken at 120 revolutions/min on a Paton orbital incubator. Agar slopes were used to inoculate 100 ml of sterile medium in 250 ml flasks, which were then incubated until mid-exponential growth (4 days). Inocula were taken aseptically and transferred to fresh media containing either [¹⁴C,³H]-mevalonic acid or diphenylamine; the latter was added in ethanol (10.0 ml/l) after autoclaving. The new cultures were incubated for a further 6 days, corresponding to the end of the exponential growth, before harvesting. The nominally (3*R*,4*R*)-[2-¹⁴C, 4-³H₁]mevalonic acid was prepared by dissolving the [¹⁴C]- and [³H]mevalonolactones, supplied as benzene solutions, in 1 M-KOH (2 ml) and incubating the solution at 22°C for 2 h to hydrolyse any lactone. Any organic solvent was evaporated under N₂ and the aqueous mevalonic acid solution was diluted with 20 mM-Tris/HCl buffer, pH 7.8 (4 ml) before being added to the medium.

Extraction of carotenoids

Cultures at stationary phase were centrifuged (15 min, 0°C, 8000g). The cell pellet was dried and weighed, then extracted in acetone (100 ml) containing the antioxidant 2,6-di-*t*-butyl-4-methylphenol (50 mg) in darkness for 18 h. These and all subsequent manipulations were performed in dull light. The acetone cell suspension was then mixed with hexane (100 ml) and saturated aq. NaCl (3 ml) was added until phase separation occurred. The aqueous

acetone layer was extracted again with hexane (100 ml) and the combined hexane extracts, which contained the bacterioruberin, were evaporated to dryness at 40°C.

Isolation of carotenoids

The carotenoids were taken up in hexane (3 ml) and applied to an activity I alumina column (10 cm × 25 mm) in hexane/acetone (4:1, v/v) eluted successively with hexane/acetone (4:1, v/v; 6 × 10 ml fractions), acetone (8 × 10 ml fractions) and methanol (4 × 10 ml fractions). The three fractions were stored, dry, at 0°C under N₂ until they were redissolved in benzene and chromatographed on a Waters high-pressure liquid chromatography semi-preparative μPorasil column (Waters Associates, Milford, MA, U.S.A.) (4 × 50 μl injections) in hexane/isopropanol [99:1, 98:2 and 95:5 (v/v) for the hexane/acetone, acetone and methanol fractions, respectively; 4 ml/min; 1.38 × 10⁵ kPa] and absorbance was monitored at 440 nm. Bacterioruberin, found in the methanol fraction, was removed from the μPorasil column using isopropanol, and crystallized on drying. All solvents were redistilled, filtered and degassed before use.

The carotenoids were identified by their absorption spectra (Liaaen-Jensen, 1960; Davies, 1976) in the appropriate solvent. Bacterioruberin concentrations were determined using $A_{1\text{cm},499}^{1\%}$ 2620 (Liaaen-Jensen, 1960; Kelly *et al.*, 1970). In addition, chemical ionization mass spectrometry and ¹H-n.m.r. spectroscopy were used to identify neurosporene (Davis *et al.*, 1966; Davies, 1970) and bacterioruberin (Liaaen-Jensen, 1969; Kelly *et al.*, 1970).

Scintillation spectrometry

The ¹⁴C and ³H present in the carotenoids were measured simultaneously by liquid scintillation counting using the methods of Milborrow (1972). Samples were counted on a Packard Tri-Carb liquid scintillation spectrometer model 2650, which gave the following efficiencies: ¹⁴C, 64.5%; ³H, 29.0%; ³H in the ¹⁴C channel, 0.09%; ¹⁴C in the ³H channel, 13.15%. All experimental samples were counted at least twice to confirm the absence of phosphorescence and to give less than 1% counting error. All samples were supplemented first with standard [U-³H]toluene (10 μl) and subsequently with [U-¹⁴C]toluene (10 μl). Correction was then made for quenching. The quenching of the ¹⁴C counts detected in the ³H channel is not the same as the quenching of the ³H counts in that channel or even that of the ¹⁴C counts in the ¹⁴C channel. This potential source of error was eliminated by reference to a calibration curve constructed from the counts of [U-¹⁴C]toluene standards quenched over a range of unlabelled bacterioruberin concentrations. In addition to this

precaution, subsamples of the [^{14}C , ^3H]carotenoids were counted over a range of concentrations so that the $^{14}\text{C}/^3\text{H}$ ratios could be determined with different degrees of quenching.

Results

Incorporation of (3*R*,4*R*)-[2- ^{14}C ,4- $^3\text{H}_1$]mevalonic acid into bacterioruberin

In order to study the stereochemistry of bacterioruberin biosynthesis, a cell preparation is required that incorporates labelled precursor into the carotenoid fraction with high efficiency. The susceptibility of *H. halobium* cells to osmotic shock is well known (Marshall & Brown, 1968) and it was found that lysis of intact cells in the low-osmotic-strength medium of Fahey & Milborrow (1978) containing [2- ^{14}C]mevalonic acid gave a cell-free system that synthesized labelled carotenoids (I. E. Swift & B. V. Milborrow, unpublished work). However, intact cells gave carotenoids of higher specific activity than did a lysate, and intact cells fed with labelled mevalonate gave the results shown in Table 1. These results indicate that the $^{14}\text{C}/^3\text{H}$ ratio for neurosporene is almost identical with that of the mevalonate supplied. This suggests that, in *H. halobium*, carotenoid biosynthesis proceeds with the same stereochemistry, at centres derived from C-4 of mevalonate, as in other micro-organisms and higher plants (Goodwin, 1971). The $^{14}\text{C}/^3\text{H}$ ratio of neurosporene, therefore, was used as a base against which other ratios were compared.

