STRUCTURAL STUDIES ON BACTERIAL CAROTENOIDS AND THEIR BIOSYNTHETIC IMPLICATIONS

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ABSTRACT

Detailed studies of the properties of carotenoids isolated from diphenylamine-inhibited cultures of *Rhodospirillum rubrum* have revealed a number of novel structures which indicate new features of carotenoid biosynthesis in the photosynthetic bacteria. The dehydrogenation of phytoene to coloured carotenoids occurs by a sequence which is different from that in higher plants in that 7,8,11,12-tetrahydrolycopene, rather than ζ-carotene, is the immediate precursor of neurosporene. Both neurosporene and 7,8,11,12-tetrahydrolycopene undergo hydration, methylation and dehydrogenation to yield spheroidene and 11′,12′-dihydrospheroidene respectively; all the intermediates in these pathways have been identified. These pathways represent alternative routes of anhydrorhodovibrin and spirilloxanthin biosynthesis.

INTRODUCTION

The carotenoid pigments, like other natural isoprenoid compounds, are formed from mevalonic acid through isopentenyl pyrophosphate. The condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate leads to the formation of geranyl pyrophosphate and this, in turn, is converted into farnesyl pyrophosphate and then into geranylgeranyl pyrophosphate. The isoprenoid chains of two C_{20}-pyrophosphate units condense, tail-to-tail, to form the first C_{40}-carotenoid precursor, phytoene, which is then dehydrogenated to form the coloured carotenoids.

PATHWAYS OF PHYTOENE DEHYDROGENATION

The only pathway of coloured carotenoid formation that has been demonstrated, and which is generally assumed to operate in all carotenogenic organisms, is the sequence of reactions first proposed by Porter and Lincoln[16] on the basis of their observations on the carotenoids of tomato fruit. Any uncertainty of the structures of the intermediates in this sequence were eliminated by the unambiguous synthesis of phytoene [1], phytofluene [2], ζ-carotene [3] and neurosporene [4] and the comparison of the synthetic compounds with natural samples isolated from carrot oil[4,5]. The structures of these intermediates indicate that phytoene undergoes a series of stepwise dehydrogenations through phytofluene, ζ-carotene and neurosporene to

lycopene [5]. At each stage of this sequence (*Figure 1*), a previously isolated double bond is brought into conjugation, thus increasing the length of the chromophore by two conjugated double bonds. The dehydrogenation steps occur alternately to the left and to the right of the central chromophore since ζ-carotene (7,8,7′,8′-tetrahydrolycopene) is a symmetrical conjugated heptaene.

