Carotenoid Biosynthesis in *Rhodopseudomonas spheroides*

S-ADENOSYLMETHIONINE AS THE METHYLATING AGENT IN THE BIOSYNTHESIS OF SPHEROIDENE AND SPHEROIDENONE

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[methyl- ^{14}C]Methionine and *S*-adenosyl[methyl- ^{14}C]methionine were incorporated into the methoxycarotenoids spheroidene and spheroidenone by *Rhodopseudomonas spheroides*. The incorporation was greatly enhanced in the presence of lysozyme. On degradation of labelled spheroidene by hydriodic acid, the ^{14}C label was recovered in methyl iodide. Degradation of spheroidenone by reduction and allylic dehydration and demethylation of the reduction product gave a mixture of unlabelled carotenoid hydrocarbons, including 3,4-didehydrolycopene and 3,4-didehydro-7',8'-dihydrolycopene. The label from [methyl- ^{14}C]methionine and *S*-adenosyl[methyl- ^{14}C]methionine was located specifically in the methoxy group of spheroidene and spheroidenone. The biosynthesis of methoxycarotenoids in *Rps. spheroides* involves methylation of the tertiary hydroxyl groups of intermediates with *S*-adenosylmethionine.

The carotenoids produced by photosynthetic bacteria of the Athiorhodaceae are characteristically acyclic, and have tertiary hydroxyl and methoxy groups at C-1 (Liaaen-Jensen, 1963). Thus the main carotenoids in *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*, grown anaerobically, are spirilloxanthin [1,1'-dimethoxy-3,4,3',4'-tetrahydro-1,2,1',2'-tetrahydro- $\psi\psi$ -carotene, (I)] and spheroidene [1-methoxy-3,4-didehydro-1,2,7,8'-tetrahydro- $\psi\psi$ -carotene, (II)] respectively. On the basis of kinetic studies, often involving use of the inhibitor diphenylamine, pathways have been postulated for the biosynthesis of spheroidene and spirilloxanthin (Liaaen-Jensen *et al.*, 1958, 1961; Eimhjellen & Liaaen-Jensen, 1964) and the recent isolation and characterization of a wide range of other carotenoids from large-scale cultures, especially of *R. rubrum* in the presence of diphenylamine (Davies, 1969, 1970a,b; Davies *et al.*, 1969; Malhotra *et al.*, 1969, 1970a,b) has led to the postulation of alternative schemes, especially for spheroidene formation via 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro- $\psi\psi$ -carotene) (Davies, 1969, 1970b). Experiments involving the use of nicotine as an inhibitor (McDermott *et al.*, 1973; Singh *et al.*, 1973), however, indicate that the pathway of spheroidene synthesis probably operating in *Rps. spheroides* is that originally proposed (Eimhjellen & Liaaen-Jensen, 1964) from neurosporene [7,8-dihydro- $\psi\psi$ -carotene, (III)]

via chloroxanthin [1,2,7',8'-tetrahydro- $\psi\psi$ -caroten-1-ol, (IV)] and demethylated spheroidene [3,4-didehydro-1,2,7',8'-tetrahydro- $\psi\psi$ -caroten-1-ol, (V)] (Scheme 1).

Irrespective of the order of formation of biosynthetic intermediates, however, three types of reaction occur that are not found in higher plants, namely introduction of the 3,4 double bond, introduction of the tertiary hydroxyl group at C-1 and methylation of this tertiary hydroxyl group. The hydroxylation and desaturation reactions have not been studied in detail, but some information has been obtained about the methylation reaction. Even before the correct structure of spirilloxanthin was established, Braithwaite & Goodwin (1958) demonstrated the incorporation of [^{14}C]formate into spirilloxanthin in *R. rubrum*, and showed by degradation that the label was located in the methoxy group; the radioactivity was recovered in methyl iodide liberated from the methoxy groups of spirilloxanthin by hydriodic acid treatment. Thus the methoxy groups of spirilloxanthin were derived from formate, which was known to be a precursor of C-1 units in other biological systems. Later work (Benedict & Beckman, 1964; Beckman *et al.*, 1965) demonstrated the incorporation of [methyl- ^{14}C]methionine into methoxycarotenoids by *R. rubrum*, and of *S*-adenosyl[methyl- ^{14}C]methionine into methoxycarotenoids by chromatophores of *R. rubrum*. In this work, however, the carotenoids were separated by