The $^{14}\text{C}/^3\text{H}$ ratio for bacterioruberin, when normalized to that of neurosporene, remained very close to that of the (3*R*,4*R*)-[2- ^{14}C ,4- $^3\text{H}_1$]mevalonic acid (10:9.78). This suggests that all the hydrogen atoms in the 4-*pro-R* position of mevalonate are retained in bacterioruberin. The same ratio would have been obtained even if the isopentenyl residue attached to lycopene had not been derived from the same pool of added mevalonate, provided that

the 4-*pro-R* hydrogen atoms of these residues were retained. Loss of ^3H from any position would have lowered the $^{14}\text{C}/^3\text{H}$ ratio to 10:8 because the bacterioruberin molecule is composed of two symmetrical halves.

Effect of diphenylamine upon bacterioruberin synthesis

Diphenylamine is a potent inhibitor of bacterioruberin biosynthesis (Fig. 2); a concentration of $5 \times 10^{-6}\text{M}$ decreased the amount formed by the cells to 43% of that of the control, and synthesis of bacterioruberin was abolished by 25 μM -diphenylamine. Cell growth was much less affected. At 5 μM the cells were orange rather than their characteristic pink because of the accumulation of uncyclized C_{40} carotenoids. The inhibition of bacterioruberin biosynthesis by diphenylamine was overcome by thiamin (250–1000 $\mu\text{g/l}$), but the vitamin did not restore the growth of the cells (I. E. Swift & B. V. Milborrow, unpublished work).

Fig. 2. Effect of diphenylamine upon growth of, and bacterioruberin concentration in, *H. halobium*

Diphenylamine in ethanol (1.0 ml) was added to sterile media (100 ml), which were then inoculated with exponentially growing cells and incubated for 6 days at 26°C. Δ , Final bacterioruberin concentration; $\bullet$, weight of cells.

Table 1. Incorporation of (3*R*,4*R*)-[2- ^{14}C ,4- $^3\text{H}_1$]mevalonic acid into bacterioruberin

(3*R*,4*R*)-[4- $^3\text{H}_1$]- + (3*S*,4*S*)-[4- $^3\text{H}_1$]Mevalonolactone (4.5 μCi) and [2- ^{14}C]mevalonolactone (0.79 μCi) were mixed and hydrolysed. The mevalonic acid (5 ml) was then added to *H. halobium* medium prior to inoculation with mid-exponential growth cells (0.05 ml). Cultures were incubated at 26°C for 6 days. The carotenoids were extracted from the cells, isolated, identified and their radioactivity was counted as described in the Experimental section.

Sample	^{14}C (d.p.m.)	^3H (d.p.m.)	$^{14}\text{C}/^3\text{H}$	Observed $^{14}\text{C}/^3\text{H}$ normalized to mevalonic acid	Observed $^{14}\text{C}/^3\text{H}$ normalized to neurosporene	Theoretical $^{14}\text{C}/^3\text{H}$ if 4- <i>pro-R</i> of mevalonic acid were retained at all positions	Theoretical $^{14}\text{C}/^3\text{H}$ if 4- <i>pro-R</i> of mevalonic acid were removed from one position during isopentenylation
Mevalonic acid	30671	264077	1:8.61	1:1	1:1.04	—	—
Neurosporene	2280	18856	1:8.27	8:7.68	(8:8)	8:8	—
Bacterioruberin	16275	131665	1:8.09	10:9.40	10:9.78	10:10	10:8

Discussion

The hydrogen atoms at C-2 and C-2' of bacterioruberin (II) have been shown by the work of Johansen & Liaaen-Jensen (1977) to have the same absolute configuration (2*R*,2'*R*) as those at the analogous positions in the C₅₀ ϵ -carotenoid decaprenoxanthin (I) and the C₅₀ β -carotenoid from *Corynebacterium poinsettiae*. We have now shown that there is no change in the ¹⁴C/³H ratio when (3*R*,4*R*)-[2-¹⁴C,4-³H₁]mevalonate is incorporated into neurosporene and bacterioruberin by *H. halobium*. We can deduce from this that the reactions involving what was C-4 of mevalonate proceed with the retention of the 4-*pro-R* hydrogen atoms during the biosynthesis of neurosporene by this organism and that they are also retained on the isoprene unit attached to C-2 and C-2' of bacterioruberin. Most importantly the hydrogen atom at these two positions (II) must have been that which occupied the 4-*pro-R* position in mevalonate. The 4-*pro-R* hydrogen is retained at C-2 in the cyclic C₅₀ ϵ -carotenoid decaprenoxanthin (Fahey & Milborrow, 1978) and has now been shown to be retained during the isopentenylolation of the acyclic C₅₀ carotenoid, bacterioruberin.

A further similarity has been found between the isopentenylolation reaction which forms bacterioruberin and the cyclization reactions, with and without isopentenylolation, that form the cyclic C₄₀ and C₅₀ carotenoids. This is the sensitivity of the reactions to diphenylamine (D. Fahey & B. V. Milborrow, unpublished work; Fig. 2) and its reversal by thiamin (I. E. Swift & B. V. Milborrow, unpublished work). The significance of this similarity cannot be assessed until other isopentenylolation reactions have been assayed.

Several analogues of bacterioruberin are known (III, IV) (Kelly *et al.*, 1970) that lack hydroxyl groups on one or both of the attached isoprene residues. All members carry hydroxyl groups at C-1 and C-1', which suggests that hydroxylation at these positions occurs concurrently with isopentenylolation.

If cyclization represents an advance from a primitive situation then this could have occurred

by the exclusion of the hydroxyl oxygen from the active site and its replacement by C-6 of the lycopene chain. It would be predicted from this that the *pro-R* methyl group at C-1 of bacterioruberin is derived from C-2 of mevalonate.

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