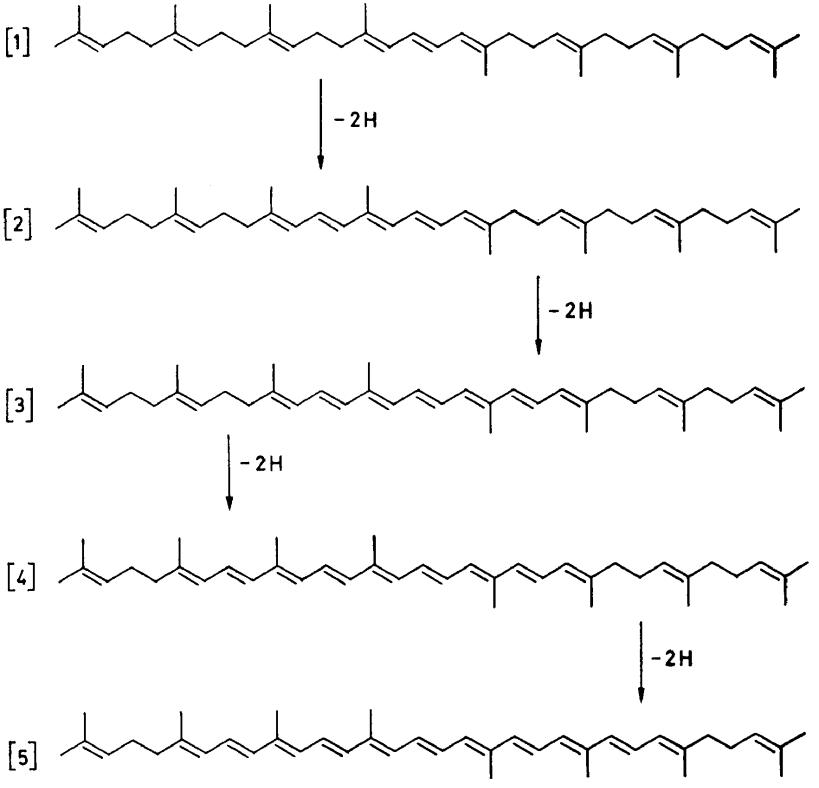


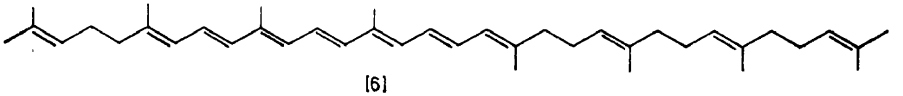
Figure 1. Dehydrogenation of phytoene in higher plants.

A similar conjugated heptaene has been isolated from diphenylamine-inhibited cultures of the purple non-sulphur photosynthetic bacterium, *Rhodospirillum rubrum*, by Goodwin and Osman.[8] Jensen and co-workers[11] showed that this compound, together with other carotene precursors, disappears from such cultures when the diphenylamine is removed, and deduced from kinetic studies that it takes part in the biosynthesis of lycopene, which is then converted into spirilloxanthin. While this conjugated heptaene from *R. rubrum* was referred to by both groups of workers as 'ζ-carotene', they described its absorption spectrum as having maxima at 376, 396 and 418 nm. in light petroleum, while the corresponding values quoted in the literature for ζ-carotene isolated from carrot oil, tomato fruit and *Phycomyces blakes-*

leeanus are slightly higher, the absorption maxima in light petroleum being at 378, 400 and 425 nm.[7,14,15] Nakayama[13] isolated a conjugated heptaene from a mutant of *Rhodopseudomonas spheroides*; this had an absorption spectrum ($\lambda_{max.}$ 377, 398 and 422 nm. in light petroleum) similar to that of the *R. rubrum* heptaene, but was referred to as θ-carotene. This is the name given by Haxo[9] to a pigment resembling ζ-carotene which he isolated from *Neurospora crassa*.

Davies and Holmes[1] have compared samples of the conjugated heptaenes isolated from carrot root and from diphenylamine-inhibited cultures of *R. rubrum* and have confirmed the difference in their absorption spectra (carrot root: $\lambda_{max.}$ 360 infl., 378, 400 and 425 nm.; *R. rubrum*: $\lambda_{max.}$ 354 infl., 374, 394·5 and 418·5 nm., both in light petroleum). They considered the possibility that the sample from *R. rubrum* might be acyclic while that from the carrot root might have the ends of the isoprenoid chain cyclised in β-ionone rings. Such a situation might arise if carotene cyclisation were to take place at an earlier stage in the dehydrogenation sequence than had previously been envisaged, since the carrot root carotenes are mostly bicyclic whilst those of *R. rubrum* are all acyclic. This possibility was eliminated by the inability of the two natural heptaenes to separate on thin layers, whilst synthetic 7,8,7′,8′-tetrahydro-β-carotene (kindly provided by Professor B. C. L. Weedon) was considerably less polar. The possibility that the 5 nm. spectral difference between the two polyenes was due to the *R. rubrum* heptaene being a *cis* isomer was ruled out by subjecting both pigments to iodine-catalysed photoisomerisation, when both were shown to have the all-*trans* configuration. The only other possibility seemed to be that the two conjugated heptaenes are different by virtue of the chromophore of the *R. rubrum* heptaene being in an off-centre position in the molecule. Indeed, the absorption spectrum reported for such an 'unsymmetrical ζ-carotene', prepared by unambiguous synthesis, was not dissimilar to that of the *R. rubrum* carotenoid[5].

Both the natural conjugated heptaenes have been examined by mass spectrometry[3]. While both show a molecular ion of mass consistent with the formula $C_{40}H_{60}$, their fragmentation patterns are different. The sample from carrot root gives peaks for (M-69) and (M-137), indicating fragmentation at the 'bis-allylic' bonds and confirming the structure of ζ-carotene as 7,8,7′,8′-tetrahydrolycopene. The conjugated heptaene from *R. rubrum*, however, shows only weak ions for (M-69) and (M-137) but gives an additional fragmentation to produce a strong ion at $m/e = 335$ (M-205). This indicates fission of the 11,12- (or 11′,12′-) bond. The only structure for the conjugated heptaene from *R. rubrum* that is consistent with the spectroscopic, chromatographic and mass spectrometric properties is 7,8,11,12-tetrahydrolycopene [6].