Scheme 1. Postulated scheme for the biosynthesis of spheroidene from neurosporene (Eimhjellen & Liaaen-Jensen, 1964)

For details see the text.

chromatography of the crude extract in a single paper chromatography system, and it is very unlikely that this procedure would give pure samples of each carotenoid; the purification of *R. rubrum* carotenoids free from non-carotenoid impurities is now known to be difficult (R. K. Singh, G. Britton & T. W. Goodwin, unpublished work). Also no degradation was attempted to confirm the location of the radioactivity in the methoxy group.

In the present work, in which rigorous purification procedures were used, the incorporation of the methyl group of methionine or *S*-adenosylmethionine into spheroidene and spheroidenone [1-methoxy-3,4-didehydro-1,2,7',8'-tetrahydro- ψ -caroten-2-one, (VI)] in *Rps. spheroides* has been demonstrated, and the location of the label in the methoxy group has been established by degradation.

Materials and Methods

[methyl-¹⁴C]Methionine and *S*-adenosyl[methyl-¹⁴C]methionine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. *Rhodospseudomonas spheroides* N.C.I.B. 8253, obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K., was cultured anaerobically in light at 28°C, on a malate-glutamate medium (Lascelles, 1956).

Incubations with [methyl-¹⁴C]methionine and S-adenosyl[methyl-¹⁴C]methionine

In experiments to compare the relative efficiencies of different modes of incubation for the incorporation of [methyl-¹⁴C]methionine, cultures (1.6 litres) were

harvested after 48 h growth, the cells were resuspended in 0.1 M-Tris-HCl buffer, pH 7.0 (20 ml), and the suspension was divided into four portions. To each portion was added ATP (6 mM), MgCl₂ (6 mM) and [*methyl*-¹⁴C]methionine (1 μ Ci). In one case, the cells were disrupted by shaking with glass beads (5 g, 0.10–0.11 mm diam.) for 2 min at 4000 rev./min with CO₂ cooling in a Braun M.S.K. homogenizer (Shandon Southern Instruments Ltd., Camberley, Surrey, U.K.); in a second case the cells were subjected to ultrasonic disintegration for 2 min at 20 kHz in a MSE 100W sonic disintegrator; to a third portion lysozyme (30 mg) was added. The fourth portion (whole-cell control) had no additions or disruptive treatment. In each case, the mixture was incubated in light at 28°C for 3 h anaerobically (under N₂) followed by 1 h of aerobic incubation. In the incubations with *S*-adenosyl[*methyl*-¹⁴C]methionine, cells from 1.2 litres of culture resuspended in 15 ml of buffer were used, and the incubation with cells disrupted by ultrasonic treatment was omitted. In each of the three incubations, 1 μ Ci of *S*-adenosyl[*methyl*-¹⁴C]methionine was used. With untreated cells incorporation was negligible.

In experiments to produce larger amounts of spheroidene and spheroidenone for degradation, the cells from 1.5 litres of culture grown for 48 h were resuspended in 10 ml of 0.1 M-Tris-HCl buffer, pH 7.0, and incubated with [*methyl*-¹⁴C]methionine or *S*-adenosyl[*methyl*-¹⁴C]methionine (5 μ Ci), lysozyme (70 mg), ATP (6 mM) and MgCl₂ (6 mM) as before. With all incubations, the reaction was terminated by the addition of acetone (25 ml).

