Stereochemistry of allene biosynthesis and the formation of the acetylenic carotenoid diadinoxanthin and peridinin (C₃₇) from neoxanthin

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Intact cells of the alga *Amphidinium carterae* (Dinophyceae), and a cell-free system prepared from it, incorporated ¹⁴C, ³H-labelled mevalonate into lycopene, β,β -carotene, zeaxanthin, neoxanthin, diadinoxanthin and peridinin. The ¹⁴C/³H ratios of zeaxanthin, neoxanthin and diadinoxanthin formed from (2*RS*,3*R*)-[2-¹⁴C,2-³H₂]mevalonate show that a hydrogen atom from C-2 of mevalonate is retained in the allene at C-8, and also at C-12 of peridinin. (3*R*,4*R* + 3*S*,4*S*)-[2-¹⁴C,4-³H₁]Mevalonate gave ¹⁴C/³H ratios in peridinin which show that C-14 is lost. The three carbon atoms excised during the formation of the C₃₇ carotenoid peridinin are C-13, C-14 and C-20 of neoxanthin.

Little is known of the biosynthesis of many of the unusual carotenoids found in marine organisms. Many of the algae which produce the compounds are difficult to grow in axenic culture and incorporate very small amounts of mevalonic acid into the carotenoid fraction. Several biosynthetic sequences have been postulated but these have been based on the occurrence of related structures and known chemical and biochemical reactions rather than on observed interconversions.

Much of the brown colouration of dinoflagellates is caused by the presence of diadinoxanthin (I) while in others large amounts of the red carotenoid peridinin (II) occur.

Plankton blooms of such dinoflagellates can be responsible for 'red tides', where the abundance of peridinin in the cells gives the sea its colour. Diadinoxanthin contains an acetylenic group while peridinin contains an allenic group. In addition, the carbon skeleton of peridinin lacks three of the carbon atoms present in the polyene chain of C₄₀ carotenoids. The absence of one of the four methyl groups normally present on the polyene chain focuses attention on the site of a putative excision. Clearly, if a methyl and two other carbon atoms were lost then one of these two latter would be the carbon carrying the methyl (i.e. C-13). This poses the question of which other carbon atom is lost? Is it C-12, derived from C-2 of mevalonate, or C-14, derived from C-4 of mevalonate?

The occurrence of relatively large amounts of diadinoxanthin and peridinin in axenically grown cells of the dinophycean alga *Amphidinium carterae* suggested that the stereochemistry of biosynthesis of

the allene group, and which carbon atoms were lost during peridinin formation, could be investigated using [2-¹⁴C]-, [2-³H₂]- and [4*R*-³H₁]mevalonic acids.

Experimental

Materials

(3*RS*)-[2-¹⁴C]Mevalonolactone (22 Ci/mol), (3*R*,4*R*)-[4-³H₁]mevalonolactone plus (3*S*,4*S*)-[4-³H₁]mevalonolactone (1.8 Ci/mmol) and (2*RS*,3*R*)-[2-³H₂]mevalonolactone (745 Ci/mol) were purchased from The Radiochemical Centre. [U-³H]Toluene (299 μ Ci/mol) and [U-¹⁴C]toluene (1.53 mCi/mol) were obtained from Packard Instruments. Aluminium oxide 60F₂₅₄ (type E) t.l.c. plates were from Merck.

Biological material

Axenic cultures of *Amphidinium carterae* Hulbert (C.S.I.R.O. number CS 21) isolated in Halifax, Canada, were grown at the C.S.I.R.O. Fisheries and Oceanography Research Institute, Cronulla, N.S.W., Australia. Batches (6 $\times$ 3 litres) of the sterile culture medium described by Guillard & Ryther (1962) were prepared except that iron sequesterene was replaced by iron citrate and EDTA (30 g/l) was added. The cells were grown under fluorescent illumination at 20°C with aeration for 5 weeks (i.e. used in the lag phase).

Preparation of a cell-free system

Cells from a 5 litre volume of culture medium were harvested by centrifugation (20 min, 0°C,

Fig. 1. Carotenoid structures

Positions occupied by ^3H atoms derived from C-2 of mevalonate are marked (●) on structure V.