[6]

The mass spectrometric confirmation of the structures of phytoene, phytofluene and neurosporene isolated from *R. rubrum*, and the apparent

absence of ζ-carotene, means that the dehydrogenation sequence in these bacteria must be revised. Phytoene [1] is dehydrogenated first to phytofluene [2] and then to 7,8,11,12-tetrahydrolycopene [6], both these steps taking place on the same side of the chromophore. These are followed by two dehydrogenation steps on the other side of the molecule, to give neurosporene [4] and lycopene [5] in turn (*Figure 2*).

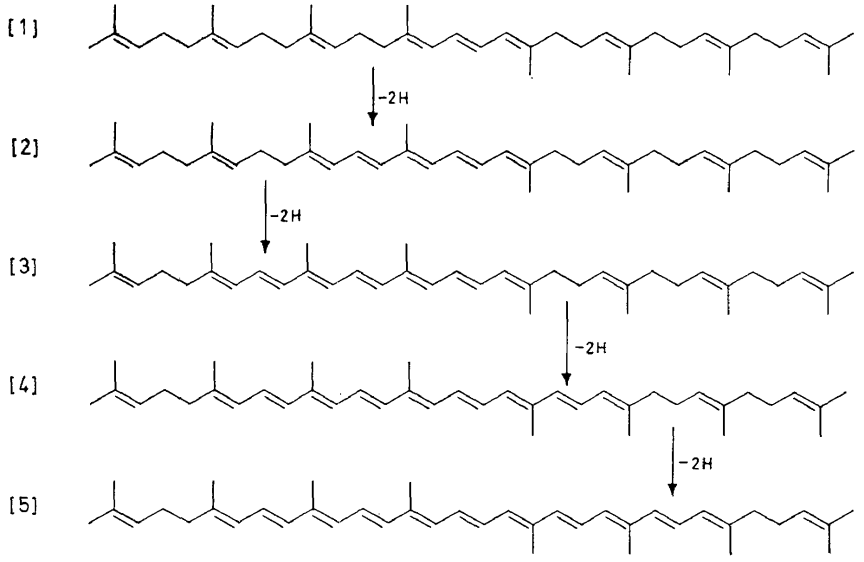


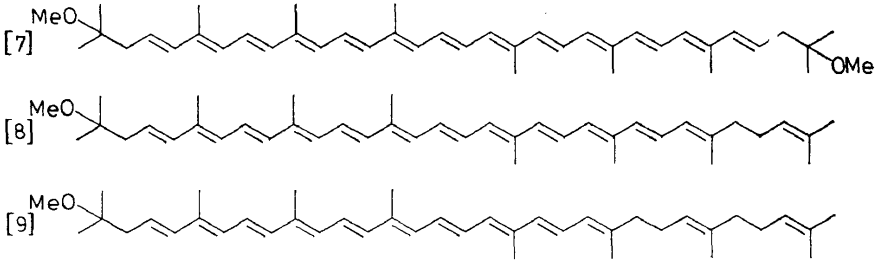
Figure 2. Dehydrogenation of phytoene in *Rhodospirillum rubrum*

So far, *R. rubrum* is the only organism that has been shown to operate this sequence, but the absorption spectrum of the conjugated heptaene isolated from other photosynthetic bacteria indicates that they, too, operate the new pathway[13,18]. Nor is this pathway necessarily confined to photosynthetic bacteria as there is strong evidence that it also operates in *Flavobacterium dehydrogenans*[17].

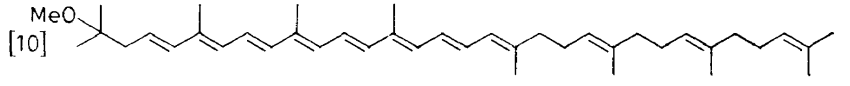
NEW METHOXY-CAROTENOIDS FROM *RHODOSPIRILLUM RUBRUM*

When *Rhodospirillum rubrum* is cultured anaerobically in the presence of diphenylamine at a concentration of 7×10^{-5} M, the precursors of spirilloxanthin accumulate. By studying the kinetics of the disappearance of these precursors on removing the inhibition, Jensen *et al.*[11,12] were able to show that lycopene [5] is converted into spirilloxanthin [7] by a number of consecutive reactions which operate first on one side of the molecule and then on the other. The hydration of an isopropylidene end group of lycopene, followed by a dehydrogenation to produce a double bond between C_3 and C_4 at the hydroxylated end and, finally, methylation of the hydroxyl group,

result in the formation of anhydrorhodovibrin [8]. The operation of the same sequence of three reactions at the remaining isopropylidene end group converts anhydrorhodovibrin into spirilloxanthin. Jensen *et al.*[12] also showed that spheroidene [9] could be produced by these same three reactions from neurosporene in *Rhodopseudomonas spheroides.*