Extraction and purification of carotenoids

After addition of acetone, the incubation mixture was left at room temperature in the dark for 15 min. The solid material was removed by filtration and re-extracted with acetone (2 $\times$ 20 ml). The acetone was removed from the combined extracts under reduced pressure in a rotary evaporator, and the residue was saponified, under N₂, for 30 min at room temperature, with 5% KOH in ethanol, to destroy bacteriochlorophyll. The carotenoids were transferred to diethyl ether (referred to as 'ether' throughout) and the ethereal solution was washed several times with water to remove alkali, and was evaporated to dryness. The residue was chromatographed on a column of neutral alumina (10 g; M. Woelm, Eschwege, Germany; deactivated to Grade III). Four fractions were collected, eluted respectively with light petroleum (the fraction b.p. 40–60°C was used throughout), 10% ether in light petroleum, 20% ether in light petroleum and ether. The second (10% ether in light petroleum) fraction, containing spheroidene, was chromatographed on thin layers of silica gel G with 10% ether in light petroleum as developing solvent,

and the main band, spheroidene (*R_F* 0.6) was further purified by successive t.l.c. on MgO-Kieselguhr G (1:1; BDH Chemicals, Poole, Dorset, U.K., and E. Merck, Darmstadt, Germany, respectively) with acetone-benzene-light petroleum (1:1:8) and on silica gel G with 15% ether in light petroleum as developing solvent. From the third (20% ether in light petroleum) column fraction, spheroidenone was purified by successive t.l.c. on silica gel G with 20% ether in light petroleum, MgO-Kieselguhr G (1:1) with acetone-benzene-light petroleum (1:1:3) and silica gel G with 25% ether in light petroleum as developing solvent.

Degradation of spheroidene

A modification of the method of Inglis (1957) was used. Labelled spheroidene (20440 d.p.m.), diluted with unlabelled spheroidene (2 mg) and spirilloxanthin (5 mg), was dissolved in phenol (AnalaR grade, 0.5 g) at 80°C (oil bath). A stream of N₂ was passed through the solution, then hydriodic acid (AnalaR, sp.gr. 1.7, 2 ml) was added, and the mixture was heated at 127°C (oil bath) for 45 min. The effluent gas stream was passed through a solution of sodium thiosulphate (5%) and CdSO₄ (5%), and the methyl iodide was condensed in a U-tube cooled in acetone-solid CO₂. After the reaction, the methyl iodide was washed out of the U-tube into a counting vial, with toluene (5 ml). Scintillator solution was added, and the sample assayed for radioactivity in the normal way in a liquid-scintillation counter (see below). The methyl iodide had an activity of 11400 d.p.m., i.e. 55% recovery.

Degradation of spheroidenone

A sample of spheroidenone (114600 d.p.m.), diluted with unlabelled carrier (0.184 mg, final sp. radioactivity 623 d.p.m./ μ g), was reduced in ethanol (20 ml) with NaBH₄ (10 mg) for 45 min. The pigments were transferred to ether, the ethereal solution was washed with water and evaporated to dryness, and the product, reduced spheroidenone, was purified by t.l.c. on silica gel G with 35% ether in light petroleum as developing solvent. The reduced spheroidenone (0.135 mg, sp. radioactivity 637 d.p.m./ μ g) was treated with HCl in chloroform (0.03 M, 4 ml) under N₂ for 8 min, after which the acid was removed by washing with water, and the products were separated by t.l.c. on silica gel G with 1% ether in light petroleum as developing solvent. Two bands (*A* and *B*) were obtained. Band *A* (yellow, *R_F* 0.5), further purified by t.l.c. on MgO-Kieselguhr G (1:1) with acetone-benzene-light petroleum (2:2:1) and on silica gel G with 5% ether in light petroleum as developing solvent had λ_{max} (light petroleum) at 426, 452, 482 nm and mol.wt. (from the mass spectrum) 534, but was not further

identified. Band *B* (orange-red, R_F 0.4) was separated by t.l.c. on MgO-Kieselguhr G with acetone-benzene-light petroleum (2:2:1) as developing solvent into two compounds, R_F 0.2 and 0.4. After further purification by t.l.c. on silica gel G with 4% ether in light petroleum as developing solvent, these were identified as 3,4-didehydrolycopene (22 μ g) and 3,4-didehydro-7',8'-dihydrolycopene (10 μ g) respectively.