9500g). The cell pellet was resuspended in 5 ml of Tris/HCl buffer, pH 7.8, containing MnCl_2 (3 mM), glutathione (5 mM), EDTA (0.2 μM), ATP (6 μM), NADH (1 μM), NAD^+ (1 μM), NADPH (1 μM) and NADP^+ (1 μM), and transferred to a chilled mortar. Celite (500mg) was added and the mixture was ground thoroughly to a homogenous slurry. This was filtered through four layers of cheesecloth and washed with 5 ml of culture medium. The combined

filtrate (9.8 ml) was divided between two flasks and either (3*R*,4*R*)-[2- ^{14}C ,4- $^3\text{H}_1$] or (2*RS*,3*R*)-[2- ^{14}C ,2- $^3\text{H}_2$]mevalonate was added.

A further 5 litres of the culture was centrifuged (20 min, 0°C, 9500g) and the cell pellet was resuspended in 10 ml of culture medium. This volume was divided equally between two flasks and either (3*R*,4*R*)-[2- ^{14}C ,4- $^3\text{H}_1$] or (2*RS*,3*R*)-[2- ^{14}C ,2- H_2]mevalonate was added to the intact cells. All

four flasks were shaken under tungsten illumination for 6 h, at 26°C.

The [¹⁴C,³H]mevalonic acids were prepared by dissolving the required [³H]- and [¹⁴C]mevalonolactones, supplied as benzene solutions, in 0.1 M-KOH (0.5 ml) and incubating at 22°C for 2 h to hydrolyse any lactone. The benzene was evaporated under N₂ and the aqueous mevalonic acid solution was diluted with Tris/HCl buffer (20 mM, pH 7.8, 2.5 ml) before being distributed amongst the cell-free systems.

Extraction of carotenoids

Intact cells or cell-free systems were resuspended in methanol (100 ml) containing the antioxidant 2,6-di-*t*-butyl-4-methylphenol (500 mg) and held in darkness at 22°C for 24 h. This extraction procedure was repeated a further three times and the methanolic extracts were combined. These and all subsequent manipulations were performed in dull light. The methanolic cell suspensions were mixed with an equal volume of diethyl ether (400 ml) and sufficient saturated aq. NaCl (80 ml) was added to cause phase separation. The ethereal layers, which contained the carotenoid and chlorophyll fractions, were collected, washed twice with water, dried over anhydrous Na₂SO₄ and evaporated at 40°C.

Isolation of carotenoids

The dried ethereal extracts were redissolved in diethyl ether (1 ml) and applied to Merck aluminium oxide t.l.c. plates, which were then developed with toluene/ethyl acetate (5:3, v/v). This solvent system gave clear separation of carotenoids and chlorophylls. The carotenoid zones were eluted with ethyl acetate, dried under N₂ and stored in darkness at 0°C. The carotenoids were chromatographed (25 µl injections) on a Waters high-pressure liquid chromatography semi-preparative µPorasil column (Waters Associates, Milford, MA, U.S.A.) in hexane/isopropanol (95:5, v/v; 4.0 ml/min; 1.4 × 10⁵ kPa) and absorbance was monitored at 440 nm. All solvents were re-distilled, filtered and degassed before use.

The carotenoids were identified by their visible absorption spectra and chemical ionization (methane) mass spectra, (Schwieter *et al.*, 1969; Bartlett *et al.*, 1969; Cholnoky *et al.*, 1969; Bonnett *et al.*, 1969; Kjosén *et al.*, 1971; Strain *et al.*, 1976). Peridinin was also identified by co-chromatography with an authentic sample.

Scintillation spectrometry

The ¹⁴C and ³H contents of carotenoids were measured simultaneously by liquid scintillation spectrometry according to the methods of Swift & Milborrow (1980). 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% and ¹⁴C in the ³H channel, 13.15%. All experimental samples were counted at least twice to confirm the absence of phosphorescence and to 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). Detailed correction was then made for quenching, as described by Swift & Milborrow (1980). A calibration curve, constructed from the counts of [U-¹⁴C]-toluene standards quenched over a range of unlabelled zeaxanthin concentrations, served to eliminate sources of error arising from the differential quenching of true ³H counts in the ³H channel and the ¹⁴C counts detected as ³H in the ³H channel. In addition, subsamples of the [¹⁴C,³H]carotenoids were counted over a range of concentrations so that the ¹⁴C/³H ratios could be determined with different degrees of quenching.

Results and discussion

Experiments with [³H]mevalonic acids: formation of the allene

The interpretations of the ¹⁴C/³H ratios of the various carotenoids formed from labelled mevalonic acids are, of course, based on the assumption that the same mechanism of biosynthesis occurs in *A. carterae* as in the other bacteria and plants that have been investigated. The ¹⁴C/³H ratios of such cyclized and uncyclized carotenoids as were investigated after feeding (4*R*)- and (2*RS*)- [³H]mevalonates are entirely consistent with previous work; there is no reason to doubt that the assumption is justified.