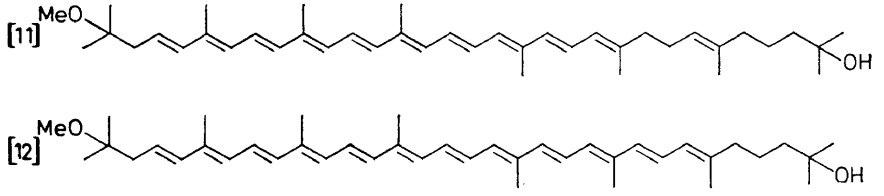
In the course of their studies on diphenylamine-inhibited cultures of *R. rubrum*, Goodwin and Osman[8] isolated two unidentified carotenoids which had absorption spectra corresponding to chromophores of 8 and 10 conjugated double bonds. Jensen *et al.*[11] designated these as P412 and P450 respectively. Davies and Holmes[2] have shown that P450 has an absorption spectrum identical with that of spheroidene isolated from *Rps. spheroides* and that it cannot be separated from authentic spheroidene by chromatography. This identification has been confirmed by mass spectrometry.[3] After comparing the chromatographic properties of P412 on thin layers of alumina with those of 7,8,11,12-tetrahydrolycopene, neurosporene, spheroidene, lycopene and anhydrorhodovibrin, Davies and Holmes[2] proposed that this pigment is 11′,12′-dihydrospheroidene [10]. The high resolution measurement of the mass of the molecular ion and a fragmentation pattern which showed the presence of a methoxyl group and revealed fragments corresponding to (M-69), (M-137) and (M-205) confirmed this structure.[3] This novel carotenoid is presumably formed from 7,8,11,12-tetrahydrolycopene in the same way that spheroidene and anhydrorhodovibrin are formed from neurosporene and lycopene respectively.



Eimhjellen and Jensen[6] demonstrated alternative pathways of spirilloxanthin formation in anaerobic cultures of *Rhodopseudomonas gelatinosa.* These operate from neurosporene through spheroidene, with spheroidene being either dehydrogenated to anhydrorhodovibrin or hydrated to OH-spheroidene [11] which is then dehydrogenated to yield rhodovibrin [12]. In each case, the normal route through lycopene is by-passed. Davies and Holmes[2] have shown that diphenylamine-inhibited cultures of *R. rubrum* also form small amounts of OH-spheroidene.

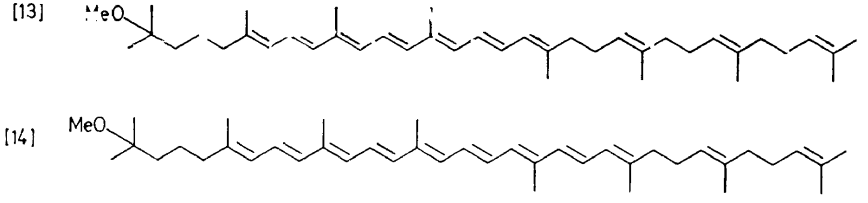
Thus it is quite conceivable that all the biosynthetic reactions demonstrated by other workers in *Rps. spheroides* and *Rps. gelatinosa* also occur in *R. rubrum.*

In addition to these, there is the new pathway from 7,8,11,12-tetrahydrolycopene leading to the formation of the novel 11′,12′-dihydrospheroidene. This could be dehydrogenated to spheroidene which could then be converted into spirilloxanthin as in *Rps. spheroides*, thus providing several alternative routes for spirilloxanthin formation in *R. rubrum*.



THE MECHANISM OF METHOXY-CAROTENOID FORMATION

When the carotenoids of diphenylamine-inhibited cultures of *Rhodospirillum rubrum* are separated by column chromatography on alumina, a number of unidentified pigments appear. Two of these have the same visible absorption spectra as 7,8,11,12-tetrahydrolycopene and neurosporene respectively and are also all-*trans* carotenoids. As they are only slightly more strongly adsorbed than the hydrocarbon polyenes to which they are obviously related, they are distinct from the monohydroxy-carotenoids[11] which are considerably more polar. When these two novel carotenoids are examined by mass spectrometry, they give high resoultion molecular ion masses and fragmentation patterns consistent with the structures 3,4,11′,12′-tetrahydrospheroidene [13][3] and 3,4-dihydrospheroidene [14] respectively.