Spectrometry and radioassay

Mass spectra were determined by Mr. J. R. Ireland (of this Department) with an A.E.I. MS 12 instrument, by using the direct insertion probe, an ion source temperature of 220°C and an ionizing voltage of 70 eV.

Absorption spectra were determined on a Unicam SP.800 recording spectrophotometer, and pigments were estimated quantitatively from these spectra by use of the $E_{1\text{cm}}^{1\%}$ values listed by Davies (1965).

Carotenoid samples were bleached by u.v. light and assayed for radioactivity in a Beckman LS200 liquid-scintillation system as described elsewhere (Britton & Goodwin, 1971). Specific radioactivities were calculated as described by Britton & Goodwin (1971).

Results and Discussion

Cells from 48 h cultures of *Rps. spheroides* incubated with [methyl- ^{14}C]methionine incorporated approx. 1.4% of the radioactivity into the unsaponifiable lipid material in 4 h (3 h of anaerobic followed by 1 h of aerobic incubation). Approx. 10% of the radioactivity incorporated was recovered in the two main

carotenoids, spheroidene and spheroidenone (Table 1). When lysozyme was added to the incubation mixture, however, a fivefold increase in total incorporation was obtained, and the radioactivity in spheroidene and spheroidenone increased tenfold. Disruption of the cells by ultrasonic treatment or by shaking with glass beads did not produce a similar increase in the efficiency of incorporation: the amounts of radioactivity incorporated into the unsaponifiable lipid material and into the two main carotenoids were similar to the values obtained in the incubation with whole cells. Lysozyme damages the cell wall of *Rps. spheroides* (Gorchein, 1968). This may facilitate the entry of methionine into the cell, resulting in the greater efficiency of incorporation into methoxycarotenoids. With the mechanical methods used, considerable cell disruption would be expected to occur. It is noteworthy that the extent of incorporation of methionine into carotenoids under these circumstances was similar to that obtained with intact cells.

In the presence of lysozyme, *Rps. spheroides* also incorporated *S*-adenosyl[methyl- ^{14}C]methionine into unsaponifiable lipid material and into spheroidene and spheroidenone (Table 1, Expt. *b*). The efficiency of incorporation in each case was somewhat less than with methionine as substrate. Cells disrupted by shaking with glass beads also produced labelled spheroidene and spheroidenone when incubated with *S*-adenosyl[methyl- ^{14}C]methionine. In all cases, the radioactivity remained associated with the carotenoids after purification on several t.l.c. systems.

These results thus establish the incorporation of methionine into methoxycarotenoids in *Rps. spheroides*, and indicate that *S*-adenosylmethionine is the methylating agent involved in the methylation of the tertiary hydroxyl groups in the carotenoids of this organism. The labelling of methoxycarotenoids in

Table 1. Incorporation of [methyl- ^{14}C]methionine and *S*-adenosyl[methyl- ^{14}C]methionine into spheroidene and spheroidenone by *Rhodopseudomonas spheroides*

All incubations were 3 h of anaerobic followed by 1 h of aerobic incubation, and contained ATP (6 mM), MgCl_2 (6 mM) and substrate (1 μCi) in 5 ml of 0.1 M-Tris-HCl buffer, pH 7.0.

	Radioactivity (d.p.m.)		
	Total incorporation into unsaponifiable lipid	Spheroidene	Spheroidenone
Expt. (a) Incubation with methionine			
(i) Cells disrupted by shaking with glass beads	26000	1150	3800
(ii) Cells disrupted by ultrasonic treatment	21700	360	1700
(iii) Cells+lysozyme	145000	7700	16250
(iv) Untreated cells	29300	870	1880
Expt. (b) Incubation with <i>S</i> -adenosylmethionine			
(i) Cells disrupted by shaking with glass beads	8470	700	1140
(ii) Cells+lysozyme	79200	7500	4350

R. rubrum by incubation with methionine or *S*-adenosylmethionine has previously been reported (Benedict & Beckman, 1964; Beckman *et al.*, 1965) but the carotenoids were not rigorously purified, and it was not conclusively established by degradation that the labelling was located in the methoxy groups. The degradation of spheroidene and spheroidenone labelled by incubation of *Rps. spheroides* with [methyl- ^{14}C]methionine and *S*-adenosyl[methyl- ^{14}C]methionine was therefore undertaken.