The biosynthesis of lycopene (Goodwin, 1971) occurs with the retention of both of the hydrogen atoms from C-2 of mevalonate in the *E* C-1 methyl and one at C-4, C-8 and C-12 of each carotenoid half molecule. Similarly, a 4-*pro-R* hydrogen atom of mevalonate is retained at C-2, C-6, C-10 and C-14. ³H atoms from labelled mevalonate will be retained at these positions unless removed by subsequent enzymic modification of the carotenoids. Loss of one or more of these atoms can be monitored by a fall in the amount of ³H in relation to ¹⁴C present in the molecule. This procedure has been used to investigate the pathway of biosynthesis of diadinoxanthin (I) and peridinin (II).

The ¹⁴C/³H ratio of lycopene (III) formed from (2*RS*,3*R*)-[2-¹⁴C,2-³H₂]mevalonate and the changes in ratio due to the losses of ³H, which were observed coincident with the losses of the expected ³H atoms, suggest that relatively little ³H is lost from what had been C-2 of mevalonate by the action of isopentenyl pyrophosphate/dimethylallyl pyrophosphate isomerase in either the intact cells or the

cell-free system of *A. carterae*. There was also a change in ratio, attributed to the loss of one ^3H atom per molecule between zeaxanthin (V) or neoxanthin and diadinoxanthin, based on the $^{14}\text{C}/^3\text{H}$ ratio normalized to mevalonate: 1.0:0.98 (cell-free system) and 1.0:0.94 (intact cells) (Table 1). This is attributed to the loss of a hydrogen atom from C-8 of neoxanthin and indicates that 0.95 of each ^3H atom from C-2 of mevalonate has been retained at each position. This suggests that either there is a relatively weak isomerase activity in relation to that of the condensing enzyme, or that the isomerase operates with a strong isotope effect favouring the retention of ^3H and the abstraction of ^1H .

The $^{14}\text{C}/^3\text{H}$ ratios of lycopene and β,β -carotene isolated from intact cells and the cell-free systems fed with mevalonic acid are very similar, as expected. There is no significant change in the $^{14}\text{C}/^3\text{H}$ ratio on conversion into zeaxanthin.

The $^{14}\text{C}/^3\text{H}$ ratios are normalized to lycopene for ease of comparison but the same conclusions are reached if the original ratio in mevalonic acid is used.

We have recently established, in *A. carterae*, that zeaxanthin is converted into neoxanthin and that neoxanthin is a precursor of diadinoxanthin and peridinin (Swift *et al.*, 1981). The presence of zeaxanthin and neoxanthin in an alga of the Dinophyceae is consistent with the scheme for the biosynthesis of the allene group proposed by Bonnett *et al.* (1969). They suggest that a 5,6-epoxy group undergoes rearrangement, hydration and dehydra-

tion to give a C-5R hydroxyl group. The hydrogen atom at C-7 is removed during the formation of the allene. A simpler version (Scheme 1) is preferred. Zeaxanthin is probably epoxidized to violaxanthin which could then be converted into neoxanthin. The chirality of the allene of neoxanthin is also consistent with the mechanism.

Another possibility is that the allene is formed by rearrangement of a C-7, C-8 acetylenic group. Carotenoids containing such groups occur in the Dinophyceae but the crucial difference between the two proposed mechanisms is that the violaxanthin pathway could retain the hydrogen atom from C-8 in the allene, whereas the participation of an acetylene would cause the loss of the C-8 hydrogen atom. The neoxanthin and peridinin (which both contain allenic groups) formed from (2*RS*,3*R*)-[2- ^{14}C , 2- $^3\text{H}_2$]mevalonic acid have the same $^{14}\text{C}/^3\text{H}$ ratios as do lycopene, β,β -carotene and zeaxanthin (Table 1). The C-6, C-7, C-8 allene, therefore, must retain the hydrogen atom derived from the C-2 of mevalonic acid at C-8. This establishes, quite unambiguously, that an acetylenic intermediate could not have participated in the biosynthesis of the allene. The data are consistent with Scheme 1.