The formation of 3,4,11′,12′-tetrahydrospheroidene can be rationalised by including this carotenoid as an intermediate in a sequence of three reactions in which 7,8,11,12-tetrahydrolycopene is converted into 11′,12′-dihydrospheroidene. In this sequence, hydration of the isopropylidene end group converts 7,8,11,12-tetrahydrolycopene into OH-7,8,11,12-tetrahydrolycopene [15]. The hydroxyl group is then methylated to yield 3,4,11′,12′-tetrahydrospheroidene which can be converted into 11′,12′-dihydrospheroidene by dehydrogenation (*Figure 3*). Similarly, 3,4-dihydrospheroidene would be an intermediate in the analogous formation of spheroidene from neurosporene.

In these two pathways, methylation precedes the dehydrogenation step, this order being opposite to that demonstrated by Jensen *et al.*[12] in the formation of spirilloxanthin from lycopene in *R. rubrum* and postulated by Eimhjellen and Jensen[6] for the formation of spheroidene from neurosporene in *Rps. gelatinosa*. However, as Jensen[10] has pointed out, the apparent order

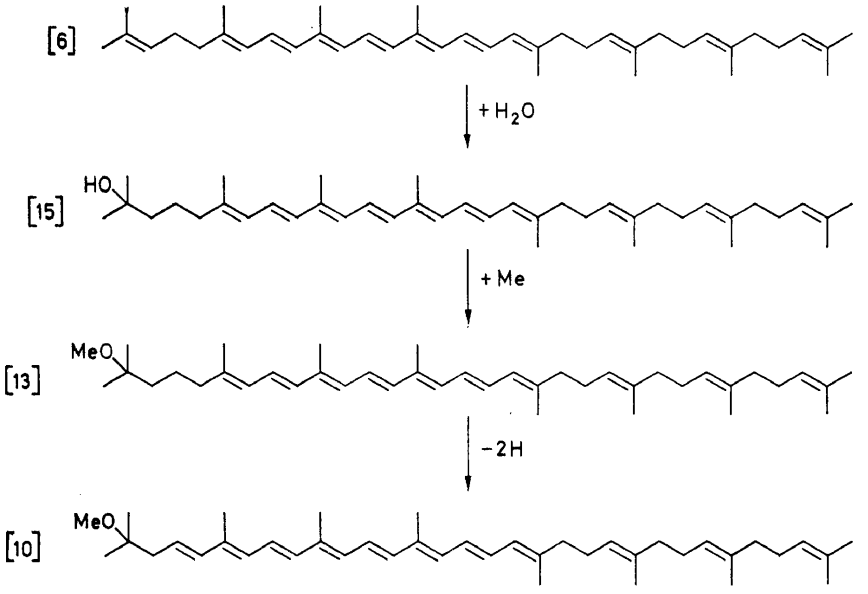
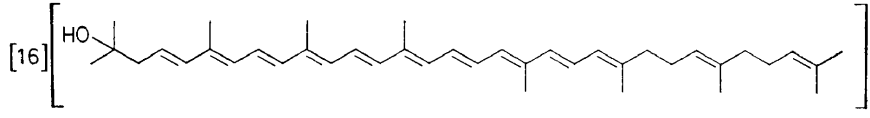


Figure 3. Formation of 11′,12′-dihydrospheroidene from 7, 8, 11, 12-tetrahydrolycopene in *Rhodospirillum rubrum*

of these two reactions is simply a measure of the relative reaction velocities and is indicated by which of two alternative intermediates appears. Thus, in the case of spheroidene formation, the apparent intermediate is 3,4-dihydrospheroidene [14] rather than the hypothetical demethylated spheroidene [16], indicating that, in this case at least, the methylation reaction is slightly faster than the dehydrogenation. This also holds true for the pathway in which 7,8,11,12-tetrahydrolycopene is converted into 11′,12′-dihydrospheroidene.