The first approach was to liberate the *O*-methyl group of spheroidene as methyl iodide by ether cleavage with hydriodic acid. This reaction had been used by Braithwaite & Goodwin (1958) to show that the methoxy groups of spirilloxanthin were labelled when *R. rubrum* was grown in the presence of [^{14}C]formate. In the work of Braithwaite & Goodwin (1958), the methyl iodide was trapped as the quinoxinium salt, which was assayed as a solid sample. In the present work, the methyl iodide was freed from iodine by passage through sodium thiosulphate and CdSO_4 (Inglis, 1957) and then assayed directly by liquid-scintillation counting. By degradation of spheroidene, labelled by [methyl- ^{14}C]methionine and *S*-adenosyl[methyl- ^{14}C]methionine, by this method 55% of the total radioactivity was recovered in the methyl iodide, a value similar to that obtained by Braithwaite & Goodwin (1958). This therefore demonstrates that it is the methoxy group of spheroidene that is labelled by incubation of *Rps. spheroides* with [methyl- ^{14}C]methionine or *S*-adenosyl[methyl- ^{14}C]methionine.

To confirm that the label was located only in the methoxy group, a second degradation method was used. In this case spheroidenone was degraded by a series of reactions that resulted in the removal of the methoxy group as methanol (not recovered), and permitted the remaining C_{40} skeleton of spheroidenone to be recovered and assayed for radioactivity. The method used involved reduction of the keto group of spheroidenone, followed by allylic dehydration and demethylation of the resulting reduced spheroidenone [1-methoxy-3,4-didehydro-1,2,7,8'-tetrahydro- ψ -caroten-2-ol, (VII)], the C_{40} carotenoid skeleton being recovered as a mixture of hydrocarbons.

A sample of spheroidenone (114600 d.p.m.) labelled by incubation with [methyl- ^{14}C]methionine was diluted with unlabelled carrier material, and reduced, in ethanol, with NaBH_4 . The product, reduced spheroidenone (86050 d.p.m.), was purified by t.l.c. and characterized by its absorption spectrum (λ_{max} in ethanol at 428, 453, 484 nm) and by its mass spectrum, which had the parent ion, M^+ at m/e 584 (4%, $\text{C}_{41}\text{H}_{60}\text{O}_2$) and fragment ions at m/e 566 (1%, $M-\text{H}_2\text{O}$), 534 (5%, $M-\text{H}_2\text{O}-\text{CH}_3\text{OH}$), 511 (1%, $M-73$, metastable ion at m/e 447; $511^2/584 = 447.1$), 478 [2%, $M-106$ (xylene)], 405 (2%, $M-106-73$, metastable ion at m/e 343; $405^2/478 = 343.1$) and 73