Formation of the acetylene

The loss of one ^3H atom during the formation of diadinoxanthin (I) from neoxanthin (IV) is attributed to the loss of the hydrogen atom of the allene at C-8, during the formation of the C-7', C-8' acetylene. The $^{14}\text{C}/^3\text{H}$ ratios are normalized to 8:12

Table 1. Incorporation of (2*RS*,3*R*)-[2- ^{14}C ,2- $^3\text{H}_2$]mevalonic acid into the carotenoids of *Amphidinium carterae* (2*RS*,3*R*)-[2- $^3\text{H}_2$]mevalonolactone (18.0 μCi) and (3*RS*)-[2- ^{14}C]mevalonolactone (1.6 μCi) were mixed and hydrolysed. The mevalonic acid (3.0 ml) was then divided equally between a cell-free system (4.9 ml) and intact cells (5 ml). Both systems were incubated under tungsten illumination for 6 h, at 26°C. The carotenoids were extracted, isolated, identified and their radioactivity was counted as described in the Experimental section.

Sample	^{14}C (d.p.m.)	^3H (d.p.m.)	$^{14}\text{C}/^3\text{H}$	$^{14}\text{C}/^3\text{H}$ normalized to mevalonic acid	$^{14}\text{C}/^3\text{H}$ normalized to lycopene
Expt. 1: cell-free system					
Mevalonic acid	4637	43 634	1:9.41	(1:2)	—
Lycopene	5473	37 381	1:6.83	8:11.62	(8:12)
β -Carotene	5989	40 426	1:6.75	8:11.48	8:11.86
Zeaxanthin	3910	26 353	1:6.74	8:11.46	8:11.84
Neoxanthin	6231	41 935	1:6.73	8:11.44	8:11.82
Diadinoxanthin	3247	20 002	1:6.16	8:10.47	8:10.84
Peridinin	8910	59 964	1:6.73	8:11.44	8:11.82
Expt. 2: intact cells					
Mevalonic acid	4637	43 634	1:9.41	(1:2)	—
Lycopene	3416	23 160	1:6.78	8:11.53	(8:12)
β -Carotene	3896	25 947	1:6.66	8:11.32	8:11.79
Zeaxanthin	2620	17 397	1:6.64	8:11.29	8:11.75
Neoxanthin	3524	23 505	1:6.67	8:11.34	8:11.81
Diadinoxanthin	2011	12 267	1:6.10	8:10.37	8:10.80
Peridinin	5651	37 636	1:6.66	8:11.32	8:11.79

for lycopene formed from (2*RS*,3*R*)-[2-¹⁴C, 2-³H₂]-mevalonate. The loss of 1.01 and 0.98 of one ³H atom derived from C-2 of mevalonate during this conversion (Table 1) is in close agreement with the expected value based on the mechanism shown in Scheme 2. Although the structures of violaxanthin, neoxanthin and the hypothetical active group of the enzymes appear to be quite different in Schemes 1 and 2, the relative three-dimensional position of the hypothetical, initiating basic group could be very close.

Formation of peridinin

The demonstration that [¹⁴C]zeaxanthin and [¹⁴C]neoxanthin (Swift *et al.*, 1981) can be metabolized to peridinin has further significance, namely that peridinin is formed by the deletion of three carbon atoms from the polyene chain of a C₄₀ carotenoid rather than by fusion of, for example, a diterpene and a sesquiterpene plus a C₂ moiety.

All the ³H atoms from (2*RS*,3*R*)-[2-¹⁴C, 2-³H₂] and (3*R*,4*R*)-[2-¹⁴C, 4-³H₁] mevalonic acids would be expected to be retained during the conversion of neoxanthin into peridinin except for one or other attached to the carbon atom on the C₃ fragment excised from the polyene chain.

Removal of a ³H atom (derived from C-2 of mevalonate) from C-12 would change the ¹⁴C/³H ratio in neoxanthin from 8:12 to 8:11 in peridinin (1:1.50 to 1:1.375). It must be remembered that the ³H atoms on C-2 of mevalonate are on the carbon atom that carries a ¹⁴C label. Consequently,

if C-12 ([2-¹⁴C]mevalonate) and its hydrogen atom were both excised as part of the C₃ fragment then the ¹⁴C/³H ratios observed would change from 8:12 to 7:11 (1:1.50 to 1:1.57 on the ratios normalized to lycopene). This change is not in agreement with the experimental results. Thus the proportion of ³H in peridinin should rise if both the ¹⁴C and the ³H at C-12 were removed. The proportion fell slightly (Table 1). If no loss of either ¹⁴C or ³H occurred from C-12 the ratio should remain as 8:12 (1:1.50). This pattern coincides closest to the experimental results and it is concluded, therefore, that C-12 is not excised and the hydrogen atom it carries is retained.