The complete pattern of carotenoid biosynthesis from phytofluene in *R. rubrum* is shown in *Figure 4.*

It must be emphasised that the novel carotenoids and some of the others in cultures of *R. rubrum* are present in very small quantities and only appear under conditions of diphenylamine inhibition. The recent isolation of carotenoids with the properties of 3,4,11′,12′-tetrahydrospheroidene and 3,4-dihydrospheroidene from normal large-scale anaerobic cultures of *Rps. spheroides*[18] indicates that these compounds are not artifacts induced by the diphenylamine inhibition. Whether the new carotenoids are important biosynthetic precursors in *R. rubrum* or merely by-products of a lack of specificity on the part of the enzymes can only be decided by many detailed and careful kinetic experiments.

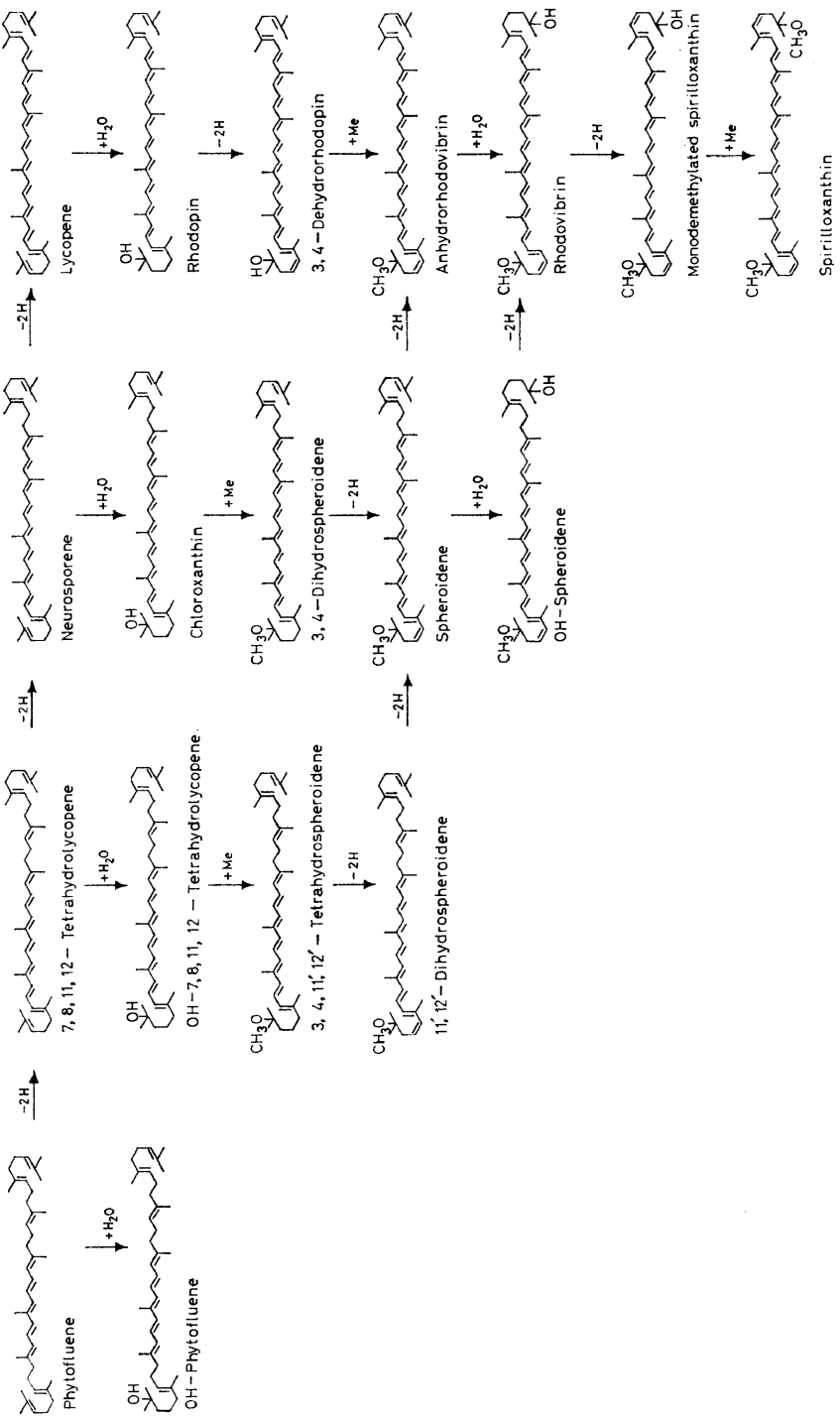


Figure 4. Pathways of methoxy-carotenoid formation in *Rhodospirillum rubrum*.

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