(100%). The specific radioactivity of this product was virtually the same as that of the starting material, thus confirming the radiochemical purity of the spheroidenone. In the reduction product, the hydroxyl group introduced is in an allylic position with respect to the main polyene chromophore, and is therefore readily eliminated by treatment with acidified chloroform. In the probable main product of this dehydration, compound (VIII), the methoxy group at C-1 is now allylic to the main polyene system, and is therefore also readily eliminated. The main product of treatment of reduced spheroidenone with acidified chloroform is 3,4-didehydrolycopene [3,4-didehydro- ψ -carotene, (IX)] as explained in Scheme 2 (Liaaen-Jensen, 1971). In the present work, three hydrocarbon products were isolated. The main one was identified as 3,4-didehydrolycopene by its absorption spectrum [λ_{max} (light petroleum) at 458, 491, 524 nm] and mass spectrum which had the parent ion, M^+ , at m/e 534 (30%, $\text{C}_{40}\text{H}_{54}$), and fragment ions at m/e 465 (1%, $M-69$, metastable ion at m/e 405; $465/534 = 404.9$), 442 [3%, $M-92$ (toluene), metastable ion at m/e 366; $442^2/534 = 365.9$] and 428 [8%, $M-106$ (xylene)]. A second product had an absorption spectrum very similar to that of lycopene, with λ_{max} (light petroleum) at 444, 469 and 500 nm, but mass spectrometry suggested that it was in fact the unsymmetrical isomer of lycopene, 3,4-didehydro-7',8'-dihydro- ψ -carotene (X). Thus the mass spectrum had the parent ion M^+ at m/e 536 (34%, $\text{C}_{40}\text{H}_{56}$), with fragment ions at m/e 444 (4%, metastable ion at m/e 368; $444^2/536 = 367.8$) and 430 (6%), owing to losses of toluene and xylene respectively, and at m/e 399 (2.5%, metastable ion at m/e 297; $399^2/536 = 297.0$), 307 (2%, metastable ion at m/e 212; $307^2/444 = 212.3$) and 293 (2%, metastable ion at m/e 200; $293^2/430 = 199.6$) owing to loss from the M^+ , $M-92$ and $M-106$ ions respectively, of a 137 mass unit fragment, by cleavage of the bis-allylic C-7',8' bond. The third product of the elimination reaction had λ_{max} (light petroleum) at 426, 452 and 482 nm. This compound, which was judged to be a hydrocarbon from its mass spectrum, which had the parent ion M^+ at m/e 534 (25%) and fragment ions at m/e 442 [1%, $M-92$ (toluene)] and 428 [3%, $M-106$ (xylene)], was not identified.

Thus the three main products of the reaction were all hydrocarbons, showing that the C-1 methoxy group had been lost in the elimination reaction. No radioactivity was detected in any of these hydrocarbon compounds. In similar experiments with *S*-adenosyl[methyl- ^{14}C]methionine as substrate, degradation by this method of reduced spheroidenone (3900 d.p.m.) prepared by reduction of spheroidenone (4320 d.p.m.) also gave hydrocarbon products that were completely unlabelled. The C_{40} carotenoid skeleton was therefore not labelled by [methyl- ^{14}C]methionine or *S*-adenosyl[methyl- ^{14}C]methionine,

Scheme 2. Degradation of spheroidenone (Liaaen-Jensen, 1971)

For details see the text.

thus confirming that the labelling in spheroidenone is located entirely in the methoxy group.

The incorporation of methionine and *S*-adenosylmethionine into spirilloxanthin in *R. rubrum* has previously been established (Benedict & Beckman, 1964; Beckman *et al.*, 1965); therefore it was reasonable to expect that these substrates would also be incorporated into other methoxycarotenoids in other bacteria. The present work is the first direct demonstration of the incorporation of methionine and *S*-adenosylmethionine into spheroidene and spheroidenone in *Rps. spheroides*, and extends the previous work by establishing, by degradation, that these substrates are incorporated specifically into the methoxy group, the C₄₀ carotenoid skeleton being completely unlabelled.

The methoxycarotenoids of *Rps. spheroides* are thus formed by methylation of the tertiary hydroxycarotenoids with *S*-adenosylmethionine as methylating agent. It appears that this methylation of the hydroxyl group occurs rapidly once the terminal double bond is hydrated, since the corresponding hydroxycarotenoids are not detected in more than trace amounts except under abnormal conditions, e.g. in the presence of inhibitors. Transmethylation reactions involving *S*-adenosylmethionine as methyl donor are well known (Greenberg, 1963), but the methylation of tertiary hydroxyl groups, as in the case of spheroidene and spirilloxanthin biosynthesis, is unusual. It is not known if the mechanism involved differs from the mechanism involved in better-known cases such as *N*-methylation and aromatic *O*-methylation.

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