If the excision reaction involved the loss of a hydrogen atom from a carbon adjacent to the excision then the use of ¹⁴C/³H ratios could give an ambiguous result. The results in Table 2 show that this apparently did not occur: one hydrogen atom from the 4-*pro-R* position of mevalonate is lost and the hydrogen atoms from C-2 of mevalonate are retained during the conversion of neoxanthin to peridinin.

We now know which ³H atoms from [2*RS*-³H₂]- and [4*R*-³H₁]mevalonic acids are retained in peridinin, and can deduce, therefore, that, during the conversion of neoxanthin into peridinin, C-12 is retained while C-13, C-14 and C-20 (C-13 methyl) are lost. Nevertheless, merely knowing which atoms are lost does not sufficiently restrict the possible alternatives so that a probable reaction mechanism can be put forward. Clearly, the nature of the deletion could give an indication of the reaction

Scheme 1. Postulated mechanism of formation of the allene in neoxanthin. Violaxanthin is the suggested precursor. [Adapted from Goodwin (1971)].

Scheme 2. Postulated mechanism of conversion of the allene in neoxanthin into the acetylene of diadinoxanthin. From the similarity between the mechanisms presented in Schemes 1 and 2, it is possible that the enzyme responsible for the formation of the acetylene has evolved from that responsible for the formation of the allene. Obviously, the allene-producing enzyme could not have evolved from the acetylene-producing enzyme.

Table 2. *Incorporation of (3R,4R)-[2-¹⁴C,4-³H₁]mevalonic acid into the carotenoids of *Amphidinium carterae**
 The same conditions as described in Table 1 were used, except that (3R,4R)-[4-³H₁]mevalonolactone (16.5 μ Ci) was mixed with (3RS)-[2-¹⁴C]mevalonolactone (1.7 μ Ci).

Sample	¹⁴ C(d.p.m.)	³ H(d.p.m.)	¹⁴ C/ ³ H	¹⁴ C/ ³ H normalized to mevalonic acid	¹⁴ C/ ³ H normalized to lycopene
Expt. 1: cell-free system					
Mevalonic acid	4917	40074	1:8.15	(1:1)	—
Lycopene	3124	25179	1:8.06	8:7.91	(8:8)
β -Carotene	6125	36811	1:6.01	8:5.90	8:5.97
Zeaxanthin	6731	40319	1:5.99	8:5.88	8:5.95
Neoxanthin	4800	28752	1:5.99	8:5.88	8:5.95
Diadinoxanthin	3621	21473	1:5.93	8:5.82	8:5.89
Peridinin	8527	43232	1:5.07	8:4.98	8:5.03
Expt. 2: Intact cells					
Mevalonic acid	4917	40074	1:8.15	(1:1)	—
Lycopene	1943	15700	1:8.08	8:7.93	(8:8)
β -Carotene	4237	25422	1:6.00	8:5.89	8:5.94
Zeaxanthin	4976	30005	1:6.03	8:5.92	8:5.97
Neoxanthin	2930	17375	1:5.93	8:5.82	8:5.87
Diadinoxanthin	2116	12633	1:5.97	8:5.86	8:5.91
Peridinin	7130	35650	1:5.00	8:4.91	8:4.95

mechanism. Whether reduction, oxidation or hydration occurs and whether the hydrogen atom of C-14 is lost to the medium are now all susceptible to experimentation in the cell-free system. Recent experiments using (3RS,5RS)-[3-¹⁴C,5-³H₂]mevalonate have shown that C-13, C-14 and C-20 are deleted (I. E. Swift & B. V. Milborrow, unpublished results).

The molecule of peridinin contains another feature of interest, namely the C-9' appears to have been oxidized to a carboxyl group which has then formed a lactone with a hydroxyl group on C-11'. The presence of this ring may indicate a possible mechanism for the deletion of the three-carbon unit during the formation of peridinin. The presence of a cyclic intermediate between C-20 and C-15 during the excision of the C₃ fragment would provide a means of holding the two parts of the molecule together while the C-14–C-15 bond was broken and reformed as a C-12–C-15 bond. One noteworthy consequence of a mechanism involving this ring intermediate is that the hydrogen atom at C-11, derived from the 5-*pro-S* hydrogen atom of mevalonate, would be lost.

The acetylation of the C-3 hydroxyl group to form peridinin may represent an end-of-biosynthesis signal that decreases the polarity of the molecule and thereby facilitates the positioning of one ring of the completed carotenoid at a hydrophilic/lipophilic interface.

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